# Final Report to Department of the Environment and Heritage on work completed for RFT 43/2004, "Experimental research to obtain a better understanding of the pathogenesis of chytridiomycosis, and the susceptibility and resistance of key amphibian species to chytridiomycosis in Australia."

## 22 May 2010

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**Executive Summary**

## ***Introduction***

The call for tenders RFT43/2004 designated 6 objectives to be addressed. In the proposal we submitted in response to RFT 43/2004, we described 9 projects, each of which addressed some or all of these objectives. All of the projects were designed to share some resources with the projects we designed in our response to RFT 42/2004, and some were partially or wholly subsumed within the work to be carried out in that submission. Similarly, some of the 9 projects outlined in our proposal for RFT43/2004 addressed data needed in our response to RFT 42/2004. Both reports therefore refer to each other where appropriate.

The tenders were called in 2004 and projects were initially designed to be completed within three years. Some projects were delayed by the need to recruit qualified staff and students, and funds were not expended as fast as initially anticipated, so the projects were extended for a fourth year. All are now complete, although some data collected remain to be written up.

RFT 43/2004 identified six major objectives. Objectives 1, 2, 4, 5, and 6 are highly interrelated and were addressed by a series of linked projects. Objective 3 was addressed by a more clearly separate project. We will present Objectives 1, 2 ,4 5, and 6, and then summarise the findings dealing with those. We will then present Objective 3 and a summary

## ***Objectives identified in RFT 43-04***

1. Build on our understanding of the pathogenesis of chytridiomycosis including the host and environmental factors that determine the ultimate outcome of infection, i.e., death, persistent infection with no obvious effect, and cure.

2. Investigate populations of frog species that have undergone widespread chytrid-associated decline to:
   i. identify the factors that maintain these populations, and
   ii. develop and assess management activities that can reproduce these factors in other populations, particularly threatened species.

3. Investigate the manifestation of chytridiomycosis by determining the physiological mechanisms associated with morbidity and mortality of affected frogs.

4. Determine whether populations that have recovered after chytrid-associated decline are susceptible to future severe effects from chytridiomycosis.

5. Determine the resistance of key frog species (how key frog species are determined must be clearly identified by the tenderer in their proposal) to chytridiomycosis using a standard laboratory model that relates to risk in the wild, particularly for species that are currently chytrid-free, or species that occur in areas where the status of chytrid is unknown.

6. Search for and examine evidence of resistance to chytrid and research techniques to increase resistance in at risk species (how at risk species are determined must be clearly identified by the tenderer in their proposal), including captive breeding and selection for restocking.

***Results addressing Objectives 1, 2, 4, 5, and 6***

We addressed these objectives simultaneously, using a combination of comparisons among natural populations, field experiments, laboratory experiments, and chemical analysis of host antimicrobial peptides.

We found that host and environmental factors interact in complex ways to determine the probability of acquiring infections by *B. dendrobatidis* and the ultimate outcome of those infections. Host behaviour, in the form of social behaviour and microenvironmental use, can have a strong effect on the probability of acquiring infections. Radio- and harmonic radar-tracking studies of three species (*Litoria genimaculata, L. nannotis, and L wilcoxii/jungguy* complex) showed that encounter rates among individuals within species varied in a way that could explain differences in the probability of becoming infected. We examined unoccupied frog retreat sites for the presence of *B. dendrobatidis* DNA using swabbing samples analysed using diagnostic quantitative PCR, and found no evidence that infective stages of the pathogen remain for even short periods after retreat sites are occupied by frogs. In laboratory experiments, we demonstrated that frogs can acquire infections from contact with water containing infective zoospores. It thus appears that either direct contact among individuals, or contact with bodies of water containing zoospores, are the usual routes by which frogs acquire infections. The species that suffered the strongest effects during initial outbreaks of chytridiomycosis, *L. nannotis*, usually spends the day in communal retreat sites in crevices in rocks in the spray zone of fast moving water. This provides an ideal environment for the transmission of the pathogen, since frogs are close together and often make physical contact or make contact with substrate immediately after it is vacated by other individuals, and the films of moisture usually present in these locations provide a means for the aquatic infective zoospores of *B. dendrobatidis* to remain viable and move from host to host. The species (*L. genimaculata*) that suffered intermediate impacts at the population level has intermediate levels of exposure to transmission via simultaneous occupation of retreat sites and contact among individuals, while *L. wilcoxii/jungguy* complex has the lowest levels. Our results make it clear that knowledge of species' social behaviour and retreat site selection can improve our ability to predict their vulnerability to acquiring *B. dendrobatidis* infections, and thus their vulnerability to epidemic outbreaks of chytridiomycosis.

In addition to affecting the probability of transmission, our tracking and experimental data indicate behaviour affects the persistence of infections and the fate of infected individuals. Previous work by our group (Woodhams et al 2003) demonstrated that elevated body temperatures can cure frogs of infection by *B. dendrobatidis*. We found that relatively short term (2 week) patterns of body temperature variation can explain a substantial proportion of both interspecific and intraspecific variation in the prevalence of infection. Across all three species, individuals who increased their body temperatures above 25 degrees C were only 1/3 as likely to carry *B. dendrobatidis* infections as those who did not. The proportion of individuals that attained those temperatures was highest in the species (*L. wilcoxii/jungguy* complex) that has the

lowest natural prevalence of *B. dendrobatidis* infections, intermediate in *L. genimaculata*, which has intermediate prevalences, and lowest in *L. nannotis*, which has the highest prevalences in the field.

To examine in more detail whether and how frog behaviour affects the transmission of *B. dendrobatidis* and the fate of infected individuals, we performed a series of experiments in standard laboratory environments. One series of experiments examined whether frogs can detect and avoid water containing *B. dendrobatidis* zoospores. He hypothesised that this might happen either because the process of zoospores encystment and infection may produce local irritation to frogs' skins, or because frogs might detect chemical cues given off by *B. dendrobatidis*, as they have been demonstrated to do for some aquatic predators. The results of these experiments indicate that two species we examined (*L. caerulea* and *L. genimaculata*) have a significant tendency to avoid water containing *B. dendrobatidis* zoospores. Some individuals of each species show this very strongly, while others do not, but overall they show a statistically significant ability to avoid infected water. This may account for some of the lower vulnerability to chytridiomycosis that these species exhibit in the field. In a second set of experiments we constructed a set of standard environmental chambers that allowed frogs to choose both temperature (along a gradient from cool to very warm) and humidity (high or low) environments. We placed infected and uninfected *L. genimaculata, L. caerulea, and L. jungguy* in these enclosures and monitored their behaviour and intensity of infection in infected individuals. We found no evidence that individuals infected with *B. dendrobatidis* altered their choice of diurnal resting sites. This suggests that none of these species exhibits the phenomenon known as "behavioural fever", in which some ectothermic animals alter their behaviour to elevate their body temperatures in response to infections. However, both infected and uninfected individuals of all three species chose microenvironments that would slow the development of *B. dendrobatidis* infections or cure infections, because they chose relatively high temperatures and low humidities at least some of the time. Any future experiments conducted to examine the potential vulnerability of species to chytridiomycosis should use experimental environments similar to ours. Confining experimental frogs to containers that offer only a constant environmental temperature (usually between 17 and 23 $^{o}$C, exactly within the optimum range for rapid growth of *B. dendrobatidis*, and saturated humidity, also ideal for *B. dendrobatidis* proliferation) provides the optimal conditions for growth of *B. dendrobatidis*. The results of experiments conducted under such conditions are likely to indicate that species are vulnerable to chytridiomycosis when they may actually be invulnerable under field conditions, where they can choose warmer and/or dryer microenvironments.

The results of this part of our study imply that elevation is not the only macroenvironmental factor that can determine the outcome of emergence of chytridiomycosis in a naïve population; it is likely that cloud cover and canopy cover also have effects, since they will alter the access of species to warmer, dryer retreat sites. Species that occur at a narrow range of elevations are more likely to survive in parts of their range with more open canopies or less cloud cover. This prediction was confirmed by our documentation of several generally unknown frog populations during this research. We carried out surveys of streams at high elevations, well above 400m, but outside the western boundary of the Wet Tropics rainforest. We initially documented populations of the endangered Waterfall Frog, *Litoria nannotis*. These populations occur at high local densities, much higher than the densities of this species

at rainforest sites it has recolonised after the local extinctions caused by chytridiomycosis in the early 1990s. They presently coexist with high prevalences of infection with *B. dendrobatidis*, and repeated sampling has demonstrated that many individuals coexist with infections for extended periods. Although their history is not known, it is likely that these populations did not suffer to the same extent as rainforest populations during the initial outbreaks of chytridiomycosis. Tracking and environmental monitoring at these sites has confirmed that at them frogs experience dryer and hotter conditions than in adjacent rainforest sites. This indicates that surveys and conservation efforts should not overlook habitats peripheral to, and even believed to be outside the range of, species under threat from chytridiomycosis. When, as is the case for rainforest stream frogs, environmental conditions at the core of species' ranges coincide with conditions favourable to chytridiomycosis, peripheral populations may be highly important for the continued survival of species, giving them access to environmental conditions that provide refuge from the disease.

The importance of the idea that peripheral populations in environmental refuges may be critical for the survival of species is reinforced by our discovery, during this research, of a previously unknown population of the Armored Mistfrog, *Litoria lorica*. This species was only known from closed-canopy rainforest sites at high elevations. It disappeared from all known sites during the epidemic outbreaks of chytridiomycosis in the early 1990s. It had not been seen since 1992, and although it was listed as critically endangered, was widely presumed to be extinct. We carried out a survey in June 2008 along a section of high elevation stream in dry sclerophyll forest outside the western boundary of the Wet Tropics on the Carbine Tableland, and discovered a population of this species, occurring at high local densities, occupying several kilometres of the stream. Swab sampling and diagnostic qPCR revealed very high rates of infection of frogs, greater than 80% in winter. Repeat sampling in 2009 showed that individuals are persisting with the infection for extended periods and the population density remains relatively constant. The area has been protected under Queensland legislation, and further research is planned to increase our understanding of how this species, and a dense sympatric population of *L. nannotis*, persist despite very high prevalence of *B. dendrobatidis*.

It has been well documented that the prevalence of infections by *B. dendrobatidis* fluctuates seasonally. It can reach very low levels during the warm summer months, but can then increase rapidly during the winter months. Because frogs appear to acquire infections either through direct contact among individuals or through contact with water containing infective zoospores, the source of this rapid increase in prevalence has been puzzling. It suggests that there is a source of zoospores other than frogs. Our examinations of frog retreat sites established that zoospores do not appear to persist in them. We also sampled a wide range of environmental substrates and potential alternative hosts. Preliminary results suggested that *B. dendrobatidis* might survive on the exoskeletons of stream-dwelling prawns and crayfish, but experimental work and extensive sampling, plus re-examination of our original data, refuted this. However, in Wet Tropics rainforests and many other habitats containing permanent water, the tadpoles of frogs are present all year. Tadpoles are known to carry infections of *B. dendrobatidis* in their keratinised mouthparts. We therefore carried out a series of detailed field studies on tadpoles. We focused primarily on tadpoles of the waterfall frog, *L. nannotis*. This species breeds in an extended period over summer, so that hatchling tadpoles enter the stream between approximately September and March in

most years. Most tadpoles remain in the stream through winter and metamorphose in the following summer, also over several months. We found the prevalence and intensity of infection fluctuated seasonally, in a manner similar to that seen in frogs but with a different mechanistic explanation. Hatchling tadpoles enter the stream in large numbers over the summer period, and are initially uninfected. Prevalence increases with residence time, so that by April/May it approaches or reaches 100%. Experiments with tadpoles maintained in containers in the stream showed that isolated individuals can acquire infections through contact with stream water, and that individuals at higher density acquire infections more rapidly than isolated individuals, so infections will build up more rapidly where tadpole populations are dense.

Because the infection occurs only in the mouthparts, infected tadpoles do not grow and develop as rapidly as uninfected conspecifics, but mortality is rare; they can survive, feed, and grow even with extensive mouthpart damage. By April/May of each year, the high densities of tadpoles, combined with high prevalences of *B. dendrobatidis* infection, form a major source of infective zoospores in the aquatic environment. It is likely that the rapid increase in prevalence among frogs at this time is in part a consequence of contact with water containing zoospores shed by infected tadpoles. In midwinter many infected tadpoles shed their mouthparts; prevalences decrease and some individuals lose their infections entirely. Uninfected individuals metamorphose earlier than infected ones, so there is always a source of infective zoospores when breeding resumes in spring. The nature of this annual cycle means that even if prevalence drops to zero or or near zero in terrestrial frogs over summer, infected tadpoles form an interspecific reservoir that maintains the pathogen in the population, and frogs reacquire infections from water as temperatures drop and tadpole prevalence increases in autumn. This result indicates that it will be difficult or impossible to eliminate *B. dendrobatidis* once it is established in a population of frogs with overwintering tadpoles; even if the infection was eliminated from all terrestrial individuals, it would persist in tadpoles and re-emerge. Successful treatment would require simultaneous elimination of the pathogen from terrestrial juveniles and adults and aquatic tadpoles. It may be possible to eliminate the infection by treatment of the terrestrial stages of frogs that occur in isolated populations and do not have overwintering tadpoles.

Although host behaviour, environment use, and intraspecific reservoirs clearly play a major role in determining susceptibility to acquiring infections and developing disease, our research also showed that aspects of the innate immune system have strong effects. It has been known for some time that frogs secrete a complex mixture of peptides from skin glands, and that some of these have antimicrobial properties (AMPs). Earlier work at James Cook University showed that, at the level of species, the effectiveness of these antimicrobial peptides against *B. dendrobatidis* was correlated with the probability of species declining during outbreaks of chytridiomycosis. The work carried out under Tender 43/2004 expanded on that earlier work by examining how the effectiveness of AMPs varies among individuals within species across sites with different histories of effects from chytridiomycosis and across seasons.

High elevation populations have been subjected to strong natural selection because they declined during outbreaks of chytridiomycosis in the 1990s. We hypothesised that if AMPs are responsible in part for the recovery of these populations, frogs in them should now have more peptides, or more effective peptides, than those in populations that did

not decline during the initial epidemics of chytridiomycosis. We found that the effectiveness of AMPs varies among sites in a manner consistent with this hypothesis. In both *Litoria genimaculata* and *L. rheocola*, a greater proportion of individuals from high elevation populations secreted peptides that inhibited the growth of *B. dendrobatidis in vitro*. We found no evidence for changes in the effectiveness of peptides per unit mass against *B. dendrobatidis*, indicating that frogs have responded to selection by producing larger quantities of the same peptides, rather than by evolving new peptides. We also found that the proportions of frogs producing effective peptides are greater in winter, when the prevalence of infection by *B. dendrobatidis* is higher and environmental factors that prevent the development of disease are less effective. The composition of peptides also varies between winter and summer, and peptides are more effective per unit mass against *B. dendrobatidis* in winter than in summer. This probably is not a response to selection via chytridiomycosis, but is likely to reflect a long evolutionary history in which frogs are more vulnerable to many infections during the cooler months, when their metabolic activity, and thus the rate of response of their adaptive immune system, is lower, and opportunities for attaining elevated body temperatures are reduced.

In addition to the work summarised above, we carried out a set of targeted field and laboratory studies on microhylid frogs. There are at least 20 species of microhylids in the Wet Tropics region, including many species with relatively limited ranges, some of which are listed because of small range or population size. No species or populations are known to have declined. Only a single individual has been reported to be infected with *B. dendrobatidis* in nature, and none have been demonstrated to be infected in captivity. We examined a total of 595 samples from different individuals of nine species of direct-developing Australian microhylids for the presence of *Batrachochytrium dendrobatidis* infection. Of these, 336 were historic samples collected between 1995 and 2001 and 259 were swab samples collected during 2005-2008. One hundred and two of the historic samples were examined histologically, and the remaining 493 samples were examined using diagnostic quantitative PCR. 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 (less than 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, well under 1%. Our data thus strongly suggest that Australian microhylids have an extremely low prevalence of Bd in nature, and are either not susceptible, or are only slightly susceptible, to chytridiomycosis. They, and perhaps some other direct-developing species, may be highly resistant to Bd because they possess antimicrobial symbionts in skin flora or in skin secretions as a means to reduce fungal infestations that would otherwise overwhelm terrestrial egg clutches.

To determine whether very powerful antimicrobial peptides might be responsible for protecting microhylids from infection by *B. dendrobatidis*, we sampled AMPs from 81 individuals belonging to six species. Analysis of the activity of their AMPs against *B. dendrobatidis* indicated that they were effective, but no more effective than the AMPs of stream-breeding species. This indicated that the factor or factors that provide the almost perfect protection from *B. dendrobatidis* infection that microhylids experience in the field must be something other than AMPs. If the protection arises from microenvironment use, it should be lost when animals are maintained in isolated

laboratory environments under constant temperature and humidity conditions that favour the growth and development of *B. dendrobatidis*. If it arises from symbiotic skin microbes, it might be reduced under laboratory conditions because the environment is more constant, environmental sources of colonising microbes are absent, and bathing frogs during exposure experiments could reduce or remove some skin microbes.

We tested this hypothesis by carrying out a laboratory infection experiment. Adult male microhylids (*Cophixalus ornatus*), and hylids (*L. wilcoxii*) were housed in similar containers in a laboratory at JCU. *Litoria wilcoxii* served as known susceptible controls. Infection experiments involved bathing all experimental and control individuals in *B. dendrobatidis* culture solution, which either contained zoospores harvested from laboratory cultures for experimental animals or was sterile for controls. We carried out a series of experimental exposures using increasing concentrations of zoospores. Exposure to concentrations of 10,000 zoospores/mL produced infections in both species. All exposed *Cophixalus ornatus* became infected. They reached maximum infection intensity rapidly (7-28 days post-exposure). Maximum intensity was relatively low, with no infected animals returning more than 78 zoospore equivalents from a standard swab sample, and only one individual *C. ornatus* remained infected 35 days after exposure. This experiment demonstrated that when maintained in environmental conditions that reduce skin microbiota and favour the growth of *B. dendrobatidis*, microhylids are not consitiutively immune to infction by *B. dendrobatidis*. However, it also demonstrates that even under those conditions, they are capable or rapidly and effectively combating the infection, not allowing it to progress to high intensity and eventually clearing it. Further work is needed to determine the mechanisms underlying the very low susceptibility of microhylids to *B. dendrobatidis*. This should focus on their skin microbiota, which may have been reduced in effectiveness by the experimental conditions but increased in effectiveness as frogs were left undisturbed, eventually clearing their infections, and on their adaptive immune defences, which is the only remaining system if AMPs, microenvironmental effects, and skin microbes are eliminated.

***Results addressing Objective 3***

We found that the development of infections on frogs in nature follows a pattern consistent with the hypothesis that they typically become infected by contact of their posterior abdomen or feet with substrates (possibly including other frogs) contaminated by *B. dendrobatidis* zoospores. As infection intensity increases, other body regions become more infected. The dorsal surface usually becomes infected last and infections on the dorsal surface typically do not reach high intensities. This result is important for the collection of diagnostic samples by swabbing; it suggests that, until more is understood, swab samplels should be collected from body parts in an order that minimises the chance of moving zoospores from more heavily infected body regions to less heavily infected ones. Our suggested swabbing patterns is dorsal surface, then feet, then thighs and posterior abdomen (Project 6.6). These results have been published as North, S., and Alford, R. A. 2008. Diseases of Aquatic Organisms 81:177-188. to ensure that they are accessible to the larger research and management communities.

In heavy infections, we found that frogs dying of chytridiomycosis lose control of their plasma electrolytes, then develop cardiac arythmia and ultimately heart failure. The results are consistent with a disturbance to the sodium-potassium ionic balance of

plasma caused by damage to the skin, particularly the ventral skin, simply causing heart failure (Projects 6.4 and 6.5). This is consistent with other work we have been involved in, which show that the symptoms that characterise the disease chytridiomycosis occur when the concentration of *B. dendrobatidis* thalli in the skin reach a critical threshold level. These results are elaborated on in the detailed report on Project 6.

***Supporting research/technique development and testing***

The work we carried out to directly address the objectives of Tender 43-04 required the development and refinement of many techniques that will now be available for further work in this field. We tested the efficacy of various reagents for denaturing the DNA of *B. dendrobatidis*, and developed criteria for the use of sodium hypochlorite; this is essential to ensure that samples taken for diagnostic purposes and epidemiological research are not cross-contaminated (Project 4.1). We examined the effects of sampling techniques for detection of the DNA of *B. dendrobatidis* using real time PCR. Our results showed that the standard swabbing technique used to detect the pathogen is highly efficient and sensitive, and that it is possible to detect it in environmental samples via water filtration (Project 4.2). Unexpectedly, we found that handling tadpoles with certain types of gloves can cause mortality (Project 4.3); those results have been published (Cashins et al., 2008. Herpetological Review 39:298-301). We examined how *B. dendrobatidis* adapts to culture. Many pathogens alter their biology in response to long-term maintenance in cultures. It is often thought that long-term culture leads to the evolution of decreased virulence. We found that cultured *B. dendrobatidis* can adapt to thermal environments between 12 and 23$^{o}$C, and that rather than decreasing virulence, it is maintained or even increased in long-term culture (Projects 6.2 and 6.3). These results highlight the importance of cryopreserving isolates as they are acquired, so that they can be studied

***Summary and recommendations***

In summary, our work shows that, in addition to the previously known effects of macroenvironment (populations at elevations below 300-400 m were not seriously affected by initial outbreaks of chytridiomycosis, while populations above those elevations were, and prevalence of *B. dendrobatidis* infections fluctuates seasonally) both behaviour and microenvironment use are important. Species that are more likely to aggregate in retreat sites and/or use relatively cool, moist retreat sites are likely to be more vulnerable to the disease, due both to increased transmission rates and increased rates of intraindividual reinfection. These effects may be exacerbated by droughts, which would force more individuals into less space. Species that occupy warmer, drier diurnal retreats are less vulnerable to infection and to the development of lethal chytridiomycosis. Microenvironmental effects may explain the precipitous, synchronous nature of the outbreaks that caused declines in the 1990s, and could occur again in populations now coexisting with the pathogen. Droughts may increase transmission rates, and prolonged periods of cloudy, moist weather might prevent frogs from experiencing elevated body temperatures and drier skins, allowing pathogen populations to accumulate on infected individuals exponentially and drastically increasing concentrations of infective zoospores in the environment and thus rates of transmission to uninfected individuals. Our results indicate that epidemic outbreaks may occur if the apparently stable host-pathogen relationships that *B. dendrobatidis* has reached with many remaining species are disrupted by climate change or even weather

fluctuations. This indicates that ongoing monitoring of apparently stable populations is needed. Our results also strongly indicate that populations on the periphery of species' ranges may be important for conservation, since they experience environmental conditions very different from populations in the core range. If core range populations are attacked by a pathogen such as *B. dendrobatidis*, they may collapse, leaving only peripheral populations. This highlights the need for full, proper surveys to document species' actual ranges, instead of relying on environmental models based on the properties of core populations.

Our laboratory data on microenvironment selection indicate that frogs do not alter their diurnal behavious in response to infection by *B. dendrobatidis*, but that if given a choice among thermal and humidity environments in captivity, many species will choose environments that decrease the probability of *B. dendrobatidis* infections building up to cause chytridiomycosis. Laboratory experiments aimed at evaluating the vulnerability of species to the disease must present animals with a range of thermal and humidity environments to choose from, or their results will not be meaningful for populations in the field. The enclosures we designed are simple to construct and maintain, and could serve as a standard for this purpose. Our laboratory data also indicate that some species may be able to avoid water containing *B. dendrobatidis* zoospores, but only after entering it, and therefore exposing themselves to infection. However, by avoiding infected water, these species may decrease the probability of infections building up to cause chytridiomycosis. Our data suggest that the innate immune defences, in the form of antimicrobial peptides, of the two species we examined (*L. genimaculata* and *L. rheocola*) have responded to natural selection exerted by chytridiomycosis. The proportions of frogs of both species that secreted peptides effective against *B. dendrobatidis* were greater in high elevation populations that have been subjected to strong selection. This suggests that selective breeding for resistance, or translocation of frogs from populations that heve developed resistance naturally, may be a means of increasing the resistance of frogs in vulnerable populations.

Our data on tadpoles indicate that they do serve as intraspecific reservoir hosts. Some proportion of tadpoles are always infected in the many rainforest species that have an annual reproductive cycle, with tadpoles persisting in the stream all year. This may help to explain the greater impact of chytridiomycosis on stream-breeding species, which tend to have long larval periods; pond breeders lack this reservoir host. It may be impractical to eliminate *B. dendrobatidis* from environments in which overwintering larvae are present, as it would need to be removed from both terrestrial and aquatic habitats. We also showed that gloves used to handle tadpoles should be carefully assessed for potential toxic effects before they are used to handle large numbers of individuals.

Our results on pathogenesis indicate that high-intensity infections disrupt the normal functioning of frog skin, leading to ionic imbalance and eventual death through heart failure. Preliminary experiments showed that it may be possible to treat frogs, even in late, symptomatic stages of infections, by providing balanced electrolytes in combination with antifungal agents. At least in the species we studied, low intensity infections occur mostly on the ventral surfaces, particularly the posterior abdomen and thighs, and to a lesser extent on the feet. We suggest that routine swabbing to collect samples for diagnostic PCR analysis should work from areas that become infected later towards those that become infected earlier, to avoid spreading infections on individuals.

We found that the prevalence of infection by *B. dendrobatidis* in frogs of the family Microhylidae is extremely low. This may in part be due to their fully terrestrial life history, which should reduce exposure to infective zoospores. It is not due to extremely effective innate immune defences in the form of antimicrobial peptides, since in laboratory experiments microhylids became infected at the same exposure levels as non-microhylids. However, microhylids cleared infections more rapidly and completely, indicating that some mechanism other than AMPs provides increased resistance. Future research should examine the skin microbiota and inducible immune system of microhylids.

We suggest that there is a need for substantial futue research on the microbial assemblage inhabiting frog skin and its interactions with *B. dendrobatidis*. This field was unknown at the time RFT 43/2004 was called and the research program reported on here was developed. Results from North American systems indicate that amphibian skin harbours many microbes that help control the growth and reproduction of *B. dendrobatidis*, that natural populations that show greater resistance to chytridiomycosis harbour more of these species, and that artificial supplementation with "beneficial" bacteria can reduce the severity of chytridiomycosis. Further development of these ideas could produce probiotic means of managing the impact of *B. dendrobatidis* on Australian frog populations, but was beyond the scope of the work we tendered for.

***Publications arising from work funded in whole or in part by Tender 43-04***

Names of authors funded by this tender in bold

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**Full report**

***Structure of the proposal and report***

The call for tenders RFT43-04 designated 6 objectives to be addressed. In the proposal we submitted in response to RFT 43-04, we described 9 projects, each of which addressed some or all of these objectives. All of the projects were designed to share some resources with the projects we designed in our response to RFT 42-04, and some were partially or wholly subsumed within the work to be carried out in that submission. Similarly, some of the 9 projects outlined in our proposal for RFT43-04 addressed data needed in our response to RFT 42-04. Both reports therefore refer to each other where appropriate.

**Table 1**. Agreed research plan, milestones, and timetable for work to be carried out in the program developed in our tender for 43-04.

| Project | Commencement* | Completion | Annual progress reports due | | Final report |
|---|---|---|---|---|---|
| | | | | | |
| 1 | start of contract | 3 years post contract start | 1 year post contract start | 2 years post contract start | 3 years post contract start |
| 2 | start of contract | 3 years post contract start | 1 year post contract start | 2 years post contract start | 3 years post contract start |
| 3 | start of contract | 3 years post contract start | 1 year post contract start | 2 years post contract start | 3 years post contract start |
| 4 | start of contract | 2 years post contract start | 1 year post contract start | | 2 years post contract start |
| 5 | start of contract | 3 years post contract start | 1 year post contract start | 2 years post contract start | 3 years post contract start |
| 6 | Note this project is separately funded and does not form part of the contract | | | | |
| 7 | start of contract | 3 years post contract start | 1 year post contract start | 2 years post contract start | 3 years post contract start |
| 8 | Note this project is part of tender 42-04 | | | | |
| 9 | Note this project is part of tender 42-04 | | | | |

The research plan, milestones and timetable have since been modified, in an amendment to the contract, which was instituted because unexpended funds remained at the close of the initial contract period, allowing additional work to be carried out. The modified version appears in Table 2.

**Table 2**. Revised research plan, milestones, and timetable for work to be carried out in the program developed in our tender for 43-04.

| Project | Commencement* | Completion | Progress reports due | | | Final report |
|---|---|---|---|---|---|---|
| | | | | | | |
| 1 | start of contract | 4 years | 1 year | 2 years | 3-1/2 years | 4 years |
| 2 | start of contract | 4 years | 1 year | 2 years | 3-1/2 years | 4 years |
| 3 | start of contract | 4 years | 1 year | 2 years | 3-1/2 years | 4 years |
| 4 | start of contract | 4 years | 1 year | | | 2 years |
| 5 | start of contract | 4 years | 1 year | 2 years | 3-1/2 years | 4 years |
| 6 | Note this project is separately funded and does not form part of the contract | | | | | |
| 7 | start of contract | 4 years | 1 year | 2 years | 3-1/2 years | 4 years |
| 8 | Note this project is part of tender 42-04 | | | | | |
| 9 | Note this project is part of tender 42-04 | | | | | |

All of the funded projects are now complete. .

Because of the complex interrelated nature of the objectives addressed by the work undertaken in this tender, and fact that the work has been separated into a series of identified subprojects, each of which is to a large extent itself an integrated, interrelated whole, plus the interrelationships between this tender and Tender 42-04, we have structured this report to briefly reiterate the objectives outlined in RFT 43-04 and summarise which of the identified projects within the tender addresses each. We then present a summary of the results of each subproject, and a summary of outputs in the form of published and submitted scientific papers, conference proceedings, and other outputs. Finally, an appendix presents published papers and completed theses resulting from this project; three theses are still being examined and are not included.

**Objectives identified in RFT 43/2004**

1. Build on our understanding of the pathogenesis of chytridiomycosis including the host and environmental factors that determine the ultimate outcome of infection, i.e., death, persistent infection with no obvious effect, and cure. **Addressed by projects 1 through 9.**

2. Investigate populations of frog species that have undergone widespread chytrid-associated decline to:
   i. identify the factors that maintain these populations, and
   ii. develop and assess management activities that can reproduce these factors in other populations, particularly threatened species.
   **Addressed by Projects 1, 2, 7, 8, and 9**

3. Investigate the manifestation of chytridiomycosis by determining the physiological mechanisms associated with morbidity and mortality of affected frogs. **Addressed by Project 6.**

4. Determine whether populations that have recovered after chytrid-associated decline are susceptible to future severe effects from chytridiomycosis. **Addressed by projects 1, 2, 5, 7, and 8.**

5. Determine the resistance of key frog species (how key frog species are determined must be clearly identified by the tenderer in their proposal) to chytridiomycosis using a standard laboratory model that relates to risk in the wild, particularly for species that are currently chytrid-free, or species that occur in areas where the status of chytrid is unknown. **Addressed by projects 1, 2, and 4.**

6. Search for and examine evidence of resistance to chytrid and research techniques to increase resistance in at risk species (how at risk species are determined must be clearly identified by the tenderer in their proposal), including captive breeding and selection for restocking. **Addressed by projects 3, 5, and 7.**

**Reports on projects identified in Tender 43-04**

***Project 1. Development of chytridiomycosis in thermal and humidity gradients.***

Project 1 addresses Objectives 1, 2, 4, and 5 of RFT 43-04. It addresses **Objective 1** by examining how microhabitat selection by frogs in controlled environments that present a range of temperature and humidity conditions affects the disease process and degree of pathogenesis in frogs of species known to be vulnerable and not to be vulnerable to chytridiomycosis-associated declines. It addresses **Objective 2** by examining the small-scale behaviour and microhabitat selection of species that have declined in association with chytridiomycosis, and how these affect the transmission and progress of the disease in these species. It addresses **Objective 4** by increasing our understanding of how the external environment affects the progress of chytridiomycosis in frogs that have suffered declines. It addresses **Objective 5** by developing and testing a standard laboratory system that allows frogs to express their behavioural preferences for thermal and humidity microenvironments. The system developed in Project will make it possible to assay the likely vulnerability of species that may be threatened by the disease under a set of standard conditions.

In **Project 1** we constructed laboratory enclosures that present animals with a range of microenvironments along gradients of temperature and humidity during 2005 and ran two series of experiments in these enclosures. These experiments suggested that infected and uninfected green treefrogs (*Litoria caerulea*) select different microenvironments, and both select microenvironments that subject them to more extreme conditions than frogs housed in typical laboratory conditions experience. In our experiments, infected frogs housed in complex environments survived at higher rates than those housed in simple laboratory environments. We did not see any signs of "behavioural fever," in fact, infected frogs chose cooler environments, on average, than uninfected frogs. However, both classes chose thermal environments that slowed the progress of *Bd* infections as compared to frogs housed in typical laboratory environments. In 2006, a second, smaller set of enclosures was constructed; these enable us to conduct experiments with more replicates. Since those enclosures were constructed we have completed experiments with *Litoria caerulea*, *L. jungguy*, and *L. genimaculata*. All three species show non-

**Figure 1.** The new, smaller enclosures that provide access to a wide range of thermal and humidity environments

random selection of at least some aspects of their microenvironments when presented with choices. Infected individuals do not appear to differ in their choice of diurnal microenvironments, however infected individuals of some species behave somewhat differently during nocturnal activity, tending to remain lower in the enclosures and spending more time on the ground.

Although infection status does not affect individuals' choice of diurnal retreat sites, two of the species, *L. genimaculata* and *L. jungguy*, chose diurnal retreat sites that were warmer and/or dryer than standard laboratory conditions a substantial fraction of the time, and individuals of all three species chose diurnal retreat sites warm enough to retard the development of chytridiomycosis. Our results therefore support the hypothesis that behaviourally-mediated microenvironment use is likely to affect the progress of chytridiomycosis in naturally infected animals, and the hypothesis that the severe effects some non-declining species experience when experimentally infected with *B. dendrobatidis* and maintained under standard laboratory conditions may result from an inability to express their normal behaviour. Full results of the microenvironment experiments have been written up in the section on Project 1.1, below.

During 2006 we we performed a series of experiments designed to test the hypothesis that frogs may be able to avoid water bodies that contain *Bd* zoospores. This is not an *ad hoc* hypothesis; several previous studies have shown that frogs discriminate among water bodies for oviposition based on the presence or absence of aquatic predators, indicating that they can use olfactory or tactile cues to determine the suitability of water bodies, and we and other researchers have noted that during experimental infections frogs appear to react differently to broth containing zoospores, making more active attempts to avoid contact with it.

The aim of the pond experiments was to evaluate whether frogs actively choose infectious or non-infectious water bodies. Each frog had a choice of two ponds. Broth from an active *Bd* culture was added to one, and sterile broth wass added to the other. Experiments were run for eight days. Every second day water was changed, *Bd* treatments were renewed, and the positions of the ponds in the enclosures were reversed. Digital images were taken at half hour intervals for determining the locations of frogs. We conducted these experiments with *L. caerulea* (captive bred), *L. genimaculata* (raised from eggs) and *L. jungguy* (raised from tadpoles). These species span a wide range of natural susceptibility to *Bd*: *L. genimaculata* declined but has recovered in the last few years. *L. caerulea* shows high susceptibility in the lab but wild populations appear not to be affected by *Bd*, and field data indicate that *L. jungguy* has very low susceptibility.

In summary the results of these experiments indicate that

- Transmission can occur via infected water (to our knowledge this had not been previously demonstrated conclusively for dilute zoospores in a naturalistic setting). Three *L. caerulea* became infected during the experiment,

- The data indicate that taken together, *L. caerulea* and *L. genimaculata* have a significant tendency to avoid water containing *B. dendrobatidis* zoospores. Some individuals of each species show this very strongly, while others do not, but overall

they show a statistically significant ability to avoid infected water. This may account for some of the lower vulnerability to chytridiomycosis that these species exhibit in the field. In our experiments, *L. jungguy* did not make enough choices to enable us to test hypotheses regarding their pond choice. A complete writeup of this project appears below as Project 1.2.

***Project 1.1. The effect of exposure to a fungal disease on anuran selection of microenvironments. A comparison of three Australian hylid frogs.***

Nicole Kenyon

Abstract
Experimental studies in the laboratory have established that elevated body temperatures similar to those experienced by basking frogs can clear individuals of chytridiomycosis, an amphibian skin disease caused by the fungus *Batrachochytrium dendrobatidis* (*Bd*). Field observations have also shown that infection status and prevalence may be correlated with microenvironment selection by frogs. We designed a laboratory environment that allowed frogs to select from a range of thermal and hydric microenvironments during disease studies, and investigated whether chytridiomycosis progresses at similar rates in frogs (*Litoria caerulea, L. genimaculata and L. wilcoxii)* given a choice of environmental conditions compared with frogs housed in constant, standard laboratory environmental conditions. We also investigated whether frog infected and not infected by *Bd* chose similar microenvironments (cold, warm or hot temperatures and low or high humidity), substrates (ground, pond or wall) and exhibited similar movement patterns (moved less or more than body length between observations or did not move). At least some individuals of each frog species selected microenvironments warm enough to clear *Bd* infections at some point during the experiment. Thermal microenvironment selection differed significantly amongst the three species studied and can be linked to some extent to the decline patterns observed in the wild. *Litoria caerulea* (vulnerable to infection with *Bd* but no population declines due to chytridiomycosis have been detected) selected warm and hot environments significantly more often compared to *L. genimaculata* (vulnerable to infection with *Bd* with highland population declines followed by recovery). Six adult *L. genimaculata* lost their infections within 18 days while housed in the laboratory at constant, standard climatic conditions, suggesting that other mechanisms, such as antimicrobial peptides, also influence the progress of chytridiomycosis.

Introduction
Anurans experience repeated changes in body temperature and water balance as they modulate their internal environment through behavioural changes and microhabitat selection to meet their physiological needs(Duellmann and Trueb, 1994; Hillyard, 1999; Beck and Jennings, 2003). Since anurans' internal environments fluctuate daily, investigating the host-disease interactions in a constant laboratory environment is likely to lead to a distorted picture (Woodhams, 2003). However, in most previous work, this important aspect of their biology has been largely ignored as the surrounding environment has been controlled and stabilised (Huchzermeyer et al., 1994; Cheatwood et al., 2003; Davidson et al., 2003)}. This may lead to inaccurate descriptions of the impact of a disease at an individual and population level.

One emerging infectious disease that has been intensively investigated in the last decade is chytridiomycosis, caused by the fungus *Batrachochytrium dendrobatidis* (*Bd*) (Berger et al., 1998; Woodhams and Alford, 2005; Voyles et al., 2007). Studies have confirmed the fatal outcome of chytridiomycosis in several Australian frog species, including *Litoria caerulea*, *L. chloris* and *Mixophyes fasciolatus,* under stable environmental

conditions (23°C and 100% relative humidity) (Berger et al., 1998; Woodhams et al., 2007) that are ideal for *Bd* growth and reproduction(Longcore et al., 1999). However, none of these species show population declines due to chytridiomycosis in the wild (McDonald and Alford, 1999).

The discrepancy between laboratory and field studies raised the question of what factors influence the host-pathogen interaction and possible alter the outcome of an infection with *Bd* (Retallick, 2004; Woodhams, 2003). Temperature (Woodhams et al., 2003; Berger et al., 2004) and humidity (Woodhams, 2003) have been shown to influence the progress of chytridiomycosis in anurans. Woodhams et al., (2003) observed that in experimentally infected *L. chloris* exposure to higher temperatures ($37^{o}$C) cured the frogs of the infection and that *Bd* is more pathogenic in high humidity (Woodhams, 2003).

In the field, greater mortality of Australian frogs infected with *Bd* during colder months (Bradley et al., 2002) and more stable populations of several susceptible species, such as *L. nannotis*, *L genimaculata* and *Nyctimystes dayi*, at lower altitudes where temperatures are higher compared to highland sites (McDonald and Alford, 1999), were observed. Relative humidity may be lower during the dry winter season compared to summer, but most likely above the threshold to reduce the progress of chytridiomycosis. The results of the laboratory studies and patterns observed in the wild suggest that climatic conditions can influence the progress of chytridiomycosis.

Since microenvironmental selection by frogs will alter the environment experienced by *Bd* in their epidermis, and it is clear that the rates of growth and reproduction of *Bd* are affected by temperature and might be affected by humidity, it is likely that frogs' microenvironment selection can affect the growth of *Bd* on individuals, or eliminate established infections. Rowley (2007) radio tracked and recorded body temperatures of wild *L. lesueuri, L. genimaculata* and *L. nannotis*, species which have experienced chytridiomycosis associated declines to different extents. Her study suggests that vulnerability of the three species may be correlated with their microenvironment selection.

Understanding the interactions of the pathogen *Bd* with wild frog populations will require a controlled and close study of the progress of chytridiomycosis in frogs exposed to a more naturalistic range of environmental conditions. This study was designed to evaluate whether infection by *Bd* alters a frog's behaviour, including microenvironment selection and movement pattern and whether this behaviour differs amongst three different anuran species that have experienced chytridiomycosis-associated declines to very different extents. All three species, *L. caerulea, L. wilcoxii* and *L. genimaculata* are from the Hylidae family. The green tree frog, *L. caerulea*, is a pond breeder and has been found positive for *Bd* in the wild but no populations have shown noticeable declines (Berger et al., 1998; Kriger and Hero, 2007a). The stony creek frog, *L. wilcoxii,* and the green-eyed tree frog, *L. genimaculata*, are rainforest stream breeders and prevalence of *Bd* is highly variable with season, location and elevation (Woodhams and Alford, 2005; Kriger and Hero, 2007b). *Litoria genimaculata* declined in the highland regions but has subsequently recovered (Richards and Alford, 2005), whereas no noticeable decline of *L. wilcoxii* has been detected throughout its range (McDonald et al., 2005). This study may assist in

explaining why some anuran species have not experienced population declines even though they are susceptible to infection with *Bd*.

## Materials and methods

### Experimental design

The experiment was designed to evaluate whether frogs of three different species that are infected with *Bd* select different microenvironments and show different movement pattern compared to uninfected frogs.

There were two different types of terraria; type I (constant environment) was designed to represent conditions normally encountered in laboratory studies where frogs have no hydric or thermal environmental choices. It consisted of a 250 x 350 x 150 mm plastic container with lid and airspace to allow air ventilation. The containers were misted every second day to maintain a constant high humidity environment and had an open plastic container (115 mm ø x 60 mm high) filled with rainwater as a pond. Type II (variable environment) allowed the frogs to select from a range of hydric and thermal environments (Figure 1). We used three 1500 x 700 x 500 mm glass terraria that were divided halfway across the longest dimension with PVC walls to create six identical 750 x 700 x 500 mm terraria for the larger sized frogs, *L. caerulea* (average snout-vent length (SVL) 61mm, mass 21g). Smaller sized frogs, *L. wilcoxii,* (average SVL 38mm, mass 5.6g) and *L. genimaculata* (average SVL 39mm, mass 3.2g) were housed in 12 600 x 200 x 300 mm terraria. All terraria were divided lengthwise with a 4.5 mm acrylic wall to create separate high and low humidity areas. Two holes (50 ø mm or 25 ø mm for the smaller terraria) were drilled between these two sections to allow the frogs to freely move between them. The high humidity environment had an acrylic lid to trap moist air and maintain high relative humidity, whereas the less humid environment had a mesh lid, which allowed air exchange with the air-conditioned room, resulting in a dryer environment. A spotlight (80 watt) was placed at one end of the terraria, centered over the wall between the high and low humidity areas, to create a thermal gradient. The larger terraria II had two halves of a PVC pipe filled with rainwater as ponds (one in the lower humidity and one in the high humidity compartment). The smaller terraria II had an open plastic container (115 mm ø x 60 mm high) in the centre of each less humid and high humidity environment compartment. The design of the varied hydric and thermal environment allowed microenvironment selection in relation to a) temperature, b) humidity and c) the combination of both to be investigated. I-button data loggers recorded the temperature and relative humidity hourly in both constant and variable environment terraria. Three digital cameras, covering all the terraria II, were programmed to automatically take pictures at half hour intervals to monitor the position of frogs in the terraria.

A total of six trials were conducted (Table 3). Four trials used the larger terraria for 12 *L. caerulea* per experiment (six in the constant and six in the variable environment) resulting in 48 frogs in total. Using the smaller terraria, one trial with 24 frogs for both, *L. wilcoxii* and *L. genimaculata*, was conducted (12 frogs of each species in the constant and 12 frogs of each species in the variable environment). Each trial lasted for minimum of eight days, except those for *L. genimaculata* which, due to an air-conditioning failure, was terminated at day 5.

All experimental trials were conducted at the freshwater compound, James Cook University, in one air-conditioned room. Room temperature fluctuated between 18-21°C at night and 21-24°C during the day. The light was automatically switched on at 7.30 am and off at 7.30 pm.

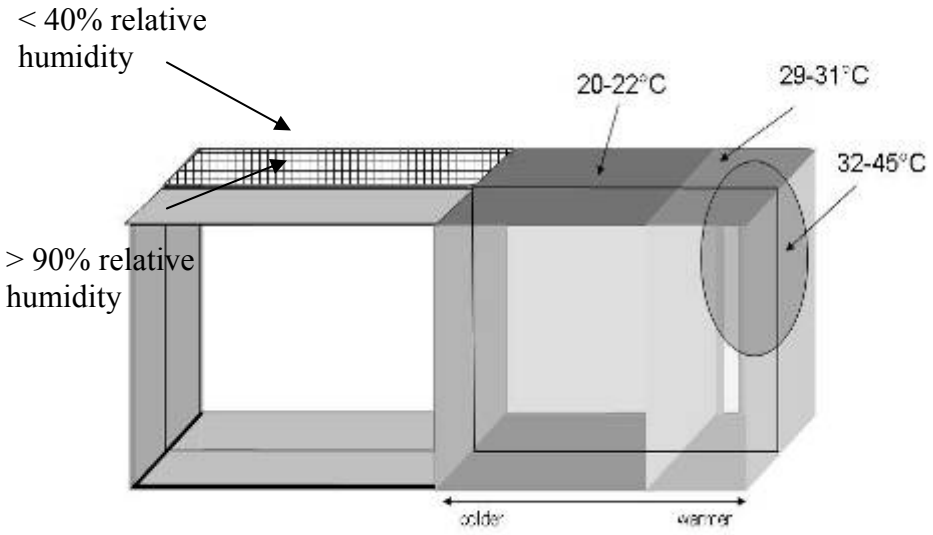


**Figure 1**. The design of variable microenvironment terraria where frogs could choose between two hydric (low and high relative humidity) and three thermal (cold, warm and hot) microenvironments.

**Table 3.** Six trials in total with three different anuran species were conducted. Each trial lasted for minimum of eight days, except those for *L. genimaculata* which, due to an air-conditioning failure, was terminated at day 5.

| Trial | Terraria II (variable environment) | Frog species | Sample size |
|---|---|---|---|
| 1 | Large | *L. caerulea* | 6 |
| 2 | Large | *L. caerulea* | 6 |
| 3 | Large | *L. caerulea* | 6 |
| 4 | Large | *L. caerulea* | 6 |
| 5 | Small | *L. wilcoxii* | 12 |
| 6 | Small | *L. genimaculata* | 12 |

Study animals

*Litoria caerulea* was the only species available as captive bred individuals at the time of the study. Subadults were obtained from captive populations maintained by Brendan Tiernan (Morphett Vale, South Australia) and Gerry Marantelli (Amphibian Research Centre (ARC), North Coburg, Victoria). In order to minimise impact on wild frog populations, I collected most *L. wilcoxii* and *L. genimaculata* from the wild in their aquatic life history stages and raised them to subadults. *Litoria wilcoxii* were raised from tadpoles collected at Crystal Creek, Paluma Range National Park, Queensland, Australia (S18°58′54″ E146°12″01″). *Litoria genimaculata* were raised from eggs or collected as adult male frogs from Birthday Creek, Paluma Range National Park (S18°58′54″ E146°10′02″). All frogs were swabbed (sterile tubed dry swab from Medical Wire and Equipment, Corsham, Wiltshire UK) before and after the experiment for diagnostic quantitative PCR analysis (Annis et al., 2004) to determine their infection status for *Bd*. In order to eliminate the possibility of unintentional infection with *Bd*, all terraria and water containers were cleaned and waste water diluted with F10 Veterinary Disinfectant (Webb et al., 2007). Gloves were worn at all times and changed between each frog and terrarium handled. Frogs received crickets (*Acheta domesticus)* twice a week; once a week 0.5 mL of a liquid supplement mixture (2mL Calcivet/100mL rainwater) was applied dorsally to each frog. Calcivet (Vetafarm) contains 33g/L calcium borogluconate, 2g/L magnesium sulphate and 25,000 i.u./L vitamine $D_3$ and appears to be more effective than coating crickets with a phosphorus free calcium and vitamin D3 supplement powder. Frogs were provided with rainwater (collected and stored in a water tank) that was changed at least once a week.

Culture of *Bd* and experimental infection of frogs

Isolates of *Bd* (*L. les donna* 06-LB-1) were cultured in the School of Veterinary and Biomedical Science, James Cook University, following the protocol of Berger et al*.,* (2005b) but using only half of the nutrients. Two different methods were used to infect the frogs with *Bd*. *Litoria caerulea* were exposed to *Bd* zoospores in a bath following the procedure of Berger et al., (2005b). Zoospores were harvested by flooding agar plates containing *Bd* cultures with 2 mL of dilute salt solution (DS) for 10 minutes (Boyle et al., 2003). The concentration of zoospores was determined by pre-sampling and counting motile zoospores with a haemocytometer in triplicate. A total concentration of 27,000 zoospores per mL was used for each *L. caerulea*. For sham infection, agar plates with no *Bd* cultures were flooded using the same amount of DS.

Unfortunately, when we tried the same procedure with *L. genimaculata* all individuals, including the control animals, died within the first four hours of the 24 hour *Bd* exposure period. Due to time constraints, we then collected 24 adult male *L. genimaculata* from the wild at the same location where we collected the eggs, so this study could be continued immediately. Birthday Creek at Paluma National Park has been monitored for several years (Richards and Alford, 2005; Woodhams and Alford, 2005) and removing 24 adult frogs would have a minimal impact on the population. Infection status was determined by swabbing and subsequent qPCr assay. However, to avoid further mortality, we exposed the uninfected *L. genimaculata* and all *L.wilcoxii* by dorsal application of *Bd* using a sterile cotton swab that had been rolled three times across agar plates with *Bd* cultures. The control group were swiped with a sterile cotton swab that was rolled on sterile agar plates. No frog died using this exposure procedure; however the disadvantage was the unknown concentration of *Bd* zoospores to which the frogs were exposed. It has been observed that frogs develop clinical signs of

chytridiomycosis at varying intensities of infection with *Bd* (Voyles et al., 2007) and for the purpose of this study it was sufficient to compare frogs positive for *Bd* (but at an unknown intensity of infection) with uninfected frogs.

Housing after experimental infection

All frogs were individually housed in constant environments (terraria I) for 10 days after their experimental exposure to *Bd*. I-button readings were used to ensure stable and constant hydric and thermal environments. Each frog was swabbed, using a sterile tubed dry swab (Medical Wire and Equipment, Corsham, Wiltshire UK) that was run across frog's hands, feet, thighs and ventral surface twice, before being released into the variable environment terrarium to measure its infection status before the start of each trial. Quantitative PCR tests for the first two runs of the experiment were carried out at the Australian Animal Health Laboratory (AAHL), Geelong, Victoria. For the remaining runs, qPCRs were carried out at the School of Veterinary and Biomedical Science at James Cook University, Townsville, Australia.

Statistical analysis

The temperature and humidity conditions in each type of terrarium were initially measured with frogs absent, by placing multiple i-button data loggers in each terrarium. In both the humid and less humid halves of the variable environment terraria, zones were determined that corresponded to three ranges of temperatures: cold, warm, and hot (during daylight hours when the heat lamps were on; temperature ranges for each zone are presented in results). For each trial of the experiment, the position of each frog within each terrarium was determined in each half-hourly photograph and recorded. I divided the data into two main groups, 1) daytime data where a thermal and hydric gradient was present and 2) night data where no thermal but the hydric gradient was present.

Frogs in the variable-environment terraria were classified by which humidity environment they were located in (high or low), which temperature zone within that humidity environment (cold, warm or hot, during the day only), what substrate it occurred on (ground, wall or pond) and how much it had moved since the previous photograph (nil movement, movement less than a body length or movement greater than a body length). These data allowed us to investigate frog microenvironment selection along several possible environmental axes alone and in combination. Combinations were included as they may not be independent. The axes and combinations we investigated were humidity alone, temperature alone, substrate alone, movement alone, temperature and humidity combined, humidity and substrate combined, humidity and movement combined. The proportion of images in which each frog was found in each combination was calculated from the digital images.

I used Blossom statistical software (version 2007 12.21) for analysis and conducted a multi-response permutation procedure (MRPP) on the proportional data for each species separately using infection status as the grouping variable. I further investigated, using the same technique, differences in frog behaviour (using all different categories, described previously) among species and between night and day.

Results

The microenvironment within terraria

Eight days of hourly i-button readings of a small and large variable terrarium designs showed similar microenvironments for frogs to select from. Relative humidity in the high humidity environment ranged from 70 to 90%, with an average of 84% for the small variable terraria and 92% for the large variable terraria. The relative humidity in the lower humidity environment was usually below 70%, with an average of 46% in the small, and 62% in the large, variable terraria (Figure 2). The relative humidity in the constant environment terraria ranged from 80-97% with an average of 91%.

The hot environment temperature fluctuated during the day from 28°C - 40°C in the small and 29.5°C -39.5°C in the large variable terraria (Figure 3). The warm environment temperature fluctuated from 23.5°C -29.7°C in the small and 25°C -30.5°C in the large variable environment terraria. The cold environment temperature fluctuated from 19.5°C -26.5°C in the small, 19°C -24.5°C in the large variable environment terraria and 19°C to 23.5 in the constant environment terraria.

During trial 6 with *L. geimaculata*, which was terminated early because of a failure of the air-conditioner in the laboratory, temperature in the terraria reached 36.5°C.

qPCR assay to determine infection status

Experimental infection with *Bd* was not always successful (Table 4). It is unclear whether frogs did not become infected when exposed to *Bd* zoospores, or rapidly cleared infections prior to swabbing for qPCR assay. Of the 24 adult *L. genimaculata* we collected in the wild, 13 were positive for *Bd* at the time of collection, but six tested negative 18 days later. During that period, frogs were housed in small containers where humidity was high and constant. Temperature in the laboratory was not recorded during that period, however there were no equipment failures, and temperatures in the same room at other times ranged from 21$^{o}$C to 26$^{o}$C, with an average of 23$^{o}$C.

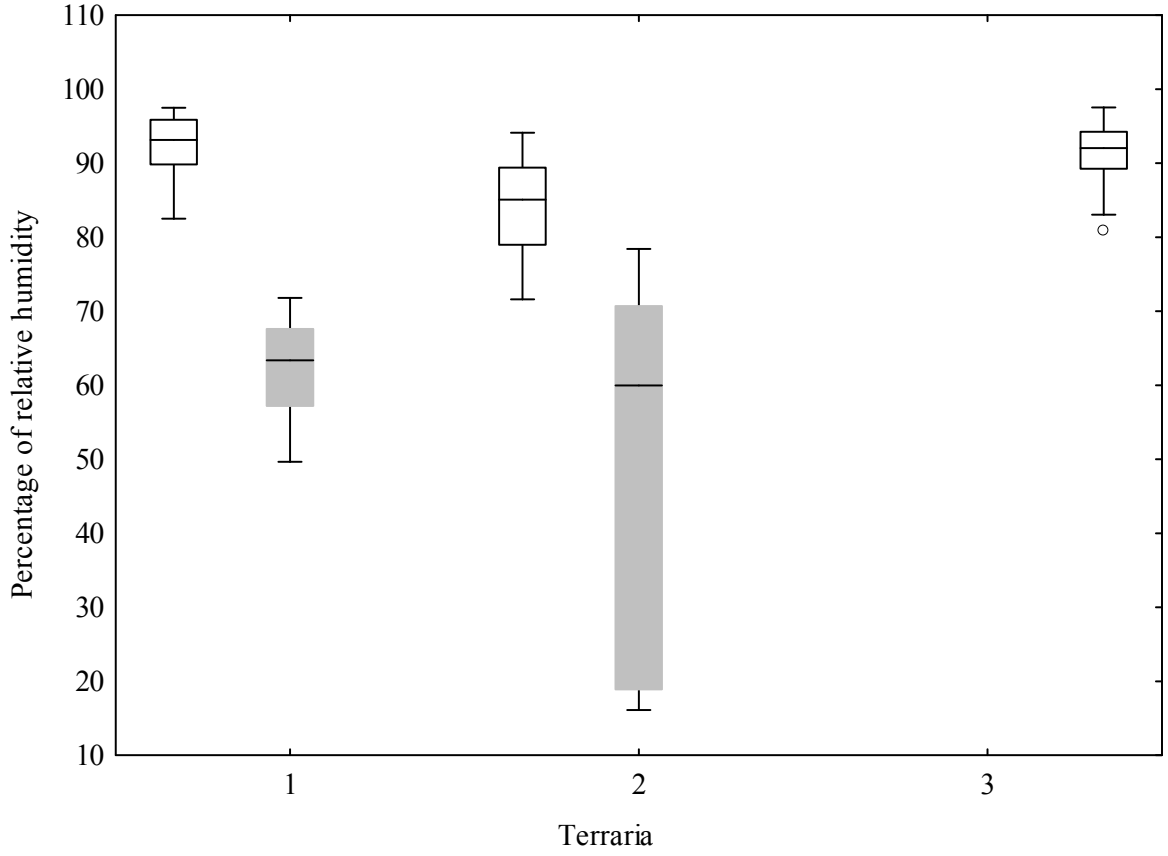


Figure 2 Tukey (1977) boxplot (vertical bars indicate ranges, horizontal bar indicates median, boxes indicate upper and lower quartile boundaries, o= outliers) of relative humidity in the terraria. 1= larger variable microenvironment terraria II, 2= smaller variable microenvironment terraria II, 3= constant microenvironment terraria I. The variable microenvironment terraria included two different relative humidities, high relative humidity and low relative humidity. The constant microenvironment included only high humidity.

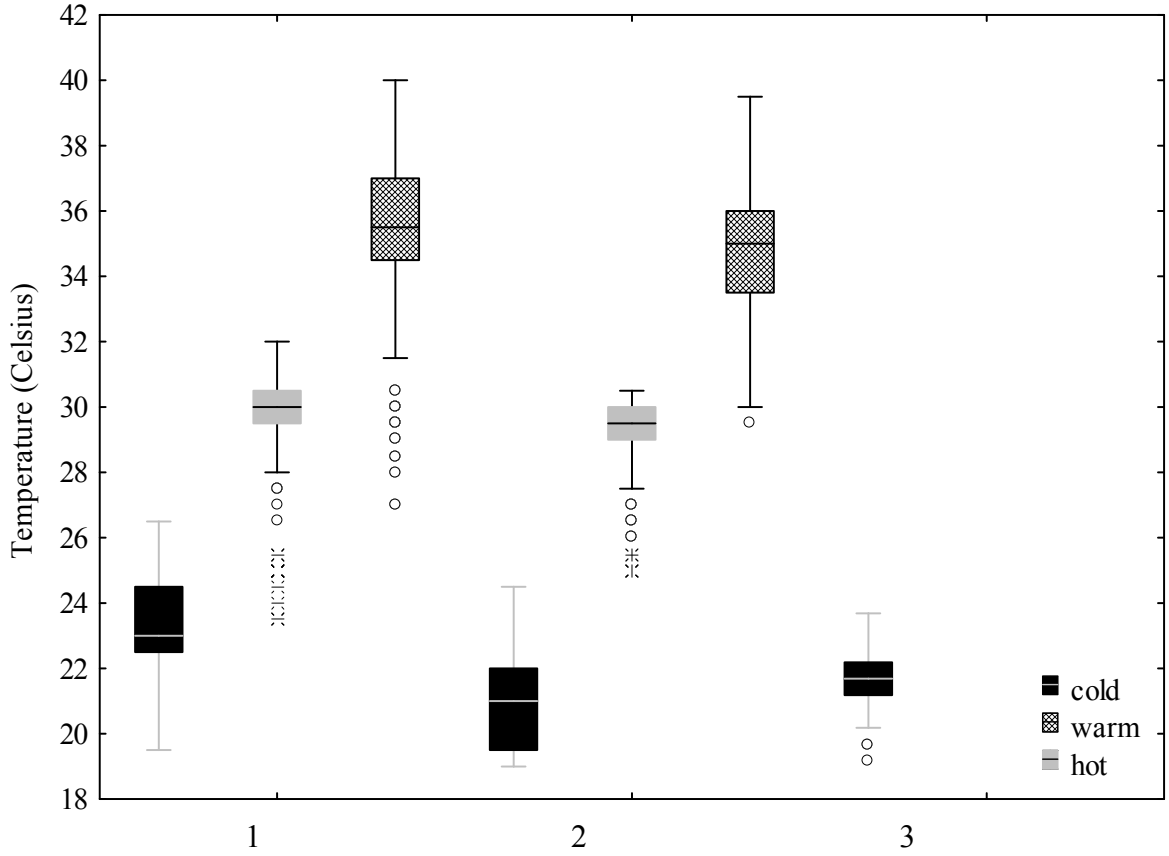


Figure 3 Tukey (1977) boxplot (vertical bars indicate ranges, horizontal bar indicates median, boxes indicate upper and lower quartile boundaries) of temperature in the three thermal microenvironments (cold, warm and hot) in the three different terraria designs. 1= larger variable microenvironment terraria II, 2= smaller variable microenvironment terraria II, 3= constant microenvironment terraria I.

**Table 4.** Infection status, determined by qPCR, of all *L. caerulea*, *L. wilcoxii* and *L. genimaculata* individuals during the microenvironment selection behaviour trials.

| Species | Total *Bd* negative- | Total *Bd* positive |
|---|---|---|
| *L. caerulea* | 15 | 8 |
| *L. wilcoxii* | 8 | 4 |
| *L. genimaculata* | 8 | 4 |

Microenvironment selection of frogs during the day

A total of 15,522 day images were analysed. Within each species, thermal and hydric environment selection, substrate selection and movement pattern did not differ significantly between infected and uninfected individuals. *Litoria caerulea* was the only species that did not choose the thermal microenvironment at random (MRBP, $\delta = 0.29$, $p < 0.01$; $\delta = 0.37$, $p = 0.25$ for *L. wilcoxii*; $\delta = 0.29$, $p = 0.14$ for *L. genimaculata*). All three species, *L. caerulea*, *L. wilcoxii* and *L. genimaculata*, selected substrates in the less humid or high humid environment in a non random pattern (MRBP, $\delta = 0.35$, $p < 0.01$; $\delta = 0.32$, $p < 0.01$; $\delta = 0.35$, $p = 0.01$, respectively; Figure 4).

The three species differed significantly in patterns of use of thermal microenvironments (MRPP, $\delta = 0.43$, $p < 0.01$). *Litoria caerulea* chose the warm and hot environments more often than *L. wilcoxi* and *L. genimaculata* (Figure 5). *Litoria wilcoxii* spent the least time in the warm and hot environments. Hydric environment selection did not significantly differ among the three species (MRPP, $\delta = 0.61$, $p = 0.20$), however, in combination with thermal microenvironments, *L. wilcoxii* spent more time in the high relative humidity environment at cold temperatures than *L. genimaculata* and *L. caerulea* (MRPP, $\delta = 0.67$, $p < 0.01$; Figure 6).

Substrate selection differed significantly among the three frog species (MRPP, $\delta = 0.14$, $p < 0.01$). *Litoria wilcoxii* was found mostly on the ground, whereas *L. caerulea* and *L. genimaculata* were mostly observed on the wall (Figure 7). The overall tendency to move differed significantly among species (MRPP, $\delta = 0.25$, $p < 0.01$): *L. genimaculata* moved (more than its body length) most frequently, and *L. caerulea* remained in the same location across the highest proportion of images (Figure 8).

Microenvironment selection of frogs during the night

A total of 13,511 night images were analysed. Infected and uninfected *L. caerulea* did not differ significantly in hydric environment selection (MRPP, $\delta = 0.55$, $p = 0.09$), substrate selection (MRPP, $\delta = 0.48$, $p = 0.37$) and movement pattern (MRPP, $\delta = 0.34$, $p = 0.26$). Infected *L. wilcoxii* moved less than uninfected individuals (MRPP, $\delta = 0.23$, $p < 0.01$) (Figure 9) but their environment or substrate selection did not differ significantly. Infected *L. genimaculata* preferred different substrates at night compared to uninfected individuals (MRPP, $\delta = 0.26$, $p < 0.01$) (Figure 10).

Substrate selection differed significantly among uninfected *L. caerulea*, *L. wilcoxii* and *L. genimaculata* (MRPP, $\delta = 0.38$, $p < 0.01$). *Litoria wilcoxii* spent most of its time at night on the ground (Figure 11). *L. caerulea* spent more time in the pond than did the other two species. A similar pattern was observed in the data for infected *L. caerulea*, *L. wilcoxii* and *L. genimaculata* (Figure 12); this pattern was also significant (MRPP, $\delta = 0.45$, $p < 0.01$).

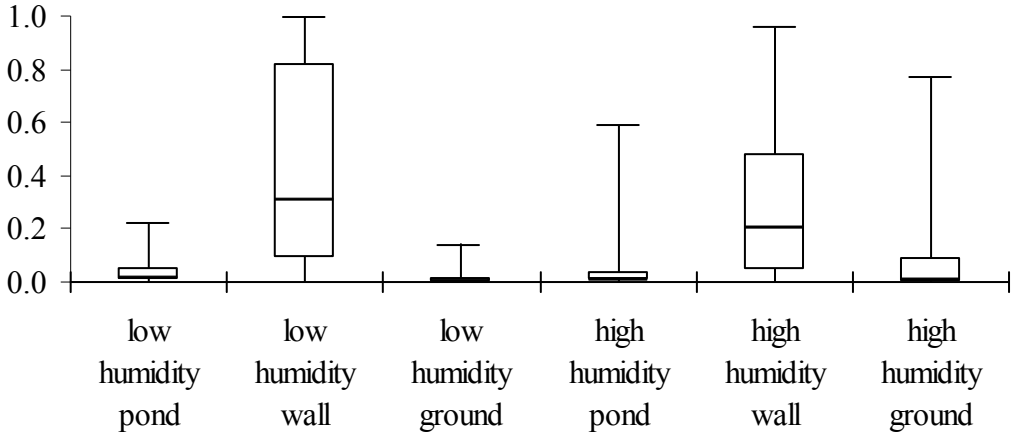


Figure 4 Tukey (1977) boxplot (vertical bars indicate ranges, horizontal bar indicates median, boxes indicate upper and lower quartile boundaries) of hydric environment and substrate selection of individual *L. caerulea*. The y-axis is the proportion of digital images of each frog in the different microenvironment categories.

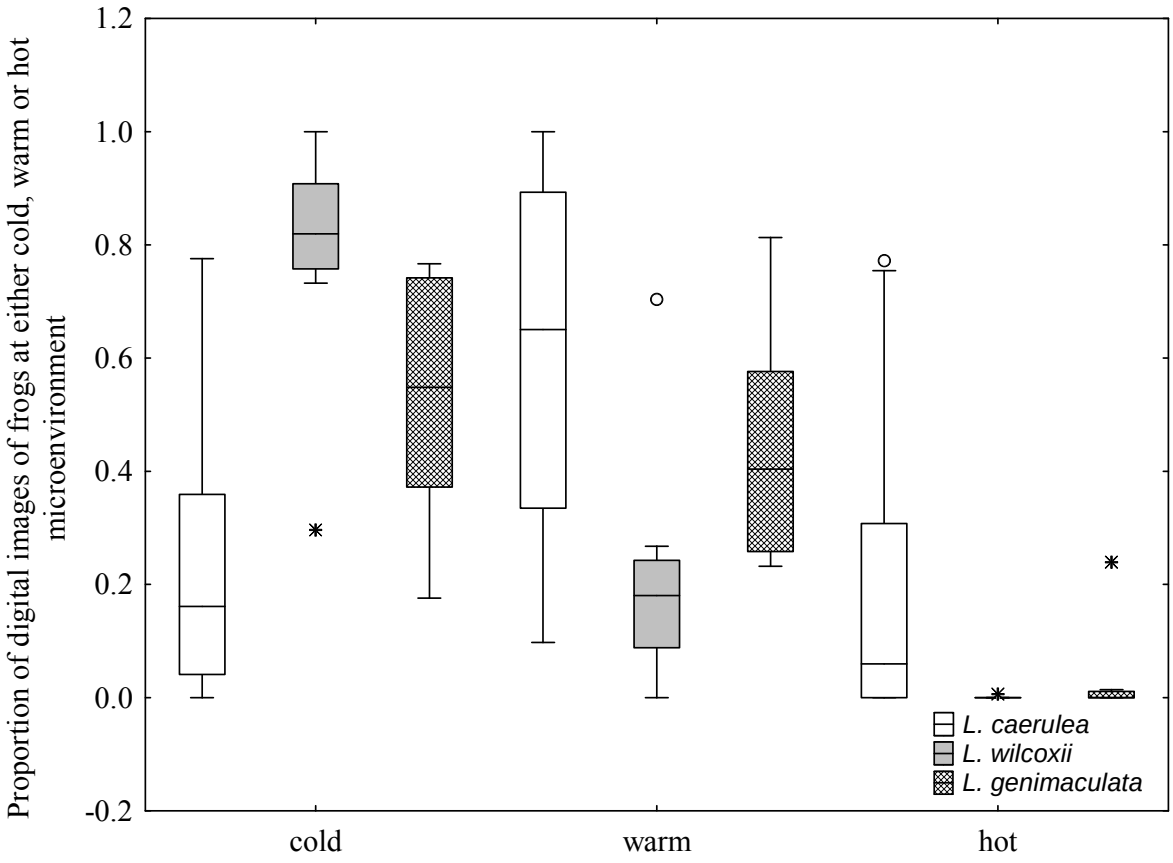


Figure 5 Tukey (1977) boxplot (vertical bars indicate ranges, horizontal bar indicates median, boxes indicate upper and lower quartile boundaries) of the proportion of time that individual *L. caerulea*, *L. wilcoxii* and *L. genimaculata* spent in each thermal environment (cold, warm and hot) during the day.

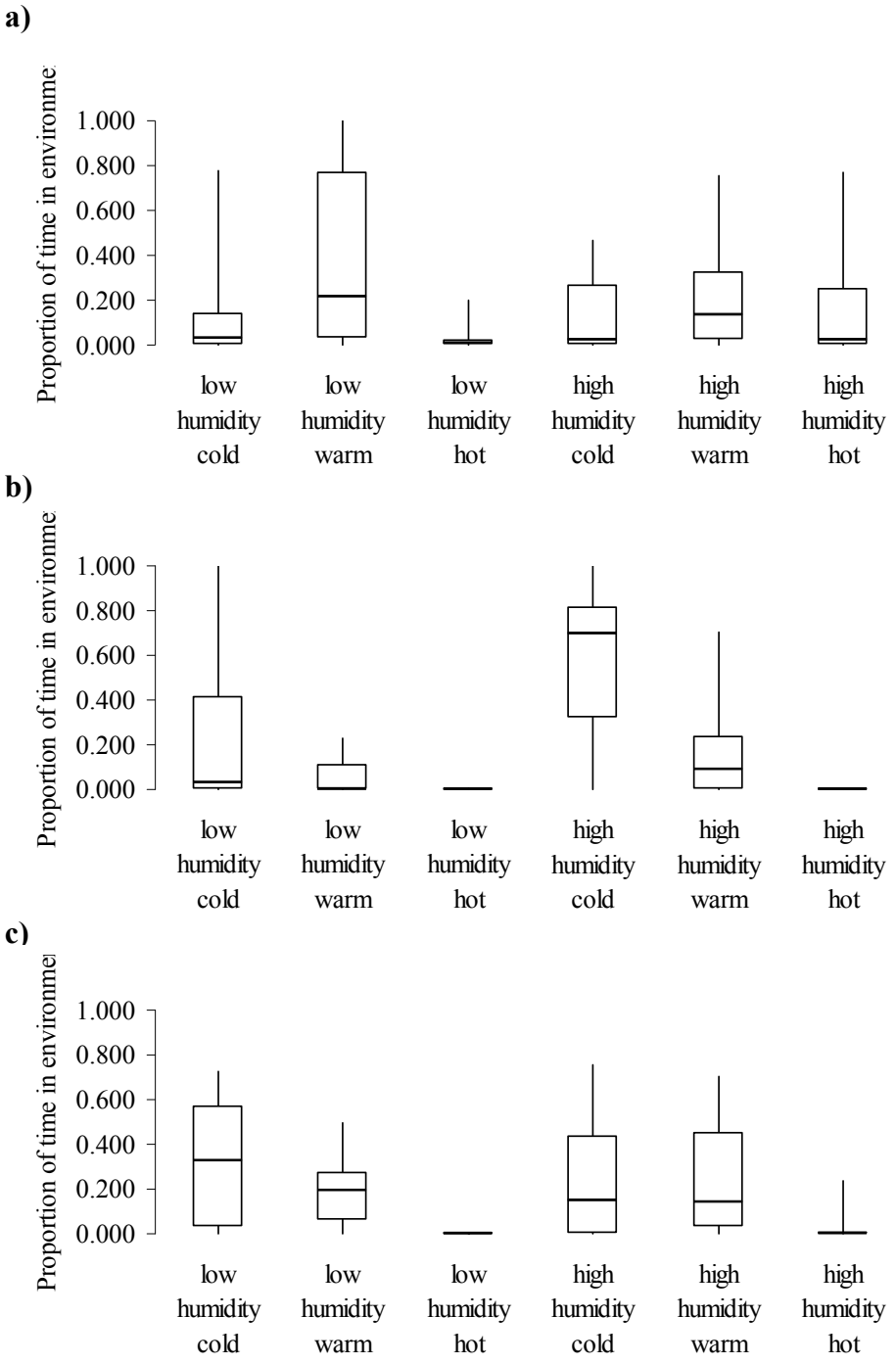


Figure 6 Tukey (1977) boxplot (vertical bars indicate ranges, horizontal bar indicates median, boxes indicate upper and lower quartile boundaries) of hermal and hydric microenvironment selection of individual a) *L. caerulea*, b) *L. wilcoxii* and *L. genimaculata.*

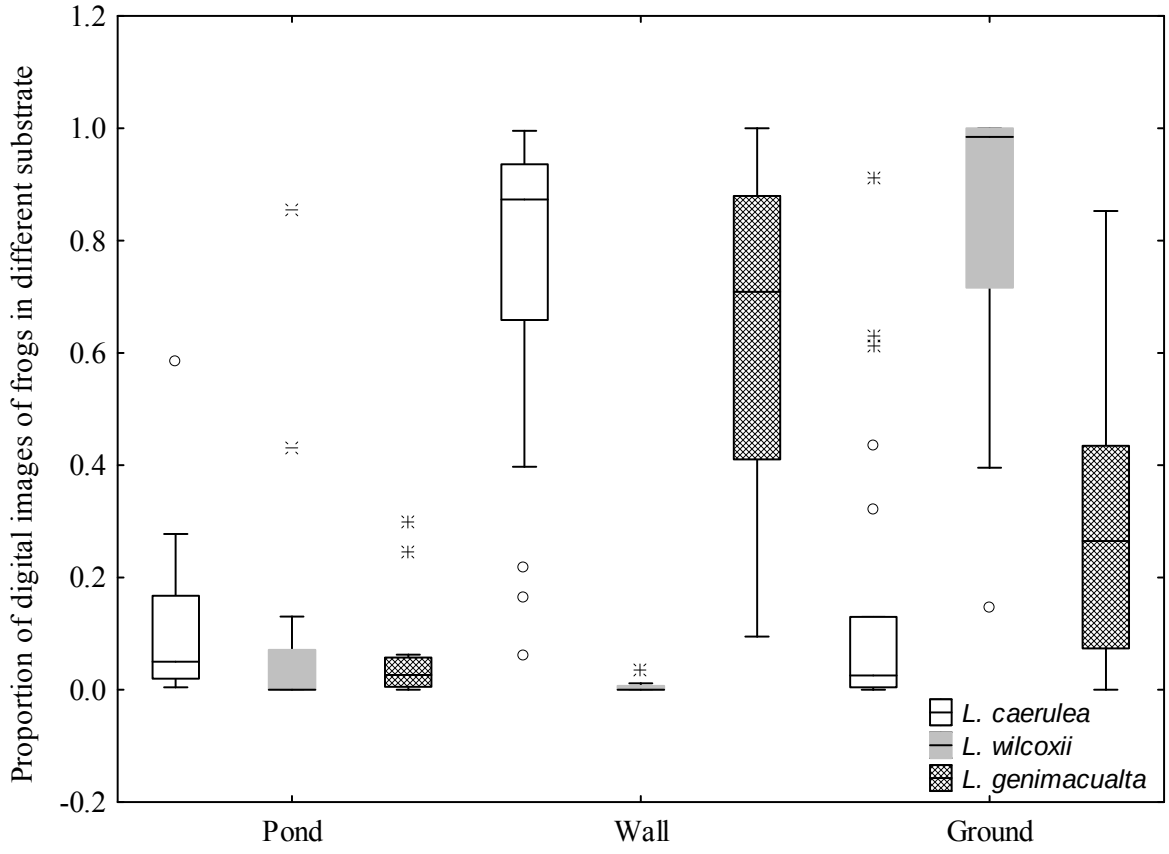


Figure 7 Tukey (1977) boxplot (vertical bars indicate ranges, horizontal bar indicates median, boxes indicate upper and lower quartile boundaries) of the proportion of time that *L. caerulea*, *L. wilcoxii* and *L. genimaculata* spent in each substrate (in the pond, on the wall or on the ground) during the day.

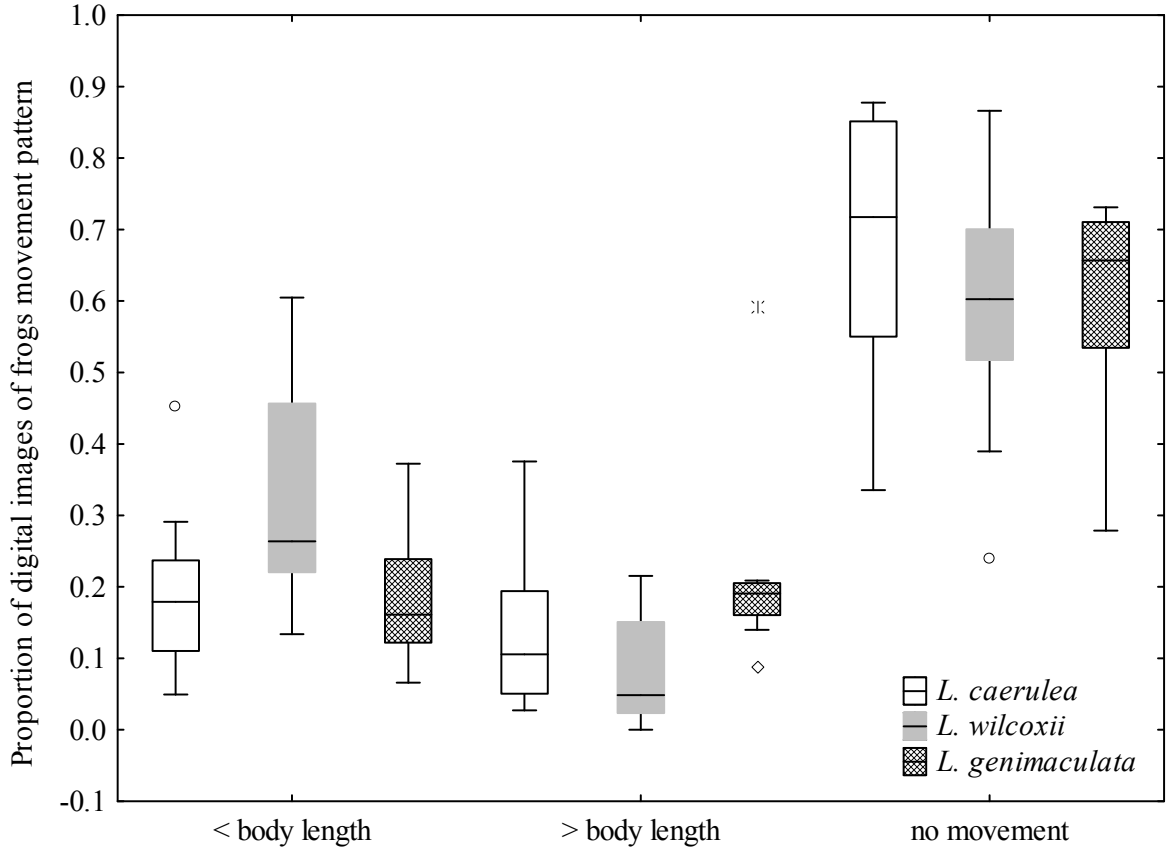


Figure 8 Tukey (1977) boxplot (vertical bars indicate ranges, horizontal bar indicates median, boxes indicate upper and lower quartile boundaries) of the proportion of *L. caerulea*, *L. wilcoxii* and *L. genimaculata* that moved < 1 body length, > 1 body length, or no movement within the terraria during the day.

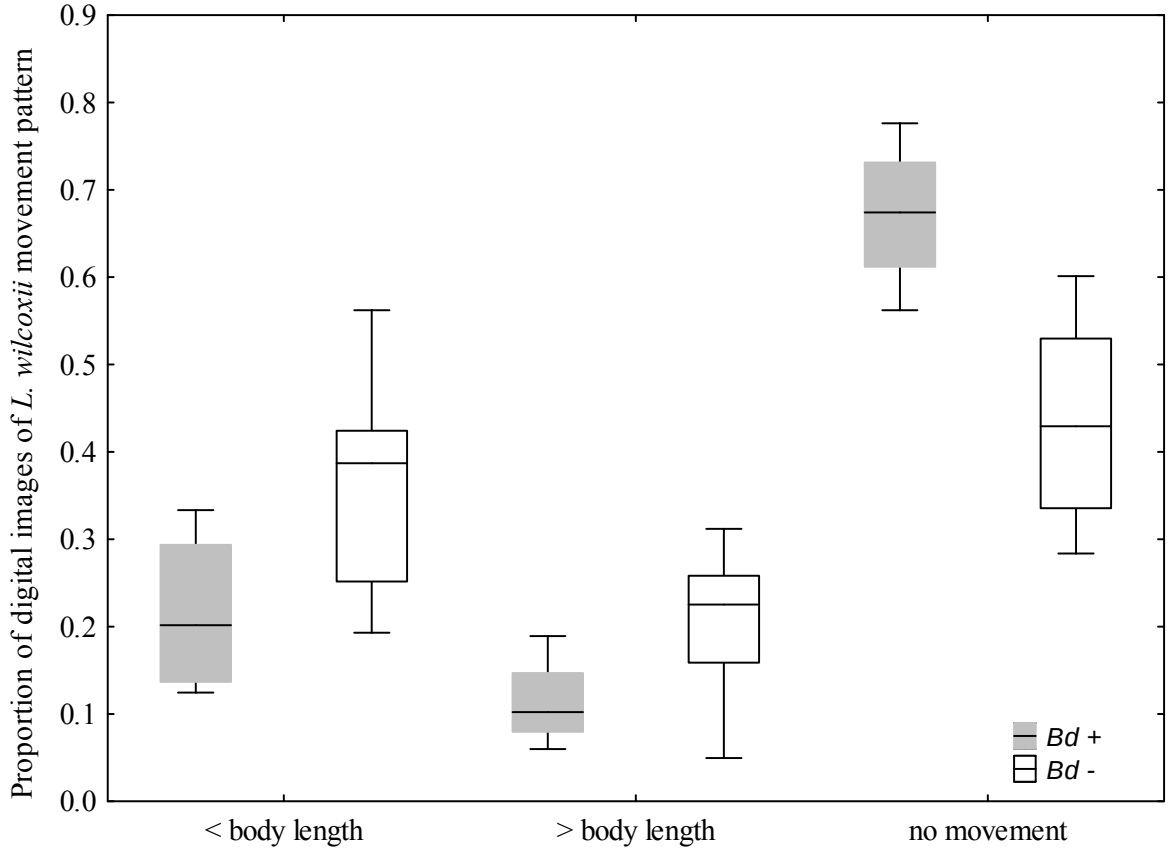


Figure 9 Tukey (1977) boxplot (vertical bars indicate ranges, horizontal bar indicates median, boxes indicate upper and lower quartile boundaries) of the proportion of time that infected (*Bd* +) and uninfected (*Bd* -) *L. wilcoxii* movement pattern (< body length, > body length or no movement) during the night.

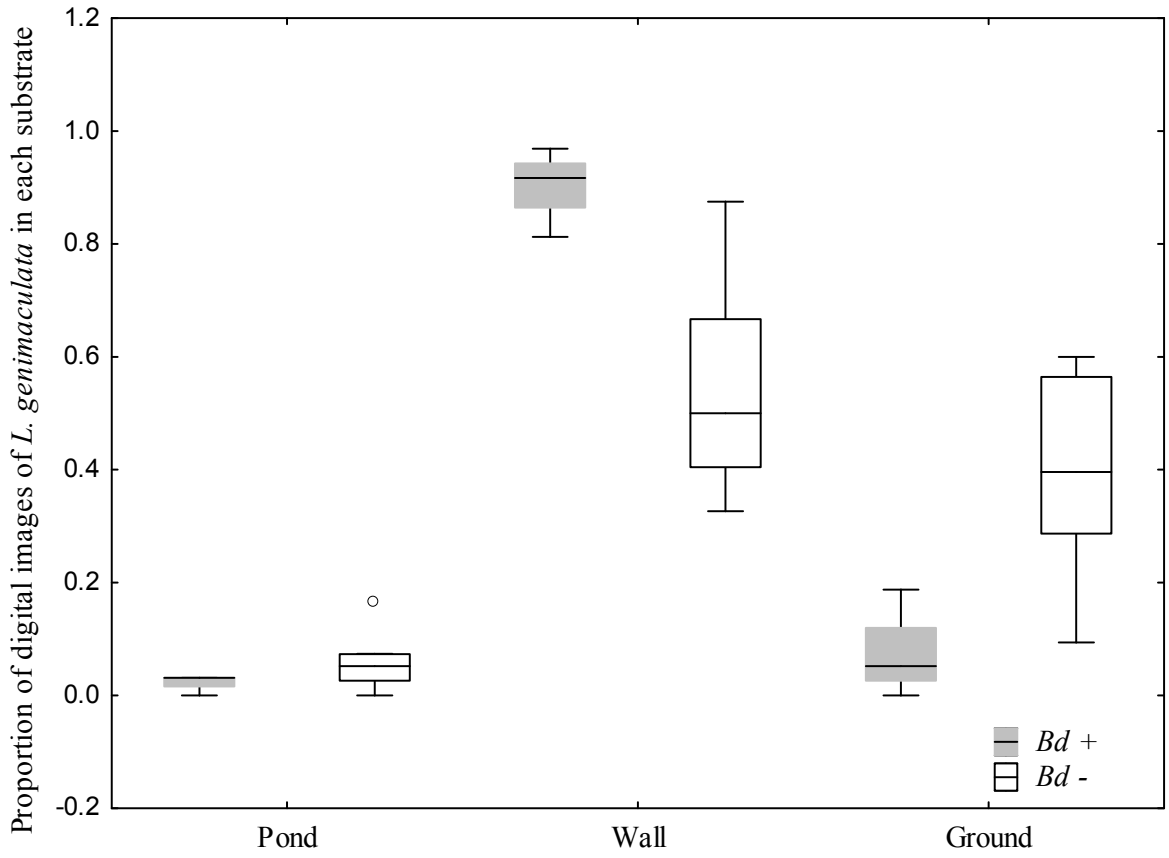


Figure 10 Tukey (1977) boxplot (vertical bars indicate ranges, horizontal bar indicates median, boxes indicate upper and lower quartile boundaries) of the proportion of time that infected (*Bd* +) and uninfected (*Bd* -) *L. genimaculata* selected each substrate (pond, wall or ground) during the night.

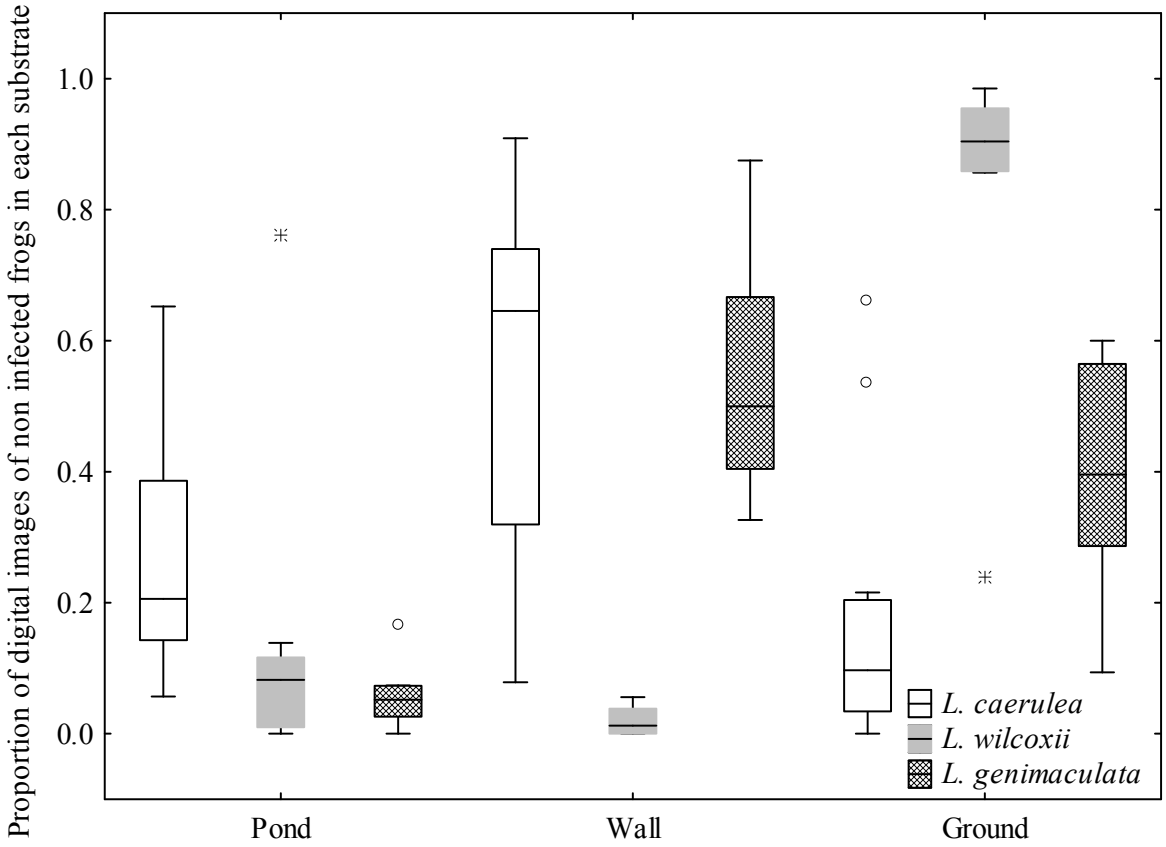


Figure 11 Tukey (1977) boxplot (vertical bars indicate ranges, horizontal bar indicates median, boxes indicate upper and lower quartile boundaries) of the proportion of time that uninfected *L. caerulea*, *L. wilcoxii* and *L. genimaculata* spent in each substrate (pond, wall or ground) during the night.

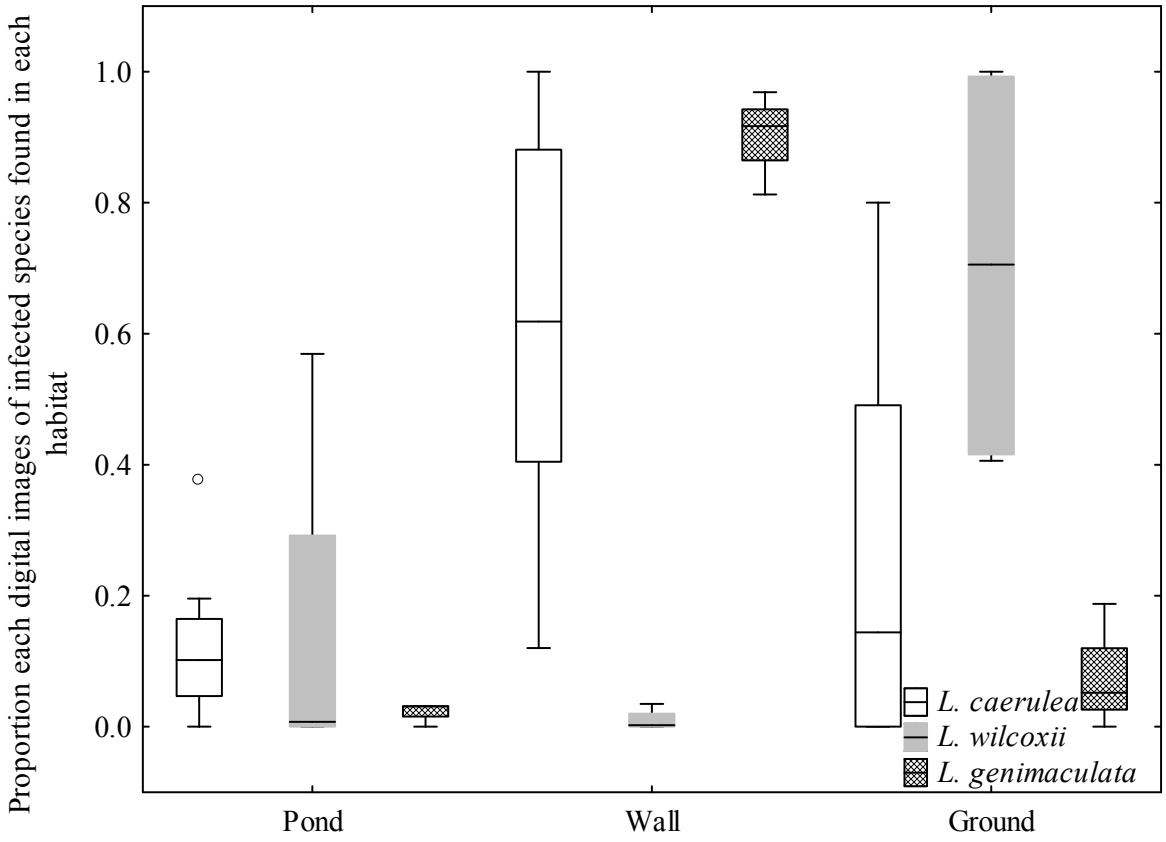


Figure 12 Tukey (1977) boxplot (vertical bars indicate ranges, horizontal bar indicates median, boxes indicate upper and lower quartile boundaries) of the proportion of infected *L. caerulea*, *L. wilcoxii* and *L. genimaculata* spent in each substrate (pond, wall or ground) during the night.

Discussion

Six *L. genimaculata* that tested positive for *Bd* at the time of capture tested negative in the laboratory 18 days later. Kriger and Herman (2006) observed recovery from *Bd* infection in wild *L. wilcoxii*. The mechanism that enables a frog to clear itself of *Bd* infection is unclear. The innate immune defences of frogs, predominantly antimicrobial peptides (AMPs), can kill *Bd* zoospores *in vitro* (Rollins-Smith et al., 2002b; Woodhams et al., 2007) this may result in a reduced rate of re-infection which may reduce the progress of chytridiomycosis sufficiently to prevent mortality. Increased body temperature, which can be achieved through warmer microenvironment selection, can also help frogs to clear infection with *Bd* (Woodhams et al., 2003). The *L. genimaculata* in this study were housed in stable climatic conditions (high humidity and an average temperature of 23ºC) that was suitable for *Bd* growth, suggesting other mechanisms than microenvironment selection, such as antimicrobial peptides, may have helped them clearing the infection. However, we have to consider that qPCR uses rDNA, which does not decompose as quickly as RNA (Matsuo et al., 1999), to determine the infection status of a frog. It is possible that the number of zoospore equivalents detected at capture of the frogs may not necessarily indicate positive infection as *Bd* zoospores do not have to be viable to cause a positive DNA reading. This aspect has been largely ignored in recent discussions on qPCR as a technique to determine the infection status of frogs with *Bd* (Kriger et al., 2007a; Smith, 2007) but certainly needs to be taken into consideration in studies where infection status is a factor in the design.

Thermal environment selection differed significantly amongst the three species and can be linked to some extent to the decline pattern observed in the wild. *Litoria caerulea*, which has not experienced any population declines due to chytridiomycosis, spent more time in the hot and warm environments compared to *L. wilcoxii* and *L. genimaculata*. *Litoria genimaculata*, which has experienced chytridiomycosis associated declines, spent little time in the hot thermal environment. However, *L. wilcoxii* most often selected the cold environment and less so the warm environment than *L. genimaculata*, even though this species has not declined due to chytridiomycosis. A radio-tracking study by Rowley (2007) found that *L. lesueuri* (which could either be *L. wilcoxii* or *L. jungguy* which are morphologically indistinguishable (Donnellan and Mahony, 2004) chose warmer temperatures than *L. genimaculata*. The average summer body temperature of *L. lesueuri* was around 25ºC (Rowley, 2007), lower than both the warm and hot thermal environments we provided. It appears, however that *L. lesueuri* and *L. wilcoxii* can select microenvironments that are suitable for *Bd* growth, and studies have shown high *Bd* prevalence in the wild (Retallick et al., 2004; Kriger and Hero, 2007b). Because this species has not shown population declines attributable to chytridiomycosis, this suggests that it has other mechanisms to survive *Bd* infection, possibly more effective antimicrobial peptides as shown by Woodhams et al., (2005), avoidance behaviour or low risk of transmission due to habitat selection that reduces the time being exposed to *Bd* (Rowley and Alford, 2007b; Rowley et al., 2007).

*Litoria wilcoxii* spent long periods of time on the ground (floor of the terraria), whereas *L. caerulea* and *L. genimaculata* spent most time on the walls of the terraria. This is similar to the results of (Rowley and Alford, 2007b), who found that *L. "lesueuri"* spent substantial amounts of time on the ground and on leaf litter, while *L. genimaculata* was more arboreal. Being above ground in a less sheltered habitat increases the frog's opportunity to increase its body temperature by basking. It also, assuming *Bd* does not

exist outside water bodies, reduces time of exposure to *Bd*. At this time there is no published evidence of the existence of viable or saprobic *Bd* outside of the stream environment, such as in the soil or forest leaf litter.

Although we did not detect a significant difference in microenvironment selection between infected and uninfected frogs during the day, individuals of all three species, *L. caerulea*, *L. wilcoxii* and *L. genimaculata*, chose thermal environments that were above 32ºC at some time during the experiment. Berger (2001) observed *Bd* zoospore mortality *in vitro* at 32 ºC. Laboratory experiments have shown a reduction of infection with *Bd* at 27 ºC in *M. fasciolatus* and elimination of *Bd* infection in *L. chloris* at 37ºC (Woodhams et al., 2003).

Most frog species, including *L. caerulea* (personal observation), *L. wilcoxii* and *L. genimaculata*, are predominantly nocturnal (Rowley and Alford, 2007b). At night, uninfected *L. wilcoxii* moved significantly more often than individuals that tested positive for *Bd*. Infected *L. genimaculata* spent significantly less time on the ground than uninfected individuals. This is the first study showing microenvironment selection differences in individuals with differing infection status. These differences may not reflect adaptive responses to infection; they could both result from generally lower tendencies of infected frogs to move substantial distances, for example. It is unclear whether these changes in behaviour would affect the course of *Bd* infection in either captive or wild frogs. Radio-tracking of frogs of these species did not demonstrate such differences in behaviour in nature (Rowley, 2007). Further studies comparing nocturnal behaviour in species that have and have not suffered declines due to chytridiomycosis, are needed to understand the possible role of microenvironment selection in the variable susceptibility to *Bd* in Australian anurans.

***Project 1.2. Do frogs avoid water that contains Batrachochytrium dendrobatidis zoospores?***

Nicole Kenyon, Ross A. Alford and Sara Bell

**Abstract**
Chytridiomycosis, caused by the fungus *Batrachochytrium dendrobatidis* (*Bd*), has been implicated as a proximate cause of many amphibian declines around the globe. However, its effects vary widely among species; some species can persist despite infection while others are driven to local extinction. Species specific variation may be partly caused by factors such as immune function and microenvironment. Less is known about factors determining the vulnerability of species to initial infection. Because infection is caused by contact of waterborne zoospores with the skin, one possible source of variation in infection risk is an ability to detect and avoid water containing *Bd* zoospores. We examined whether frogs of three species, *Litoria caerulea, L. genimaculata* and *L. wilcoxii,* which have suffered to different degrees from chytridiomycosis, avoid water contaminated with *Bd* zoospores. Individual frogs were presented with a choice of *Bd*-contaminated and non-contaminated water bodies (ponds) to use for hydration. The initial choice of all three species appeared not to be influenced by pond contamination, however, significantly more *L. caerulea* subsequently chose non-contaminated ponds more often, and *L. genimaculata* showed a similar but not statistically significant trend. Frogs that initially chose a pond without *Bd* also tended to spend more time submerged in water than did individuals that initially chose a pond containing *Bd* zoospores. Overall, *L. caerulea* spent significantly more time submerged in water than *L. genimaculata* and *L. wilcoxii* and was the only species in which some individuals became infected by *Bd* during the experiment.

**Introduction**
Chytridiomycosis is an amphibian skin disease caused by the fungus *Batrachochytrium dendrobatidis* (*Bd*). It has been implicated as a proximate cause of many amphibian declines, many in relatively pristine habitats around the globe (Berger et al., 1998; Mutschmann et al., 2000; Ron and Merino, 2000; Bosch et al., 2001; Bradley et al., 2002; Garner et al., 2005; Lips et al., 2006). In Australia, chytridiomycosis was listed in 2002 as a key threatening process under the Environment Protection and Biodiversity Conservation Act (EPBC) because approximately 57 native anuran species had been found with *Bd*, of which approximately 30% have suffered declines (Speare and Berger, 2005).

Frog populations in northern Queensland, Australia were monitored before, during and after outbreaks of *Bd* in the early 1990's (McDonald and Alford, 1999). This revealed that the impact of *Bd* on populations varied among species. Some species, such as *Litoria caerulea, L. xanthomera*, *L. wilcoxii* and *L. jungguy* suffered no detectable ill effects, while at elevations above 400m in the Wet Tropics, *L. genimaculata* declined substantially and *L. nannotis* declined to local extinction (Richards et al., 1993; McDonald and Alford, 1999; McDonald et al., 2005). Several years after the declines, upland populations of *L. genimaculata* recovered, whilst others, such as *L. nyakalensis* and *L. lorica,* failed to reappear (Ingram and McDonald, 1993; McDonald et al., 2005). Similar patterns of variation in susceptibility have also been reported from North and South America (Hale et al., 2005; Lips et al., 2005b; Garcia et al., 2006) and New Zealand, where the terrestrial *Leiopelma archeyi* appeared to decline due to *Bd*, while a

sympatric semi-aquatic species, *L. hochstetteri,* showed no dramatic declines (Bell et al., 2004).

Variation among amphibian species in their susceptibility to declines caused by chytridiomycosis is often thought of as being related to differences in the fate of infected animals (Daszak et al., 1999; Lips et al., 2003; Lips et al., 2005b). For example, species may have stronger or weaker innate immune responses (Rollins-Smith and Conlon, 2005; Woodhams et al., 2006), or may inhabit microenvironments more or less favourable to the development of chytridiomycosis (Rowley and Alford, 2007b). Less thought has been devoted to another source of variation; differences in rates of exposure to *Bd*. Richards et al. (1993) first noted that declining Wet Tropics frog species, such as *L. nyakalensis, L. nannotis or L. genimaculata* were associated with streams, and many authors since have suggested that stream-breeders may experience higher rates of exposure to *Bd* compared to terrestrial species (McDonald and Alford, 1999; Lips et al., 2003). However, there is substantial variability even among stream-breeding species (McDonald and Alford, 1999); some of this may be caused by differences in transmission rates caused by effects of behavior on exposure.

A variety of mechanisms may affect the exposure of frogs to *Bd* zoospores and thus their risk of infection. These include general levels of interaction with other frogs (Rowley and Alford, 2007a) and possible avoidance of contact with infected frogs or contaminated water bodies. It is known that *Bd* can be transmitted to frogs and tadpoles via contaminated water (Parris, 2004; Rachowicz and Vredenburg, 2004). If infection is often via contaminated water bodies, it is possible that anurans could reduce the probability of acquiring an infection by detecting and avoiding water bodies containing *Bd* zoospores. Parasite avoidance behavior has been observed in several mammal species (Lozano, 1991; Gilbert, 1997; Hutchings et al., 2001a); Hutchings et al. (1998) observed an increase in this behavior in parasitized animals.
Several studies (Resetarits and Wilbur, 1989; Egan and Paton, 2004; Rieger et al., 2004) have demonstrated that anurans can detect predator cues and use them to avoid ovipositing in water bodies containing predators. This detection is generally assumed to be via olfaction (Duchamp-Viret et al., 1996; Jorgensen, 2000). Given that frogs may detect the presence of predators in water through olfaction, it is possible that at least some frog species may detect *Bd* metabolites (Ibelings et al., 2004) and use them as cues to discriminate between contaminated and non-contaminated water bodies. Additionally, it is possible that frogs may experience some degree of skin irritation as *Bd* encyst on and infect the epidermis (Berger et al., 2005a) and use this cue in a similar way.

Our aim was to determine whether frogs of three species (*L. wilcoxii, L. genimaculata* and *L. caerulea*) that are susceptible to *Bd* but have suffered from chytridiomycosis to different extents discriminate among water bodies used for rehydration based on whether they contain *Bd* zoospores.

**Materials and Methods**

In order to minimise impact on frog population dynamics, our permit conditions required us to collect *L. wilcoxii* and *L. genimaculata* from aquatic life stages, as these two species were not available from captive breeders. *Litoria wilcoxii* were raised from tadpoles (no eggs were found) collected at Crystal Creek, Paluma Range National Park, Queensland, Australia (S18°58′54″ E146°12′′01″). This species is part of the *Litoria lesueuri* group, which was recently split into three species (*L. wilcoxii*, *L. jungguy* and *L. lesueuri*) and occurs in rainforest habitat along the east coast of Australia (Donnellan and Mahony, 2004). Individuals of the sympatric species, *L. genimaculata*, were raised from eggs collected at Birthday Creek, Paluma Range National Park (S18°58′54″ E146°10′02″). *Litoria caerulea* is a widespread Australian pond breeder (Barker, et al., 1995) and subadults were supplied from a captive bred population (Brendan Tiernan, Morphett Vale, South Australia). All frogs were swabbed using a sterile tubed dry swab (Medical Wire and Equipment, Corsham, Wiltshire UK) that was run across frog's hands, feet, thighs and ventral surface twice, before and after each trial for diagnostic quantitative PCR analysis, which was carried out at the School of Veterinary and Biomedical Sciences, James Cook University.

Isolates of *Bd* (Tully-*L. rheocola* 06-LB-1) were cultured on agar plates in the School of Veterinary and Biomedical Science, James Cook University, following the protocol of Longcore et al., (1999), using half of the amount of nutrients as this appeared to increase culture growth. Zoospores were harvested by flooding plates with 2 mL of dilute salt solution (DS) (Boyle et al., 2003) for 10 minutes. For the control ponds, plates without *Bd* zoospores were flooded with the same amount of DS. The concentration of zoospores was determined by counting live and moving zoospores in three subsamples using a haemocytometer.

The experiment was designed to determine whether the presence of *Bd* zoospores affected the use of ponds by frogs and each trial ran for 8 days. During each trial, frogs were individually housed in glass terraria, each provided with two containers ("ponds"; 115 mm diameter X 60 mm high), one at each end of the long axis and centered on the short axis. Each pond contained 120-200 ml for smaller sized species (*L. genimaculata* and *L.wilcoxii*) or 500-600ml for larger sized species (*L. caerulea*) of rainwater. One pond was denoted the infectious pond and the other the non-infectious pond. The infectious pond was inoculated with 8,000-12,000 *Bd* zoospores per mL of water. The concentration of *Bd* zoospores in Australian rainforest streams is unknown. We chose to inoculate ponds with this concentration of zoospores because exposure to zoospores at this concentration has been used successfully to infect Australian frogs with *Bd* (Woodhams et al., 2003; Woodhams et al., 2007). Dilute salt solution with *Bd* zoospores was added to the rainwater to create the infectious pond whilst an equal volume of DS solution without *Bd* zoospores was added to the same volume of rainwater to create the non-infectious pond. The amount of DS added varied each time ponds were inoculated, as it depended on the number of zoospores per mL harvested from the agar plates. To exclude biases of frogs preferring particular sides of terraria, location of the infectious pond (either right or left side of terrarium) was determined randomly at the beginning of each trial. To minimise cross-contamination and ensure the presence of living *Bd* zoospores, the water (with or without fresh *Bd* zoospores) and pond container were changed, and the positions of experimental and control ponds were reversed, every second day. Terraria and pond containers were disinfected between trials using F10 Veterinary Disinfectant which has been shown to be highly effective at

killing *Bd* (Webb, et al., 2007). Three digital cameras (Pentax Optio 33WR) were used to photograph the locations of frogs within the terraria at 30 minute intervals. A pilot study indicated that this was sufficient to capture most frog movements (Kenyon and Alford, unpublished). The first three trials on *Litoria caerulea* were conducted using six large terraria (750x350x500mm) with one frog per terrarium. In order to increase sample size per experiment, we used twelve smaller (600x200x300mm) terraria for all other trials. All trials were conducted in an air-conditioned room at 23°C. A total of eight trials were run; four (three using larger terraria, one using smaller terraria) on *L.* caerulea (n=30, average SVL 61mm, range 43-70mm; mass 21g, range 10.5-28.5g), two on *L. genimaculata* (n=24, average SVL 28mm, range 21-33mm; mass 2g, range 1-3.3g), and two on *L. wilcoxii* (n=24, average SVL 39mm, range 35-44mm; mass 4g, range 3.4-4.9g).

**Statistical analysis**
We analyzed data combined across all trials of the experiment for each species. Any effects of the change in the size of the terraria during the trials with *Litoria caerulea* should contribute to the error variation in these analyses, so our results are conservative. We also performed additional analyses, where sample size permitted, within species in which we separately examined the data from individuals with different *Bd* infection histories. The analysis proceeded through several stages.

1) Each photograph was examined and the location of the frog was noted. Frogs were scored as having chosen a pond if they made contact with the interior of the pond container (either submerged or not submerged in water) and if they were not in contact with that pond during the previous digital image. In most analyses we included data only for frogs for which we recorded five or more choices during the eight days of the trial; this is the minimum number needed for any bias to be detectable if the null hypothesis is that pond choice by individuals is a binomial random variable with $p = 0.5$.

2) There is no simple way to simultaneously test the hypothesis across all frogs that individuals show biases towards one end of the terrarium. We therefore carried out independent binomial tests on the data for each frog to determine whether it showed a significant bias. We then used Fisher's combined probability test (Sokal and Rohlf, 1995) to evaluate the overall hypothesis that there is a tendency for frogs to exhibit a bias.

3) We next carried out a similar test to determine whether the presence of *Bd* affected frog pond choice throughout the trial, calculating the binomial probability of the observed number of choices of ponds with *Bd* for each frog that made more than five choices, and using a combined probability test to produce a chi-squared statistic for each species. We did not use data for frogs that made less than five choices because the individual binomial probabilities for such frogs could never be less than 0.05.

4) We next tested the hypothesis that the presence of *Bd* might affect each frog's initial choice of pond. We did this to definitively eliminate the influence of any possible bias that might have influenced subsequent choices (e.g., after initially choosing the pond at one end of the terrarium, based on the presence or absence of *Bd*, the frog might have continued to choose the pond at that end of the terrarium regardless of the presence of *Bd*), using a simple chi-squared goodness-of-fit test. All frogs that made at least one choice were included in that analysis.

5) For the next three analyses we included data where frogs were observed to be submerged in the pond water rather than simply making contact with the pond container. First, we used Wilcoxon's matched-pairs tests on the data for each species to determine whether the time spent in water with and without *Bd* zoospores differed consistently within individuals. We illustrated this graphically by subtracting the total number of images of each individual frog submerged in water without *Bd* from total images of each individual frog submerged in water with *Bd*. Second, we compared by using Kruskal Wallis test, whether *L. caerulea* with different infection status spent different amount of times in ponds without *Bd*. Third, we determined whether individuals that initially chose a pond without *Bd* zoospores spent more time in the pond

with or without *Bd*, compared to individuals that individuals that initially chose ponds with *Bd* zoospores, using Wilcoxon's matched-pairs tests.

6) Finally we determined whether the total time spent in water of any sort (log transformed) differed among the three frog species using a Kruskal-Wallis test.

**Results**

The quantative PCR showed that no *L. genimaculata* or *L. wilcoxii* were infected with *Bd* before or after the trials (Table 3). Seven *L. caerulea* tested positive for *Bd* infection before the trial; of these two tested negative at the end. Twenty-three *L. caerulea* were initially uninfected; of these three tested positive at the end of the trial.

1) No *L. wilcoxii* made five or more choices of ponds, so data for this species were excluded from most analyses.

2) Individuals of *L. caerulea* and *L. genimaculata* that chose a pond more than five times showed a tendency to choose the pond at one end of the terrarium (preferred pond) more frequently, regardless of whether *Bd* zoospores were present, than would be expected by chance (*L. caerulea*, n= 12, $\chi^2$= 57.63, df= 24, p≤ 0.001; *L. genimaculata*, n=4, $\chi^2$= 25.70, df= 8, p≤ 0.002; Figure 13).

3) Despite tending to prefer one end of the terrarium, seven *Litoria caerulea* individuals chose ponds that did not contain *Bd* significantly more frequently, two chose both types with equal frequency and three chose contaminated ponds more often (n= 12, $\chi^2$=37.47, df= 24, p= 0.04; Figure 14). Although *L. genimaculata* did not show a significant tendency to avoid ponds containing *Bd* (n= 4, $\chi^2$=4, df= 8, p= 0.64), in both species more than twice as many individuals chose non-contaminated ponds more frequently than ponds that contained *Bd* (Figure 14).

4) Two *L.caerulea* were not observed to visit the pond throughout the experiment. The initial pond choice of each species was not significantly influenced by the presence of *Bd* (*L. caerulea*, n= 28, $\chi^2$=0.03, df= 2, p= 0.99; *L. genimaculata* n= 24, $\chi^2$= 0.75, df= 2, p= 0.69; *L. wilcoxii*, n= 19, $\chi^2$= 0.05, df= 2, p= 0.98).

**Table 5.** Quantative PCR results of *Litoria genimaculata*, *L. wilcoxii* and *L. caerulea* before and after trials.

| | | | After trial | |
|---|---|---|---|---|
| | | | + for *Bd* | - for *Bd* |
| *Litoria genimaculata* | Before trial | + for *Bd* | 0 | 0 |
| | | - for *Bd* | 0 | 24 |
| *Litoria wilcoxii* | Before trial | + for *Bd* | 0 | 0 |
| | | - for *Bd* | 0 | 24 |
| *Litoria caerulea* | Before trial | + for *Bd* | 5 | 2 |
| | | - for *Bd* | 3 | 20 |

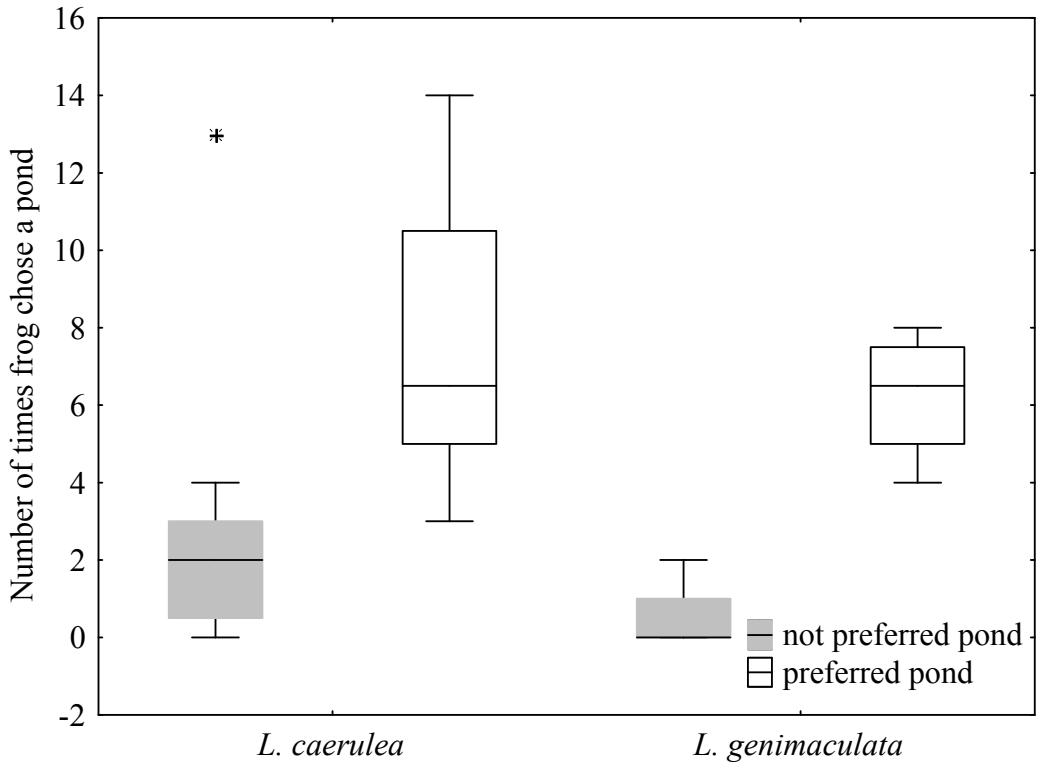


**Figure 13** Tukey (1977) boxplot (vertical bars indicate ranges, horizontal bar indicates median, boxes indicate upper and lower quartile boundaries) of *Litoria caerulea* and *L. genimaculata* individuals that chose a pond (frog being either submerged or not submerged in water) more than five times. The preferred pond was the pond that was chosen by an individual more frequently compared to the other pond (not preferred pond).

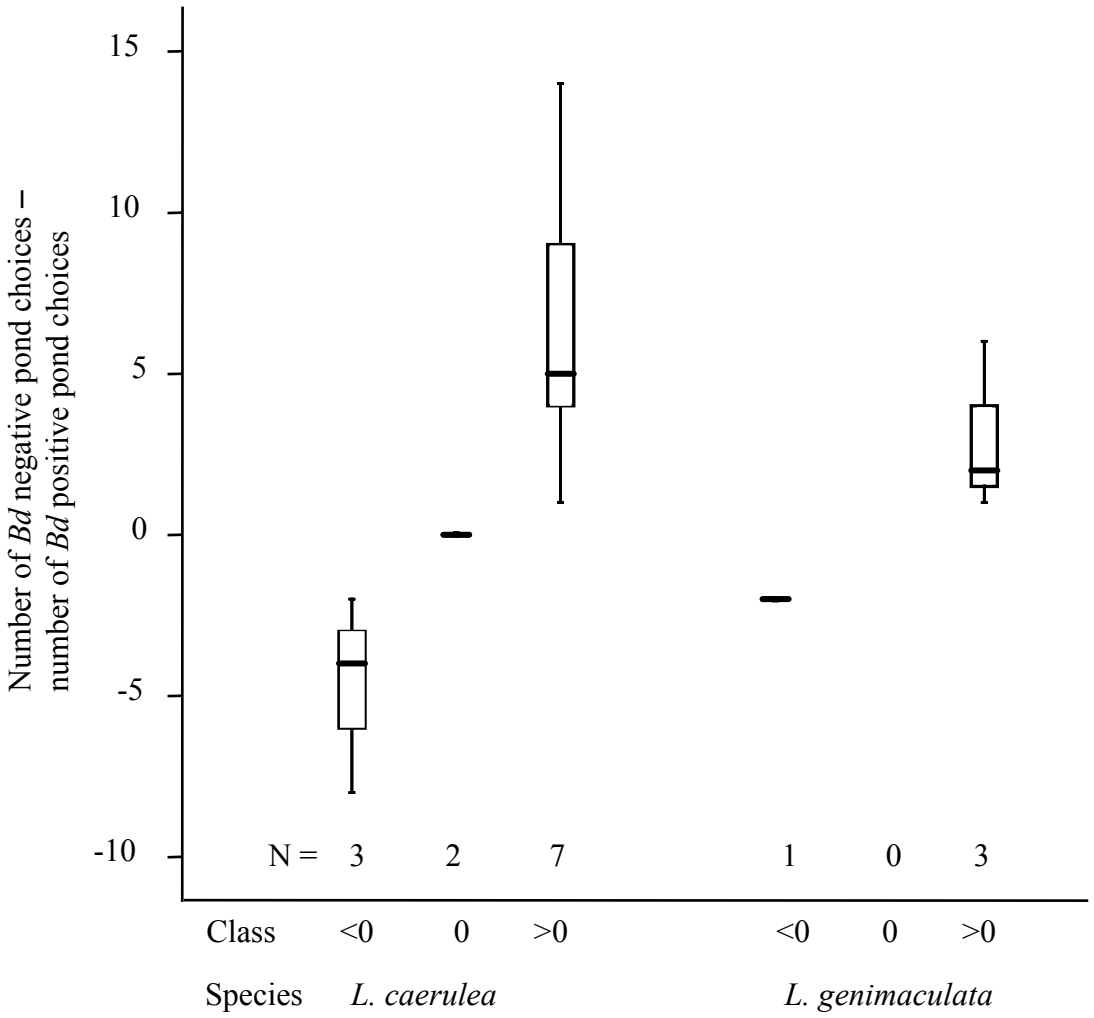


**Figure 14** Tukey (1977) boxplot (vertical bars indicate ranges, horizontal bar indicates median, boxes indicate upper and lower quartile boundaries) of distributions of the differences between the numbers of times individual *L. caerulea* and *L. genimaculata* chose ponds without and with *Bd* zoospores present. Separate distributions (classes) are shown for frogs that chose ponds with *Bd* more often (<0), frogs that chose ponds without *Bd* more often (>0), and those that chose both types with equal frequency (=0).

5) *L. caerulea*, *L. wilcoxi* and *L. genimaculata* individuals did not spend significantly more time in non-contaminated ponds than in ponds containing *Bd* zoospores (Wilcoxon's matched pairs tests, n=28, z=0.33, p=0.74; n=19, z=0.20, p=0.84; n=24, z=0.36, p=0.72, respectively). However, *L. caerulea* that were infected before the trial but not after (*Lc* infected B), spent significantly more time in ponds without *Bd*, than did *L. caerulea* that were infected before and after the trial (*Lc* infected B+E) (Kruskal Wallis test, n=7, H= 3.75, p=0.05; Figure 15). The Wilcoxon matched pairs tests indicated that the amount of time spent in water with or without *Bd* did not significantly depend on the initial experience of individual *L. genimaculata* (Wilcoxon matched pairs test, n=19, z=0.17, p=0.89) and *L. wilcoxii* (Wilcoxon matched pairs test, n=24, z=0.14, p=0.89). On the other hand, *L. caerulea* individuals that initially chose a pond without *Bd* zoospores spent significantly more time submerged in water than did individuals that initially chose a pond with *Bd* zoospores (Wilcoxon matched pairs test, n=28, z=1.57, p=01; Figure 16).

6) Overall, *L. caerulea* spent significantly more time in water than did *L. genimaculata* or *L. wilcoxii* (Kruskal Wallis test, n=71, H= 25.82, p < 0.01) (Figure 17).

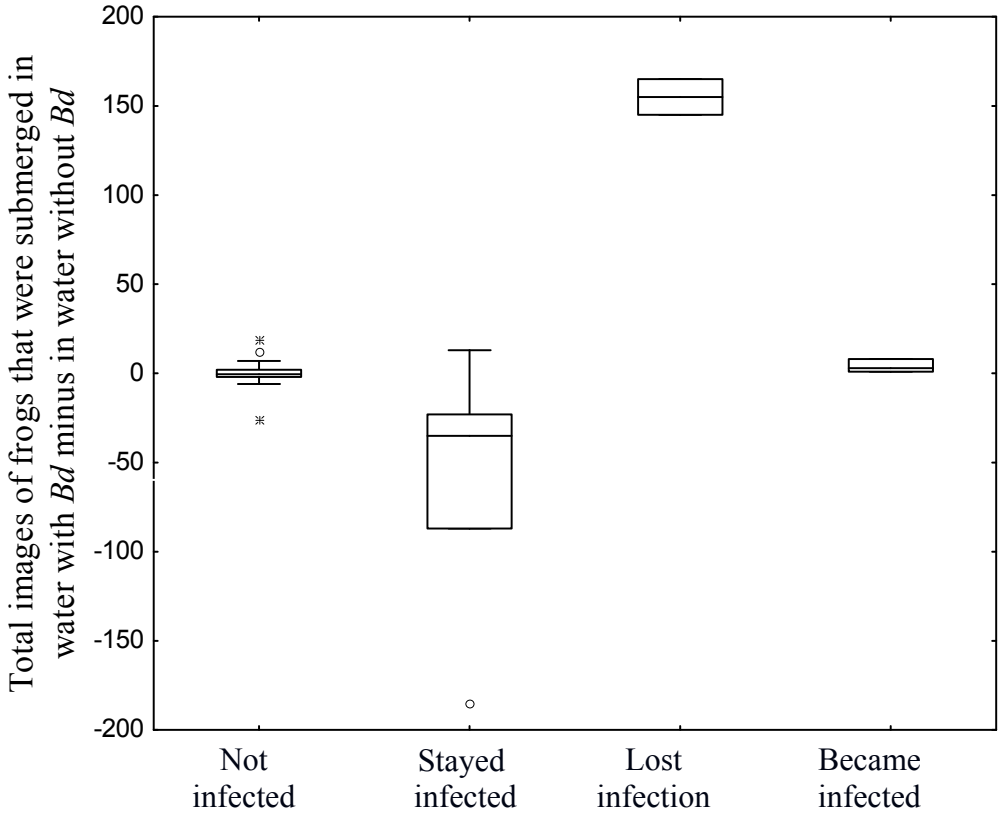


**Figure 15** Tukey (1977) boxplot (vertical bars indicate ranges, horizontal bar indicates median, boxes indicate upper and lower quartile boundaries) of the differences between the count of images where frog was in water with *Bd* minus count of images where frog was in water without *Bd.* Consequently, if in the positive, frogs selected ponds without *Bd* more often. Individual *Litoria caerulea* were grouped according to their infection status before and after the trials, determined by qPCR.

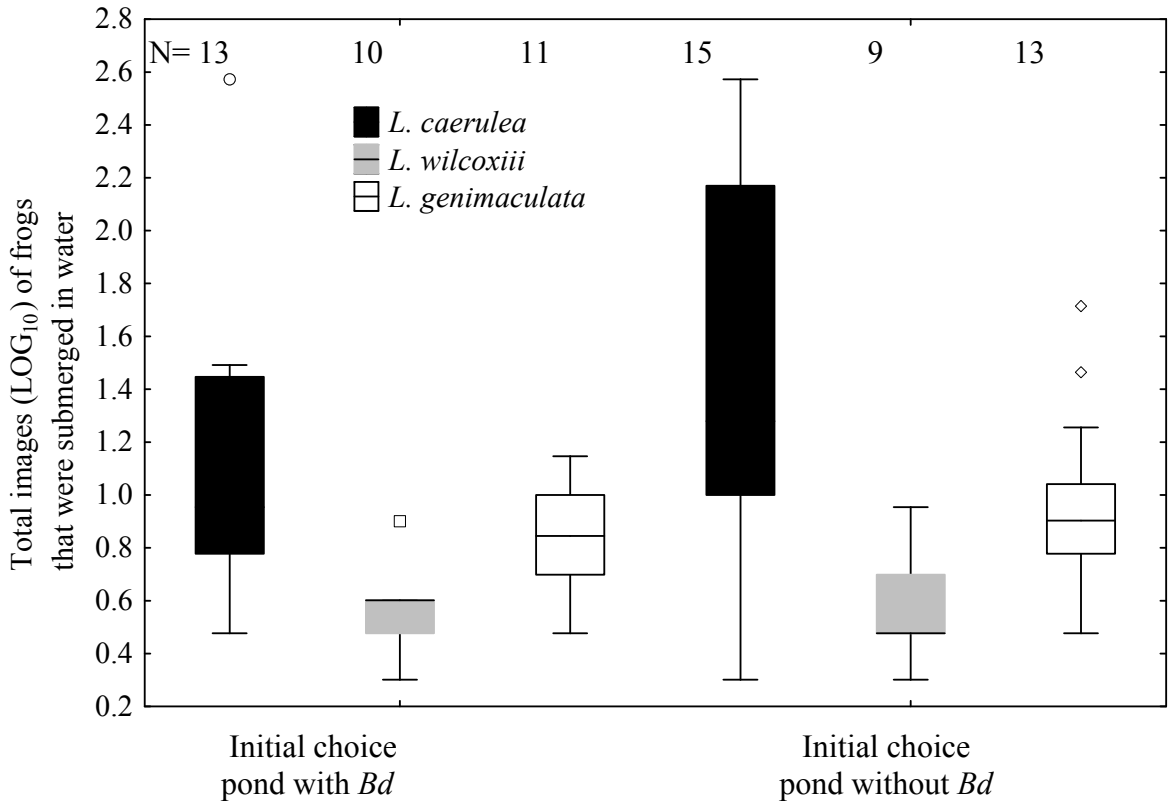


**Figure 16** Tukey (1977) boxplot (vertical bars indicate ranges, horizontal bar indicates median, boxes indicate upper and lower quartile boundaries) comparing the frog's initial choice of pond (either with or without *Bd* zoospores) and time spent in water (=total count of images where frog was submerged in water).

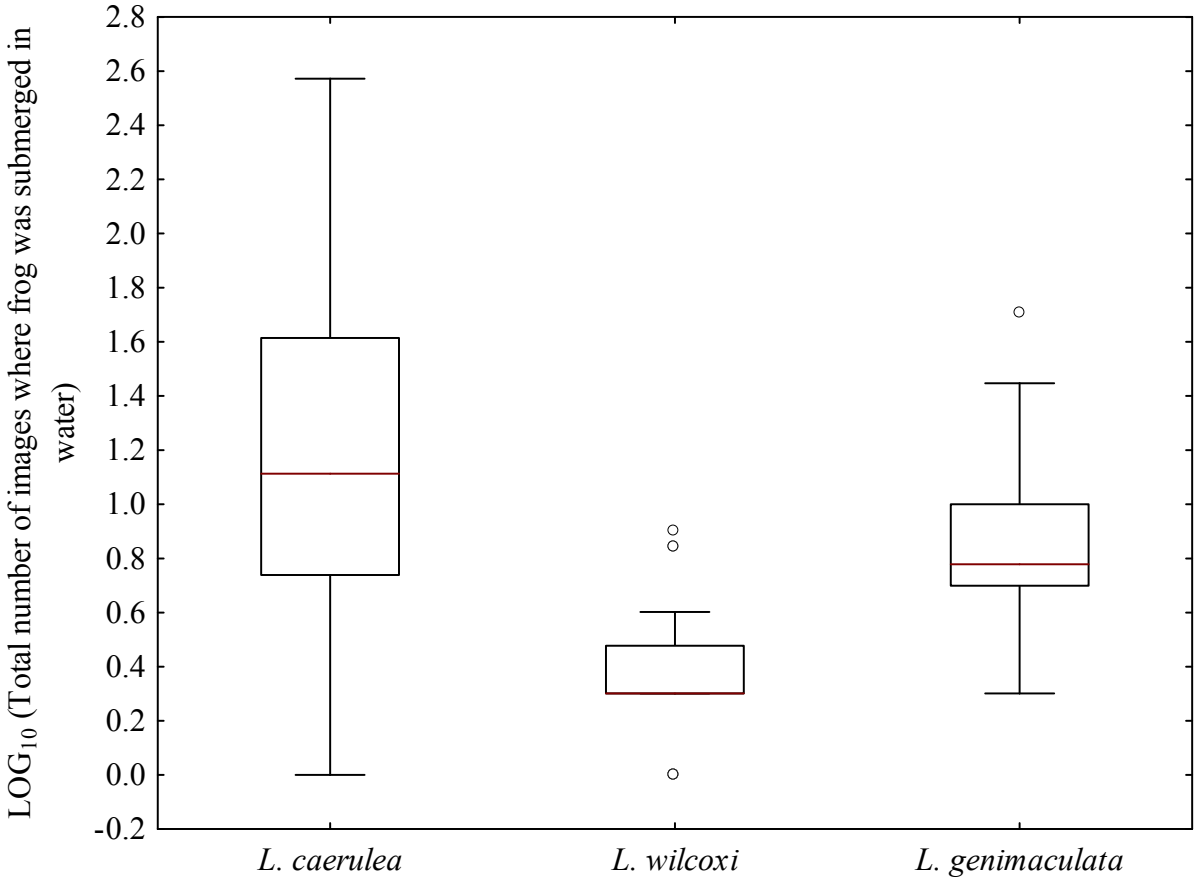


**Figure 17** Boxplot (vertical bars indicate standard deviation, horizontal bar indicates mean, boxes indicate upper and lower standard error) of total images of frogs submerged in water. (*Lc* not infected= *L. caerulea* negative with *Bd* throughout the trials, *Lc* infected B E= *L. caerulea* positive with *Bd* throughout the trials, *Lc* infected B= *L. caerulea* positive with *Bd* at the beginning but not at the end of the trial, *Lg* not infected= *L. genimaculata* negative with *Bd* throughout the trials).

**Discussion**

Because we sampled the behaviour of frogs by determining their locations using photographs taken every 30 minutes, our data may underestimate the number of times they visited water. We also cannot determine whether animals that avoided water containing *Bd* zoospores actually did not enter the water at all, or entered it for a short period only. Although our data do not show any *L. wilcoxii* making five or more decisions to become associated with ponds, it is possible that they visited ponds for short periods of time only. However, our pilot study on *L. wilcoxii*, using four minute intervals, recorded the same number of pond visit and does suggest that this species visits water infrequently. This has also been observed in the field; Rowley and Alford (2007b) found that individuals of the *Litoria lesueuri* complex (a mixture of *L. wilcoxii* or *L. jungguy*) only infrequently made contact with water.

Individuals of both of the other species tended to select the pond at one end of the terrarium significantly more often than the other pond. Because the locations of ponds containing and not containing *Bd* zoospores were swapped each time water was changed, this bias would not have led to frogs selecting ponds with and without *Bd* at different rates. Selecting ponds at random or consistently choosing the pond at one end of the terrarium would both have led to equal numbers of choices for ponds with and without *Bd*. However, side fidelity did not prevent *L. caerulea* from significantly avoiding ponds containing *Bd* zoospores and *L. genimaculata* showed a similar but not statistically significant trend.

The initial experience of frogs visiting a pond affected their subsequent behaviour. *Litoria caerulea* individuals that initially chose a pond without *Bd* zoospores tended to spend more time submerged in water than did individuals of the same species that initially chose a pond with *Bd* zoospores. The reasons for this tendency remain unclear. Chemical cues exuded by *Bd* may play a role in avoidance of infectious water bodies. However, the detection of *Bd* would have to be rapid enough to reduce rates of colonization of zoospores. Slight physical skin irritation that could result from zoospores attaching to the epidermis of frogs might also result in frogs spending less time in the water. Skin irritation might be more detectable to a frog in the still water of these artificial ponds than when encountered in a fast flowing creek. Several frog species, such as *L. nannotis,* that prefer fast flowing water have shown more severe declines than species such as *L. genimaculata* that inhabit slower flowing parts of the same creeks (McDonald and Alford, 1999).

*Litoria caerulea* was the only species that became infected with *Bd* during the study even though *L. genimaculata* or *L. wilcoxii* are susceptible in the wild (McDonald and Alford, 1999) and all individuals were at some point submerged in contaminated water containing a higher concentration of zoospores than is known to occur in natural water bodies (Kirshtein et al., 2007). It is possible that the availability of non-contaminated water may reduce the severity of infections. *Litoria caerulea* that were infected before the trial but not after spent significantly more time in the non-contaminated pond; suggesting that sitting in *Bd*-free water may reduce the rate at which zoospores reinfect the host individual to such an extent that the final qPCR readings were negative. None of the frogs had access to external heat sources and the room temperature was maintained at 23°C, a temperature where infection with *Bd* has been shown to be fatal to several frog species, including *L. caerulea* (Voyles et al., 2007).

Overall, *L. caerulea* spent significantly more time than other species sitting in water. This is consistent with the hypothesis that species that spend more time in water are more likely to become infected. None of our species completely avoided water containing *Bd* zoospores, possibly in part because their pond choices were influenced by side fidelity, and possibly also because they needed to enter ponds to determine their contamination status. However, *L. caerulea* significantly avoided water containing *Bd* zoospores, suggesting that detection and avoidance of contaminated water may play a role in determining the vulnerability of some species to infections by the amphibian chytrid.

*Acknowledgments*--
We thank Stephen Garland at the School of Veterinary and Biomedical Science at James Cook University, Townsville, Australia for qPCR analysis, Scott Cashins for raising *L. genimaculata* tadpoles and Ashley Pearcy for taking care of the frogs. Animals were obtained and trials conducted under Animal Ethics Approval A960 granted by James Cook University Animal Ethics Committee and Scientific Purposes Permits WITK01932505 and WISP01764304, granted by Queensland Parks and Wildlife Service.

***Project 2. Microenvironmental profiling of frogs in the field and how microenvironmental history affects the development of chytridiomycosis.***

Project 2 addresses **Objective 1** by determining how frogs of species known to be vulnerable and not to be vulnerable to chytridiomycosis-associated declines select and use thermal and humidity environments in nature, and constructs daily and seasonal profiles of the thermal and humidity environments experienced by these species over time. It also determines whether simulating the temperature and humidity profiles recorded for species in the field in controlled controlled environments produces results consistent with those observed in field populations. Project 2. addresses **Objective 2** by determining how remaining populations of species that have undergone chytridiomycosis-associated declines use thermal and humidity microhabitats in nature, comparing their use of these habitats with that of species that have not declined, and determining, using laboratory experiments, how the thermal and humidity profiles measured in the field affect the growth of *Bd*. It addresses **Objective 4** by producing comparative behavioural and ecological data profiling microenvironmental conditions encountered by frogs of vulnerable and less vulnerable species at sites where declines have and have not occurred, and determining how microenvironments following these these time profiles affect the growth of *Bd* in the laboratory. It addresses **Objective 5** by developing and testing an alternative more intensive approach, which could be used to assay the vulnerability of species of special concern, by first profiling their exposure to microenvironments using field studies, then simulating that exposure in controlled environment chambers.

Data collection and initial writeup for **Project 2** was completed in 2006, and the student, Jodi Rowley, completed and submitted her Ph.D. thesis in December 2006. That thesis is available at http://eprints.jcu.edu.au/1828/2/02whole.pdf. The experimental phase of this project was been replaced by additional field work being carried out by Robert Puschendorf, because the technical resources and animals needed for the experimental work were too great, and the risk of carrying out work with very uncertain results on an endangered species was considered to be too great. The results of this project were largely summarised in previous annual reports; a revised and clarified version of this summary is repeated below.

In Project 2, a total of 117 rainforest frogs of three species, one (*Litoria nannotis*) that has suffered severe population declines and local extinctions in association with *Bd*, one (*Litoria genimaculata*) that has suffered local declines and subsequently recovered, and one (*Litoria lesueuri*) that is apparently not affected, have been tracked, and their microenvironment use and infection status have been monitored, at upland and lowland sites in the wet and dry seasons. The techniques we developed for this work were sufficiently novel that they have resulted in four publications (Rowley and Alford 2007a, b, c; Rowley and Alford 2010). All three species used a wider range of microenvironments than might have been expected from their usual description as "stream-associated" frogs, venturing relatively long distances from water, and (in two species) far into the canopy. All three showed patterns of behaviour and microenvironment use consistent with their relative vulnerabilities to *Bd*-associated population declines. Table 6 presents a broad summary of how our measurements of frog behaviour and microhabitat use would be expected to affect the vulnerability of these species to chytridiomycosis. In every measured factor except humidity, *L. lesueuri*, the species least affected by *Bd*, behaves and uses the environment in ways that should make it least vulnerable to chytridiomycosis, while for most factors *L.*

*genimaculata* should be intermediate and *L. nannotis* should be most vulnerable. It appears possible that much of the variation in vulnerability among these species is related to behaviour and microenvironment use.

Table 6. Summary of field data on how behaviour and microenvironmental relations of tracked frogs should affect their vulnerability to chytridiomycosis. The species are ordered from left to right by increasing vulnerability to population declines and extinctions in association with chytridiomycosis. Ranks are ordered so that 3 is the species that should be at lowest risk, as determined by the factor under consideration, while 1 is the greatest risk. Tied ranks are averaged.

| Factor class | Factor | *L. lesueuri* (no declines) | | *L. genimaculata* (declines) | | *L. nannotis* (local extinctions) | |
|---|---|---|---|---|---|---|---|
| | | description | rank | Description | rank | description | rank |
| Transmission | Frog contact rate | low | 3 | Medium | 2 | high | 1 |
| | Water contact rate | low | 2.5 | Low | 2.5 | high | 1 |
| Movement and habitat use | Mobility | high | 3 | Medium | 2 | low | 1 |
| | Habitat specificity | low | 3 | Medium | 2 | high | 1 |
| | Association with streams | low | 3 | Medium | 2 | high | 1 |
| Microenvironment suitability for Bd | Thermal | poor | 3 | Medium | 2 | good | 1 |
| | Humidity | medium | 2 | Low | 3 | good | 1 |
| Rank summary | | low | 2.8 | Medium | 2.2 | high | 1 |

In addition to recording instantaneous data on the environment as experienced by individual frogs, the results presented above incorporate data collected using a novel technique we developed and validated (Rowley and Alford 2010) that enables us to place physical models of frogs in sites they have been observed to occupy in the field, and record the range of body temperatures they could be exposed to in those positions, along with potential rates of evaporative water loss. We have used these models to thoroughly characterise the ranges of environmental conditions available to frogs of the species we have been tracking. A summary of the data from these models appears in Figure 18. This demonstrates that retreat sites occupied by *L. lesueuri* and *L. genimaculata* spend substantial periods of time at temperatures above the optimal growth range for *Bd* during the cool, dry season, when the threat of development of chytridiomycosis appears to generally be the highest due to cool environmental temperatures. Some of the retreat sites we monitored for *L. lesueuri* also spent substantial periods above the temperature threshold (30$^{o}$C) at which *Bd* mortality begins to occur; frogs in those sites would probably have lost any infection they might have. Our data appear paradoxical in that maximum temperatures of retreat sites of both species were lower during the summer wet season; this occurred because cloud cover was greater during those tracking periods, and may also reflect sampling variation, since we placed the models in retreat sites used by frogs during each tracking episode. The

retreat sites of the most vulnerable species, *L. nannotis*, were almost always within the optimal growth temperature range for *Bd*.

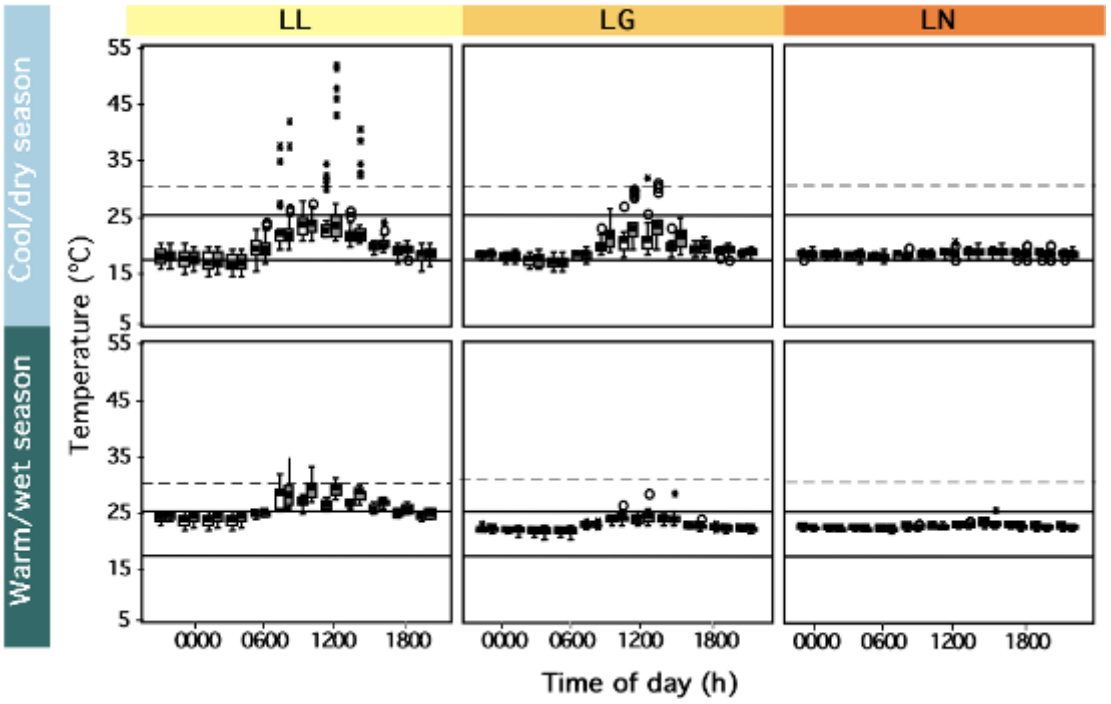


Figure 18. Boxplots of temperatures measured at diurnal retreat sites of frogs of each species using physical models. Clear boxes indicate the interquartile range for models that lost water evaporaively at maximal rates, solid boxes indicate the interquartile range for impermeable models. Bars indicate typical ranges, while points indicate outliers.

The data presented in Figure 18, with the addition of data on the nocturnal temperatures of our models, provide detailed, 24-hour per day profiles of the ranges of thermal environments experienced by individuals. Our measurements of weight change of the permeable models also provide us with a picture of the humidity environments the animals experience (Rowley and Alford 2009).

In the course of tracking frog behaviour in Project 2, we extensively sampled the immediate environment of frogs for quantitative PCR analyses to detect possible persistence by *Bd* in the environment, in association with the saprobic behaviour work addressing objective 1 of tender 42-04. We also sampled potential alternative hosts, including freshwater shrimp. All of our samples from frog retreat sites were analysed during 2006, and the results were negative . Initial diagnostic PCR results for two shrimp species indicated that *Bd* was present on their exoskeletons (Rowley et al., 2006). During 2007 we carried out a program of work designed to test and extend these results. We performed diagnostic PCR analyses on a larger set of field samples, conducted a series of laboratory experiments in which we attempted to culture *Bd* on freshwater shrimp and crayfish, and carried out additional genetic analysis on the original samples in an attempt to confirm the results of the initial diagnostic PCRs. All of this work produced negative results; we did not detect any additional naturally infected animals, could not culture *Bd* on crustaceans in the laboratory, and failed to

amplify any *Bd* DNA from our original samples. A paper based on this work (Rowley et al. 2007) suggests that freshwater shrimp may not serve as alternative hosts for *Bd*, leaving the question of environmental reservoirs outside of amphibians still unanswered.

Further details of the work undertaken in Project 2 appear in the Ph.D. thesis by J.J.L Rowley, http://eprints.jcu.edu.au/1828/2/02whole.pdf.

***Project 3. Determining the vulnerability and mechanisms of resistance to chytridiomycosis of microhylid frogs.***

Kim Hauselberger

Project 3 addresses **Objective 1** by determining the reasons why one family of Australian frogs, the Microhylidae, appears not to be negatively affected at the population level by chytridiomycosis, despite occurring at high elevations and having low rates of reproduction and restricted ranges, which have been associated with declines in species belonging to other families. It addresses **Objective 6** by searching for evidence of resistance to chytrid in the microhylid frogs, the only family so far unaffected by declines associated with chytridiomycosis. It will determine the mechanism or mechanisms of this resistance; this knowledge may suggest methods for increasing the resistance of species at risk.

- Calling data from one site (Paluma) have been collected for four entire wet seasons since 1995. These recordings have been analysed and suggest that the abundance of the two species of microhylid that inhabit the area have not changed considerably (Figure 19), although population declines occurred in other species in this area in the 1990s.

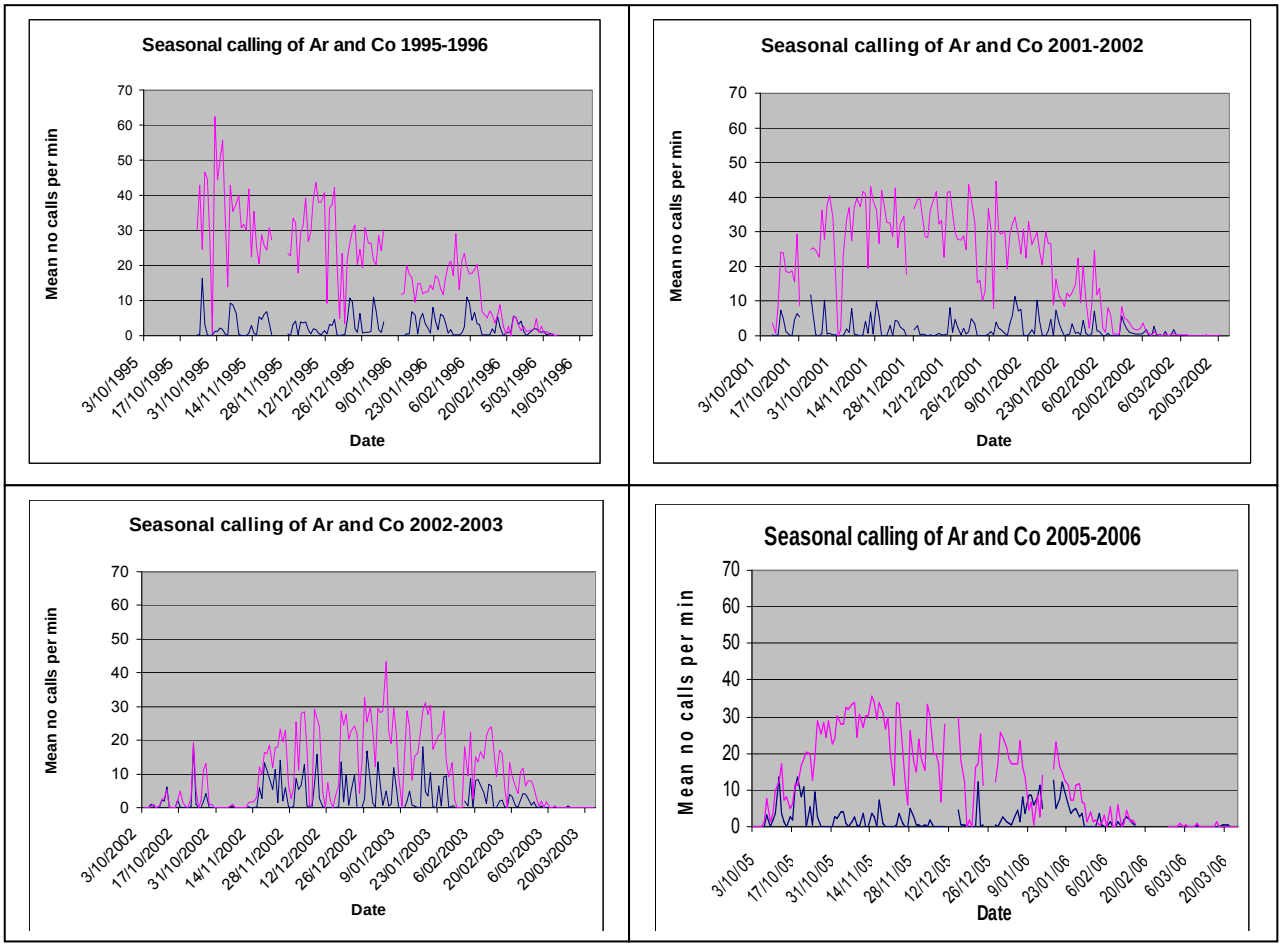


**Figure 19.** Calling activity by *Austrochaperina robusta* (Ar, dark lines) and *Cophixalus ornatus* (Co, light lines) along a 550 m transect near Paluma. Data are mean number of calls heard per minute at each of 7 (1995-96 and 2001-02) or 3 (2003-03 and 2005-06) recording stations. Peak numbers for both species remained relatively constant over the 11-year period of data collection.

## ***Project 3.1 Resistance of the Australian Microhylidae to Batrachochytrium dendrobatidis in nature***

Kim F. Hauselberger and Ross A. Alford

Abstract
The emerging infectious disease chytridiomycosis has been implicated in the declines and disappearances of amphibian populations around the world. However, the extent of pathological effects appears to vary among hosts, and frogs and salamanders with life-histories that include parental care of direct-developing terrestrial eggs may tend to be less susceptible. We examined a total of 595 samples from nine species of direct-developing Australian microhylids for the presence of *Batrachochytrium dendrobatidis* (Bd) infection. Of these, 336 historic samples were collected between 1995 and 2004; 102 were analysed histologically and 234 were examined using diagnostic quantitative PCR. Swab samples from 259 frogs were collected during 2005-08 and were examined using diagnostic quantitative PCR. 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 (less than 1%). Even if only the data from the more powerful diagnostic qPCR tests are used, the upper 95% confidence limit for prevalence is 0.0074 (less than 1%). Our data thus strongly suggest that Australian microhylids have an extremely low prevalence of Bd in nature, and are either not susceptible, or are only slightly susceptible, to chytridiomycosis. They, and perhaps some other direct-developing species, may be highly resistant to Bd because they possess antimicrobial symbionts in skin flora or in skin secretions as a means to reduce fungal infestations that would otherwise overwhelm terrestrial egg clutches.

Introduction
Pathogenic organisms are frequently cited as important drivers of community dynamics (Anderson and May 1986; McCallum and Dobson 1995; Cleaveland *et al.* 2001; Hudson *et al.* 2001). Disease emergence can have drastic effects on wildlife populations, and pathogens have been implicated in the decline and extinction of a wide variety of species (Warner 1968; van Riper III *et al.* 1986; Berger *et al.* 1998; Cunningham and Daszak 1998; Daszak *et al.* 1999). Pathogens can cause mass mortality of species, and can convert species rich systems into depauperate communities that are dominated by a few resistant species (Burdon 1991). This phenomenon has been observed in island birds (Warner 1968; van Riper III *et al.* 1986), forest trees (Burdon 1991), and rainforest frogs (Lips *et al.* 2006). When this occurs, some species experience severe declines, others decline less severely, and others suffer no losses. Host susceptibility may differ greatly among and even within species (Wakelin 1978), and is influenced by pathogen transmission and the progress and outcomes of infections (Warner 1968; van Riper III *et al.* 1986; Lips *et al.* 2006). Disease dynamics are affected by multiple factors related to the host, the pathogen, and the environment.

Chytridiomycosis is an emerging infectious disease which is caused by *Batrachochytrium dendrobatidis* (Bd), a chytrid fungus that parasitises the mouthparts of larvae and the keratinised epidermis of post-metamorphic amphibians (Berger *et al.* 1998; Longcore *et al.* 1999). This pathogen has been associated with population declines of amphibian species in many regions (Berger *et al.* 1998; Carey *et al.* 1999; Daszak *et al.* 1999; Bosch *et al.* 2001; Bradley *et al.* 2002; Rachowicz *et al.* 2006), including at least 14 threatened Australian species (Speare and Berger 2004a). Bd causes mortality in a large range of amphibians, however, the extent of its pathological effects appear to vary greatly among host species (Blaustein and Wake 1990; Wake 1991; Blaustein and Wake 1995; Fisher and Shaffer 1996; McDonald and Alford 1999). Where outbreaks of chytridiomycosis have occurred, it is common for some species to suffer local extinction, while others decline and some suffer no population-level effects (McDonald and Alford 1999; Lips and Donnelly 2002; Retallick *et al.* 2004; Lips *et al.* 2006). Experimental data has also demonstrated that significant inherent differences exist within (Davidson *et al.*

2003), and between species infected with Bd in a laboratory setting (Woodhams *et al.* 2007). In Australia there have been four patterns of effects of Bd on frog species:

It is presumed to have caused the extinction of several species. Some species have suffered extinctions of local populations, but have escaped global extinction. Some species have suffered declines or local extinctions of populations that have subsequently recovered or recolonised and now show apparently stable associations with Bd. Populations of many species have shown no apparent effects of Bd and now exist in apparently stable associations with it. The association of Bd with frog species thus provides an excellent model system in which to study variation in disease susceptibility.

Variation in susceptibility to Bd can be associated with ecological and life-history traits of host species (Blaustein and Wake 1990; Williams and Hero 1998; Lips *et al.* 2003; Retallick *et al.* 2004; Woodhams and Alford 2005). The fungus requires a moist environment to multiply and release zoospores, and it is these waterborne zoospores which are infective to amphibians (Powell 1993; Pessier *et al.* 1999). The habitat or microhabitat used by individual species may alter the extent or likelihood of initial contact with Bd (Rowley and Alford 2007), and thermal and hydric environments chosen by the host species may have a large influence on disease development (Woodhams 2003; Woodhams *et al.* 2003). Host behaviour is likely to affect pathogen transmission, depending on the frequency of contact between individuals, infected water bodies, or contaminated environmental substrates (Lips *et al.* 2006; Rowley and Alford 2007). Frog species that do not appear to be susceptible to Bd in the wild may simply not come into contact with Bd zoospores, or may exist in environments that do not permit the growth of the fungus.

Several studies have found correlations between species that have exhibited mass mortalities and their behavioural, locational or life-history characteristics. High-elevation populations suffer more severely from declines (Blaustein and Wake 1990; Wake 1991; Richards *et al.* 1993; Lips 1998; McDonald and Alford 1999); affected species tend to occupy restricted geographic ranges (Murray and Hose 2005); they usually have aquatic larvae associated with streams and spend a large proportion of their time in or near streams (Lips 1998; McDonald and Alford 1999; Lips *et al.* 2003; Woodhams and Alford 2005); and they may have relatively smaller clutch sizes (Williams and Hero 1998). However, these relationships are difficult to interpret, and of the studies mentioned, only Murray and Hose (2005) carried out phylogenetically independent contrasts of correlates of decline, and found that when phylogeny was accounted for, geographic range size was the only variable correlated with the probability of decline in Australian frog species.

Frog species with life-histories incorporating direct development, which therefore have no tadpole stage exposed to water bodies, do not appear to suffer greatly from population declines associated with Bd. Of 60 amphibian species of upland (<300m) areas in eastern Australia, only one species (*Philoria frosti*) out of 20 with direct development has undergone declines, whilst 20 of the 40 species with an aquatic stage in their life-history have experienced population declines (Hero *et al.* 2005). Declining species have also tended to have aquatic larvae in Brazil (Eterovick *et al.* 2005), Ecuador (Ron *et al.* 2003: Bustamante, 2005 #350), Venezuela (La Marca 1995), Costa Rica (Crump *et al.* 1992; Pounds *et al.* 1997; Lips 1998), Panama (Lips *et al.* 2006; Brem and Lips 2008), Spain (Bosch *et al.* 2001), and the USA (Muths *et al.* 2003). World-wide, the presence of Bd infection in terrestrial, direct-developing frogs that bypass a free-swimming tadpole stage is low. Very few examples of population crashes of species with terrestrial oviposition and direct development have been recorded in the literature (Appendix 1), and even fewer can cite the presence of Bd as a possible cause of the declines. Examples come from Central America (Lips *et al.* 2006; Brem and Lips 2008), Puerto Rico (Burrowes *et al.* 2004), and New Zealand (Bell *et al.* 2004).

The apparently lower vulnerability of direct-developing species may be due to the absence of tadpoles, which may often be involved in transmission of Bd, because they are likely to be exposed to the fungus' aquatic zoospores (Daszak *et al.* 1999). In addition to the absence of a tadpole stage, direct-developing species commonly exhibit some form of parental care of the developing egg clutch (Duellman and Trueb 1994; Crump 1995). Egg brooding may prevent predation (Kluge 1981), desiccation (Forester 1984; Townsend *et al.* 1984), and fungal infestation of the developing embryos (Forester 1979; Simon 1993), and studies have shown that clutches suffer increased mortality when brooding adults are removed from nests (Forester 1979; Townsend *et al.* 1984; Simon 1993).

Amphibians that exhibit brooding behaviour can possess bacterial flora that inhibit the growth of a range of bacteria and fungi, including Bd (Austin 2000; Harris *et al.* 2006). Egg brooding adults may reduce microbial attacks on eggs by providing either or both of antimicrobial skin secretions or antimicrobial substances produced by their skin microbiota (Harris *et al.* 2006). In either case, adaptations that normally serve to protect developing egg clutches may predispose terrestrial breeders to be relatively resistant to infection by Bd.

Despite extensive research on the extent and effect of Bd in Australia, data on the distribution of Bd is patchy, and is largely derived from opportunistic collection of sick or dead frogs (Berger *et al.* 2004), or surveys of archived specimens (Speare and Berger 2004a; McDonald *et al.* 2005; Kriger and Hero 2007). Unfortunately, little sampling of Bd from non-declining populations has been undertaken; with the result that frog decline has been used as a proxy for Bd appearance (Lips *et al.* 2008). Currently, Bd has been found on 57 Australian species from three families (Hylidae, Myobatrachidae and Bufonidae; Speare and Berger 2004a; Kenyon 2008).

The family Microhylidae is the only native family of Australian frogs for which no declines related to chytridiomycosis have been reported. Most species occur in the Wet Tropics (WT) region (Cogger 1996), where extensive declines have occurred in other species, and the pathogen is now endemic (Berger *et al.* 2004; McDonald *et al.* 2005; Woodhams and Alford 2005). Microhylids inhabit environments that should be favourable for Bd, as they occur in cool, moist habitats, and they also possess ecological traits that characterise susceptible species. These include occurring at high altitudes and having restricted distributions (Laurance *et al.* 1996; Lips 1998; McDonald and Alford 1999; Murray and Hose 2005). Despite the absence of extensive population data, it does not appear that this family have undergone population losses, as population densities appear to be high (Hauselberger 2001; Hauselberger and Alford 2005; Williams 2007).

Until recently, only two Australian species of microhylid (*A. robusta* and *C. ornatus*) from populations near Paluma, Queensland had been surveyed histologically for infection by Bd. These surveys failed to detect any infected animals (D. Mendez pers. comm.; Hauselberger 2001). Kriger and Hero (2006) collected a sample from a single *Cophixalus ornatus* in September 2005 that tested positive for Bd DNA using a diagnostic quantitative PCR assay. This indicates that microhylids can be infected by Bd in nature, however the absence of positive samples from extensive histological surveys suggests that infection may be rare.

It is important to establish the extent to which Australian microhylids are infected with Bd in nature, as this will increase the certainty of predictions regarding their future persistence. If this family is susceptible to the pathogen, the persistence of several species may be in doubt due to their extremely small range sizes. As small changes in environmental conditions have greatly affected the prevalence and virulence of the pathogen in other species (Pounds *et al.* 2006), alterations in the ambient environment from factors such as climate change could affect entire species over a very short period of time. Given the recent, dire predictions regarding possible responses of narrowly-distributed species to climate change in the WT (Williams *et al.* 2003), the added pressure of an invasive disease could result in the extinction of microhylid species. If

this group are naturally highly resistant to infection by Bd, then understanding the mechanism of their resistance may aid in captive management and treatment of other species of frogs that are vulnerable to the pathogen.

## Materials and methods

### *Diagnostic quantitative PCR*

Whenever possible, samples were analysed using the qPCR assay developed by Boyle *et al.* (2004). Comparison of qPCR and previous methods of Bd detection such as histology, demonstrate that it is a much more sensitive assay (Boyle *et al.* 2004; Hyatt *et al.* 2007), and is highly specific, approximating 100% (Boyle *et al.* 2004; A. Hyatt unpublished observations). 451 samples were analysed at JCU, Townsville by R. Campbell, S. Garland and A. Phillot, and 42 samples were analysed at CSIRO, Geelong under the supervision of A. Hyatt.

### *Historic samples*

Historic samples of seven microhylid species (*Cophixalus aenigma*, *C. bombiens*, *C. hosmeri*, *C. infacetus*, *C. neglectus*, *C. ornatus* and *Austrochaperina robusta*) were available from previous research. The samples consisted of toe-clips and tissue sections preserved in 70% ethanol, or prepared slide sections of toe clips, stored at James Cook University (JCU) and the University of Queensland (UQ). Samples dated back to 1995, and came from five separate research studies (Brooke *et al.* 2000; Hauselberger and Alford 2005; Felton *et al.* 2006; Williams 2007; C. Hoskin pers. comm.).

All ethanol-preserved tissue samples were processed for the detection of Bd DNA at JCU using qPCR. Each sample was removed from its storage vial using forceps and transferred to an individually labelled Eppendorf tube containing 50% ethanol. Forceps were sterilised, and any DNA that might have adhered to them was destroyed, by submerging in 100% ethanol and flaming between each use. Brooke *et al.* (2000) collected several hundred toe-clips from *C. ornatus* at Paluma during the 1995-96 wet season; these had been stored at JCU. 201 samples included more than one toe clip, and these were used in qPCR analysis for detection of Bd DNA. Williams (2007) collected toe-clips from various microhylid species throughout the WT in Queensland during 2000-2004; we were able to use 18 samples from 5 species for diagnostic qPCR. We also examined historic samples from *C. ornatus* and *C. neglectus* that were originally collected by J. Milton and C. Hoskin and were stored at UQ. Several frogs collected from Mt. Bartle Frere in early 2001 were brought back to UQ in Brisbane and died in captivity shortly thereafter. These individuals were thought to have died from chytridiomycosis, and were stored in 70% ethanol. We obtained 15 tissue samples from these individuals, each consisting of a strip of skin cut from the ventral thigh or abdominal region. They were treated in the same manner as toe clips.

Samples that existed as prepared histological slides (originally used for skeletochronological determination of age) were examined using a light microscope to detect Bd infection by searching for fungal structures in the *stratum corneum*. Felton *et al.* (2006) collected toe-clips from 47 *C. ornatus* at Paluma from 1998-1999, and Hauselberger and Alford (2005) collected toe-clips from 55 *A. robusta* in 2000-01 at Paluma. These samples had been prepared histologically as 7um sections, stained in heamotoxylin and eosin, and mounted on microscope slides.

### *Field data*

Skin swabs from seven species (*A. fryi*, *A. pluvialis*, *A. robusta*, *C. aenigma*, *C. hosmeri*, *C. neglectus*, *C. ornatus*) of microhylid frogs were collected to determine if this family of frogs is susceptible to Bd infection in the wild. Samples were collected at various locations within the WT during 2004-2008 (Table 7). A standard method for sampling frogs was employed (Skerratt *et al.* 2008), and strict hygiene protocols for the handling of individual frogs and for disinfection of equipment between field sites were followed (NSW Parks and Wildlife Service 2001).

Table 7. Location of collection sites within the WT region of north Queensland.

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

Each frog was handled with a new pair of latex gloves and was held by the hind legs to prevent escape. A sterile medical cotton swab (Medical Wire & Equipment Co. Bath Ltd., Wiltshire, UK) was run over the dorsum, including the surfaces of the forelegs, hindlegs, and back, for approximately 10 seconds. The frog was then inverted so that its ventral surface was exposed, and the forelegs, hindlegs, and the ventral surfaces of the head and body were swabbed for 10 seconds. The swab was also placed near the toes of each of the feet of the individual so that it grasped the swab with its toe pads. This procedure generates the highest sensitivity using the qPCR assay for detection of Bd DNA (Hyatt *et al.* 2007). It allows sampling of all dorsal and ventral surfaces, is non-invasive and not harmful to the individual. To prevent contamination, each swab was placed directly into its individual container after the swabbing procedure, and stored in a refrigerated environment as soon as possible after collection (<3hr). When a refrigerator was not available, samples were placed on ice in a portable, insulated container. Each frog was swabbed twice so that a second sample would be available for analysis, should it be required.

*Batch samples*

Due to the cost of qPCR analysis, some samples were analysed in batches. One hundred historic samples from Brooke *et al.* (2000) were analysed in batches of ten, where ten toe clips were placed into a single container for analysis. This was only carried out on samples that contained more than one toe clip, so that if a positive result occurred, individual toes could be retested to determine which ones were positive. 80 field samples collected from 2004-08 were run in batches of four. All field samples collected consisted of two swabs per individual, so that if a positive result was found in the qPCR analysis, the second set of swabs could be individually retested. Hyatt *et al.* (2007) showed that the maximum number of swabs that can be pooled without lowering the sensitivity of the qPCR assay is five. Batching groups of greater than five lowered the sensitivity of the test in laboratory trials, as it produced negative results in 40% of samples that had less than 10 zoospores. No negative results were produced in samples of any size that contained 100 zoospores (Hyatt *et al.* 2007). Because the batch samples that were run in groups of 10 were actual toe clips rather than skin swabs, we believe it is unlikely that the sensitivity of the test was reduced for historic samples.

*Statistical Analysis*

We calculated Clopper-Pearson 95% binomial confidence limits for population prevalence of Bd in a variety of populations. Using the formula of DiGiacomo and Koepsell (1986), a minimum of 72 individuals from each population needed to be sampled, with none positive, to support the idea that the prevalence in the population is less than 5% with 95% confidence. It

would require 367 negative samples, with none positive, to indicate that true prevalence is less than 1% with 95% confidence.

Sample sizes and Clopper-Pearson 95% binomial confidence limits were calculated for all areas surveyed both in field and historic sampling. For field sampling, results for the three years of surveys (2004-08) were collated, and confidence limits for infection prevalence in populations at each of the sites surveyed during this time period were estimated. For the historic data, samples were not collated, and confidence limits were calculated independently for each sample at each location.

## Results

### *Historic data*

336 historic samples, taken from seven species, were analysed for the presence of Bd using histological and qPCR techniques. All samples were negative (Table 8). This includes a group of *C. neglectus* and *C. ornatus* individuals that were collected from Mt Bellenden Ker in early 2001 and died in the laboratory shortly after and stored in ethanol. It had been assumed these individuals succumbed to chytridiomycosis, however analysis of samples indicated no fungal DNA. The small sizes of many samples have led to high upper 95% confidence limits for prevalence, but the three larger samples have upper 95% confidence limits below 10%, and the sample for Paluma in 1996, which was analysed using the sensitive qPCR assay, has an upper 95% confidence limit of 1.8%. Aggregated across all historical samples, the upper 95% confidence limit for prevalence is 1.1%.

Table 8. Historical samples analysed for the presence of Bd using qPCR and histological techniques. All samples were negative for the presence of Bd.

| Species | Date | Location | Collector | Analysis | Number | Upper 95% CL |
|---|---|---|---|---|---|---|
| A. robusta | 2001 | Pal | Hauselberger | Histology | 55 | 0.065 |
| C. aenigma | 2004 | AU6 | Williams | qPCR | 4 | 0.602 |
| C. bombiens | 2004 | AU4 | Williams | qPCR | 1 | 0.975 |
| C. hosmeri | 2004 | AU2 | Williams | qPCR | 3 | 0.708 |
| C. infacetus | 2004 | AU4 | Williams | qPCR | 6 | 0.459 |
| C. neglectus | 2001 | BF13 | Hoskin | qPCR | 1 | 0.975 |
| | 2004 | BK | Williams | qPCR | 4 | 0.602 |
| C. ornatus | 1995 | Pal | Brooke | qPCR | 201 | 0.018 |
| | 1998 | Pal | Felton | Histology | 47 | 0.075 |
| | 2001 | BF1 | Hoskin | qPCR | 4 | 0.602 |
| | 2001 | BF7 | Hoskin | qPCR | 4 | 0.602 |
| | 2001 | BF13 | Hoskin | qPCR | 4 | 0.602 |
| | 2001 | Hai | Hoskin | qPCR | 1 | 0.975 |
| | 2001 | Mau | Hoskin | qPCR | 1 | 0.975 |
| Total | | | | | 336 | 0.011 |

### *Field data*

None of the 259 swab samples taken from seven species during our surveys in 2004-07 tested positive for the presence of Bd using diagnostic qPCR (Table 9). The small sample sizes at many sites mean that upper 95% confidence limits for prevalence are high, but at the three sites with larger samples they are below 10%. Aggregating all the field samples taken during 2004-2007 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%

Table 9. Field samples (2004-2007) analysed for the presence of Bd using qPCR techniques in 2004-08, and upper Clopper-Pearson 95% binomial confidence limits for population prevalence of Bd across field sites. All samples were negative for the presence of Bd.

| | 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% confidence limit | 0.71 | 0.84 | 0.98 | 0.06 | 0.28 | 0.6 | 0.98 | 0.08 | 0.03 | 0.014 |

Finally, if we combine all of the data for diagnostic qPCR assays of historical and survey samples (0 positives from 493 individuals), the upper 95% binomial confidence limit for prevalence in this sample of WT microhylids is 0.75% (Table 10). Adding the results of the negative histological assays of an additional 102 individuals gives an upper 95% binomial confidence limit of 0.62%.

Table 10. Sample sizes and Clopper-Pearson 95% binomial confidence limits for population prevalence of Bd across qPCR samples, histological samples, and total samples.

| | qPCR samples | Histological samples | Total |
|---|---|---|---|
| Positive swab for Bd | 0 | 0 | 0 |
| Number of trials | 493 | 102 | 595 |
| Upper 95% confidence limit | 0.0075 | 0.0355 | 0.0062 |

## Discussion

Of all the field and historic samples analysed in this study, none tested positive for the presence of Bd DNA. At two sites (Bellenden Ker and Paluma) sample sizes were large enough to suggest very strongly that Bd was probably absent from particular microhylid populations. The absence of Bd from microhylid populations at Paluma is an interesting finding, as Bd has occurred in the area since at least 1990, and individuals of at least two frog species (*Litoria caerulea* and *L. genimaculata*) have been found infected with Bd (Kenyon 2008; S. Chapman and R. Alford pers. comm.). Smaller sample sizes make the results for other sites less conclusive, however, treating either the 493 individuals that returned negative diagnostic qPCR results, or the total of 595 individuals (including those examined only histologically) as a random sample representative of all WT microhylids, the upper 95% binomial confidence limit for the true prevalence of infection is less than 1%. These data indicate that the prevalence of Bd in WT microhylids is very low in nature, and suggests that microhylids have very low susceptibilities to infection by the pathogen.

It is possible that our results may include some false negatives. Low infection intensity, inhibitors, and degradation of fungal DNA can all reduce the accuracy of qPCR results. Inhibitors may be present in a sample if foreign material such as dirt or detritus is picked up on a swab, or may be present in skin secretions of some species or life stages (Hyatt *et al.* 2007). However, standard protocol in both the JCU and AAHL/CSIRO PCR laboratories includes a positive control in each PCR run that should reveal the presence of inhibition. No evidence of inhibition was detected in our samples. Some samples, taken in remote areas, were not immediately refrigerated, which could have led to degradation of fungal DNA. However, this effect was minimised, as air temperatures at the sampling sites were typically below 30$^{o}$C, and

swabs were always refrigerated or stored on ice as soon as possible after the samples were taken. Hyatt *et al.* (2007) demonstrated that storing swabs at 23°C in the laboratory for at least six months did not reduce detection sensitivity. All historical samples analysed using qPCR were fixed and stored only in ethanol, and it is known that this does not degrade the sensitivity of PCR tests (Boyle *et al.* 2004). Hyatt *et al.* (2007) demonstrated that in all but the very early stages of infection, qPCR assays of toe-clips have very similar sensitivity to swab samples. Our 493 diagnostic qPCR results are thus unlikely to include many false negatives. In summary, although it is not possible to rule out with 100% certainty the possibility that inhibition or sample degradation may have affected some of our qPCR results, they are very unlikely to have affected a high proportion. Diagnosis via histology is less sensitive than qPCR (Hyatt *et al.* 2007). Our results for the 102 samples diagnosed histologically may have suffered from a higher rate of false negatives than our qPCR samples. However, they form a relatively minor part of our data, and even if false negative rates are assumed to be high, our conclusion that prevalence in WT microhylids must be very low will not be affected.

Our results demonstrate that that Bd infection is very rare in Australian microhylids in the field. There is one report (Kriger and Hero 2006) of infection detected by qPCR in a single individual *Cophixalus ornatus* in the WT. Our data suggest that this individual was unusual and that prevalence in nature is usually very low. One possible explanation for the absence or extremely low prevalence of Bd infections in Australian microhylids in nature is that they may simply not come into contact with fungal zoospores. From their observations of a mass die-off event at El Cope, Panama, Lips *et al.* (2006) postulated that chytridiomycosis emerges at a site and 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 and Cornelius 2004). Riparian species may be more susceptible to transmission of the disease because zoospores can survive in water, however, exclusively terrestrial species can also be infected (Burrowes *et al.* 2004; Lips *et al.* 2006; Puschendorf *et al.* 2006a). Lips *et al.* (2008) found that Bd was present in the environment during an epidemic, and this supports the hypothesis that Bd can be transmitted by contaminated environmental substrates. This suggests that Australian microhylids should not completely escape infection through lack of opportunity for transmission. Although they are not known to enter water, they occur commonly within a few meters of streams (pers obs.) , and the substrates they come into contact with are frequently transited by more aquatic species as they move into and out of the forest (Rowley and Alford 2007).

Although reduced opportunities for transmission may contribute to the extremely low prevalence or absence of Bd infection from Australian microhylids, it seems likely that they are also highly resistant to infection by the pathogen. Resistance to infection is probably determined by many factors, including environmental conditions, host behaviour, and pathogen and host biology. Innate or acquired immune responses may provide microhylids with inherent protection from infection by Bd. Like other vertebrates, amphibians have well-developed immune defences, featuring both adaptive and innate responses (Duellman and Trueb 1994; Carey *et al.* 1999; Apponyi *et al.* 2004). Innate immune defences in amphibian skin may be important in conferring resistance to chytridiomycosis, as limited lymphocytic infiltration in the chytrid-infected skin of frogs suggests that these animals have a poor cell-mediated immune response against Bd (Berger *et al.* 1998; Pessier *et al.* 1999), and acquired immunity to fungal pathogens has not been demonstrated (Carey *et al.* 1999). Innate immune defences such as the production of antimicrobial peptides have been studied in some detail, and secreted peptide mixtures from the skin of a range of amphibian species can inhibit Bd growth and may be important in maintaining protection from infection (Rollins-Smith *et al.* 2002; Woodhams *et al.* 2005). 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 which inhibit the growth of a range of microbes and fungi, including Bd (Austin 2000; Harris *et al.* 2006). Immune responses, whether they are innate or acquired, are expected to vary among lineages (Carey *et al.* 1999). Amphibian species

that lay eggs in terrestrial nests that are brooded or tended by parents may be preadapted to being highly resistant to Bd because they 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 brooded egg clutches (Austin 2000).

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 resolve the reasons for the absence or very low prevalence of Bd infection in this family, and to determine whether its cause or causes may be transferable to more susceptible taxa.

Acknowledgements
This research was supported by funding from the Australian Rainforest Co-operative Research Centre, and by tender 43/2004 from the Australian Department of Environment and Heritage (R. Alford, principal investigator), 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). Animal ethics permission was obtained from the James Cook University Animal Care and Ethics Committee (A931 and A960). KFH was supported by an Australian Postgraduate Research Scholarship. Thanks also to R. Hobbs, C. Steel and many volunteers for assistance in the field; and thanks to A. Felton, C. Hoskin, P. Brooke and Y. Williams for access to historic samples.

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Appendix 1.1. Global records of Bd infection in terrestrial, direct-developing frog species

| Family | Species | Location | Reference |
|---|---|---|---|
| Plethodontidae | *Bolitoglossa colonnea* | Panama | (Lips *et al.* 2006) |
| | *B. schizodactyla* | Panama | (Lips *et al.* 2006) |
| | *Oedipina collaris* | Panama | (Lips *et al.* 2006) |
| Leptodactylidae | *Craugastor azueroensis* | Panama | (Lips *et al.* 2006) |
| | *C. bransfordii* | Panama | (Lips *et al.* 2006) |
| | | Costa Rica | (Puschendorf *et al.* 2006a) |
| | *C. bufoniformis* | Panama | (Lips *et al.* 2006) |
| | *C. cerasinus* | Panama | (Lips *et al.* 2006) |
| | *C. crassidigitus* | Panama | (Lips *et al.* 2006) |
| | | Costa Rica | (Puschendorf *et al.* 2006a) |
| | *C. fitzingeri* | Costa Rica | (Puschendorf *et al.* 2006a) |
| | *C. gollmeri* | Panama | (Lips *et al.* 2006) |
| | *C. megacephalus* | Panama | (Lips *et al.* 2006) |
| | *C. noblei* | Panama | (Lips *et al.* 2006) |
| | *C. podiciferus* | Panama | (Lips *et al.* 2006) |
| | | Costa Rica | (Puschendorf *et al.* 2006a) |
| | *C. punctariolus* | Panama | (Lips *et al.* 2006) |
| | *C. tabasarae* | Panama | (Lips *et al.* 2006) |
| | *C. talamancae* | Panama | (Lips *et al.* 2006) |
| | | Costa Rica | (Puschendorf *et al.* 2006a) |
| | *Eleutherodactylus aurilegulus* | Honduras | (Puschendorf *et al.* 2006b) |
| | *E. emcelae* | Panama | (Speare and Berger 2004b) |
| | *E. caryophyllaceus* | Panama | (Lips *et al.* 2006) |
| | *E. coqui* | Puerto Rico | (Burrowes *et al.* 2004) |
| | | Hawaii | (Beard and O'Neill 2005) |
| | *E., cruentus* | Panama | (Lips, 2006; (Speare, 2004) |
| | *E. diastema* | Panama | (Lips *et al.* 2006) |
| | *E. karlschmidti* | Puerto Rico | (Burrowes *et al.* 2004) |
| | *E. melanostictus* | Costa Rica | (Lips *et al.* 2003) |
| | *E. museosus* | Panama | (Lips *et al.* 2006) |
| | *E. ridens* | Panama | (Lips *et al.* 2006) |
| | *E. saltator* | | (Speare and Berger 2004b) |
| | *E. vocator* | Panama | (Lips *et al.* 2006) |
| | *Gastrotheca cornuta* | Panama | (Lips *et al.* 2006) |
| Leiopelmatidae | *Leiopelma archeyi* | New Zealand | (Speare and Berger 2004b) |

## ***Project 3.2 Anti-fungal skin peptides of microhylids***

Kim F. Hauselberger

Introduction

Infectious agents are forces that generate and maintain biodiversity, and are important drivers of community dynamics (Anderson, 1986; McCallum, 1995; Cleaveland, 2001; Hudson, 2001). Chytridiomycosis is an emerging infectious disease of amphibians caused by the chytrid fungus *Batrachochytrium dendrobatidis* (Bd), and is responsible for causing mass mortality, population declines and extinctions of amphibian species world-wide (Berger, 1998; Daszak, 1999; Lips, 1999; Longcore, 1999). The intensity of infection and the extent of pathological effects appear to depend strongly on the host species, as the nature of population losses has varied among species and regions of the world (Wake, 1991; Fisher, 1996; McDonald, 1999). Some species have declined to the point of extinction, some have declined and then recovered, and others have appeared completely unaffected by the fungus (Blaustein, 1990; Retallick, 2004; Lips, 2006). These observations indicate that amphibian species have varying levels of resistance to this pathogen.

Multiple factors are likely to underlie disease resistance, including environmental conditions and pathogen and host biology. Certain aspects of a species' physiology may either kill Bd, prevent infection, or impede fungal growth and the ability to cause disease (Carey, 1999; Pessier, 1999; Woodhams, 2005). As in all vertebrates, amphibian skin is the primary barrier against infection by environmental pathogens, and skin is the site of infection by Bd. Limited lymphocytic activity in the skin of chytrid-infected frogs suggests that they have a poor cell-mediated immune response against Bd (Berger, 1998; Pessier, 1999), and acquired immunity to fungal pathogens has not been demonstrated (Carey, 1999). This lack of acquired immune responses of frogs to Bd suggests that innate immune mechanisms may be major factors contributing to disease resistance.

Multiple innate defense mechanisms appear to be involved in the resistance of amphibians to chytridiomycosis (Woodhams, 2007), however, one mechanism that may be particularly important in reducing or preventing infection is antimicrobial peptides (AMPs) (Rollins-Smith, 2002; Rollins-Smith, 2002; Rollins-Smith, 2005; Woodhams, 2005). Granular glands in the dermal layer of amphibian skin produce and store an array of host-defensive, bioactive substances, including AMPs, which form an integral part of amphibian innate immune systems (Nicolas, 1995; Rinaldi, 2002; Apponyi, 2004; Pukala, 2006). AMPs are small (10-46 amino acid residues), cationic, hydrophobic compounds which are active against bacteria, yeast, fungi, protozoa, and viruses (reviewed in (Nicolas, 1995; Rinaldi, 2002; Apponyi, 2004)). Each species produces its own unique suite of peptides that may act individually or synergistically against a variety of pathogens (Erspamer, 1994; Simmaco, 1998; Rinaldi, 2002; Apponyi, 2004; Pukala, 2006; Woodhams, 2006). Little is known about the natural triggers of peptide secretion (Rollins-Smith, 2002), however, alarm responses to predators or injury may activate the sympathetic nervous system (Nicolas, 1995; Simmaco, 1998). This, in turn, stimulates adrenergic receptors, which causes granular glands to release their contents on the surface of the skin, often in large quantities (Rollins-Smith, 2002; Rollins-Smith, 2005). Electro-stimulation of the skin, or exposure to adrenergic agents such as epinephrine or norepinephrine, are techniques that artificially induce secretion of the

contents of the granular glands in amphibians (Benson, 1969; Tyler, 1992). Whilst the concentrations of AMPs present on the skin of healthy, resting amphibians are unknown, the concentrations released in skin secretions following artificial stimulation can exceed 1mg/ml (Tyler, 1992). After depletion, skin peptides may be resynthesized within days to weeks (Erspamer, 1994).

Over 300 amphibian AMPs have been isolated and described, and *in vitro* experiments show that a range of peptides are active against Bd (Rollins-Smith, 2002; Woodhams, 2005). Natural peptide secretions of four Australian frog species (*Litoria cearulea*, *L. chloris*, *Mixophyes fasciolatus* and *Limnodynastes tasmaniensis*) have inhibited Bd growth *in vitro*, and the effectiveness of AMPs was significantly correlated with species' resistance to chytridiomycosal infection (Woodhams, 2007). Population trends associated with chytridiomycosis in Australian rainforest frogs have also been correlated with the effectiveness of skin peptide defenses against Bd. Natural peptide mixtures from five stream-associated Queensland species (*Nyctimystes dayi*, *Litoria genimaculata, L. leseuri, L. nannotis,* and *L. rheocola*) inhibited growth of Bd *in vitro*, and effectiveness of peptides was significantly negatively correlated with the degree of decline of species (Woodhams, 2005). That is, the species with the most potent AMPs (*L. leseuri* and *L. genimaculata*) did not suffer from population declines, whereas the species with the least potent AMP activity (*L. rheocola* and *N. dayi*), have experienced population declines associated with chytridiomycosis (Woodhams, 2005).

In addition to physiological factors, interspecific behaviour is an aspect of host biology that may explain why some frog species succumb to chytridiomycosis when other species do not. Microhabitat use and thermoregulatory behavior may alter the extent or likelihood of initial contact with Bd or may affect the growth rate of pathogen populations on infected hosts, and may thus alter susceptibility to chytidiomycosis (Woodhams, 2003; Rowley, 2007; Rowley, 2007). Frog species with a life-history that involves direct development of terrestrial eggs into froglets, and which excludes a tadpole stage, have not suffered from extensive population declines in comparison with aquatic breeding species (Lips, 1999; McDonald, 1999; Lips, 2006; Hero, 2005; Woodhams, 2005; Puschendorf, 2006). Tadpoles are believed to be an important life-stage with respect to disease transmission, as they stand a high chance of being exposed to the fungus' aquatic zoospores (Daszak, 1999). In addition to the absence of a tadpole stage in development, direct-developing species commonly exhibit some form of parental care of the developing egg clutch, and brooding adults may prevent fungal attack of eggs by spreading anti-microbial skin secretions around the eggs, or by providing antimicrobial substances produced by their skin microbiota (Duellman, 1994; Crump, 1995; Harris, 2006). Studies have shown that when amphibian embryos of species that brood eggs are deserted, or adults are removed from nests, embryos often succumb to fungal infections (Forester, 1979; Townsend, 1984; Simon, 1993). Having antifungal agents to protect developing egg clutches may predispose terrestrial breeders to having stronger resistance to Bd than more aquatic species.

Whilst research has focused on measuring the AMP effectiveness of species that have been affected by Bd, little work has been carried out on species that have not appeared to suffer from declines associated with chytridiomycosis. Determining the effectiveness of AMPs in species that have not undergone population declines is necessary to determine whether resistance in these species is conferred by innate immune responses.

The Australian Microhylidae exhibit direct development, and adults brood eggs, which are laid in terrestrial nests (Zweifel, 1985). They inhabit environments that are favourable to Bd, and occur where sympatric species have suffered from population declines associated with chytridiomycosis (Richards, 1993; McDonald, 1999; Richards, 2005), however, they appear to be unaffected by the disease. A large survey of wild populations of seven microhylid species found no Bd infection (Project 3.1), and no microhylids appear to have suffered from population declines in northern Queensland, as numbers appear to be stable (Project 3.1; Richards, 2005; Williams, 2007). In laboratory experiments, at least one microhylid species (*Cophixalus ornatus*) has been successfully infected, but infection levels were low, and frogs appeared to clear infection over time (Project 3.3). This study aimed to determine whether Australian microhylids possess innate immune mechanisms, in the form of AMPs, that prevent infection by Bd and the development of chytridiomycosis.

## Methods

Skin peptide samples from six species of microhylid frogs were collected and used in challenge assays(Rollins-Smith, 2002; Rollins-Smith, 2006) to determine whether they could inhibit the growth of Bd.

### Skin peptide induction and collection procedure

Beginning in 2005, four 500-m transects were surveyed for microhylids within the Wet Tropics bioregion (WT) of northern Queensland (Table 11). Individual frogs were located via their mating calls, and were captured in an inverted, labeled, plastic sandwich bag with a handful of leaf litter. Call sites were individually marked, to allow release of individuals at the point of capture. To minimise contact and disturbance, males were released at their calling sites as soon as possible after measuring, identification, and peptide collection had taken place (usually <3hr).

Table 11. Locations of collection sites within the Wet Tropics region of northern Queensland.

| Label | Location | Latitude S | Longitude E | Elevation (m) |
|---|---|---|---|---|
| AU10 | Charmillin Ck | 17°40.86' | 145°31.19' | 1000 |
| CU8 | Mt Lewis | 16°35.38' | 145°17.35' | 800 |
| CU10 | Mt Lewis | 16°35.21' | 145°16.36' | 1000 |
| Pal | Paluma | 19°00.42' | 146°12.43' | 900 |

Individuals were weighed using a digital balance (Sper Scientific, China, pocket balance 100009) to the nearest 0.01g. Snout vent length (SVL) was measured three times using dial callipers (to the nearest 0.1mm), and an average of the three readings was calculated. To ensure that Bd was not transported among frogs, or field sites, strict hygiene protocols were followed (Hygiene Protocol for the Control of Diseases in Frogs (NSW Parks and Wildlife Service, 2001; Speare, 2004)). Tools used for measurement and identification purposes were disinfected with 100% ethanol and flaming (where possible) and individuals were captured in new plastic bags, and handled using disposable gloves. Boots and field equipment were washed and allowed to sun-dry between field sites.

To obtain AMP samples, frogs were subcutaneously injected dorsally (0.01 mL/g frog weight) with norepinephrine bitartrate salt (Sigma N-5785, St. Louis, USA) at 10nM concentration in phosphate buffered saline, using a 30 gauge (0.30mm) x 8mm needle (BD Ultra-Fine$^{TM}$ II, Becton, Dickinson & Co., NJ, USA)(Rollins-Smith, 2005). This method provides a standardised stimulus to induce skin secretions from small amphibians. At this moderate level of stimulation, the granular glands release significant quantities of material, but peptide stores are not fully depleted and are naturally replenished over a relatively short period(Rollins-Smith, 2005).
After injection, frogs were placed in a disposable 250ml specimen jar with 50 ml of collecting buffer (50 mM sodium chloride, 25 mM sodium acetate, pH 7.0), and were bathed in this solution for 15 min while skin secretions accumulated. Individuals were observed to ensure that they remained largely submerged in solution during sample collection. Animals were then removed from the container, washed with distilled water, and replaced into individual bags for later release at the point of capture. The collecting buffer solution was acidified with 1ml of 50% HCl immediately on removal of the frog, in order to inactivate endoproteases that are also secreted by the skin (Resnick, 1991).

Partial purification of skin peptide mixtures
Skin peptides were partially purified as described by (Goraya, 1998; Goraya, 2000; Woodhams, 2005). Using a sterile, 60mL disposable syringe (Livingstone International Pty. Ltd., NSW, Australia), the collecting buffer solution containing skin peptides was pushed through an activated $C_{18}$ Sep-Pak Cartridge (WAT020515, Waters Corporation, Milford, MA, USA). Sep-Pak filters were activated by pushing 10 mL of methanol and 10 mL of Buffer A (0.1% HCl) through the filter. Once filters were activated they were stored in 20mL specimen jars with approximately 2 mL of Buffer A to keep moist. Peptides in the molecular weight range of about 500-10,000 are retained on the Sep-Pak cartridges, whilst other skin proteins pass through and were discarded. Sep-Pak cartridges carrying the peptides were stored in 20mL vials with approximately 2mL Buffer A to keep moist, and transported in an insulated, styrofoam container with ice, until they could be refrigerated at -10°C and stored at James Cook University (JCU) herpetology laboratory.

Elution of sep-pak filters
Peptides bound to the Sep-Pak cartridges were removed from the filters by eluting them with Buffer B (70% acetonitrile, 29.9% water, 0.1% triflouroacetic acid (TFA; v/v/v)). Sep-Pak filters were connected to a peristaltic pump (Gilson Minipuls 2), and 10mL of Buffer A was pushed through the filters at a speed of ~2.5 ml/min. Each individual filter was placed into a labeled 50mL centrifuge tube, and had 21mL of Buffer B pushed through at a speed of ~0.65 ml/min. Sep-Paks were then removed from the pump and stored in their original specimen jars, which were refrigerated (at 4°C), for future reference. Samples were collected immediately after agitating the centrifuge tube by spinning it with an automatic vortex, so that peptides would be evenly distributed in solution. A 1ml aliquot of each peptide solution was pipetted into a labeled Eppendorf tube. These 1ml samples were stored in a freezer (-10°C) for use in protein assays, and the remaining 20mL peptide solutions were stored at -10°C for rotary evaporation and freeze drying.

Rotary evaporation and freeze drying of peptide samples
The 20mL peptide solutions were concentrated using a rotary evaporator, to remove the acetonitrile added during elution. Samples were heated in a water bath at 50ºC, and

were reduced by evaporation from 20mL to 4-8mL. Samples were then stored at -80°C until they were freeze-dried. A Dynavac freeze drier (Vic, Australia) set at -40°C was used to reduce the water content of samples, and samples were reduced from ~4-8ml to <1mL. As peptides are partially hydrophobic, samples were freeze-dried to a volume that would allow reconstitution with water.

Protein assays

The total skin peptides per sample recovered after Sep-Pak purification were determined by MicroBSA Assay (Pierce, Rockford, IL, USA). The 1mL aliquot samples taken from eluted Sep-Paks were diluted with sterile, high-performance liquid chromatography (HPLC) water to either: 1:2, 1:3, 1:5, or 1:10 of their original concentrations depending on the amount of peptides in the sample. Two dilutions from each sample were prepared in triplicate, so they could be compared. We used a MicroBSA Assay kit, following manufacturers instructions (Biotechnology, 1997), except that we used bradykinin synthetic peptides (RPPGFSPFR; Sigma Chemical, St. Louis, MO, USA) to establish a standard curve, instead of bovine serum albumin (BSA), because bradykinin synthetic peptides allow better detection of small antimicrobial peptides (Rollins-Smith, 2002). After pipetting samples into 96-well, flat-bottomed microtiter assay plates (Costar 3596, Corning Inc., NY, USA), plates were incubated at 37°C for two hours, and read on a spectrophotometer (Multiscan Ascent, Thermo Electron Corporation, Shanghai, China) at an absorbance of 540 nm. Readings from each of the two dilutions for each sample were averaged, giving a measurement of the concentration of protein in each sample.

Rehydration and purification of peptides to a known concentration

After determining the concentration of total protein in samples using the protein assays, peptide samples were reconstituted to a known concentration. The peptide samples were reconstituted with HPLC water to either 1mg/mL or 5mg/mL depending on the volume of the sample. Each peptide sample was thawed, and measured to the nearest µl by using a micropipetter to draw up the fluid until the entire sample was removed. The amount of the total peptide sample was then subtracted from the total protein per sample as determined by the protein assay, to give the amount of sterile HPLC water that needed to be added to the sample. Using this value, the required amount of sterile HPLC water was added to each sample to make a known concentration of peptides. This mixture was then further purified using a sterile 0.22 µm filter (Millex GV, Millipore Corp., Bedford, MA, USA), and transferred into labeled, sterile, 2ml o-ring microtubes (Sarsedt, Germany).

Serial dilutions of peptide mixtures

Once peptides were rehydrated to a known concentration, a set of serial dilutions of each sample was prepared for challenge assays. Depending on the total amount of peptide solution available for each sample, a range of dilutions were created using sterile HPLC water as the diluting agent. Dilutions of 1:1, 1:2, 1:4, and 1:8 were prepared, with each sample having a minimum of one and a maximum of four dilutions prepared. Dilutions were prepared to determine the minimum concentration of peptide solution that would cause an inhibition of Bd growth in the challenge assay.

Culture and maintenance of *Bd*

A single isolate of Bd was used in growth inhibition assays and all work associated with the fungus was carried out under sterile conditions in R. Speare's laboratory in the School of Veterinary and Biomedical Sciences at JCU. Type isolate (GibboRiver-

L.lesueuri-00-LB-1) was collected and isolated from a diseased *Litoria leseurii* in Gibbo River, Queensland by L. Berger in 2000. This isolate was cryoarchived soon after isolation, and the revival date was 6/3/08. The isolate was cultured following the protocol of (Longcore, 1999) but it was grown in 50% nutrient tryptone-gelatine hydrolysate-lactose (TGhL) broth medium (8 g tryptone, 2 g gelatin hydrolysate, 1 g lactose, 1000ml distilled water) as this increased Bd culture growth.
Bd was grown in tissue culture flasks (TPP9025, JRH Biosciences) to ensure the maximum number of zoospores in solution. Stock culture was passaged at intervals of 4-7d to assure that cells were in an active phase of growth. After 4-7d incubation at 22-23°C, the maximum number of zoospores in culture was reached, and the culture flasks were scraped using a TPP cell scraper (CSL Biosciences). 10mL of refrigerated (3°C) stock of 50% nutrient TGhL broth was pipetted into a labeled, sterile tissue culture flask, and left at room temperature for 10min allowing the broth to warm up. The Bd culture was passaged by pipetting 1mL of the stock solution into the new broth flask which was then incubated at 23°C.

Production of zoospores
To collect Bd zoospores for inoculation in assays, stock solution was transferred from 50% TGhL broth and grown on 50% nutrient TGhL agar (8 g tryptone, 2 g gelatin hydrolysate, 1 g lactose, 10 g agar, 1000ml distilled water) in 9-cm culture dishes (Sarstedt, Australia, Inglefarm, SA). After culture flasks were scraped (as described above), 0.75mL of stock solution was transferred to agar plates with a sterile transfer pipette. Innoculated dishes were left open for approximately 5-10 minutes in a laminar flow hood until the added broth was dry. Petri dishes were labeled, sealed with Parafilm®, and incubated at 23°C for a minimum of three days to achieve maximum zoospore release levels.

Harvesting zoospores
Zoospores were harvested using methods described by (Rollins-Smith, 2002). Agar plates were flooded with 3ml 50% nutrient TGhL broth and were left to sit for 20min while zoospores accumulated in the liquid. Supernatant from the agar plate was collected using a sterile transfer pipette, and was vacuum filtered through an autoclaved 20 μm spectra/mesh nylon filter (Spectrum 722-05067-000) to remove mature zoosporangia that might influence culture growth. This procedure provides a zoospore population that is 99% pure (Rollins-Smith, 2002). Zoospores may stay motile (thus infective) for up to 24 hours, however, most encyst before 24 hours (Longcore, 2008); all zoospores collected from agar plates were used in assays <2hr after harvesting.

Zoospore concentration
The concentration of zoospores collected from agar plates was estimated by counting live and moving zoospores using a haemocytometer. Five counts were completed on 1 square of 0.005mm$^2$ area, and this was replicated twice. The two results were averaged, to create the final count of zoospores, and this was multiplied to give a zoospore count per mL of culture. A concentration of approximately 1x10$^6$ zoospores/mL was used in challenge assays, so the zoospore count as estimated by a haemocytometer was diluted to the relevant concentration using 50% nutrient TGhL broth as the diluting agent.

*Bd* growth inhibition assay
Serial dilutions of natural peptide mixtures were used in a challenge assay for their ability to inhibit growth of Bd. The assay followed methods as described by (Rollins-

Smith, 2002; Rollins-Smith, 2002; Rollins-Smith, 2002). To determine minimum inhibitory concentration (MIC µg/ml) and percent chytrid zoospore growth inhibition at a peptide concentration of 50µg/ml, approximately $1x10^6$ zoospores in 50µl of broth were plated in five replicates onto 96-well, tissue culture treated microtitre assay plates (Costar 3596, Corning Inc., NY, USA) to which 50µl of the serial dilutions of the peptides were added. Each plate contained three peptide samples at each of the four dilutions (five replicates each) with Bd, ten replicates of Bd culture at 5×105 mL-1 zoospores (positive control), five replicates of non-viable Bd culture that was heat-treated for 30 minutes at 60 ºC (negative control), and 19 replicates of the TGhL culture medium (background absorption correction; Figure 20).

| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| A | + | + | + | + | + | Empty | empty | + | + | + | + | + |
| B | + | + | + | + | + | * | * | * | * | * | * | * |
| C | Peptide sample 1 (µg/ml) | | | | Peptide sample 2 (µg/ml) | | | | Peptide sample (µg/ml) | | | |
| | 1000 | 500 | 250 | 100 | 1000 | 500 | 250 | 100 | 1000 | 500 | 250 | 100 |
| D | ↓ | ↓ | ↓ | ↓ | ↓ | ↓ | ↓ | ↓ | ↓ | ↓ | ↓ | ↓ |
| E | | | | | | | | | | | | |
| F | | | | | | | | | | | | |
| G | | | | | | | | | | | | |
| H | * | * | * | * | * | * | * | * | * | * | * | * |

Figure 20. 96-well plate layout for Bd growth inhibition assay.
+ = positive control (50µl *Bd* + 50µl HPCL water)
- = negative control (50µl *Bd* heat killed + 50µl HPCL water)
* = background absorption correction (100µl TGhL)

Plates were sealed with Parafilm®, and incubated at 23°C for up to 10 days. Plates were read every 24hr on a spectrophotometer (Thermo Electron Corporation, Shanghai, China) at an absorbance of 492 nm. The optical density (OD) of wells was recorded until the positive control samples reached a growth plateau, which usually occurred within 7-9d and did not exceed 10d. In comparison to controls, wells with higher OD readings were interpreted a cell growth, and lower readings were interpreted as an indication of inhibitory peptide bioactivity. Any contaminated wells were removed from the analysis.

Calculation of percentage inhibition in samples
The percentage inhibition of Bd growth in challenge assays was determined by comparing OD readings of the positive control wells on each plate on the day of maximum Bd growth (Dx), with the OD readings of the positive control wells at day zero (D0). Dx was determined by plotting the positive control readings from each plate against time and determining the day where the OD reading reached a plateau. Once this day was determined, the following equation was calculated using the equation formulated by (Kenyon, 2008):
% inhibition = ( 1-[(Dx - D0)/(Dx – D0) + mean positive control)]} x 100
Inhibition of Bd growth was also observed manually by viewing plates under a light microscope and looking for wells that had limited or complete loss of Bd growth. When wells were observed where the fungus showed no signs of growth, the sample was recorded as having 100% inhibition. For each sample that produced 100% inhibition of Bd growth, several further calculations were undertaken.

Minimum inhibitory concentration

The minimum inhibitory concentration (MIC) in μg/ml was calculated by determining the lowest concentration of peptide sample that caused approximately 100% inhibition of Bd growth in the assays. That is, where sample microplate readings were not significantly greater than those observed for negative controls. Where a sample had a substantially greater OD value than the negative control, inhibitory effects were confirmed or denied by visual observation.

Total protein secretion

The total peptide secreted by each individual frog was approximated using the following equation (Woodhams, 2005):

$\sum$ protein secretion ($\mu g/cm^2$) = Protein concentration (μg) / Surface area of frog ($cm^2$)

The concentration of peptides in each sample was determined using the MicroBSA protein assay. The surface area for each frog was approximated using a mathematical equation developed by (McClanahan, 1969) which is calculated using body weight. The equation is as follows:

Surface Area ($cm^2$) = 9.9 x (weight in g)$^{0.56}$

Overall protection of each frog

To determine the overall protection that was afforded to an individual frog, the effectiveness of skin peptide secretions was calculated and compared. This was carried out using an equation that was based on the MIC value of peptide samples and the amount of peptides per surface area (Kenyon, 2008).

$$\text{Overall protection (ml at MIC per cm}^2) = \text{Total mass of peptides} \times \frac{1}{\text{Surface area (cm}^2) \quad \text{MIC}}$$

Statistical analysis

The standard statistic considered in medical and toxicological literature is the LC50, the concentration of an agent that is lethal to 50% of the target population (Sanchez-Bayo, 2007). However, this seems irrelevant in the study of AMPs as a frog surrounded by a concentration of AMPs that reached the LC50 for *Bd* zoospores could presumably still become infected and possibly die as a result of chytridiomycosis. I therefore calculated and analysed the IC100, the concentration necessary to completely inhibit the growth of *Bd.* This is the same as the MIC as described by Rollins-Smith et al. (2003).

To compare the proportion of samples that did and did not show complete inhibition, Fisher's exact tests were used. To determine whether protein secretion was correlated with body mass, I regressed the amount of peptide secretion (μg) on body mass (g). To compare the total peptide secretion of microhylid species I conducted a one-way ANOVA on total peptide secretion ($\mu g/cm^2$) of individuals with complete inhibition of Bd growth. The average total protein secretion in microhylid species was compared with average results for Queensland rainforest frog species *Litoria genimaculata* and *L. rheocola,* as reported by (Kenyon, 2008), using a Student's t-test.

The MIC (μg/ml) of peptide samples that caused 100% inhibition of Bd growth were compared among microhylid species using one-way ANOVAs. Mean MIC equivalents of microhylids were also compared with four south-east Queensland frog species reported by (Woodhams, 2007), using a Student's t-test.

To test for differences in the effectiveness of skin peptides, the overall protection of each individual frog as MIC (μg/ml) equivalents per $cm^2$ surface area was calculated. Overall protection was compared among species using a one-way ANOVA. Average protection values of microhylids were also compared to values for other Queensland rainforest frog species reported by (Woodhams, 2007) and (Kenyon, 2008) using a Student's t-test.

Results

Inhibition of growth of Bd

Natural mixtures of skin peptides were collected from 81 microhylid frogs from six species throughout 2005-07. 16 of the 81 (19.75%) skin peptide samples showed no inhibition of the growth of Bd, 51 samples (63%) showed some inhibition of Bd growth, and 14 samples (17.3%) showed complete inhibition of growth. These results are summarized in Table 12.

Table 12. Results of assays for inhibitory activity of skin peptide samples against the growth of Bd.

| Species | *N* | No inhibition | Partial inhibition | Complete inhibition | Mean % inhibition |
|---|---|---|---|---|---|
| *A. fryi* | 7 | 2 | 4 | 1 | 34.64 |
| *A. pluvialis* | 3 | 1 | 2 | 0 | 12.19 |
| *A. robusta* | 21 | 6 | 12 | 3 | 40.16 |
| *C. aenigma* | 2 | 1 | 1 | 0 | 12.69 |
| *C. neglectus* | 25 | 3 | 18 | 4 | 42.35 |
| *C. ornatus* | 23 | 3 | 14 | 6 | 50.74 |
| Total | 81 | 16 | 51 | 14 | |

In each species that was sampled, some individuals produced peptide secretions that caused at least some inhibition of the growth of Bd. No samples taken from *Austrochaperina pluvialis* or *Cophixalus aenigma* produced complete inhibition of growth, however, sample sizes for these species were low (*N*=3 and *N*=2 respectively). AMPs that completely inhibited Bd growth occurred in four species at four locations (Table 13). For these samples, the percentage inhibition of Bd growth ranged from 92.01-100% in challenge assays.

Table 13. Detaled results for skin peptide samples that showed complete inhibition of Bd growth in challenge assays.

| Species | Date | Site | Protein assay (μg) | % inhibition | MIC (μg/ml) | SA (cm2) | Peptide secretion (mg/mL) | Protection |
|---|---|---|---|---|---|---|---|---|
| *A. fryi* | 21/01/26 | CU8 | 1223.5 | 92.94 | 500 | 15.86 | 77.14 | 0.15 |
| *A. robusta* | 24/01/07 | Pal | 3320 | 94.69 | 1000 | 9.90 | 335.35 | 0.34 |
| *A. robusta* | 24/01/07 | Pal | 1693 | 100 | 500 | 10.34 | 163.80 | 0.33 |
| *A. robusta* | 24/01/07 | Pal | 1959.5 | 95.31 | 1000 | 11.66 | 168.01 | 0.17 |
| *C. neglectus* | 7/12/05 | BK | 902.5 | 100 | 250 | 12.24 | 73.75 | 0.30 |
| *C. neglectus* | 7/12/05 | BK | 1537.5 | 97.01 | 250 | 14.84 | 103.61 | 0.41 |
| *C. neglectus* | 7/12/05 | BK | 1290.5 | 100 | 250 | 9.45 | 136.58 | 0.55 |
| *C. neglectus* | 7/12/05 | BK | 1167.5 | 99.10 | 250 | 11.57 | 100.95 | 0.40 |
| *C. ornatus* | 8/03/07 | AU10 | 1644 | 100 | 500 | 8.68 | 189.49 | 0.38 |
| *C. ornatus* | 23/01/07 | Pal | 2814.5 | 100 | 1000 | 9.04 | 311.38 | 0.31 |
| *C. ornatus* | 24/01/07 | Pal | 1327.5 | 99.11 | 100 | 8.74 | 151.94 | 1.52 |
| *C. ornatus* | 24/01/07 | Pal | 1411.5 | 92.01 | 500 | 9.73 | 145.03 | 0.29 |
| *C. ornatus* | 25/01/07 | Pal | 1946.5 | 98.08 | 1000 | 7.91 | 246.05 | 0.25 |
| *C. ornatus* | 25/01/07 | Pal | 1185.5 | 100 | 500 | 9.33 | 127.03 | 0.25 |

The proportion of peptide samples that caused total, partial or no inhibition of the growth of Bd did not differ significantly among species (Fisher's exact test $p>0.05$; Figure 21). However, within *C. ornatus*, the proportion of peptide samples that did and did not completely inhibit the growth of Bd differed significantly among sites. The proportion of samples from *C. ornatus* at Paluma that inhibited Bd was significantly greater than the proportion of samples froom site AU10 (Fisher's exact test $p=0.03$) (Table 13).

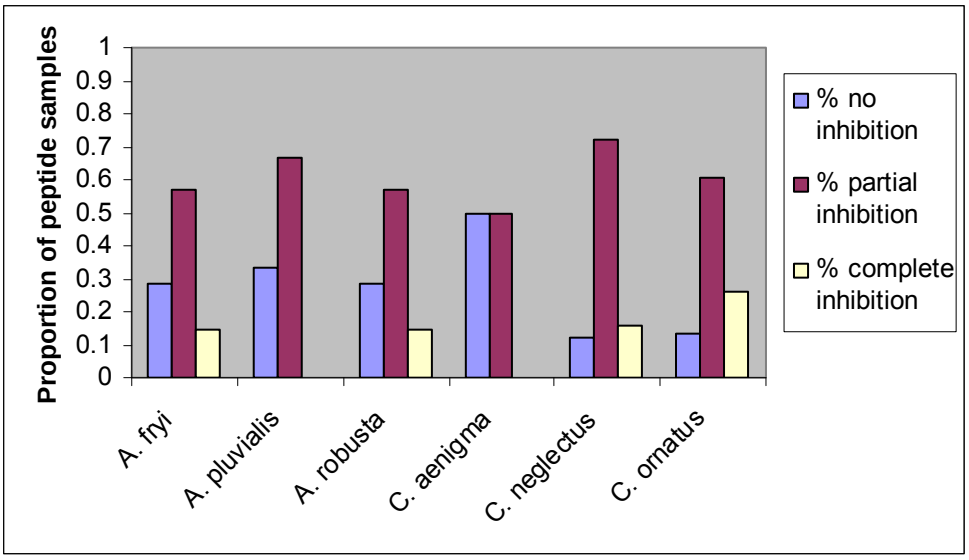


Figure 21. Proportion of peptide samples that showed complete, partial, or no inhibition of Bd growth during challenge assays.

Total skin peptides secreted

For peptide samples that produced partial inhibition of Bd growth (*N*=51), the total amount of peptides secreted (ignoring species identity) was not correlated with body mass ($r^2$=0.01; p=0.49; Figure 22). Separate correlation analyses within species with more than 5 samples showing complete inhibition also showed no significant correlations (*A. robusta* $r^2$=0.04; *C. neglectus* $r^2$=0.02; *C. ornatus* $r^2$=0.17, all P > 0.05).

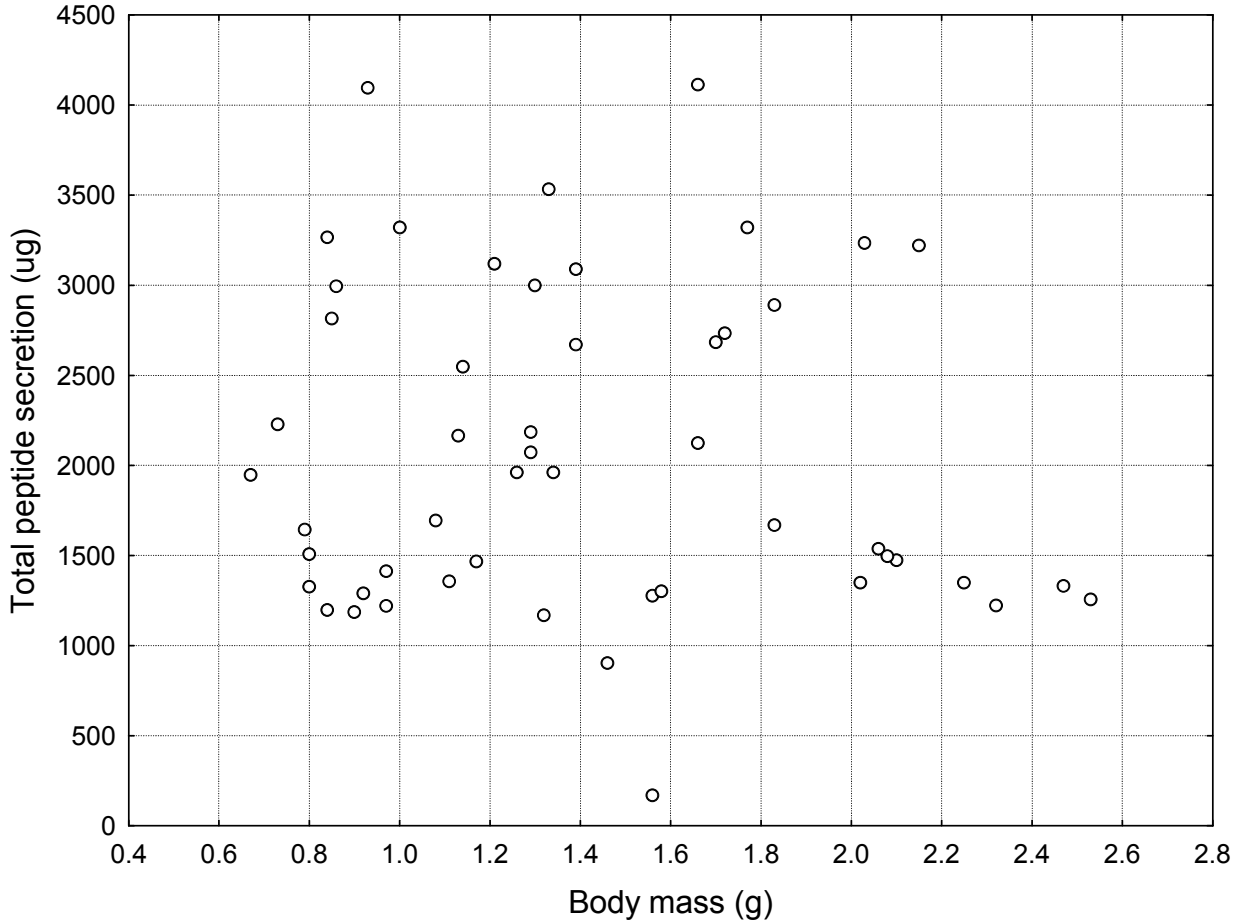


Figure 22. Relationship between total peptide secretion (μg) and body mass (g) in all microhylid species with peptide samples that produced some inhibition of Bd growth (*N=51,* r=-0.10, $r^2$=0.01, P=0.49).

Protein secretion (μg) per $cm^2$ of microhylids that completely inhibited Bd growth, differed significantly among species (ANOVA, $F_{3,14} = 5.409$, P = 0.011). *A. robusta* had the highest average protein secretion per unit area, followed by *C. ornatus*, *C. neglectus*, and *A. fryi* (Figure 23. Table 14).

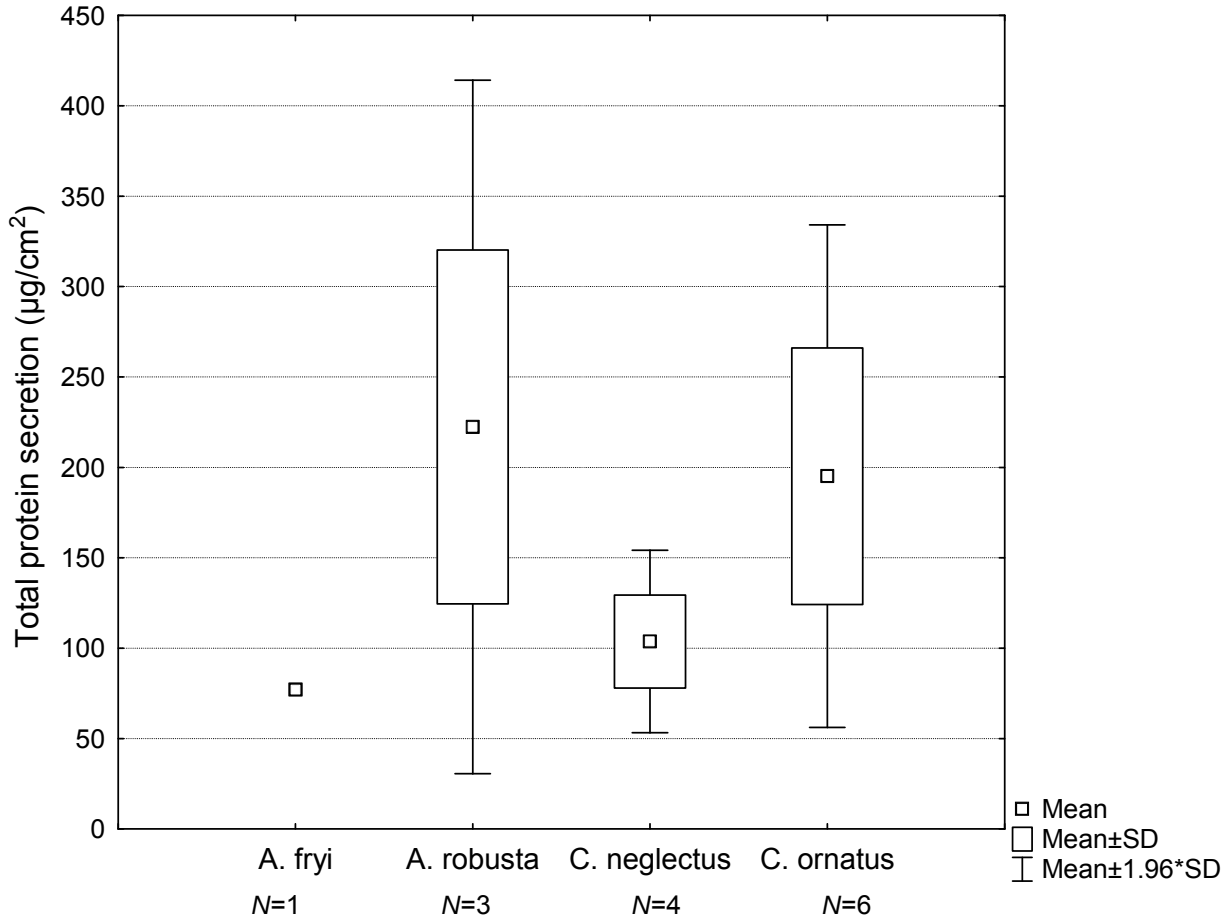


Figure 23. Total protein secretion (µg) per surface area ($cm^2$) of microhylids that produced complete inhibition of Bd growth. Differences among species are significant (ANOVA, $F_{3,14} = 5.409$, $P = 0.011$).

The average total protein secretion (µg) per $cm^2$ of samples that completely inhibited Bd growth were not significantly different from those reported by (Kenyon, 2008) (Fig 24) ($t_4 = 2.776$, $P = 0.891$).

Table 14. Average total protein secretion (µg) per surface area ($cm^2$) for microhylid species and for northern Queensland rainforest frog species as reported by (Kenyon, 2008).

| Species | *N* | Total secretion (µg) per SA |
|---|---|---|
| *Austrochaperina fryi* | 1 | 77 |
| *Auatrochaperina robusta* | 3 | 222 |
| *Cophixalus neglectus* | 4 | 104 |
| *Cophixalus ornatus* | 6 | 195 |
| *Litoria genimaculata* | 160 | 130 |
| *Litoria rheocola* | 56 | 185 |

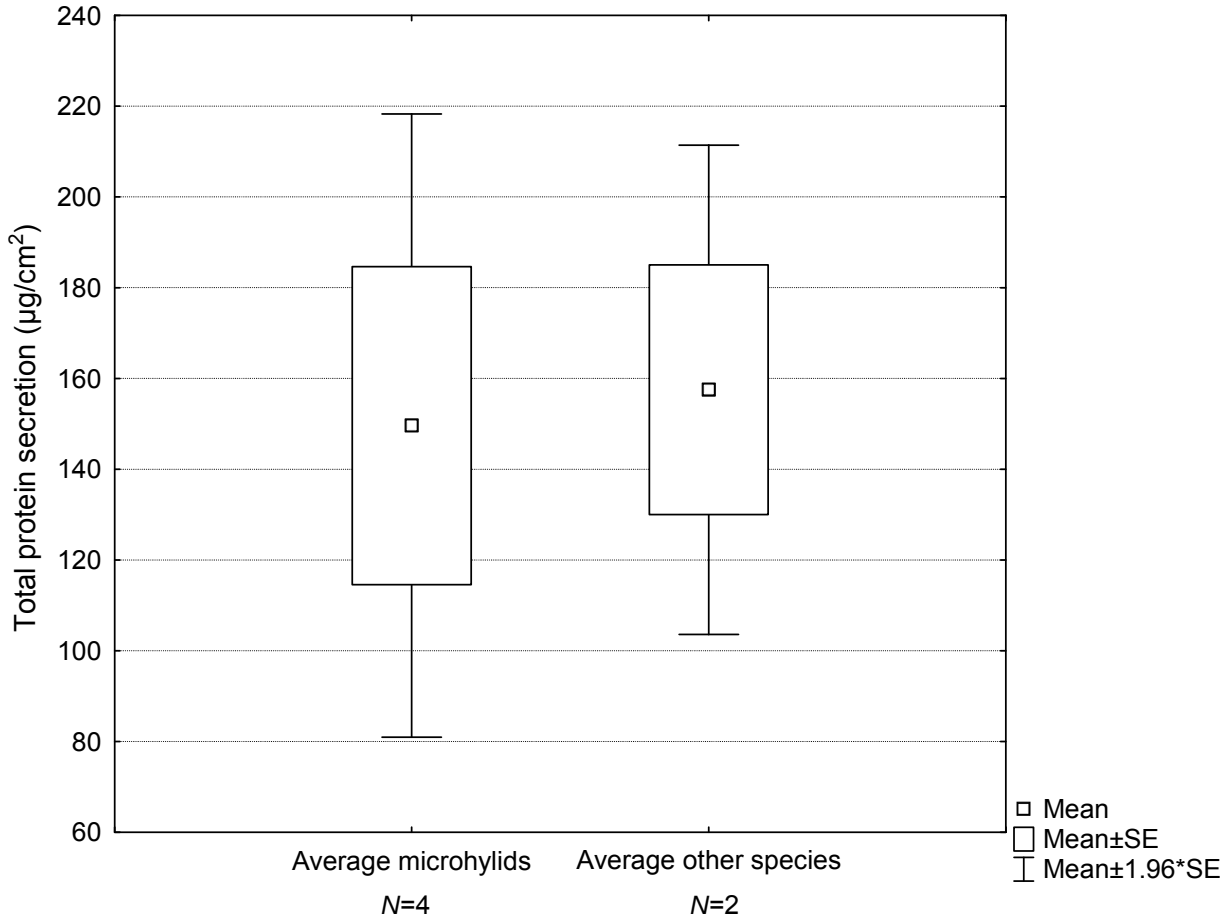


Figure 24. Average total protein secretion of microhylid species (*N*=4), and other (non-microhylid) Queensland rainforest frog species (*N*=2) reported by (Kenyon, 2008). Values were not significantly different among species groups ($t_4 = 2.776$, P = 0.891).

Effectiveness of AMPs (MIC)

For samples that inhibited Bd, the concentration of peptides necessary to completely inhibit growth of the fungus ranged from 100-1000 µg/ml (Table 13). The most potent natural peptide mixture occurred in a secretion from *C. ornatus* that inhibited zoospore growth at a concentration of 100 µg/ml. *A. robusta* had the highest mean MIC value of 833 µg/ml, followed by *C. ornatus* (mean MIC = 600 µg/ml), *A. fryi* (mean MIC = 500 µg/ml), and *C. neglectus* (mean MIC = 250 µg/ml; Table 15). Average MIC values did not differ significantly among microhylids (ANOVA, $F_{3,10} = 2.685$, P = 0.103; Figure 25). A comparison of the overall mean MIC for microhylids with other (non-microhylid) species reported by (Woodhams, 2007) showed that values for microhylids are significantly higher than those species ($t_6 = 2.447$, P = 0.047; Figure 26).

Table 15. Average MIC (µg/ml) values for microhylid species, and south-eastern Australian species as found by (Woodhams, 2007).

| Species | *N* | Average MIC (µg/ml) |
|---|---|---|
| *Austrochaperina fryi* | 1 | 500 |
| *Austrochaperina robusta* | 3 | 833 |
| *Cophixalus neglectus* | 4 | 250 |
| *Cophixalus ornatus* | 6 | 600 |
| *Limnodynastes tasmaniensis* | 20 | 133 |
| *Litoria caerulea* | 20 | 271 |
| *Litoria chloris* | 20 | 261 |
| *Mixophyes fasciolatus* | 20 | 272 |

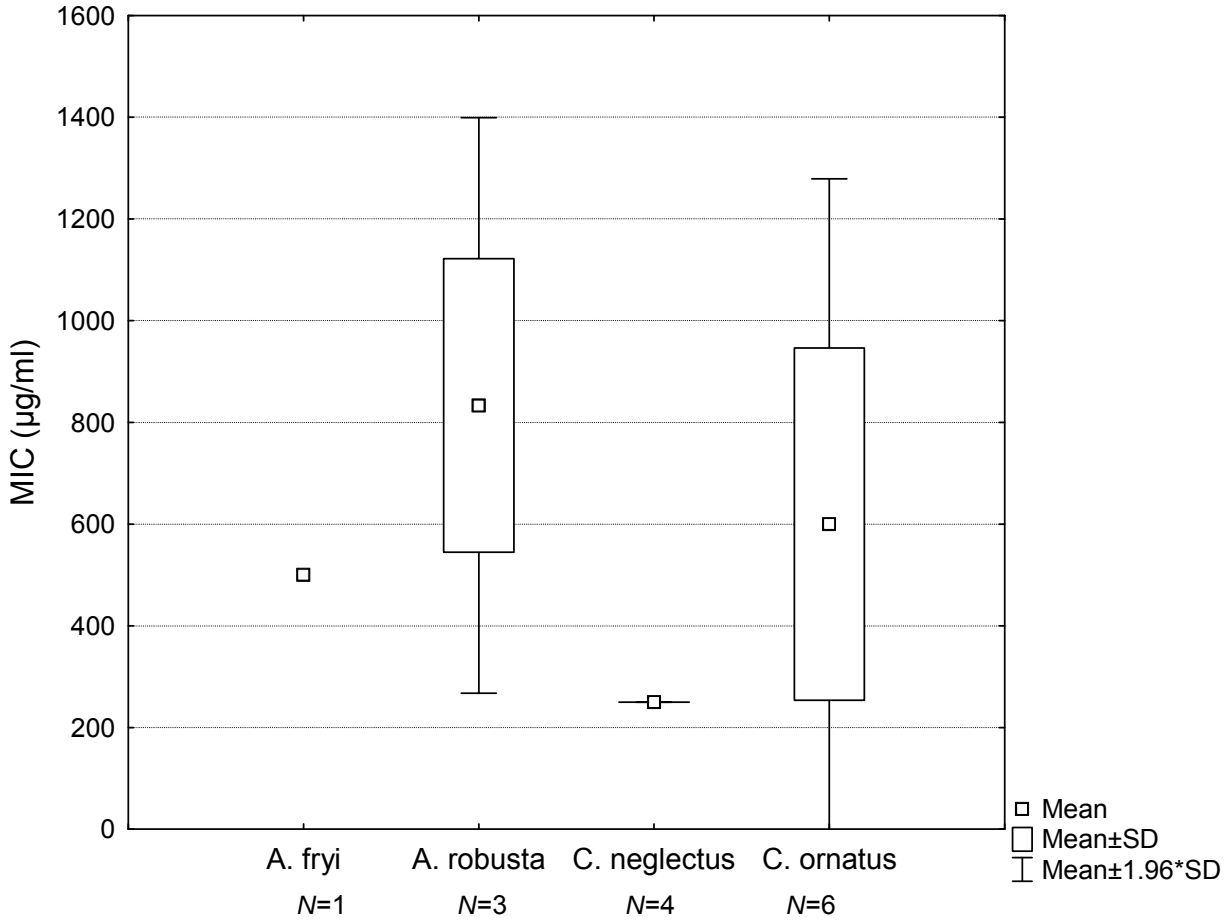


Figure 25. MIC (µg/ml) values for microhylid species that produced complete inhibition of Bd growth. Values are not significantly different between species (ANOVA, $F_{3,10} = 2.685$, $P = 0.103$.

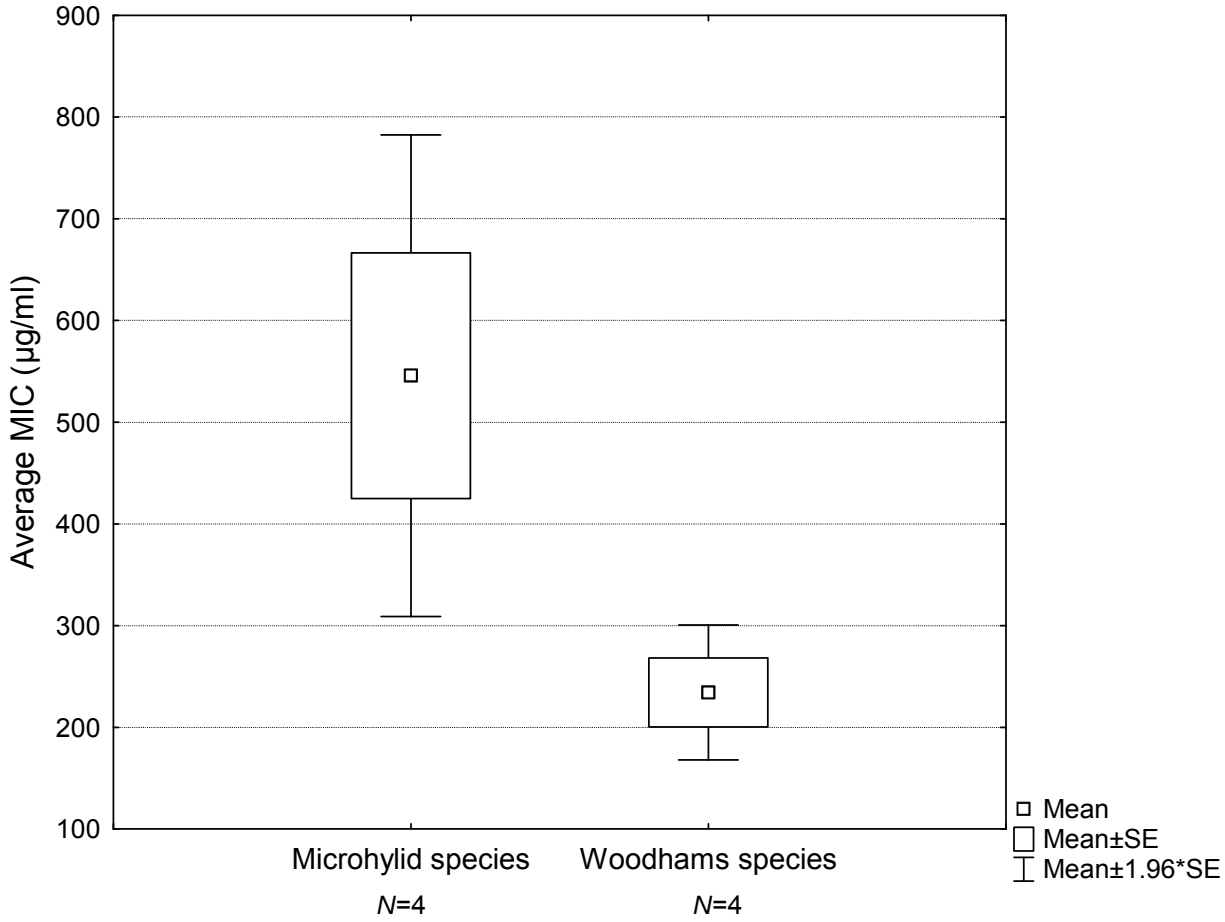


Figure 26. Average MIC (µg/ml) values for microhylid species, and four south-eastern Australian species reported by (Woodhams, 2007). Values for microhylid species were significantly higher than those reported by (Woodhams, 2007) ($t_6 = 2.447$, P = 0.047).

Overall protection of species

When average protection of microhylid species was compared with the results for other Queensland (non-microhylid) frog species (Woodhams, 2007; Kenyon, 2008), values for microhylid species were lower than other species, but were not significantly different ($t_9 = 2.262$, P = 0.122; Figure 27; Table 16). When values for microhylids were compared only with (Woodhams, 2007) results there was no significant difference ($t_7 = 2.365$, P = 0.241; Figure 28), however, when microhylid values were compared to the protection values of (Kenyon, 2008), microhylid protection values were significantly lower ($t_4 = 2.776$, P = 0.002; Figure 29).

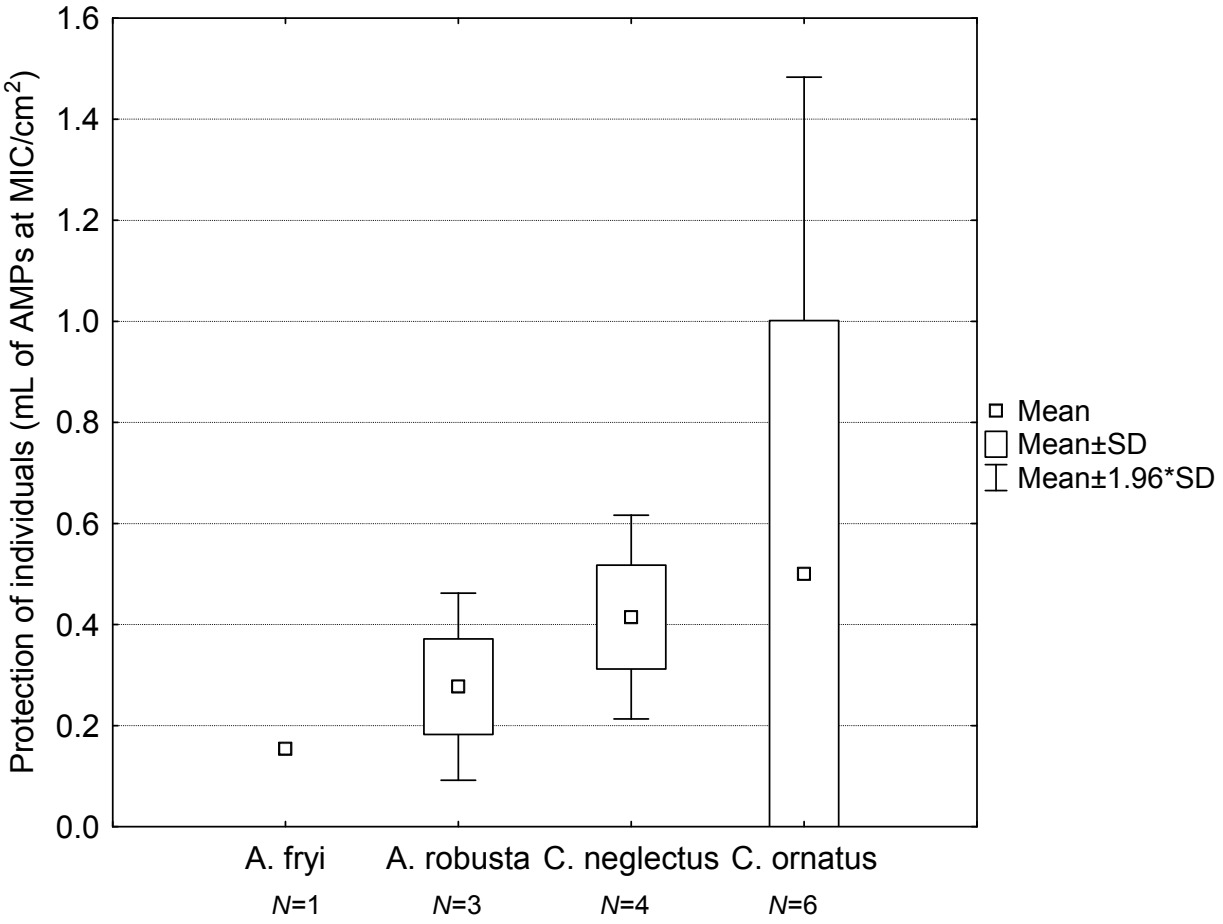


Figure 27. Overall protection afforded by AMPs (mL of AMPs at MIC/cm$^2$) in *A. fryi*, *A. robusta*, *C. neglectus* and *C. ornatus*. There were no significant differences between species (ANOVA, $F_{3,10} = 0.424$, $P = 0.740$).

Table 16. Average overall protection (mL of AMPs at MIC/cm$^2$) microhylid species, and Australian rainforest frog species as found by (Woodhams, 2007) and (Kenyon, 2008).

| Species | *N* | Protection | Researcher |
|---|---|---|---|
| *Austrochaperina fryi* | 4 | 0.15 | Hauselberger |
| *Austrochaperina robusta* | 3 | 0.28 | Hauselberger |
| *Cophixalus neglectus* | 18 | 0.41 | Hauselberger |
| *Cophixalus ornatus* | 14 | 0.50 | Hauselberger |
| *Litoria genimaculata* | 160 | 1.19 | Kenyon |
| *Litoria rheocola* | 56 | 1.35 | Kenyon |
| *Litoria genimaculata* | 20 | 0.31 | Woodhams |
| *Litoria leseuri* | 20 | 0.48 | Woodhams |
| *Litoria nannotis* | 20 | 0.35 | Woodhams |
| *Litoria rheocola* | 20 | 0.5 | Woodhams |
| *Nyctymistes dayi* | 20 | 0.8 | Woodhams |

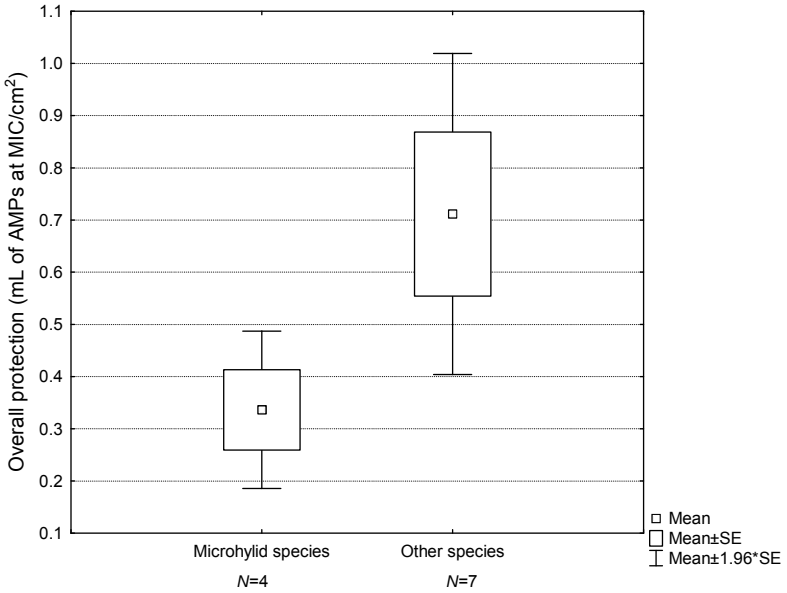


Figure 28. Average overall protection (mL of AMPs at MIC/cm$^2$) of microhylid species, and Australian rainforest frog species (Woodhams, 2007) and (Kenyon, 2008). There was no significant difference between microhylids and other species ($t_9 = 2.262$, P = 0.122).

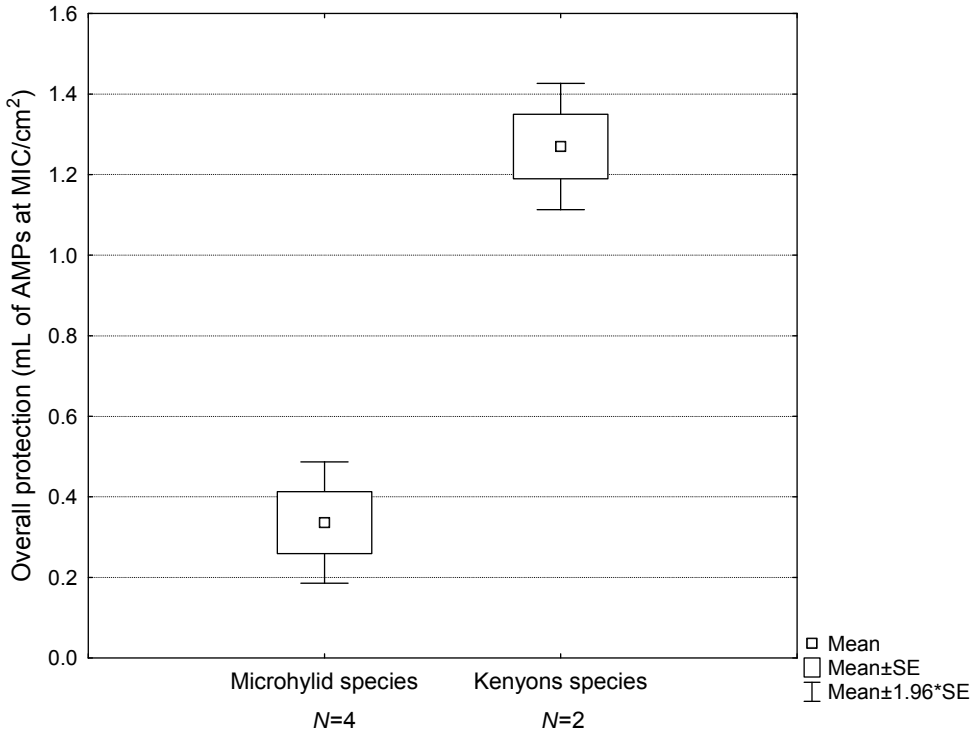


Figure 29. Average overall protection (mL of AMPs at MIC/cm$^2$) of microhylid species, and Australian rainforest frog species as found by (Kenyon, 2008). The values for overall protection in microhylid species were significantly lower than those reported by (Kenyon, 2008) ($t_4 = 2.776$, P = 0.002).

## Discussion

Most amphibian species express an array of peptides as part of their innate immune function (Vanhoye, 2003), and a more complete understanding of the effectiveness of the AMP repertoire of each species can be reached by examining the activity of natural

mixtures of skin peptides. As amphibian chytridiomycosis is characterized by the colonization of epidermal cells with Bd sporangia, AMPs may be a particularly important line of defense in preventing chytridiomycosis. In this study, 65 of 81 individuals sampled produced AMPs with at least some activity against Bd, and 14 (17%) showed 100% inhibition of Bd growth.

Bd infects and replicates within skin cells via motile, reproductive zoospores which attach to the keratinized outer layers of an amphibian host. The zoospores encyst and enlarge to form a zoosporangium which produces up to 300 infectious zoospores (Pessier, 1999). At maturity, the contents of the zoosporangium are released to the exterior of the skin, and the zoospores frequently infect near-by areas of the skin of the same host (Berger, 1998; Pessier, 1999; Longcore, 1999). As skin is the primary site of Bd infection, AMPs may be a particularly important line of defense in preventing infection and progression of chytridiomycosis. Each species produces its own unique AMP repertior (Apponyi, 2004), and peptides may act individually or synergistically against a variety of pathogens (Erspamer, 1994; Simmaco, 1998; Rinaldi, 2002; Pukala, 2006; Woodhams, 2006), Since it is unknown exactly how AMPs act to inhibit Bd, I have examined all possible effects by using natural mixtures of peptides in frog skin secretions, and observing different measures of AMP effectiveness in terms of total secretion, MIC and protection of individuals.

There is great variation in the intensity of infection of frogs with Bd, and not all species will suffer from chytridiomycosis. Several north Queensland, frog species persist in populations that show stable infections with Bd but do not suffer from the effects of chytridiomycosis. For example, *L. genimaculata* shows high prevalence of Bd infection, but apparently a balance between the host and pathogen has been achieved, and individuals do not succumb to the disease (Speare, 2000). Another species, *Taudactylus eungellensis* has suffered from extensive population declines due to chytridiomycosis in the past, but remnant populations of frogs persist with stable infections of Bd (Retallick, 2004). Frog species from the family Microhylidae, however, are particularly unusual among Queensland species, as they not only appear to be highly resistant to chytridiomycosis, but they also seem to be highly resistant to infection by Bd. It is thus of interest to compare the AMP secretions, and the various measures of AMP effectiveness, between microhylids and other species that are more susceptible to this disease. I have compared various measures of AMP effectiveness for microhylids to those calculated for other Queensland frog species, in order to determine whether AMPs may be a reason for the protection from Bd infection that microhylids seem to experience.

There were no significant differences among species in the proportion of peptide samples that completely inhibited Bd, indicating that the level of activity of AMPs was similar amongst microhylids. Whilst samples that produced complete inhibition of Bd growth were not that common for the majority of species and sites (0-26.01%), there was one exception. Five out of six *C. ornatus* samples taken from Paluma showed complete inhibition in challenge assays, and this site had a significantly higher proportion of samples from *C. ornatus* that completely inhibited Bd compared to the site AU10. It is possible that *C. ornatus* produces AMPs that are particularly effective at one site within the WT due to specific environmental traits. Repeated samples of *C. ornatus*, taken on different days, showed high inhibition compared to other sites.

When the samples that completely inhibited Bd growth were compared, there was no significant difference in total protein secretion per $cm^2$ among species. When the overall average protein secretion for each of the four microhylid species was compared with results for *L. genimaculata* and *L. rheocola* (Kenyon, 2008), the results were not significantly different. Thus, microhylids are apparently producing peptides per surface area in quantities similar to other Queensland rainforest frogs.

Microhylid species did not differ significantly in terms of the lowest concentration of peptides that caused 100% inhibition of Bd growth (MIC). The average MIC (μg/ml) values of microhylid peptides that caused complete inhibition of Bd growth was significantly higher than those for four Australian frog species (*Limnodynastes tasmaniensis*, *Litoria caerulea*, *Lit. chloris*, and *Mixophyes fasciolatus*) (Woodhams, 2007). Thus, greater concentrations of the peptides secreted by microhylids are required to inhibit the growth of Bd compared to these other Queensland frog species.

The average level of protection of microhylids (MIC; $\mu gmL^{-1}$) did not differ significantly among microhylid species, nor was it different from the average level of protection found for five Queensland rainforest species *Lioria genimaculata*, *L. leseurii*, *Lit. nannotis*, *Lit. rheocola* and *Nyctimystes dayii* (Woodhams, 2005). However, the average level of protection of microhylids was significantly lower than for two other Queensland rainforest frog species (*L. genimaculata* and *L. rheocola*) (Kenyon, 2008). It is probably more reasonable to compare my results with (Kenyon, 2008) than (Woodhams, 2007), because the procedures used to determine inhibition of Bd growth in (Kenyon, 2008)'s study were the same as mine. Comparing my results with Kenyon (date) suggests microhylids have lower overall protection by AMPs from Bd infection. Although the concentrations of peptides used in challenge assays were consistant with those used in other studies of AMPs in Australian frog species, in most cases (82.72%), peptide samples from microhylids did not completely inhibit the growth of Bd *in vitro*. Although less than complete inhibition could provide protection from the development of chytridiomycosis in infected frogs, and thus provide immunity to the disease, it should not by itself provide immunity against infection by the pathogen, which is the feature that distinguishes microhylids sampled in nature from other Queensland rainforest frogs. Microhylid species appear to produce peptides in amounts comparable to at least two other sympatric frog species (*L. genimaculata* and *L. rheocola*), however, the overall protection of microhylid frogs provided by AMPs was significantly lower than for these two species. The effectiveness of microhylid peptides was also significantly lower than for four additional south-eastern Australian frog species.

Microhylid species do not appear to have AMPs that are particularly effective against Bd, so their resistance to infection in nature is likely to be due to some other factor. Experimental evidence suggests that at least one species of microhylid (*C. ornatus*), can be infected by Bd but is highly resistant to the development of chytridiomycosis (Project 3.3). Experimental exposures of *C. ornatus* to high levels of Bd zoospores produced infections, but no individuals developed chytridiomycosis, and all individuals cleared infections at five weeks post exposure. This indicates that a different mechanism enables microhylids to clear themselves of Bd infections.

AMPs are not the only innate immune response in amphibians. Other epithelial defenses, such as regulation of skin sloughing, and commensal microflora may influence Bd zoospore survival and encystment rates (Woodhams, 2008). Species of

bacteria that live on the epidermis of amphibians produce antimicrobial agents effective against pathogenic fungi, and that, in some cases, inhibit the growth of Bd (Austin, 2000; Harris, 2006). Some species of amphibians that lay eggs in terrestrial nests that are brooded or tended by adults, posses anti-fungal flora that can inhibit the growth of fungi that are pathogens of embryos (Austin, 2000). These species may not suffer from severe population declines associated with chytridiomycosis because they may be more likely to harbor bacteria that inhibit the growth of Bd (Harris, 2006). It is possible that microhylids possess antimicrobial skin flora that inhibits the growth of Bd, and confers at least partial resistance to chytridiomycosis. More research is needed to determine the ability of microhylids to resist infection by Bd, as it appears that this family's AMPs are not the most important innate immune defense offering them protection from infection by Bd. Additional studies to further understand the microbial ecology of amphibian skin are needed to determine if other innate immune responses are responsible for the apparent resistance of microhylids to chytridiomycosis.

Acknowledgements
This research was supported by funding from the Australian Rainforest Co-operative Research Centre, and by tender 43/2004 from the Australian Department of Environment and Heritage (RFT 43-04, R. A. Alford P.I.), 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). Animal ethics permission was obtained from the James Cook University Animal Care and Ethics Committee (A931 and A960). KFH was supported by an Australian Postgraduate Research Scholarship. Thanks to R. Hobbs, C. Steel and many volunteers for assistance in the field.

## ***Project 3.3 Experimental Infection of Cophixalus ornatus, an Australian direct developing frog, with* Batrachochytrium dendrobatidis**

Kim F. Hauselberger

### Introduction

Amphibian populations are declining around the world; current estimates suggest that 43% of all known amphibian species are experiencing some form of population loss (Stuart, 2004). Global amphibian declines currently rank among the most critical issues in conservation biology (Stuart, 2004; Mendleson, 2005), and factors thought to be responsible include habitat loss, climate change, overexploitation, chemical contaminants and emerging infectious disease (Collins, 2003). One major cause of declines is chytridiomycosis, an invasive skin disease of amphibians caused by the chytrid fungus *Batrachochytrium dendrobatidis* (Bd) (Berger, 1998; Longcore, 1999). Chytridiomycosis has been associated with mass mortality, population declines, and extinctions of amphibian species world-wide, and has been found in the Americas (Muths, 2003; Lips, 2003; Lips, 2004; Ron, 2000; Barrionuevo, 2006; Carnaval, 2006; Lampo, 2006; Felger, 2007); Puerto Rico (Burrowes, 2004), Cuba (Díaz, 2007), the West Indies (Alemu, 2008), Europe (Bosch, 2001; Cunningham, 2005; Garner, 2005); Africa (Hopkins, 2003), Australia (Laurance, 1996; Berger, 1998), and New Zealand (Waldman, 2001).

Chytridiomycosis causes mortality across a broad range of amphibian species that live in a variety of habitats. In many cases, population declines attributed to Bd have been dramatic, resulting in the rapid extinction of up to 50% of amphibian species at a particular site (Lips, 2006). However, species that have suffered from declines commonly coexist with non-declining species, and the prevalence and intensity of infections and the extent of pathological effects appear to differ strongly among host species (Retallick, 2004; Lips, 2006). High-elevation populations suffer more severely from declines (Wake, 1991; Laurance, 1996; Lips, 1998; McDonald, 1999). Affected species generally occupy restricted geographic ranges, and they usually have aquatic larvae associated with streams and spend a large proportion of their time in or near streams (Lips, 1998; McDonald, 1999; Lips, 2003; Woodhams, 2005). Tadpoles are believed to be an important life-stage with respect to disease transmission, as they stand a high chance of being exposed to the fungus' aquatic zoospores (Daszak, 1999).

While most anurans mate and deposit eggs in water, numerous frog species possess terrestrial reproduction, depositing their eggs on land. Many of these species undergo direct development, which is characterised by the absence of a free-living, aquatic larval stage and the direct, embryonic formation of adult features (Jennings, 1998). Direct development has evolved independently within each of the three extant orders of amphibians (frogs, salamanders, and caecilians). It characterises hundreds of living species in over a dozen families including the Australian microhylids, and it is the predominant reproductive mode in some clades, e.g., plethodontid salamanders (Wake, 1996).

Although the proportion of amphibian species with direct development is comparatively small, evidence of declines of this group of amphibians is disproportionately scarce. In Australia, a study of 60 frog species from eastern, upland (<300m) areas showed that only one species (*Philoria frosti*) out of 20 (5%) with direct development was known to have undergone declines, whilst 20 out of 40 (50%) species that have an aquatic stage in their life-history have experienced population declines (Hero, 2005). A study of 30 Costa Rican species showed that of 15 with direct development, 2 were in decline (13%), and of the 15 species with an aquatic stage in their development, 9 were in decline (60%) (Puschendorf, 2006). There are very few examples of population crashes of species with direct development in the literature, and even fewer can cite the presence of Bd as a possible cause of the declines (Pounds, 1999; Bell, 2004; Burrowes, 2004; Lips, 2006 #418, Brem, 2008). Two examples come from Panama where

direct-developing frogs and salamanders have declined and disappeared from sites during epidemic outbreaks of chytridiomycosis (Lips, 2006; Puschendorf, 2006).

Species of terrestrial, direct-developing salamanders have been found with Bd infections in the wild (Lauer, 2007; Harris, 2009), and experimental infections with Bd have been produced in atleast two plethodontid species (*Plethodon metcalfi* and *P. cinereus*). Mortality was only observed in *P. metcalfi*, suggesting that *P. cinereus* is not vulnerable to the effects of chytridiomycosis (Vazquez, 2007; Harris, 2009). Salamanders appear to be less affected by chytridiomycosis than anurans (Lips, 2006). There is evidence that direct-developing frog species from the genus *Eleutherodactyus* and *Craugastor* are able to coexist in stable populations with Bd (Puschendorf, 2006), and data shows that atleast one species (*E. coqui*) does not show a significant response of mortality in experimental exposure of Bd under laboratory conditions (C. Carey pers. comm.).

In Australia, the family Microhylidae includes 19 direct developing species that represent 9% of the entire anuran species diversity (Hoskin, 2004). Microhylids commonly occur in moist, tropical environments at high altitudes that experience cooler temperatures in comparison with the lowlands. No microhylids species appear to have suffered population losses, despite inhabiting environments that are favourable to Bd. In many locations were they are found, sympatric species have undergone population declines associated with Bd (Woodhams, 2005; McDonald, 2005). Despite the absence of extensive population data, it does not appear that microhylids have undergone population losses, as anecdotal observations suggest that numbers of individuals of most species appear to be high (Williams, 2007). Data on the number of calling individuals along a transect within the WT from 1995-2007 showed that populations of two microhylid species were relatively stable over 12 years.

The ability of Bd to infect direct-developing microhylids under standard laboratory conditions is unknown. Prior to my project, two Australian species of microhylid (*Austrochaperina robusta* and *Cophixalus ornatus*) had been extensively surveyed histologically for chytrid infection, with negative results (Hauselberger, 2001) (D. Mendez pers. comm.). However, these surveys were carried out at only one location (Paluma in northern Queensland). (Kriger, 2006) reported a single positive diagnostic PCR assay on a sample from an Australian microhylid, however, this result may be dubious, and it is possible that the sample was contaminated. The results of extensive surveys of nine Australian microhylid species indicate that infection by Bd is extremely rare or absent in nature.

The absence or near absence of Bd infections from Australian microhylids could have several causes. The first is that the terrestrial environment may not be a competent reservoir for Bd because it is drier and warmer than riparian habitats (Brem, 2008). Thus, as microhylids do not reproduce aquatically, and are not known to enter stream water, they may not be exposed to infective zoospores (e.g., (Rowley, 2007)). The second is that microhylids as a group may be preadapted to being highly resistant to infection by Bd because they have particularly effective innate immune defences as a means to reduce fungal infestations that would otherwise overwhelm terrestrial egg clutches. These species may secrete antimicrobial peptides (AMPs) and alkaloids that can inhibit the growth of pathogenic fungi (Simmaco, 1998; Rollins-Smith, 2002). They may also possess symbiotic cutaneous microbial flora which produce antibiotic substances that inhibit the growth of fungal species which are pathogens of embryos (Rollins-Smith, 2002; Harris, 2006; Woodhams, 2007).

If the apparent immunity of microhylids to chytridiomycosis is due to AMPs or symbiotic microbiota, there may be potential to use these agents to control Bd in other amphibians in the laboratory or even in the field (eg. (Harris, 2009)). A first step towards discovering what is responsible for the extremely low prevalence or absence of infection in Australian microhylids in nature is to determine whether they are constitutively immune to infection by Bd. I therefore carried out a series of laboratory experiments to determine whether the most common species,

*Cophixalus ornatus*, can become infected by Bd, and if so, under what conditions this may occur.

## Methods

### Experimental design

15 frogs were used in an infection experiment. Seven *C. ornatus* and five *L. wilcoxii* (as positive controls, since this species is known to be vulnerable to infection by Bd) were sequentially exposed to increasing numbers of Bd zoospores (100 on day 0), while and three *C. ornatus* were used as negative controls. The relatively low level of replication was due to permit restrictions, but sufficed to demonstrate effects. A single isolate of Bd (Tully-L.rheocola 06-LB-1) was used, and each exposure to individuals consisted of administering 20μL of dilute salts solution (DS) (Boyle, 2003), either sterile (control animals) or containing a set number of Bd zoospores (experimental animals) directly onto the posterior dorsal surface of the frog using a mico-pippetter. Animals were held at constant temperatures of 21-24.5°C, and were exposed to 100, 1000, and 10,000 living Bd zoospores on days 0, 17, 46, and 66 of the experiment.

### Swabbing for diagnostic qPCR

Diagnostic samples were collected using a standardized technique. A sterile medical cotton swab (Medical Wire & Equipment Co. Bath Ltd., Wiltshire, UK) was run over the dorsum, including the surfaces of the forelegs, hindlegs, and back, for approximately 10 seconds. The frog was then flipped over so that its ventral surface was exposed, and the forelegs, hindlegs, and the ventral surfaces of the head and body were swabbed for another 10 seconds. The swab was also placed near the toes of each of the feet of the individual so that it grasped the swab with its toe pads. All swabs underwent qPCR analysis for the presence of Bd DNA. qPCR was carried out on samples to determine whether infection occurred, and the relative level of infection. Previous research has shown that qPCR can detect Bd infection in frogs as early as 7 d post infection (Boyle, 2004).

### Collection of experimental animals

10 calling adult male *Cophixalus ornatus* were collected from rainforest on either side of the "H track" in Paluma (19°00.42'S, 146°12.43'E) during the 8-10$^{th}$ February 2008. All weighed between 0.76-1.03g, with snout-vent lengths (SVLs) from 18.4-22.03mm. Individuals were located via their mating calls, captured in an inverted, plastic sandwich bag with a handful of leaf litter, and swabbed for detection of the presence of Bd DNA using real-time TaqMan polymerase chain reaction assay (qPCR) (Boyle, 2004). They were placed in a small insulated cooler to preclude rapid changes in ambient environmental temperature during transport to the laboratory. Collection sites were flagged with fluorescent flagging tape and labeled with the frog's individual number so that individuals could be released at the point of capture after the experiment ended and they were known to be uninfected.

### Animal housing and enclosures

Animals were housed in a laboratory at the James Cook University freshwater compound that is maintained at approximately 23°C. Daily measurements revealed that temperatures ranged from 21-24.5°C. Relative humidity ranged from 60-75%. The laboratory is lighted by a large window, and animals therefore experienced a natural diurnal light cycle. Frogs were acclimatised to these experimental conditions for 14 days before infection.

Ten *C. ornatus* were housed individually in 1.6L transparent plastic enclosures (18x11x12cm) with ventilated lids (Critter Keeper$^{TM}$, Rolf Hagen & Co. Inc: Mansfield, USA). Containers were filled with 2-3cm of autoclaved peat soil which was moistened weekly. Containers also had autoclaved leaf litter and small pieces of wood which had previously been removed from Paluma, to provide shelter for frogs and to make enclosures more closely resemble natural habitat. The swabs taken from all animals at the time of collection tested negative for Bd DNA, indicating that all animals were uninfected prior to the experiment.

Five *Litoria wilcoxii* individuals were also used in infection experiments as positive controls. These frogs were raised from tadpoles collected at Crystal Creek, Paluma Range National Park, Queensland, Australia (S18°58′54″ E146°12′'01″) by N. Kenyon (Kenyon, 2008). *Litoria wilcoxii* individuals were housed in 32x21x17cm plastic containers with removable lids that had holes drilled in them for ventilation, and were accompanied with a small amount of autoclaved leaf litter. They were also provided with a 12x6cm plastic tub of rainwater (collected and stored in a water tank) that was changed weekly. *Litoria wilcoxii* individuals were swabbed for detection of the presence of Bd DNA using qPCR prior to the experiment taking place. All were uninfected.

All experimental frogs were fed cultured crickets (*Acheta domesticus)* weekly *ad libitum*. *Cophixalus ornatus* individuals were fed baby, "pin head" crickets and *L. wilcoxii* individuals were fed adult crickets. Once every month, 0.5 mL of a liquid supplement mixture (2mL Calcivet/100mL rainwater) was applied dorsally to each *L. wilcoxii*.

Culture and maintenance of *Bd*

All work associated with Bd was completed at the School of Veterinary and Biomedical Sciences at James Cook University in the laboratory of R. Speare, and was carried out under sterile conditions in a laminar flow hood. Transport of zoospore solutions from the Speare laboratory to the frog enclosures was conducted in a Styrofoam insulated "esky" which had an ice pack included to keep the environment cool. Experiments were carried out with appropriate sterilisation of clothing, equipment, and effluent using established protocols (e.g., (Speare, 2001)) to prevent the escape of the pathogen to the environment or any cross-contamination of animals. These techniques are well-established and effective. To prevent cross-infection, disposable latex gloves were worn at all times and changed between frogs and animal enclosures.

A single isolate of Bd was used in this study (Tully-L.rheocola 06-LB-1). It was grown in 50% nutrient tryptone-gelatine hydrolysate-lactose (TGhL) broth medium (8 g tryptone, 2 g gelatin hydrolysate, 1 g lactose, 1000ml distilled water) in 25cm$^3$ tissue culture flasks (TPP9025, JRH Biosciences). Stock culture was passaged at intervals of 4-7d to assure that cells were in an active phase of growth. After 4-7d incubation at 23°C, the maximum number of zoospores in culture was reached, and the culture flasks were scraped using a TPP cell scraper (CSL Biosciences) in order to get as many zoospores into suspension as possible. 10mL of refrigerated (3°C) stock of 50% nutrient TGhL broth was pipetted into a labeled, sterile tissue culture flasks, and this was left at room temperature for 10min allowing the broth to warm up. The Bd culture was passaged by pipetting 1mL of the stock solution of Bd into the new broth flask which was then incubated at 23°C.

Production of zoospores

To collect Bd zoospores for inoculations, stock solution was transferred to and grown on 50% nutrient TGhL agar (8 g tryptone, 2 g gelatin hydrolysate, 1 g lactose, 10 g agar, 1000ml distilled water) in 9 cm Petri dishes (Sarstedt, Australia, Inglefarm, SA). After culture flasks were scraped (as described above), 0.75mL of stock solution was transferred to agar plates with a sterile pipette. Inoculated dishes were left open for approximately 5-10 minutes in a laminar flow hood until the added broth was dry. Petri dishes were then labeled, sealed with Parafilm®, and incubated at 23°C for a minimum of three days, to enable the growth of a dense culture of Bd at maximal zoospore release.

Harvesting zoospores

Zoospores were harvested using the methods described by (Rollins-Smith, 2002). Agar plates were flooded with 3ml of DS solution and were left to sit for 20min while zoospores accumulated into the liquid. The supernatant was then collected using a sterile pipette and transferred into a labeled Eppendorf tube. A sterile TGhL agar plate was also flushed with DS

solution and the supernatant collected in the same way, to be used as a control in the experiment. Zoospores may stay motile (and thus infective) for up to 24 hours, however, most encyst before this time (Longcore, 2008); in this study, all zoospores were used in experimental trials <2hr after harvesting.

Zoospore concentration

The number of zoospores in the DS solution was estimated by counting live and moving zoospores using a haemocytometer as described by (Boyle, 2004). Five counts were completed on 1 square of 0.005mm$^2$ area, and this was replicated twice. The two results were averaged, to create the final count of zoospores, and this was multiplied out to give a zoospore count per mL of culture. Using this result, the zoospore solution was diluted to the level needed in the experimental trials with DS solution; the control solution was also diluted to the same extent.

Infection experiments

The infection experiments consisted of four trials in which frogs were exposed to increasing numbers of zoospores (100, 1000, and the final two at 10,000 each time). These trials were continued until the majority of exposed frogs became infected. The experiment included 15 frogs, five *L. wilcoxii* individuals who acted as the positive control (this species is susceptble to Bd in the wild but has not suffered from declines (Retallick, 2004) and has previously been infected under standard laboratory conditions (R. Alford pers. comm.), and ten *C. ornatus* individuals, three of which were control subjects and were not exposed to Bd.

Experimental trials

Frogs underwent the first infection trials on day 0, 25/2/2008. Seven *C. ornatus* and five *L. wilcoxii* individuals were exposed to approximately 100 Bd zoospores from broth culture Tully-L.rheocola 06-LB-1-p24, cultured and collected as described above. Zoospores were administered in 20µL of DS solution pipetted from an Eppendorf tube with a concentration of approximately 5000 zoospores per mL, which equates to approximately 100 zoospores being administered to each individual. The three control *C. ornatus* were administered 20µL of dilute salt solution that had been flushed over a sterile TGhL plate. Because the *C. ornatus* and *L. wilcoxii* individuals were usually quite moist, frogs were first blotted dry using paper towel, to ensure that the solution would adhere to their skins.

In this and subsequent trials, all frogs were checked daily, fed weekly, and were swabbed 7 and 14 days after exposure for diagnostic qPCR to determine whether *Bd* DNA was present.
The second trial of the experiment occurred on day 17 (13/3/2008). A dose of approximately 1000 zoospores per frog was prepared and administered to experimental frogs as outlined for trial 1. Control frogs were reexposed to control inoculum. The concentration of the 20µL of Bd-containing DS solution administered to each experimental frog was 50,000 zoospores per ml. The third trial was carried out on day 46 (11/4/2008). A dose of approximately 10000 zoospores per frog was prepared and administered to experimental frogs as outlined for trial 1. Control frogs were reexposed to control inoculum. The concentration of the 20µL of Bd-containing DS solution administered to each experimental frog was 500,000 zoospores per ml. The fourth trial was carried out on day 66 (1/5/2008). Exposures and controls were treated identically to the third trial, with a second dose of 10000 zoospores administered to each experimental frog. Following the fourth trial, animals were swabbed 7, 14, 21, 28, and 35 days post-exposure to follow the progress of infections produced.

Disposal of frogs

The majority of animals used in infection experiments were clear of infection by the end of the experimental phase. Individuals that still showed the presence of infection were treated with heat after the experimental study was completed to clear their infections. This consisted of placing enclosures in a position in the laboratory that received direct sunlight for 1h each day for five consecutive days. Whilst undergoing this treatment, animals were observed, and temperatures of the frogs never exceeded 26°C. Animals were swabbed at weekly intervals, and

when 2 consecutive samples showed a negative result for the presence of Bd DNA, *C. ornatus* were released at the point of capture. All *L. wilcoxii* individuals were maintained in the laboratory for future use.

### Data analysis

Overall mean numbers of zoospore equivalents for all positive samples were calculated for each species and reported, with standard deviations. These numbers could not be used to compare intensity of infection between species since repeated measures within individual frogs are not independent. Intensity of infection was compared between species by calculating the mean number of zoospore equivalents estimated for Bd-positive swabs from each individual that tested positive at least once. These individual means were compared between species using a Mann-Whitney U test. Data for samples that did not test positive in all three of three replicate wells during qPCR were omitted from numerical comparisons; this may bias estimatres of intensity of infection slightly towards higher intensities.

## Results

No animals became infected in trials 1 and 2, at doses of 100 or 1000 zoospores (Table 17). Trial 3, the first trial in which animals were exposed to 10000 zoospores, produced relatively light infections in two *C. ornatus* and four *L. wilcoxii* individuals (Table 17). The apparent infection in *C. ornatus* individual 10 at seven days after exposure in this trial was positive in less than three of the replicate wells for the qPCR, and might have been caused by the continuing presence of DNA from the initial infective dose of zoospores, instead of indicating an actual infection. In, Trial 4, the second inoculation with 10000 zoospores, all 7 *C. ornatus* individuals showed infection at 14 days following exposure (Table 17), and 2 of the *L. wilcoxii* individuals showed infection (Table 17). B y 35 days after exposure in trial 4, only one *C. ornatus* appeared to remain infected (Table 17), while two of the positive controls retained relatively intense infections. Because frogs were maintained at high humidities and temperatures close to 23$^{o}$C, environmental temperature and humidity could not be responsible for the observed losses of infections.

Table 17. Results of the infection experiment. Doses of 100, 1000, 10,000, and 10,000 zoospores, respectively, were applied on days 0, 17, 46, and 66 of the experiment in trials 1, 2, 3, and 4, respectively.

| Trial | | | 1 | | 2 | | 3 | | 4 | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Day in experiment | | | 7 | 14 | 24 | 31 | 53 | 60 | 73 | 80 | 87 | 94 | 101 |
| Days since exposure | | | 7 | 14 | 7 | 14 | 7 | 14 | 7 | 14 | 21 | 28 | 35 |
| Species | Frog | Exposed or Unexposed | qPCR results (number of zoospore equivalents) | | | | | | | | | | |
| *C. ornatus* | 1 | U | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| | 2 | U | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| | 3 | U | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| | 4 | E | 0 | 0 | 0 | 0 | 6 | 0 | 4 | 4 | 0 | 7 | <2* |
| | 5 | E | 0 | 0 | 0 | 0 | 0 | 0 | 34 | 67 | 0 | 6 | 0 |
| | 6 | E | 0 | 0 | 0 | 0 | 0 | 5 | 11 | 11 | 46 | 0 | 0 |
| | 7 | E | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 3 | 0 | 77 | 0 |
| | 8 | E | 0 | 0 | 0 | 0 | 0 | 0 | <3* | <3 | 0 | 20 | 0 |
| | 9 | E | 0 | 0 | 0 | 0 | 0 | 0 | <2* | 2 | <4 | <4* | 0 |
| | 10 | E | 0 | 0 | 0 | 0 | <2* | 0 | 15 | 2 | 0 | 24 | 0 |
| *L. wilcoxii* | 1 | E | 0 | 0 | 0 | 0 | 785 | 1064 | 22 | 34 | 312 | 176 | 106 |
| | 2 | E | 0 | 0 | 0 | 0 | 24 | 12 | 213 | 135 | 135 | 308 | <3 |
| | 3 | E | 0 | 0 | 0 | 0 | 554 | <1* | 0 | 0 | 0 | 0 | 0 |
| | 4 | E | 0 | 0 | 0 | 0 | 0 | 2919 | 0 | <3* | 0 | 0 | 0 |
| | 5 | E | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |

*only 1 or 2 of three replicate wells positive

Results of the qPCR analysis of skin swabs show that the mean number of zoospore equivalents in samples from infected *C. ornatus* individuals that were positive in all three replicate wells was 19.1 (S.D. = 22.7, N = 18) and the maximum number was 77, whereas the mean from positively *L. wilcoxii* was 453 (S.D. = 747.0, N = 15), with a maximum of 2919 (Table 17). Mean numbers of zoospore equivalents within individuals for samples that were positive in all three wells were 5.3, 35.7, 18.3, 40.0, 20.0, 2, and 13.7 for *C. ornatus* individuals 4-10, respectively, while mean numbers within infected *L. wilcoxii* individuals were 357.0, 137.8, 554, and 2519 for individuals 1-4, respectively. These individual means were compared between the two species using a Mann-Whitney U test, because they appeared unlikely to satisfy the assumptions of parametric tests. This showed that mean number of zoospore equivalents in positively infected individuals differed significantly between the species ($U = 28$ with 7 and 4 d.f., $P = 0.006$). Figure 30 illustrates the distributions of mean number of zoospore equivalents for individuals of each species that tested positive at least once for Bd.

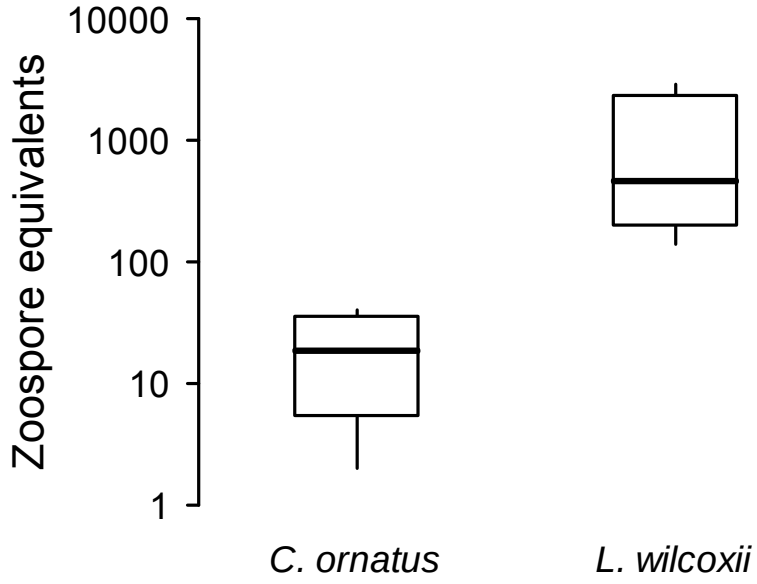


Figure 30. Boxplots of mean numbers of zoospore equivalents recovered from positive swab samples for each individual *C. ornatus* and *L. wilcoxii* shown to be positively infected.

## Discussion

This is the first demonstration that an Australian microhylid species can become infected with Bd under standard laboratory conditions. All seven experimental *Cophixalus ornatus* clearly became infected, showing increasing numbers of Bd zoospore equivalents over successive swab samples during trial 4. Four of the five *Litoria wilcoxii* positive controls tested positive for relatively high numbers of zoospore equivalents at some point during trials 3 and 4. None of the control *C. ornatus* ever tested positive for Bd.

Positive qPCR results were not recorded after doses of 100 or 1000 zoospores in either *C. ornatus* or the positive control species, *L. wilcoxii*. The first dose of 10000 zoospores produced light positive infections in two *C. ornatus*, and a possible positive in a third, while it produced intense infections in four of the five positive control animals. This suggests that *C. ornatus* may have greater innate resistance to acquiring Bd infections than does *L. wilcoxii*. After a second exposure to 10000 zoospores, all *C. ornatus* showed positive qPCR results for the presence of Bd DNA. Levels in all individuals increased at some time after day 7 following exposure, making it very unlikely that positives were caused by residual DNA remaining from the zoospores used to expose the frogs.

This research indicates that *C. ornatus* can become infected by Bd when exposed to relatively high numbers of zoospores. However, it also suggests that once acquired, infections are cleared relatively rapidly, and are unlikely to persist or develop into chytridiomycosis. The number of zoospore equivalents detected on swabs taken from *C. ornatus* was always relatively low, and on many individuals it fluctuated to low levels during days 7-28 following the second exposure to 10000 zoospores. In all but one individual, the infection appeared to be cleared by day 35, and the single individual that remained positive on day 35 returned a very weak result, positive in less than three replicate wells. In contrast, two of the four *L. wilcoxii* that became infected in trial 3 remained infected until day 101, at least 55 days after the trial 3 exposure and 35 days after the trial 4 exposure. *Litoria wilcoxii* also appeared to develop more intense infections, with mean numbers of zoospore equivalents in samples from infected *L. wilcoxii* more than 10 times as high as in *C. ornatus* (Figure 30).

The results of the present study support the idea that Australian microhylids are highly resistant to infection by Bd and to chytridiomycosis. Although experimental conditions were within the range thought to be optimal for the growth of the fungus (Piotrowski, 2004), *C. ornatus* resisted initial infection and rapidly cleared infections once they were acquired. Under similar laboratory conditions, many species that have not suffered from declines in the wild are highly susceptible to Bd (Berger, 1999; McDonald, 1999; Woodhams, 2005). Only a single Australian microhylid has been found infected in the wild, a *C. ornatus* individual sampled by (Kriger, 2006). It is not clear whether the diagnostic PCR run on that sample was replicated, and if it was not, the result could be due to cross-contamination of samples. The results of an extensive sampling program in which 595 individuals across nine microhylid species were sampled, indicated that all were negative for Bd infection (K. Hauselberger, unpublished data). This suggests that if infections occur in the field, they are extremely rare, and the results of the present study indicate that rarity is likely to be caused by a combination of low rates of initial infection and high rates of clearance.

Innate or acquired immune responses may confer protection from infection by Bd, and may contribute to disease resistance. Like other vertebrates, amphibians have a well-developed immune defense, featuring both an acquired and an innate response (Duellman, 1994; Carey, 1999; Apponyi, 2004). Acquired immune functions are pathogen specific, and are based on the antigen/antibody complex, which develops slowly and improves with repeated exposures. Innate immune functions are rapid, are not pathogen-specific, and include macrophage, neutrophil and natural killer cells, cytokines, acute phase reactants, and antimicrobial peptides (AMPs) (Carey, 1999). AMPs are thought to be particularly important in the defense against Bd (Rollins-Smith, 2002; Woodhams, 2007), and are produced in granular glands in the skin of amphibians and are active against bacteria, yeast, protozoa, viruses and fungi including Bd (Rinaldi, 2002; Rollins-Smith, 2002; Rollins-Smith, 2005; Woodhams, 2005; Woodhams, 2007). The effectiveness of AMP skin secretions in Australian frog species has been significantly correlated with resistance to chytridiomycosis (Woodhams, 2007), and population trends associated with chytridiomycosis in Australian rainforest frogs has been correlated with the effectiveness of AMP defenses against Bd (Woodhams, 2005).

It is possible that innate immune functions such as AMPs were preventing chytridiomycosal infection in both species at the doses of 100, 1000, and in some individuals 10000 zoospores. It is also possible that some frogs' were unable to mount an effective defense on the second dose of 10000 zoospores, as the concentration of the pathogen was too high. This result has been observed in *Mixophyes fasciolatus* individuals that were exposed to varying levels of Bd. Juveniles that were exposed to a dose of 10 zoospores did not suffer from chytridiomycosis, where as those that were exposed to 100 zoospores suffered 100% mortality between 35 and 47 days post exposure, and those that were exposed to 1000 zoospores suffered 100% mortality between 23 and 38 days post exposure (Berger, 1999). In addition to innate immune responses there may also be some element of acquired immune response occurring in *L. wilcoxii*, as two of the four individuals that became infected in trial 3 of the experiment lost the infections and did not become re-infected in trial 4. Acquired immunity may be occurring in these individuals or innate immune defences such as AMPs may functioning at higher levels, making reinfection less likely over a short time period.

The significantly different responses of the two species to Bd infection in the presence of zoospores in skin swabs could be due to interspecific immune responses. Experimental data has demonstrated that significant inherent differences exist in four Australian frog species (*Litoria caerulea, L. chloris, Mixophyes fasciolatus* and *Limnodynastes tasmaniensis*) infected with Bd in a laboratory setting, as measured by survival time, number of deaths, behavior and pathogen load (Ardipradja, 2001). The effectiveness of AMPs in these four species were also correlated with their resistance to chytridiomycosis (Woodhams, 2007). As innate or acquired immune defenses are adaptive, they are expected to vary among lineages, and AMP defenses may principally account for the disparity in disease susceptibility among species (Woodhams, 2007).

It is possible that the innate immune defense of *C. ornatus* is more effective at preventing Bd infection than *L. wilcoxii.*

In addition to immune responses having an effect on the susceptibility of amphibian species to Bd, interspecific behaviour may be another factor which explains why species differ in their response to disease. Species that are more susceptible to the disease may behave in a manner favourable to the transmission, growth and survival of Bd, whilst the least susceptible species may behave in a manner which is not conducive to Bd transmission, growth and survival (Rowley, 2007). An aspect of the life history of microhylids, such as terrestrial breeding may be a reason why these species do not appear to suffer from the effects of chytridiomycosis in nature (pers. obs.).

Microhylid species such as *C. ornatus* have a life-history that involves direct development of terrestrial eggs into froglets, and exhibit some form of parental care of the developing egg clutch (Zweifel, 1985). This species may posses AMPs and bacterial flora which inhibit the growth of a range of bacteria and fungi, including Bd (Austin, 2000; Harris, 2006). Egg brooding may prevent fungal attack of eggs, by adults applying anti-microbial skin secretions to the eggs, by providing antimicrobial substances produced by their cutaneous micro-flora to the eggs, or by some combination of these (Simmaco, 1998; Rollins-Smith, 2002; Rollins-Smith, 2005; Harris, 2006). Evidence for egg-brooding species possessing anti-fungal agents comes from studies that show that when amphibian embryos of species that attend nests and brood eggs are deserted, or adults are physically removed from nests, embryos often succumb to fungal infections (Forester, 1979; Townsend, 1984; Simon, 1993).

The effectiveness of AMPs has been studied for Australian microhylids, and it appears that natural peptide mixtures isolated from frogs are not extremely effective against the growth of Bd. This suggests that other innate immune responses may be responsible for the apparent resistance of *C. ornatus* to chytridiomycosis. We hypothesize that species that brood their embryos have evolved to maintain a mutualistic antifungal skin flora in order to protect their embryos, and that they are less likely to succumb to chytridiomycosis than species that do not attend nests. The inhibition of Bd growth by natural skin flora of amphibians has been investigated, and bacteria from nest-attending plethodontid salamanders are successful in inhibiting the growth of the fungus (Harris, 2006). Possession of microbial skin flora to protect developing egg clutches, in addition to normal immune defences may predispose terrestrial breeders to having a higher resistance to Bd.

The majority of population declines associated with Bd have occurred in species that inhabit aquatic environments, and the few population declines noted in terrestrial species may be due to the possession of anti-fungal microflora. However, a lower prevalence of infection in terrestrial species may also be due to a reduced transmission of Bd (Lips, 2006). Completely terrestrial species such as the Microhylidae may simply have a reduced probability of coming into contact with Bd in nature and if they do become infected, they may provide an environment that is not conducive to the growth of the fungus. As these frogs are able to spend the majority of their time away from water sources, they may be able to dry out their skin to a level that would make growth of Bd difficult or impossible.

Despite the importance of transmission in the epidemiological process, very little is known about the transmission of Bd, particularly in the field. Bd zoospores swim less than 2cm before they encyst, so the pathogen is likely to be spread between individuals during close contact during aggregation or mating (Piotrowski, 2004). It has been postulated that transmission may occur via contact with infected individuals or contaminated environmental substrates (Berger, 1999; Daszak, 1999; Lips, 2006), and this has been shown in the laboratory (Davidson, 2003), and in field mesocosms (Parris, 2004). Because zoospores survive in water, riparian species are more susceptible to transmission of the disease (Piotrowski, 2004). However, completely terrestrial species have been found infected with the fungus (Burrowes, 2004; Lips, 2006), and

(Lips, 2006) found that Bd was present on a wet rock during an epidemic in Panama, which supports the hypothesis that Bd is capable of transmission via contaminated environmental substrates. This suggests that Australian microhylids should not completely escape infection through lack of opportunity for transmission. They inhabit areas where sympatric species have suffered population declines, and individuals inhabit environments that come into contact with water bodies where the fungus is likely to be spread (pers. obs.). This may explain the why *C. ornatus* are able to be infected with Bd under laboratory conditions that favour the growth of the fungus, but they are not found infected with Bd in nature. It is possible that a combination of life-history and behavioural traits coupled with an effective immune response is responsible for the presence of limited infection of *C. ornatus* with Bd, and the ability to this species to lose infection over time. Further research is required to determine if microhylid species possess skin flora which inhibits the growth of Bd.

Despite the high sensitivity of the qPCR assay, there are still limitations to the technique. Its inability detect Bd early in the infection period (less than 4 d), and the reduction of sensitivity due to the presence of an inhibitor in the sample, or the degradation of fungal DNA are limitations, but these have been minimised in this study. It is also not clear whether animals that have been kept in stable laboratory environments accurately reflect the tested condition, and therefore, susceptibility of animals in nature to Bd. Wild amphibians will vary greatly in condition within a cohort and between years, and this is likely to cause substantial variation in their ability to mount an anti-Bd response (Fisher, 2007). In this case, what appears to be a species which is able to be infected by Bd in the laboratory may not be at risk in nature. Further work on Bd is needed to determine how other species from the family Microhylidae respond in laboratory settings and how transmission occurs. Knowledge gained from these investigations will be useful in preventing further population crashes, and in the management of other wildlife species.

## 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, R. A. Alford P.I.), 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). KFH was supported by an Australian Postgraduate Research Scholarship. Thanks also to R. Hobbs, C. Steel and many volunteers for assistance in the field.

***Project 4. Effects of water movement on infection and reinfection by the amphibian chytrid.***

Scott Cashins

Project 4 addresses **Objective 1** by determining how the buildup of B.d. infections within individuals is affected by water flow. Current laboratory models almost all have used still water, which might be expected to allow higher than natural rates of infection and reinfection as it should maximise the chance that B.d. zoospores will re-encounter hosts after they exit zoosporangia. It addresses **Objective 5** by determining the effects of water flow on the buildup of disease and its transmission among individuals. If these effects are important, it will be necessary to modify the standard laboratory system designed in Project 1 to incorporate appropriate flow regimes.

The results of six months of fieldwork investigating the prevalence of infection in tropical stream dwelling tadpoles (reported on in the Year 2 report for RFT 42/2004) suggested that contact with zoospores in the water column may not be the primary method by which stream-dwelling tadpoles acquire *Bd* infections. Only the mouthparts of tadpoles are known to become infected with *Bd*, so it might be expected that tadpoles that feed primarily by filtration from the water column would become infected at the highest rates. However, field survey data indicated that torrent tadpoles, which are usually attached to substrates using oral disc suction, and feed primarily by scraping material from those substrates, become infected at the highest rates. This suggests that contact of the tadpole with the substrate during feeding is the most likely path of transmission. In light of these field results, we decided it was necessary to examine, in the laboratory, how contact with infected substrates may affect transmission to torrent tadpoles. Understanding how transmission occurs is a prerequisite to examining the effects of waterflow and zoospore density on transmission and infection progress.

To understand how contact with the substrate during feeding may affect transmission we developed a protocol to grow *Bd* on the food of tadpoles. Using this protocol we conducted an experiment comparing modes of transmission in 40 *Litoria caerulea* tadpoles. Although *L. caerulea* tadpoles do not occur in streams of the Wet Tropics, these tadpoles have a similar feeding mechanism to *Litoria genimaculata* tadpoles (pool tadpole). One half of the tadpoles were allowed to graze on feeding plates with *Bd* actively growing on it (Figure 31). The remaining 20 tadpoles were exposed to *Bd* suspended in the water column. Tadpoles were exposed to *Bd* for six days, at which time all tadpoles were transferred to clean containers for the remainder of the experiment. All tadpoles were swabbed prior to and following exposure at regular intervals through to metamorphosis. Faeces of tadpoles were collected immediately following exposure on filter membranes in order to determine if tadpoles are capable of ingesting *Bd* and excreting it in a form detectable with PCR.

Figure 31. Tadpoles grazing on food plate containing *Bd*

Immediately following exposure, 7 of 20 tadpoles that fed on Bd tested very slightly positive compared to 0/20 tadpoles that were exposed only to *Bd* in the water column. The faeces of some of these PCR positive tadpoles also contained low numbers of *Bd* zoospores. However, a week later no tadpoles tested positive. It is likely that the initially positive tadpoles had zoospores "stuck" to their mouthparts as a result of contact during grazing. However, the contact may not have been sufficient to result in infection. This is important as it may help explain why some frog species are able to rebound following an epidemic and others are not. If tadpoles of a species are infected at a high prevalence and then maintain the infection through metamorphosis, this would likely result in decreased metamorph survival as the metamorphs of many species are especially susceptible to chytridiomycosis. Over time this loss of recruitment would hinder a species from successfully repopulating an area following a crash. In the Wet Tropics this pattern has, in fact, been observed. In general the frogs with torrent-adapted tadpoles have not faired as well as frogs whose tadpoles develop in pools. At the moment this is an untested hypothesis and more work is needed to test this hypothesis further including rerunning the above experiment with both torrent-adapted and pool-adapted tadpoles simultaneously, investigating if infected torrent-adapted tadpoles maintain infection through metamorphosis and if this infection results in increased metamorph mortality.

In order to address some of these important questions we developed a tadpole enclosure suitable for placement in fast flowing sections of creek where torrent adapted tadpoles can be safely maintained and individually monitored through development. We conducted experiments in these enclosures in 2007. These experiments showed that larval *Litoria nannotis* contracted *B. dendrobatidis* infections even when initially uninfected tadpoles were alone in enclosures, indicating that they acquired infections from stream water. Tadpoles placed in groups that contained an initially infected tadpole contracted infections more rapidly and predictably than tadpoles placed in groups that did not initially contain an infected individual, indicating that the presence

of infected animals increases the rate of acquisition of new infections. Infected tadpoles lost body condition over the winter months, and many shed all or a large fraction of their keratinized mouthparts; many of the animals that shed their mouthparts subsequently regrew them and regained body condition, and some lost their infections entirely. This is the first experimental demonstration that infection by *B. dendrobatidis* directly causes a loss of body condition, and therefore reduces the growth rate and survival rate, of Australian stream tadpoles. It is also the first to suggest that shedding the keratinized mouthparts may be an adaptive defence against the disease. Its results are presently being written up for submission to an international journal.

In addition to the experiment detailed above, in 2007 we did a series of technique-oriented experiments that were required as a result of our preliminary results. In one series of trials, we showed conclusively that handling tadpoles of several species with latex gloves produces severe local tissue dmage and mortality, as a result of reactions of tadpole's tissues to contact with latex. This is potentially very important to conservation, since the requirement to handle animals with gloves for the purposes of hygiene could lead to mass mortality, which could remain undetected if animals are handled and immediately released, since the symptoms take a short time to develop.

***Project 4.1. Sodium hypochlorite denatures the DNA of the amphibian chytrid fungus Batrachochytrium dendrobatidis****

* Modified version of: Cashins, S.D., Skerratt, L.F., Alford, R.A., 2008. Sodium hypochlorite denatures the DNA of the amphibian chytrid fungus *Batrachochytrium dendrobatidis*. Diseases of Aquatic Organisms. 80, 63-67.

Abstract

*Batrachochytrium dendrobatidis*, an aquatic amphibian fungus, has been implicated in many amphibian declines and extinctions. A real-time polymerase chain reaction (PCR) TaqMan assay is now used to detect and quantify *B. dendrobatidis* on amphibians and other substrates via tissue samples, swabbing and filtration. The extreme sensitivity of this diagnostic test makes it necessary to rigorously avoid cross-contamination of samples, which can produce false positives. One technique used to eliminate contamination is to destroy the contaminating DNA by chemical means. We tested three concentrations of sodium hypochlorite (NaOCl) (1%, 6%, 12%) over four time periods (1, 6, 15 and 24 hours) to determine if NaOCl denatures *B. dendrobatidis* DNA sufficiently to prevent its recognition and amplification in PCR tests for the fungus. Soaking in 12% NaOCl denatured 100% of DNA within an hour. 6% NaOCl was on average 99.999% effective across all exposure periods, with only very low numbers of zoospores detected following treatment. 1% NaOCl was ineffective across all treatment periods. Under ideal, clean, conditions treatment with 6% NaOCl may be sufficient to destroy DNA and prevent cross contamination of samples, however, we recommend treatment with 12% NaOCl for an hour to be confident all *B. dendrobatidis* DNA is destroyed.

Introduction

Chytridiomycosis, caused by the highly virulent fungus *Batrachochytrium dendrobatidis*, has caused declines of natural amphibian populations and deaths in captive populations around the world (Berger et al. 1998, Pessier et al. 1999, Lips et al. 2006, Rachowicz et al. 2006, Skerratt et al. 2007). *Batrachochytrium dendrobatidis* is a highly transmissible pathogen requiring proper hygiene protocols, including disinfection of equipment, to prevent its spread (Speare et al. 2004). Many disinfectants are very effective at killing *B. dendrobatidis* on a range of substrates (Johnson et al. 2003, Webb et al. 2007). However, for researchers collecting and processing samples for diagnostic PCR, killing *B. dendrobatidis* is not enough; it is necessary to destroy its DNA to prevent cross contamination of samples that may lead to false positives.

DNA-based PCR tests are now commonly used diagnostic tools to detect *B. dendrobatidis* due to their sensitivity and specificity (Annis et al. 2004, Boyle et al. 2004, Hyatt et al. 2007). When using diagnostic PCR it is important to prevent contamination of samples. Even very low levels of DNA contamination on sampling equipment can create false positives. Recent papers have raised concerns regarding the generation of false positives via shared equipment (Kirshtein et al. 2007, Woodhams et al. 2007). While it is now standard practice to disinfect field equipment with bleach, > 70% ethanol or other treatments to prevent the transfer of live *B. dendrobatidis* between individual amphibians and sites (Johnson et al. 2003, Speare et al. 2004, Webb et al. 2007), these protocols may not denature *B. dendrobatidis* DNA. This would make the results of diagnostic PCR unreliable. Dipping instruments in alcohol and burning off

the residue is an effective sterilisation technique and may render *B. dendrobatidis* DNA undetectable by PCR. However, this has not yet been tested and flaming is an impractical solution for larger field tools such as collection trays and animal enclosures and equipment that would be destroyed by flames such as fabrics and plastics.

The antimicrobial properties of sodium hypochlorite (NaOCl) are well known and it is widely used as a disinfectant in medicine (Eventov et al. 1998), endodontics (Gomes et al. 2001), water treatment, and around the home. Archaeologists and forensic scientists have discovered its usefulness for destroying contaminating DNA prior to PCR amplification of target DNA from teeth and bones (Kemp and Smith, 2005). NaOCl has also been used to denature pathogens and allergens. Sehulster et al. (1981) found that NaOCl eliminates Hepatitis B antigenicity, Matsui et al. (2003) reduced the immunogenicity of a cat allergen, and Martyny et al. (2005) denatured the fungal allergen *Aspergillus fumigatus* on environmental substrates, significantly reducing its recognition by ELISA. We experimentally tested three concentrations of NaOCl over four exposure periods to determine its effectiveness at denaturing *Batrachochytrium dendrobatidis* DNA as determined through a real-time PCR assay.

Materials and Methods

We maintained *Batrachochytrium dendrobatidis* culture in TGHL broth (16 g tryptone, 4 g gelatine hydrolysate, 2 g lactose, 10 g agar, and 1000 ml distilled $H_2O$) in 75 $cm^2$ tissue culture flasks (Sarstedt Inc. USA) at 20 °C. After four days, the flask bottom was scraped with a cell scraper to dislodge all encysted zoospores and zoosporangia. The entire cell suspension (~30 ml) was emptied into a 50 ml centrifuge tube and centrifuged at 1,100 g for five minutes at 4 °C. The supernatant, containing the reproductive zoospores, was then transferred to a clean tube and the pellet, consisting mostly of zoosporangia, was discarded. The tube containing the supernatant was gently inverted two or more times to ensure mixing. Three separate aliquots of the suspension were then removed and counted on a haemocytometer. The concentration of zoospores was then diluted to $4.0 \times 10^5$ zoospores/ml in TGHL broth. We added 200 µl of this suspension to each well of a 96 well plate and the first 5 columns of a second 96 well plate. A visual inspection of the wells using an inverted microscope confirmed that all wells contained similar numbers of active zoospores. The 96 well plates were then placed in a 20°C incubator.

After three days, microscopic examination confirmed that there was considerable growth in each well, with mature zoosporangia and active zoospores. We removed the broth from each well and replaced it with 200 µl of one of three NaOCL concentrations; 1%, 6%, 12%, or a control (TGHL). Each sodium hypochlorite dilution at each exposure period had seven replicates and the control group had ten replicates.

In order to prevent *B. dendrobatidis* in the control group from continuing reproduction, controls were processed immediately after addition of the TGHL. The 200 µl of TGHL was removed and 200 µl of a dilute saline solution added (DS; Boyle et al. 2003). The bottom and sides of each control well were scraped 16 times, and the corner where the sides meet the bottom of the well was scraped four times with a sterile wooden dowel. The 200 µl aliquot of DS was then removed and placed in a 1.5 ml Micro tube (Sarstedt Inc. USA). A second 200 µl aliquot of DS was then added to the well and the process was repeated. All samples were immediately centrifuged at 16,100 g for three minutes

to form a pellet of *Bd*. Most of the supernatant (280 µl) was removed and discarded and the tube placed in a minus 60°C freezer.

Following 1, 6, 15, and 24 hours of exposure of *Batrachochytrium dendrobatidis* to NaOCl, the procedure detailed above for the control group was repeated with the three NaOCl dilution treatments. As with the TGHL in the controls, the NaOCl solution was removed and discarded from each well prior to the addition of DS. Before the addition and removal of the NaOCl solution, a visual inspection of each well was conducted to assess fungal condition. Quantitative real time TaqMan® (Applied Biosystems) PCR assays were run on all samples using a Rotor-Gene™ 6000 (Corbett Life Sciences) as described by Boyle et al. (2004) with some modifications. In order to test for possible inhibition by residual NaOCl a repeat triplicate analysis was performed on four samples containing the highest concentration of NaOCl (12%), thus the most likely to inhibit, and four controls containing no NaOCl by incorporating the TaqMan® Exogenous internal positive control (IPC) (0.6x Exo IPC Mix, 0.6x Exo IPC DNA) into the assay. Inhibition is indicated by Ct values significantly higher that those obtained for the negative control.

Examination of the data made it clear that some results did not require statistical hypothesis testing. When results were not certain by examination, hypotheses were tested using one-way ANOVA to compare differences among exposure periods within NaOCl treatments, and Bonferroni-adjusted *t*-tests were used to determine whether groups of NaOCl treatments that did not differ significantly among themselves differed significantly from controls. All statistical analyses were performed using Statistica 7.1 (StatSoft Inc.).

Results

The 1% sodium hypochlorite solution did not differ significantly in effectiveness across the four exposure periods (ANOVA, $F = .777$, $df = 3, 27$ $p = 0.519$), and comparison with the control treatment indicated that it did not denature *Batrachochytrium dendrobatidis* DNA effectively enough over any of the four exposure periods for use as a denaturing agent ($t = -.340$, $df = 36$, $p = .736$; Figure 32). Only very low numbers of zoospores were detected in the 6% NaOCl treatment (Figure 32). These did not differ among exposure period treatments (ANOVA, $F = .531$, $df = 3, 27$ $p = .665$), and were significantly reduced compared to controls ($t = 3.088$, $df = 36$, $p = .004$). The 12% NaOCl treatment was 100% effective and denatured all *Batrachochytrium dendrobatidis* DNA across all exposure periods (Figure 32). No inhibition from the 12% NaOCl was detected with the internal positive controls (mean Ct value 12% NaOCl = 28.12, mean Ct value control = 28.61, $t = -0.985$, $df = 6$, $p = .363$). Visual inspection of each well prior to the removal of NaOCl revealed that zoosporangia and zoospores from the 6% and 12% NaOCl treatments were severely fragmented and completely unrecognisable. The 1% NaOCl treatment wells contained shrivelled and shrunken but recognizable zoospores and zoosporangia.

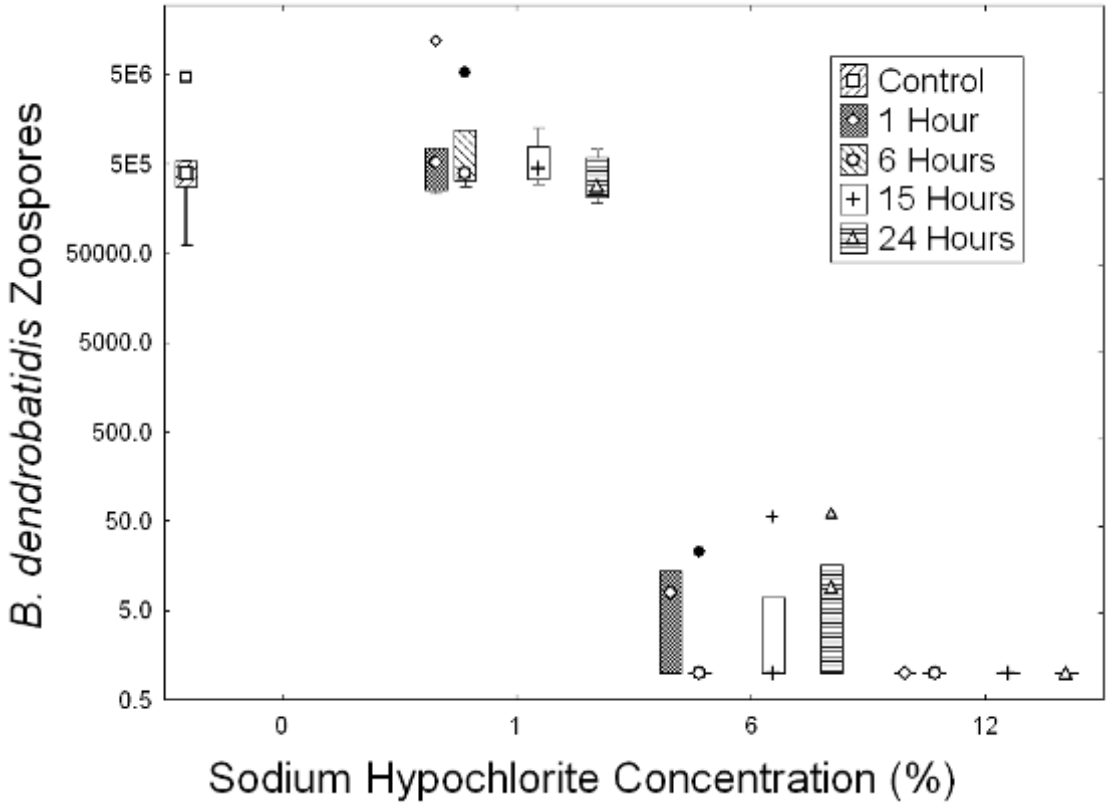


Figure 32.. Numbers of Batrachochytryium dendrobatidis zoospores detected in controls and at three concentrations of sodium hypochlorite after varying periods of exposure. Symbols within boxes represent the median, boxes represent the upper and lower quartiles, whiskers represent the non-outlier spread and symbols above boxes represent the outliers.

Discussion

We found that 12 % sodium hypochlorite can be effectively used to denature 100% of even very high densities of *Batrachochytrium dendrobatidis* within one hour. Similarly, 6% NaOCL was extremely effective, with only 6-13 zoospore equivalents detected across all exposure periods (Figure 32). This represents a mean reduction from the controls of 99.999%. As the PCR assay can detect as little as one zoospore (Boyle et al. 2004), a minimum of approximately 100,000 contaminant zoospores would need on average to be present for a false positive result following treatment with 6% NaOCl. In practice, the number of *B. dendrobatidis* zoospores contaminating equipment is likely to be a fraction of the levels examined in this study. For example, the highest level of *B. dendrobatidis* reported on a swab sample from an infected animal is 545,000 zoospores on a *Mixophyes fasciolatus* (Hyatt et al. 2007). Any contamination of equipment is likely to be at levels far below those of severely infected animals. It is possible then, that a 6% NaOCl solution can be used to denature DNA on clean equipment sufficiently to prevent false positives through PCR. However, as field equipment often contains soil, plant material and other particulates which may either shield *B. dendrobatidis* or decrease the efficiency of NaOCl (LeChevallier et al. 1988) it is far safer and advisable to use 12% NaOCl to prevent cross contamination. Prior to soaking in NaOCl equipment should be cleaned to reduce the negative impact of any attached particles.

The 1% NaOCl solution did not effectively denature *B. dendrobatidis* zoospores at any of the exposure periods and should not be used to prevent sample contamination.

Most household bleach products contain between 4.00 - 6.15% NaOCl and the most common concentration of commercially available bulk NaOCl is 12%, making the concentrations tested here readily available to most researchers. Exactly how sodium hypochlorite deactivates micro-organisms has never been experimentally shown (Gomes et al. 2001). However, it is believed that cell death is a result of oxidation of sulfhydryl groups and amino acids on the exterior of the cell by $OCl^-$ (Eventov et al. 1998). Sodium hypochlorite destroys DNA in a similar manner through oxidative damage (Ohnishi et al. 2002, Kemp & Smith 2005), resulting in the breakdown of DNA into segments shorter than that recognized by the PCR assay (Prince & Andrus 1992). Because PCR is now the preferred diagnostic test for chytridiomycosis (Hyatt et al. 2007) it is crucial to minimise contamination so that results are comparable and reliable. Just as great care should be taken in the laboratory during processing of samples to prevent contamination, equal care must be taken in the field during the collection of samples. When sampling amphibians for infection, each individual should be captured with a separate pair of gloves or plastic bag. If animals are captured in a bag, a fresh pair of gloves should be worn during each swabbing and each swab should be housed individually. These precautions should be sufficient to prevent cross contamination of DNA during field surveys where the animals are captured by hand and returned to their natural habitat following swabbing. However, as researchers move from documenting occurrence and prevalence of chytridiomycosis into more manipulative experimental work, the risk of contaminating samples through shared contact presents an important problem. For example, reusing experimental equipment such as containers, enclosures, sorting trays and nets in conjunction with PCR can result in contamination of samples. Also, equipment used to search for *B. dendrobatidis* in the environment such as filter holders and tubing could cross contaminate samples (Kirshtein et al. 2007). As NaOCl is toxic, great care should be taken to avoid release or spillage of bleach into water bodies, drains or drainages when in the field. Used bleach that needs to be disposed of should always be carried back to the lab or, if absolutely necessary, spread onto a flat surface such as a paved road to evaporate. NaOCl evaporates quickly and breaks down to water, oxygen and table salt (NaCl). However, other chemicals may be added during production, particularly in household cleaning products (Clarkson & Moule 1998). Bleach already forms a part of many researchers' tool kit for the purposes of disinfection. Our experiment shows that NaOCl can also be used to denature *B. dendrobatidis* DNA to prevent its recognition by PCR and reduce the likelihood of cross contamination of samples.

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***Project 4.2. Effect of Sample Collection Techniques on Sensitivity of a Real Time PCR Assay for Detecting the Amphibian Pathogen Batrachochytrium dendrobatidis***

Cashins, S.D., Skerratt, L.F. & Alford, R.A., (in Press). Effect of Sample Collection Techniques on Sensitiviy of a Real Time PCR Assay for Detecting the Amphibian Pathogen Batrachochytrium dendrobatidis. Veterinary Microbiology.

Abstract

*Batrachochytrium dendrobatidis* is a fungal amphibian skin pathogen responsible for the death and decline of hundreds of species of frog across the globe in captivity and in the wild. It has likely caused the extinction of dozens of species from the wild. The World Organisation for Animal Health (OIE) has recently listed chytridiomycosis, the disease caused by this pathogen, as a "notifiable" disease and will establish guidelines to screen animals involved in the international amphibian trade. When selecting a diagnostic tool for the detection of a pathogen, the test should be highly sensitive in order to avoid false negatives. This is particularly true when the test is used to prevent the movement of the pathogen into new areas. Here we test the effect of swab and filtration sample protocols on sensitivity by a real time PCR assay. We find that swabs, used to sample amphibian skin and larval mouthparts, are a very sensitive sampling tool with a low risk of generating false negatives. Two filter types with two pore sizes used to screen water samples were also tested and found to exhibit reduced sensitivity at lower zoospore concentration. These results should assist researchers and international agencies in the use of screening tools and management of chytridiomycosis.

Introduction

The disease chytridiomycosis, caused by the amphibian fungal pathogen *Batrachochytrium dendrobatidis* [Phylum Chytridiomycota, Class Chytridiomycetes, Order Rhyzophydiales, Family not yet placed; (James, et al., 2006; Longcore, et al., 1999) has caused an unprecedented, global loss of amphibian biodiversity (Skerratt, et al., 2007). *Batrachochytrium dendrobatidis* zoosporangia produce aquatic zoospores (2 – 4 μm diameter) that invade the superficial epidermis of the stratum corneum and stratum granulosum of amphibians, resulting in mild skin lesions including hyperplasia, hyperkeratosis and excessive skin sloughing (Berger, et al., 1998; Berger, et al., 2005; Pessier, et al., 1999). Heavy infections appear to disrupt normal cell function, causing osmotic imbalance from loss of electrolytes. A decrease in electrolytes, such as seen in severely infected frogs, can impair cardiac function resulting in death (Voyles, et al., 2007)

This pathogen has emerged globally over the past 30 years (Skerratt, et al., 2007) and evidence implicates human mediated transport of infected hosts as a likely significant driver of spread into new areas (Fisher, Garner, 2007). While many species are highly susceptible to chytridiomycosis, individual hosts can carry sub-lethal infections for long periods before death or even indefinitely with no overt symptoms rendering any amphibian a potential carrier (Daszak, et al., 2004). At least 28 species of introduced amphibians have been known to carry *B. dendrobatidis*, many asymptomatically (Fisher, Garner, 2007). Even heavily infected frogs can appear behaviorally and physiologically normal until just a few days or hours before death (Berger, et al., 2007;

Berger, et al., 2004; Voyles, et al., 2007). As a result, infected frogs have been found within every major industry that moves amphibians, including the pet (Mutschmann, et al., 2000; Speare, 2000), bait (Picco, Collins, 2007), zoological (Pessier, et al., 1999; Raverty, Reynolds, 2001), scientific (Parker, et al., 2002; Weldon, et al., 2004) food stocking (Hanselmann, et al., 2004) and food trading industries (Daszak, et al., 2006; Mazzoni, et al., 2003). Often, transported amphibians escape and establish viable populations. Infected feral bullfrogs (*Rana catesbeiana*) have been found on three continents (Garner, et al., 2006) and were the first wild amphibians found carrying the pathogen in Britain (Cunningham, et al., 2005).

Despite tens of millions of frogs being transported annually (OIE, 2006), the amphibian trade has remained largely unregulated. More stringent inspection of this global market is important to prevent further spread of this and other amphibian diseases. Experts in the field, national threat abatement plans and international action plans urgently recommend screening of transported amphibians at the national and international level as a key regulatory practice to prevent continued spread through trade (Australian Government Department of the Environment and Heritage, 2006; Cunningham, et al., 2001; Daszak, et al., 2000; Daszak, et al., 2007; Fisher, Garner, 2007). The World Organisation for Animal Health (OIE) listed chytridiomycosis as a "wildlife disease of concern" in 2001 (OIE, 2006) and has recently listed chytridiomycosis as a "notifiable disease" (pers. comm. Peter Daszak, member OIE Amphibian Disease Committee). Testing of amphibians for import and export will now be required under OIE guidelines. For screening to be an effective control strategy, the diagnostic test used to identify infected individuals must have high sensitivity, specificity, repeatability, reproducibility and recoverability. The quantitative real-time PCR (qPCR) assay developed for *B. dendrobatidis* has been validated to be highly specific, repeatable, reproducible and sensitive in sampling known infected individuals (Boyle, et al., 2004; Hyatt, et al., 2007). However, how the recoverability (extraction efficiency) of *B. dendrobatidis* DNA from the various sampling materials (swabs or filters) affects sensitivity is unknown. The effect of sampling technique on quantitation from a diagnostic qPCR assay is important as it indicates how likely the assay will be to detect a target organism successfully collected during sampling and how accurately it determines the amount of organism present. Quantifying this is particularly important to be able to confidently accept a negative qPCR result, which is essential, particularly when screening the animal trade.

Potentially infected amphibians are typically sampled using cotton swabs. Swabs are systematically passed over the skin of adults or mouthparts of larvae to collect *B. dendrobatidis* zoosporangia and zoospores present within the superficial epidermal layer. The cotton bud is snapped off the applicator stick into the reaction tube and processed through PCR (Hyatt, et al., 2007).

Filtration can also be used to sample amphibians (Hyatt, et al., 2007) and has recently emerged as an important technique to detect *B. dendrobatidis* in the aquatic environment (Kirshtein, et al., 2007; Walker, et al., 2007). While there are a variety of possible filtration methods, the quickest and least complicated method is to simply push water samples through a filter membrane. The trapped zoospores are extracted and processed through PCR in the same manner as swab samples. An important application of filtration may be to screen samples of water transported within and between regions. *Batrachochytrium dendrobatidis* can survive up to six weeks in sterile water and moist

sand (Johnson, Speare, 2003). Therefore, transported water previously in contact with an infected amphibian, even weeks prior, could harbor the pathogen and should be screened or disinfected (for treatments see: Johnson, et al., 2003; Webb, et al., 2007). Here we compare five different sampling materials used to collect *B. dendrobatidis*; one swab, and four filters (two filter types x two pore sizes) across four dilutions of zoospores to determine how efficiently DNA is extracted from each and to provide recommendations for the best filtration materials.

Materials and Methods

We established that swabs validated for sampling amphibians (Hyatt, et al., 2007; tubed dryswab, Medical Wire & Equipment Co (Bath) Ltd.) could absorb 10 µl of fluid with no loss. Therefore all treatments (swabs and filters) and controls were inoculated with 10 µl of *B. dendrobatidis* culture. Innoculations of *B. dendrobatidis* zoospores were prepared by seeding two ml of actively growing culture in TGhL broth (16 g tryptone, 4g gelatin hydrolysate, 2g lactose, 1000ml ultrapure water) onto two TGhL agar plates. Plates were sealed with Parafilm® and incubated at 21°C. Following five days, zoospores were harvested by flooding each plate twice with two ml DS (dilute salts solution; $10^{-3}$M $KH_2PO_4$, $10^{-4}$ M $MgCl_2$ and 2 x $10^{-5}$ M $CaCl_2$) and leaving for two minutes. The eight ml zoospore suspension was centrifuged at 4500 x g for five minutes to pellet the zoospores. The supernatant was removed and DS added to create a concentrated suspension. Four independent zoospore counts were made with a haemocytometer. The final concentration was 5.72 x $10^6$ zoospores/ml. This was serially diluted 1:10 three times to form four concentrations. Ten microliters from the four dilutions (for total zoospore counts of: 57,200; 5,720; 572 and 57) were subjected to each of the following five sample collection treatments: "Swab" and four different membrane "Filters" (two filter types x two pore sizes). Each treatment was replicated three times prior to qPCR processing. There was also a group (designated "Control") in which the above dilutions were directly processed for qPCR without application of a sample collection treatment and served as a comparison for the treatments. All samples were stored at -30°C until DNA extraction and qPCR processing.

<u>*Swab*</u>: Ten µl was applied directly to each swab. Swab tips were then snapped off into an eppendorf tube for DNA extraction.
<u>*Filters*</u>: The following four 25mm filter membranes were tested: MF 1.2 and 5.0 µm Millipore ™ membranes (mixed cellulose esters) and IP 2.0 and 5.0 µm Isopore ™ Millipore membranes (polycarbonate). Each membrane was held in a Swinnex ® filter holder (Millipore). Five ml DS was added to five ml syringes. The filter holders were attached to the syringe tip and 10 µl of zoospore dilution was added to the DS. The syringe plunger was inserted and the syringe inverted five times to evenly distribute zoospores before pushing the liquid through the filter. This was repeated once with clean DS to rinse. The membranes were removed with sterile forceps, allowed to dry and placed in Eppendorf tubes for DNA extraction.
<u>*Control*</u>: Ten µl of pure culture at the four dilutions above were added directly to an Eppendorf tube for DNA extraction.

All samples were bead-beaten with 30 – 40 mg zirconium/silica beads. DNA was extracted in PrepMan Ultra and processed with real-time TaqMan® (Applied Biosystems) quantitative PCR assay following the protocol described by Boyle *et al*. (2004). The analysis was performed on the Rotor-Gene™ 6000 (Corbett Research)

using Gene-Disc 100 tubes. The sample extract and negative control were diluted 1 in 10. Triplicate analyses were performed for each sample, negative control and no-template-control, and quadruplicate analyses were undertaken for each standard (100, 10, 1, 0.1 zoospore equivalents). For statistical analyses we use the cycle threshold (Ct) value at a threshold of 0.01. If a sample contains more initial DNA it will reach the threshold of detection at a lower Ct value. Hypotheses were tested using two-way ANOVA to compare differences in Ct values among collection materials and zoospore concentrations. *Post hoc* tests (Tukey's HSD) were used to identify which treatments differed. All statistical analyses were performed with Statistica 7 (Statsoft, Tulsa OK, USA).

Results

There was no significant difference in extraction efficiency and therefore sensitivity between the swab samples and the pure culture controls at any of the four *B. dendrobatidis* concentrations (Figure 33; df = 48. 57 zoospores, p = 1.000; 572 zoospores, p = .926; 5,720 zoospores, p = 1.000; 57,200 zoospores, p = 1.000). The same was not true of the filters. When averaged across dilution treatments, less *B. dendrobatidis* was detected from each of the four filters than from the pure culture controls (Figure 34; df = 48. IP 2.0, p = .0008; IP 5.0, p = .0003; MF 1.2, p = .0001; MF 5.0, p = .0003). However, when we looked at the interaction between filter type and zoospore concentration separately we found that only at the lower concentrations were fewer zoospores detected compared with pure culture controls. All four filter types from the lowest concentration (57 zoospores) had Ct values significantly higher than controls (df = 48. IP 2.0, p = .0008; IP 5.0, p = .0273; MF 1.2, p = .0049; MF 5.0, p = .0126). The only other filter treatment with a Ct value significantly higher than the controls was the MF 1.2 filter at the 572 zoospore concentration(df = 48, p = .0040). There was no difference among filters at any concentration level.

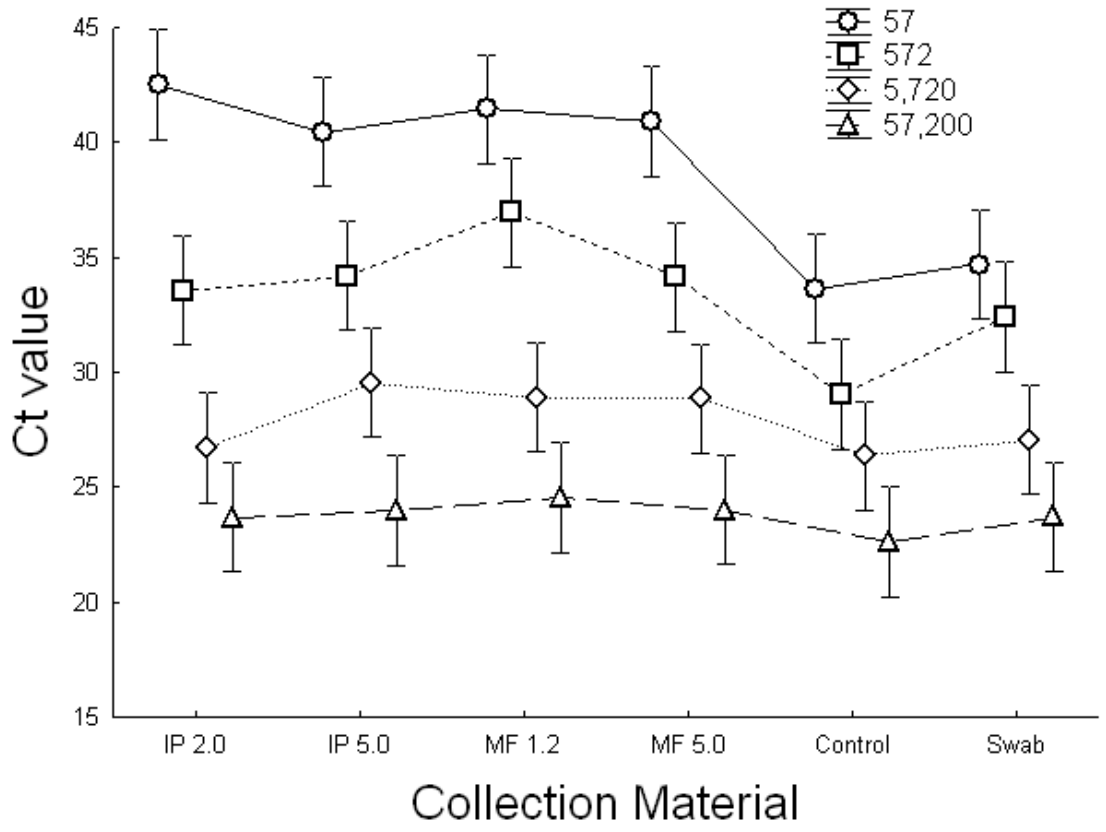


**Figure 33. Interaction diagram between sample material and zoospore concentration. Symbols represent the mean cycle threshold value (Ct) and whiskers represent the 95% confidence interval. Connecting lines are included to aid in visualization. IP 2.0 = Isopore 2.0 µm, IP 5.0 = Isopore 5.0 µm, MF 1.2 = Mixed Cellulose Esters 1.2 µm, MF 5.0 = Mixed Cellulose Esters 5.0 µm.**

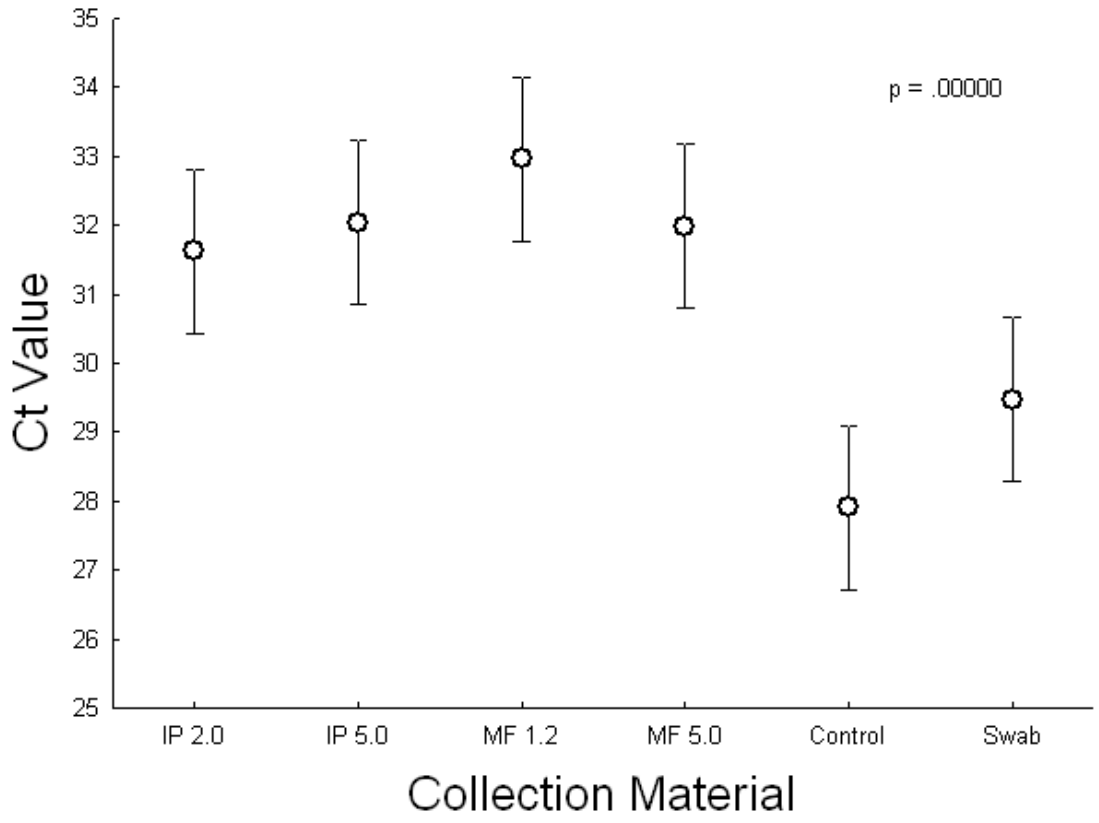


Figure 34. Mean cycle threshold value (Ct) by sample material averaged across zoospore concentration. Symbols represent the mean Ct value and whiskers represent the 95% confidence interval. IP 2.0 = Isopore 2.0 µm, IP 5.0 = Isopore 5.0 µm, MF 1.2 = Mixed Cellulose Esters 1.2 µm, MF 5.0 = Mixed Cellulose Esters 5.0 µm.

Discussion

The Ct value of swab samples was not significantly different from the controls indicating a very high percentage of *B. dendrobatidis* DNA present on swabs was recovered during extraction. Therefore, when *B. dendrobatidis* is successfully collected on a swab and processed following the protocol of Boyle et al (2004), the end result will be efficient extraction and amplification of DNA resulting in a highly sensitive test. Therefore, a negative qPCR assay accurately reports the absence of *B. dendrobatidis* DNA on the swab in the absence of other factors such as inhibition of qPCR. This high level of DNA recoverability, in addition to the demonstrated high level of sensitivity, specificity, repeatability and reproducibility (Hyatt, et al., 2007) make this PCR-based swab assay an ideal diagnostic tool to screen animals involved in the global amphibian trade and for research efforts to study this disease.

We found some loss of DNA during filtration, extraction and qPCR assay and, predictably, this loss had a greater relative impact when *B. dendrobatidis* density was low. This loss resulted in, on average, 7.7 additional amplification cycles before reaching the Ct threshold at the 57 zoospore concentration, 5.7 additional cycles at 572 zoospores, 2.2 cycles at 5,720 zoospores and 1.4 cycles at 57,200 zoospores. This makes the lowest consistently detectable number of zoospores (a positive reaction in

each of three wells) with these filters, approximately 40 zoospores under clean conditions (ie. no inhibition). In turbid or sediment-laden water more zoospores are likely needed in the sample to achieve consistent positive results (Kirshtein, et al., 2007; Walker, et al., 2007). Particles in water samples can cause inhibition of PCR. DNA soil extraction kits such as MoBio Power Soil can be used to purify the sample and reduce inhibition (Walker, et al., 2007). Alternatively, increasing extraction dilutions from 1 in 10 can also remove inhibition. However, both techniques will decrease sensitivity requiring more initial target DNA to be present.

The levels of *B. dendrobatidis* detected in studied pond sites has been relatively high (between .5 to 454 zoospore equivalents l-1Kirshtein, et al., 2007; Walker, et al., 2007), however, at many sites, such as fast flowing streams, levels of *B. dendrobatidis* are likely to be much lower as a result of the constant flushing and dispersal of zoospores in the current. Increasing the volume of water sampled will improve the chances of detecting low levels of *B. dendrobatidis*. Accordingly, we tested filters with pore sizes above and below either end of the zoospore diameter spectrum (2 – 4 μm), in order to maximize the volume of liquid sampled. We detected no difference between filter types or pore sizes. Millipore Isopore membranes (polycarbonate) contain a uniform pore size, trapping all particles larger than the pores on the surface. This allows easier extraction of sample material for microscopic assessment. These filters are non-absorbent, resulting in faster drying time following filtration and less absorption of reagents during processing. In comparison, the Millipore MF (mixed cellulose esters) membranes do not have a uniform pore size, instead particles are trapped in a matrix of interwoven fibers rendering most sample material inaccessible for microscopic assessment. These filters are absorbent, resulting in longer drying times and increased adsorption of reagents. As all filters were found to be equally efficient, we recommend the Millipore Isopore 5 μm membrane for *B. dendrobatidis* filtration to maximize volume of water sampled and reduce drying time in the field and absorption of reagents in the laboratory.

We have shown *B. dendrobatidis* DNA collected on swabs is extracted efficiently and quantified accurately using the real time qPCR assay developed for this organism and therefore is a sensitive sampling tool. The global movement of amphibians appears to be an important driver of spread of amphibian pathogens such as *B. dendrobatidis*, into new areas. Following the recent recognition of chytridiomycosis by the OIE as a notifiable disease and the expected increased screening of the amphibian trade, it is important to have confidence in negative results generated by the diagnostic test. Our results show that such confidence is warranted when sampling by swab. We have also shown that extraction of DNA from filters is less efficient than from swabs. At lower zoospore concentrations, significantly fewer zoospore equivalents were detected on filters than controls. At higher concentrations, there was no significant difference between filters and controls or among filter types and pore sizes. As a result, we suggest maximizing sample volume and minimizing reagent loss by using the Millipore Isopore 5.0 μm membrane.

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## ***Project 4.3. Lethal Effect of Latex, Vinyl and Nitrile Gloves on Tadpoles***

Modified version of: Cashins, S.D., Alford, R.A., Skerratt, L.F., 2008. Lethal Effect of Latex, Vinyl and Nitrile Gloves on Tadpoles. Herpetological Review 39, 298-301.

### Introduction

Tadpoles are studied in a variety of fields including husbandry, developmental physiology, toxicity testing, and basic biological and ecological research. In many instances it is necessary to use gloves when handling tadpoles or during water changes to protect the experimenter (e.g. teratology research) or to promote hygiene and prevent the transfer of pathogens between tadpoles (Retallick, et al., 2006; Sobotka and Rahwan, 1999). While investigating aspects of the virulent amphibian fungal pathogen, *Batrachochytrium dendrobatidis*, we discovered that a variety of gloves can be lethal to tadpoles. We present here two case studies, one in the lab, one in the field, and two experiments, all demonstrating the lethal effect of gloves on tadpoles. Following exposure to the various glove treatments, all tadpoles were categorized as either fine, listless, or dead.

### Case study 1: laboratory

*Batrachochytrium dendrobatidis* infects the skin of frogs, but only the mouthparts of tadpoles (Knapp and Morgan, 2006; Marantelli, et al., 2004). During a laboratory experiment investigating *B. dendrobatidis* infection in *Litoria genimaculata* and *Litoria nannotis* tadpoles, each tadpole was to be measured, weighed and its mouthparts swabbed with a sterile cotton swab to test for *B. dendrobatidis* by diagnostic PCR (Boyle, et al., 2004). A new pair of latex gloves (SuperMax, low powder) were worn when handling each tadpole to prevent transmission of *B. dendrobatidis* between individuals and to prevent DNA contamination of swab samples. Each tadpole was scooped out of its container with a gloved hand. The tadpole was secured, ventral surface up, in between the index and middle fingers by gently depressing the thumb to the base of the tail. A swab was gently passed over the mouthparts repeatedly to collect *B. dendrobatidis* DNA on the cotton fibers. Each tadpole was in hand for approximately 30 to 90 seconds before being returned to its container.

Thirty-six *L. genimaculata* had been processed in this way when we observed that some of the earliest handled tadpoles appeared listless, could not remain upright and had difficulty using their tails for locomotion. Upon closer inspection the tails of the listless tadpoles were gray and dead in appearance at the locations where gloved fingers held them in place during swabbing. At that time, we suspended tadpole handling. Within 24 hours 26 of the 36 tadpoles died. The surviving ten tadpoles did not appear listless, showed no overt adverse effects and survived 4 – 6 weeks to metamorphosis (Figure 35A). Although care was taken to handle tadpoles gently, the observed mortality could possibly have been due to mechanical damage, so we initiated a series of experiments. Based on the results of these experiments (see below) we switched from latex to vinyl gloves for the remainder of the lab study. Ten unhandled *L. genimaculata* and *L. nannotis* tadpoles were processed as previously described except with vinyl instead of latex gloves. Following 24 hours of observation no mortality or ill effects were noted. Satisfied that vinyl gloves were safe for tadpoles of these species, the remaining 13 *L. genimaculata* and 22 *L. nannotis* tadpoles were processed using vinyl gloves (Figure

35A). All tadpoles appeared unaffected after handling. In total, 26/36 *L. genimaculata* died following handling with latex gloves, while 0/23 *L. genimaculata* and 0/32 *L. nannotis* died following contact with vinyl gloves.

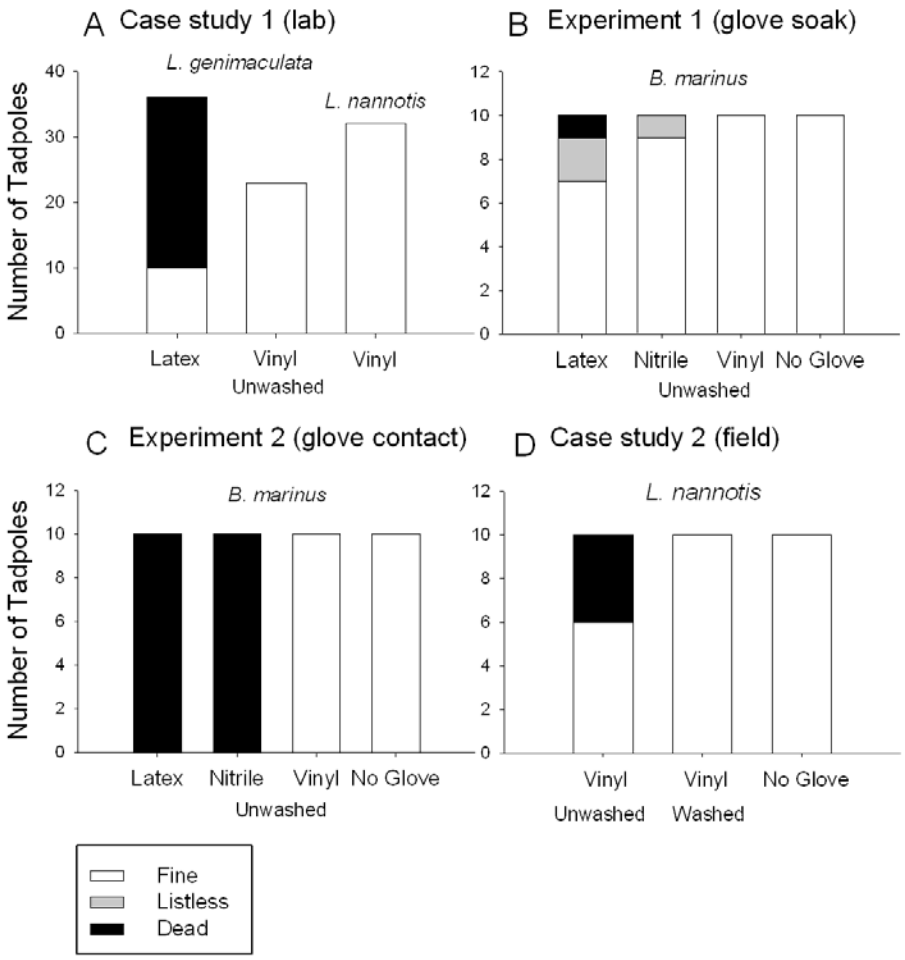


Figure 35. Tadpole mortality in response to disposable glove exposure. (A) Case study 1: laboratory; Number of *Litoria genimaculata* and *Litoria nannotis* tadpole deaths within 24 hours following contact with latex or vinyl gloves for 30 – 90 seconds. (B) Experiment 1: glove soak; Number of *Bufo marinus* tadpole deaths within 72 hours following contact with water containing latex, vinyl and nitrile gloves soaked for 5 minutes. (C) Experiment 2: glove contact; Number of *B. marinus* tadpole deaths within 24 hours following direct contact with latex, vinyl, and nitrile gloves and bare hands for 60 seconds. (D) Case study 2: field; Number of *L. nannotis* deaths within 24 hours following direct contact with unwashed vinyl gloves, washed vinyl gloves and bare hands for 30-90 seconds.

Experiment 1: glove soak

In order to determine the best gloves for handling tadpoles we conducted an experiment testing the three most common glove types; latex (SuperMax, low powder), vinyl (Livingstone, clear, low powder) and nitrile (Livingstone, low powder). Forty non-native *Bufo marinus* tadpoles were captured from a local pond and allowed to rest in individual 1000 mL containers with 500 mL of collected rainwater and a pinch of powdered tadpole chow (3:1 alfalfa pellets:fish food, ground and passed through a 250 μm sieve). After 24 hours one of each glove type was draped over the edge of the container for five minutes so that the five fingers of each glove were submerged. Ten control containers had no contact with a glove. The condition of each tadpole was recorded at 2, 12, 24, and 72 hours following removal of the glove, however, tadpole condition did not change beyond the two hour post-exposure point. One of ten tadpoles exposed to the latex gloves died and two more were listless and floating awkwardly within two hours of glove exposure. One of the tadpoles exposed to nitrile gloves was listless while all of the vinyl and control treatments appeared unaffected (Figure 35B). The listless tadpoles remained in an impaired state for the full 72 hours of observation and appeared permanently affected. These listless tadpoles were euthanized and preserved in 70% ethanol. Although three of the ten *B. marinus* tadpoles exposed to latex gloves experienced deleterious effects, the rate of mortality was lower than we expected given the high level of mortality we previously observed in *L. genimaculata*. We hypothesized this difference was related to the different methods of glove exposure. The *L. genimaculata* in "Case study 1" were in direct physical contact with the gloves during measuring and swabbing while the *B. marinus* in "Experiment 1" were in water in which gloves were soaked. This indirect glove contact may have yielded a lower dose of the toxic compound(s).

Experiment 2: glove contact

To determine whether direct glove contact increases mortality we ran a second experiment in which *B. marinus* tadpoles were handled in the same manner as previously described for *L. genimaculata* and *L. nannotis*. Each tadpole was gently held in place at the base of the tail between the thumb and index finger for 60 seconds with one of the three types of gloves as treatments or with an ungloved hand as a control. A new glove was worn for each tadpole and the treatments were interspersed, with each glove type and the bare hand treatment applied in succession. Prior to the no glove treatment, hands were rinsed in tap water and dried with a paper towel to remove any residual powder from the previous glove treatment.

Within two hours of handling, all tadpoles that had been in contact with latex or nitrile gloves were dead or listless. Those that were listless died within 24 hours (Figure 35C). Listless tadpoles had little to no tail function and the usually dark black tail had a discolored, dead-looking, gray appearance. This discoloration was most pronounced where direct contact with the gloves occurred. Particles in the water soon began to attach to the epidermis of the dying tail, giving it a fuzzy appearance. None of the tadpoles handled with either vinyl gloves or bare hands suffered noticeable ill effects and all survived to metamorphosis (Figure 35C).

Case Study 2: field

We applied our conclusion that vinyl is the safest glove material to "Case Study 1: lab" (described above) as well as a field study monitoring *B. dendrobatidis* in the wild. Individual tadpoles were to be captured, handled with vinyl gloves, measured, swabbed

for *B. dendrobatidis* infection and returned to the stream unharmed. During initial field sampling, individuals were processed and kept temporarily in a holding tray to monitor condition following swabbing. Unexpectedly, of the first ten *L. nannotis* tadpoles processed, four became listless and died within one hour (Figure 35D). The remaining six tadpoles appeared normal and did not develop signs over the following 24 hours. As a test, the next ten captured tadpoles were processed with bare hands and suffered no ill effects, suggesting the gloves and not the handling were the cause of mortality. The next ten captured tadpoles were processed with vinyl gloves that were rinsed in a bucket of water prior to handling. All of these tadpoles survived and appeared normal suggesting that a substance on the outside of the vinyl glove was toxic and that rinsing successfully removed it. All tadpoles were held for 24 hours for observation. From this point on we incorporated the rinsing of vinyl gloves into the standard field protocol. Vinyl gloves were rinsed in a 10 L bucket of water which was changed after at most ten tadpoles. This was adequate to ensure the glove-wash residue did not attain a high enough concentration to cause harm. To date over 2500 tadpoles have been handled with washed vinyl gloves with no ill effects. On a few occasions, the rinsing step was accidentally skipped and many of these tadpoles became listless and died.

The fact that the same type and brand of vinyl glove did not cause mortality in *L. nannotis* tadpoles in the laboratory trials but did cause mortality in the field suggests that the presence or level of the toxic compound(s) may vary among boxes of gloves. This may be a result of varying conditions during glove fabrication. During production of disposable gloves a large number of chemicals are added including vulcanizers, accelerators, colorants, preservatives, stabilizers and antistatic agents (Boman, et al., 2004). These chemicals are typically the cause of glove sensitivity in humans. The type and quantity of these compounds can vary widely among manufacturers and possibly even production runs (Boman, et al., 2004).

Our results show that unwashed latex, nitrile and vinyl gloves can be toxic to tadpoles. Unwashed latex and nitrile gloves caused up to 100% tadpole mortality following only 30 – 90 seconds of direct contact (Figure 35C). Rapid, localized necrosis of tissue at the point of contact was observed grossly. Even five minutes of partial glove submersion was sufficient to cause mortality in the latex and nitrile treatments (Figure 35B).

Despite a thorough literature search, only two references to the toxic effects of gloves on tadpoles were found and both of these were published in toxicological journals, likely to have low readership by herpetologists. In a letter to the editor, Sobotka and Rahwan (1999) reported that water from unwashed latex gloves (American Dental Association, Safeskin brand) and washed latex gloves (Baxter Pharmaseal Flexam) soaked for 24 hours caused mortality in *Xenopus laevis* tadpoles. However, water from washed vinyl gloves (Baxter Triflex) did not. Gutleb et al. (2001) reported 100% mortality in *Xenopus laevis and Rana temporaria* tadpoles exposed to water from unwashed latex gloves (Becton-Dickinson) soaked for 24 hours. Even very dilute solutions of glove- soaked water (0.29% for *X. laevis* and 0.15% for *R. temporaria*) caused 100% mortality. Gutleb et al. (2001) found that vinyl gloves (Becton-Dickinson) soaked for 24 hours also killed tadpoles, but only at relatively high concentrations: 33% and above. Mortality was 100% at or above this concentration but 0% below this concentration.

Our results, together with the results from these published studies, demonstrate the potentially high toxicity of latex gloves to tadpoles. Different brands of latex gloves, different exposure methods, and tadpoles of different species were used in each study. Sobotka and Rahwan (1999) tested washed and unwashed latex gloves. The end result, however, was the same: significant tadpole mortality. This suggests that glove toxicity may be associated with many different brands of disposable latex glove and tadpoles of many different species are likely to be affected. Ours is the first report that nitrile gloves can also be extremely toxic to tadpoles, producing 100% mortality in *B. marinus* following direct glove contact.

We found that unwashed vinyl gloves can also cause mortality, however, at a lower rate than either latex or nitrile gloves. This finding is supported by Gutleb et al. (2001) who found that vinyl glove-soaked water caused mortality only at dilutions over 110 times more concentrated than latex glove soaked water. Importantly, by rinsing the vinyl gloves in water we eliminated any obvious toxicity.

As a result of the apparently more toxic nature of latex and nitrile gloves compared with vinyl, and the ability to eliminate toxicity in vinyl gloves through rinsing, we recommend the use of well rinsed vinyl gloves when handling tadpoles or cleaning aquaria. However, all glove brands and types are potentially toxic and should not be used until proven safe with tadpoles of the particular species being handled. Even then, handled tadpoles should be observed carefully as toxicity may vary between production runs.

It is important to note that gloves have not been found to negatively affect juvenile or adult amphibians. The use of gloves to handle amphibians is widespread in the field and lab. Changing gloves between amphibians remains an important hygiene measure to prevent transmission of infectious agents such as *B. dendrobatidis* and ranaviruses between individual amphibians and aquaria. However, given our tadpole results, it would be useful to formally investigate potential non-lethal effects of gloves on adult and juvenile amphibians to ensure that gloves really are entirely non-injurious.

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## ***Project 4.4 Tadpole Ecology: the context of interactions with B. dendrobatidis***

Scott D. Cashins

Introduction

Understanding a host-pathogen interaction within individual hosts and, more broadly, within a community requires knowledge of the ecology of each component. By approaching the study of disease from within the context of their respective ecologies, and the elements of the environment they interact in, we gain a greater ability to properly interpret and integrate data across a range of levels; from microbe to host to population to landscape. This integrated approach is needed to address many aspects of disease ecology including persistence, transmission, seasonality, virulence, resistance and recovery.

Tadpoles are a strictly growth and energy-gathering stage of adult frogs. The primary aim of tadpoles, therefore, is to consume resources, and survive to metamorphose at as large a size and as quickly as possible within the physical and physiological limits of the species (Wilbur 1980). Accordingly, a tadpoles' "fitness" is based on the physical condition the individual achieves at the transition to the terrestrial phase. Individuals that metamorphose at a size, time and condition that increases reproductive success as adults have a selective advantage. A larger size and early season emergence are shown to lead to a larger size and increased survival rate at first breeding, a decreased time to reach reproductive maturity, and larger egg clutches (Scott 1994, Altwegg & Reyer 2003, Chelgren et al. 2006). These traits contribute to an increase in offspring over the course of a lifetime.

To achieve maximum fitness, tadpoles, like most communities, partition both seasonally and spatially, thereby maximizing access to resources and reducing competition (Schoener 1974, Heyer 1976). Variation in larval partitioning is driven and maintained ultimately by the reproductive success of individuals in the adult life stage. This selective pressure on tadpoles to maximize condition at metamorphosis has led to an impressive diversity of larval body forms and strategies to exploit the spectrum of aquatic microhabitats (Altig & Johnston 1989). These divergent strategies, while important in maximizing nutrient acquisition, may result in differential risk of exposure and differential response to infection following the emergence of a virulent pathogen such as *Batrachochytrium dendrobatidis*.

Much of the study of tadpole ecology has focused on lentic communities. Stream environments have been comparatively neglected, particularly within the tropics. However, see: Inger et. al (1986) and Eterovick and Barata (2006). Regional forces of species formation, geographic dispersal, and past chance evolutionary events establish the species composition for local processes to act upon (Ricklefs 1987). An interaction of local processes, including competitive exclusion (Alford & Crump 1982), competition for food resources (Morin 1983) predation (Heyer et al. 1975), abiotic parameters (Dunson & Travis 1991) and adult breeding behavior (Kopp & Eterovick 2006) determine partitioning of the assemblage in time and space. Frequently, a combination of factors is important (Eterovick & Barata 2006). However, in some cases, one dominant factor such as can drive partitioning (Inger et al. 1986). I studied aspects of the behavior and the seasonal and spatial distribution of tadpoles at two

streams in the Australian tropics over two years. These data are combined with patterns of *B. dendrobatidis* infection to contribute to the understanding of *Bd* in streams and the epidemiology of chytridiomycosis in tadpoles.

Site and Species Descriptions
Habitat

Sampling occurred between February 2006 and February 2008 at two lowland rainforest streams in northeast Queensland, Australia: the creek at bridge 7 in Murray Upper National Park (145° 52.116 E 18° 11.750 S, 210 m asl) and an unnamed creek in Tully Gorge National Park (145° 38.747 E 17°46.340 S, 130 m asl, Figure 36-37). Both sites are surrounded by simple notophyll vine forest. The creeks are generally fast flowing and creek beds are characterized by medium to large granite boulders. Small waterfalls, riffles and runs (> 5cm/$s^2$ flow rate; Figures 39-41) are the dominant stream habitat. Climate is highly seasonal, allowing in-stream "connected" pools (< 5 cm/$s^2$ flow rate; Figure 38) to form in the deeper sections, particularly during the cool/drier winter months (May – Nov) when water levels drop. The warm/wet summer (Dec - April) brings monsoonal rains, increasing water flows, and causing periodic spates where water levels rise dramatically, scouring rock surfaces and redistributing boulders and debris. Due to streambed topography that includes both steep and flat sections (Figures 39-41), a large variation in water velocity exists, resulting in a range of microhabitats and creek bed substrates. In the higher velocity sections, the water current prevents leaf litter, or detritus from settling. The rock surfaces in these habitats appear bare, but support a community of microorganisms (biofilm), including plankton, diatoms, protozoa, bacteria and fungi (Lear et al. 2008). The material that remains suspended and passes over the runs and riffles settle in the pools. Here, sand, leaf litter and detritus accumulate. At Tully, the recession of stream water after heavy rain left stream side rock depressions filled with water. Leaves and other plant material can fall into these isolated pools following formation, introducing nutrients. At times, however, they can remain free of external organic inputs.

Figure 36. Location of Field Sites in the Wet Tropics, Queensland Australia.

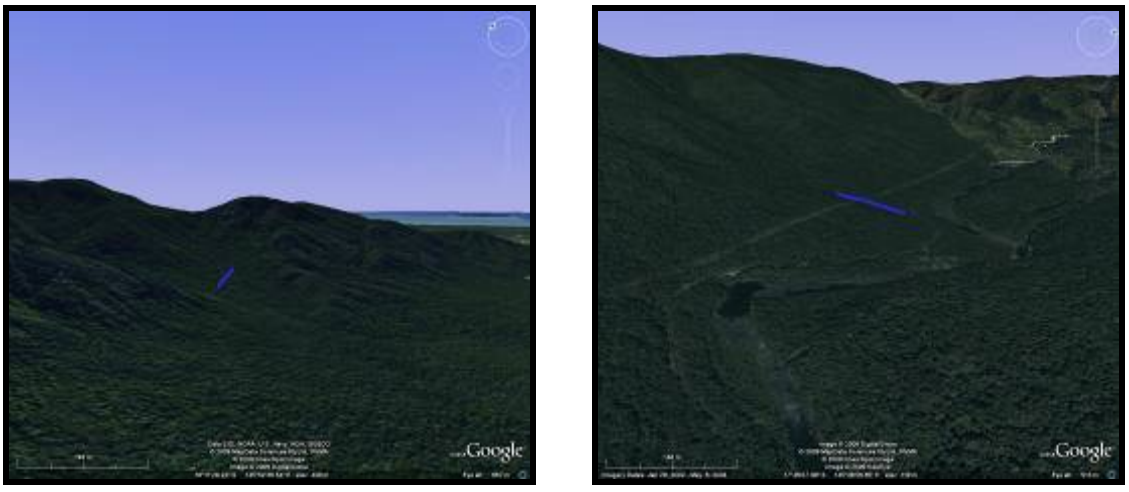

Figure 37. Close up of field sites. Left: Murray Upper National Park, Right: Tully Gorge National Park. Blue lines indicate location of transects.

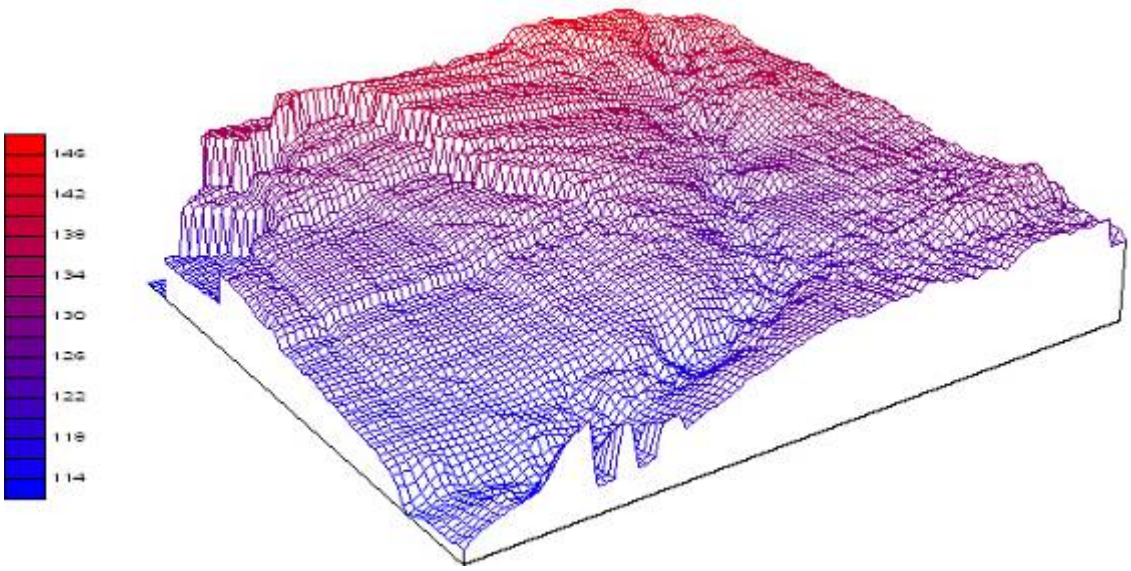


Figure 38. Topography of Tully Gorge National Park Site. Graph key represents elevation in meters.

Figure 39. Tully Gorge National Park. A series of riffles and small cascades.

Figure 40. Murray Upper National Park.  A series of riffles ending with a connected pool.


Figure 41. Tully Gorge National Park following heavy rain.

Species

The amphibian community within these two creeks is comprised of five species (*Litoria nannotis, L. rheocola, L. genimaculata*, and *N. dayi*) that utilize the stream throughout the year for habitat, foraging, breeding and egg deposition. These species declined throughout their range around the time *Bd* first emerged and all, except *L. genimaculata*, disappeared above 400 meters elevation (Berger et al. 1999, McDonald & Alford 1999). Below 400 meters, these species now persist with *Bd* endemically, but suffer seasonal mortality due to chytridiomycosis (Andrea Phillott pers. com.). A fifth species, *Taudactylus acutirostris*, has not been seen at these sites since the early 1990's. Evidence indicates their disappearance was due to the emergence of chytridiomycosis (Schloegel et al. 2006).

Additional species utilize the stream habitat primarily for breeding and are occasionally found along the stream, but spend a significant proportion of their time on land. These frogs are not known to have declined and include the *Litoria leseuri* complex, *L.* xanthomera and *Mixophyes schevilli*. The non-native *Chaunus marinus* is occasionally found stream side and can breed in slow streams, but typically deposits eggs in shallow puddles and ephemeral pools. Terrestrial and direct developing microhylids can be found in the surrounding forest. None of these frogs appear to have declined.

Tadpoles of *L. nannotis*, *L. rheocola*, *N. dayi* and the extinct *T. acutirostris* can be classified as members in the "lotic-suctorial" ecomorphological guild (Altig & Johnston 1989). They are considered to be "torrent-adapted" because they inhabit the faster flowing (>.05 m/sec), turbulent sections of the stream (Liem & Hosmer 1973, Richards 1992). Torrent-adapted tadpoles are characterized by a hydrodynamic, depressed body shape, large ventral oral disc, and muscular tail with low fins (Richards 2002) (Figure 42 A-C). These adaptations allow them to adhere to boulder surfaces in fast-flowing habitat, exploit the resources present, and avoid being swept downstream during flood events (Figure 41).
Torrent tadpoles adhere to substrates in fast flowing water, using keratinized jaw sheaths and tooth rows (Figure 42 D) to scrape and extract the nutritional material present on rock surfaces. Studies examining specifically what food source suctorial tadpoles derive most of their nutrition from are lacking. However, tadpoles are believed to assimilate not only the algae and diatoms ubiquitous in gut content analyses, but also the associated protozoa, bacteria, and fungi (Whiles et al. 2006, Altig et al. 2007). During the course of this study, *N. dayi* tadpoles were difficult to capture and even more difficult to observe. Therefore, most observations and data on torrent tadpoles focuses on the more abundant *L. nannotis* and *L. rheocola*.

*Litoria genimaculata* tadpoles are members of the "clasping" ecomorphological guild and are adapted to the slow-flowing (< .05 m/sec) connected-pool stream environment (Figure 40). They are less streamlined in shape than the torrent-adapted tadpoles, have smaller mouthparts and deeper tail fins. The ability to maintain position with oral disc is minor.

*Litoria xanthomera* is in the "nektonic" ecomorphological guild (Altig & Johnston 1989). Tadpoles are found in ponds and isolated pools adjacent to rainforest creeks. Adult *L. xanthomera* descend from the tree canopy during heavy rains to breed and deposit eggs before, presumably returning to the tree tops (Barker et al. 1995).

Frogs from the *L. leseuri* complex and *M. schevilli* are known to deposit eggs in connected stream pools. Over the course of this study only one tadpole of these species was ever found (a single *M. schevilli* at Murray Upper National Park). As a result, I will not discuss these, or *C. marinus* tadpoles.

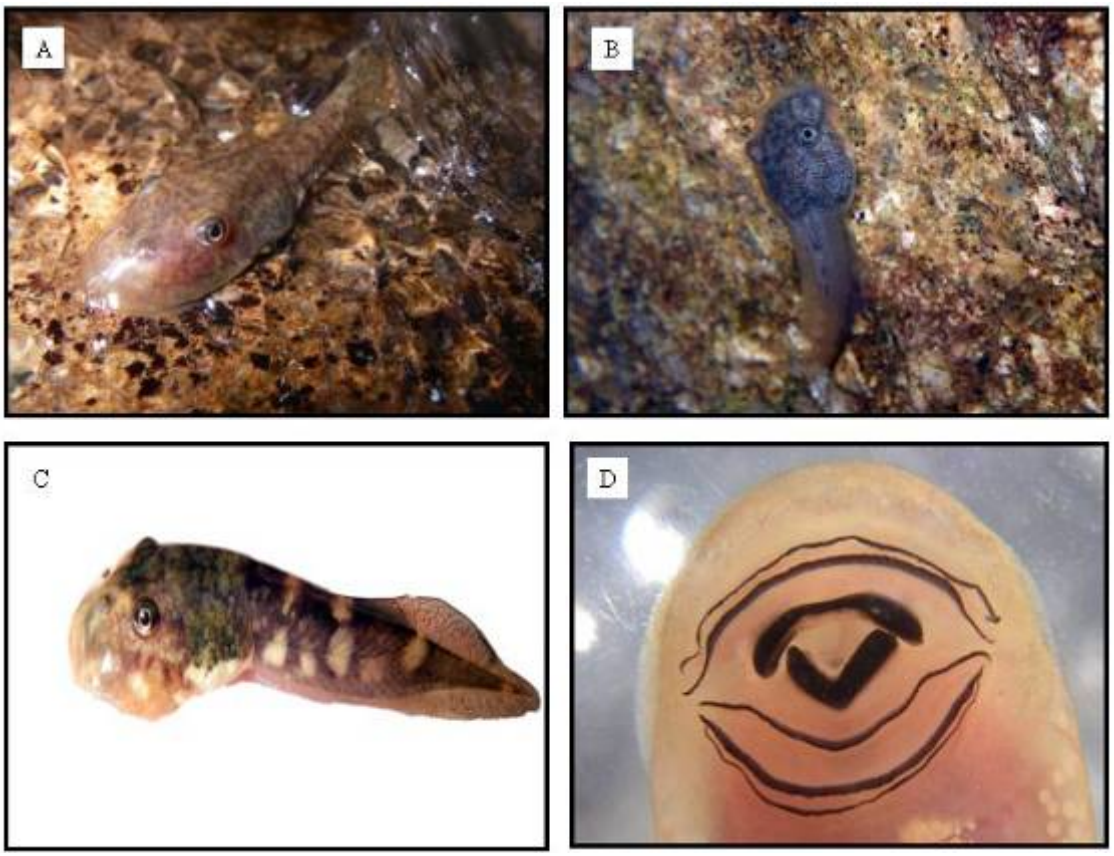

Figure 42. Torrent adapted Tadpoles. A) *L. nannotis* B) *L. rheocola* C) *N. dayi* D) Ventral view of the large, suctorial mouthparts of *L. nannotis* consisting of two anterior and three posterior keratinized tooth rows and a keratinized jaw sheath.

Materials and Methods

Tadpole Behavior Experiment (observational)

Individual tadpoles were visually identified through the water column and observed for five-minute periods. The number of times the individual tadpole broke contact with the substrate and entered the water column was recorded. In total, 50 *Litoria nannotis*, 50 *L. rheocola* and 25 *L. genimaculata* tadpoles were observed.

Cross-Sectional Tadpole Survey

Sampling occurred approximately every four weeks beginning in February 2006 at both sites and then approximately every two weeks beginning April 2007, to increase sampling precision, at the Tully Gorge National Park site only.

At both sites a 200-meter transect along the creek was established. The transects were mapped and then stratified by habitat type into 18 possible mesohabits as proposed by Hawkins et al. (1993). These 18 mesohabitats were then condensed into two broad

habitats for sampling purposes, termed "riffle" (fast water) and "pool" (slow water) and numbered sequentially (Hawkins et al. 1993). Habitats were sampled proportionally to their abundance. For each visit, a random integer between 1 and 4 was selected to identify the first habitat to be sampled. Every 4$^{th}$ habitat was then sampled until all habitats of each type were exhausted (Hartwell et al. 1997). This method ensures valid statistical comparisons between habitat types and sampling periods by ensuring $\geq 1$ sampled habitat of each type, approximately equal sampling effort within each habitat, good spatial coverage across the transect, and independence of samples among habitats (Hartwell et al. 1997). It was not feasible to capture every tadpole within a selected habitat due to their high density, cryptic nature and ability to move into and out of adjacent habitat units. Therefore, each habitat was sub-sampled via three one-minute dip-net sweeps interspersed with at least a 5 minute break between sweeps. Due to differences between habitat types (eg: flow rate) and the behavior of tadpoles in those habitat types, slightly different sweep techniques were employed. In pools, the-dip net was swiftly, but gently "bounced" between the substrate and the water column, disrupting tadpoles within or on the substrate into the water column for capture. In riffles, the dip-net was quickly scraped across the rock surfaces. When appropriate, upstream rocks were disrupted to dislodge hidden tadpoles into the downstream net. Captured tadpoles were held and released at the upstream portion of the sampling area following the third sweep. Along the Tully transect, two stream-side isolated pools, suitable for *L. xanthomera* tadpoles, formed following adequate rainfall. As there were only two isolated pools available, both were sampled during each visit. Isolated pools were surveyed for the first year only.

Following capture, tadpoles were emptied into a sorting tray where they were quickly scooped into individually numbered ziplock bags until processing. For processing, a new pair of well-rinsed vinyl gloves were worn for each tadpole (Cashins et al. 2008). Each individual was measured, and swabbed with a thin tipped cotton swab (Medical Wire & Equipment Co. MW 100–100) to determine *B.* dendrobatidis infection status using a real time Taqman PCR assay (Boyle et al. 2004) Beginning in the second year tadpole mass and a score of mouthpart and jaw loss was recorded.

Mouthpart and Jaw Loss

Torrent tadpole mouthpart condition was assessed visually through the ziplock bags (Figure 43). Pool tadpoles were examined in hand, with a 10X lens if necessary. The loss of keratinized tooth structures and jaw sheath were assessed on site and ranked from 0-5 using individual scales as follows. **Teeth loss scale** (Figure 44). 0: No loss. 1: Discoloration. 2: Less than 25% loss. 3: 25% - 50% loss. 4: 51%-75% loss. 5: Greater than 75% loss. **Jaw loss scale**. 0: No loss. 1: Thinning of jaw. 2: Small gap in jaw. 3: Medium gap in jaw. 4: Large gap in jaw. 5: Jaw completely missing.

Figure 43. *Litoria nannotis* tadpole in a ziplock bag for mouthpart loss analysis

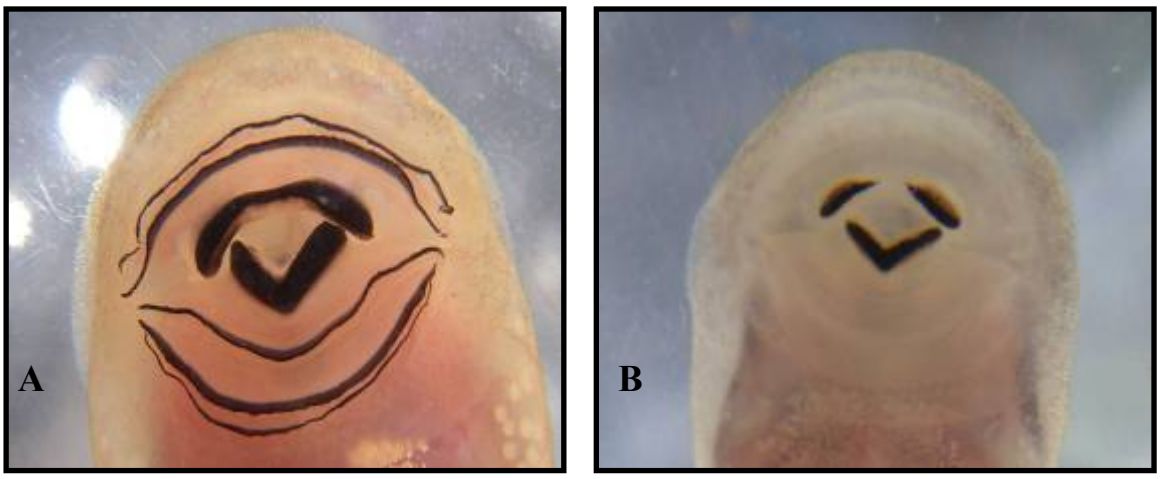


Figure 44. Mouthpart Loss. A) *Litoria nannotis* tadpole with mouthpart loss score of 0 (no tooth loss) and a jaw loss score of 0 (no jaw loss). B) *L. nannotis* tadpole with a mouthpart loss score of 5 (≥ 75% tooth loss) and a jaw loss of 4 (large gap in jaw).

Measurements

Body length was measured from the tip of the snout to the base of the tail where the axis of the tail myotomes contacts the body wall (Altig 2007) to 0.1mm precision using vernier calipers. Body lengths were condensed into 9 size classes for some analyses; 1: 0-2.9 mm, 2: 3-4.9mm, 3: 5-6.9mm, 4: 7-8.9mm, 5: 9-10.9mm, 6: 11-12.9mm, 7: 13-14.9mm, 8: 15-16.9mm, 9: 17mm +). These size classes where used with tadpoles of all

species. Developmental stage (Gosner 1960) was not recorded as the hind limbs of *L. nannotis*, *L. rheocola*, and *N. dayi* develop in sheaths beneath the epidermis until late in development and cannot be determined without dissection. Mass was determined using a digital balance accurate to 0.01g.

Environmental Measurements

A range of environmental variables were measured including water flow, water temperature, air temperature and relative humidity.

Water flow

Following each net sweep, three water flow measurements were measured with a digital flow meter (Model # FP101, Global Water Instrumentation Inc.). Values were recorded in meters/second to the nearest 0.01 and were applied to those tadpoles captured in the associated sweep.

*Water temperature*
Thermochron iButton temperature dataloggers (DS1921Z-F5, Dallas Semiconductor) were chosen to record water temperature due to their proven accuracy (±0.5°C) precision (±0.4°C) and affordability (Johnson et al. 2005). Dataloggers were individually placed in ziplock bags within water tight metal canisters and placed underwater. Canisters were anchored to streamside supports with cable wire to prevent dislodgement downstream. The iButtons were programmed to record temperature every hour. Two iButtons were placed in pools and two were placed in riffles at different locations along the transect.

*Air temperature and relative humidity*
Two temperature (Cº) and humidity datalogging stations (Tinytag Plus, Gemini Data Loggers Ltd.) were set up at the 50 meter and 150 meter marks of the transects to record every hour.

*Additional Environmental Variables*
Additional environmental variables for the two sites were obtained from the Australian Bureau of Meteorology "Data Drill" database (http://www.longpaddock.qld.gov.au/silo/). This database provides spatially interpolated observational data collected from all available Bureau weather stations using a trivariate, thinplate, smoothing spline with latitude, longitude and elevation as independent variables. Interpolated data minimize the variability and error of single station data records and can provide an accurate record of local climate in locations without a nearby weather station or in locations surrounded by multiple weather stations. Available data include minimum/maximum air temperature (Cº), daily rainfall (mm), evaporation (mm), solar radiation ($MJ/m^2$) and relative humidity (%). Jeffrey et al. (2001) provide a detailed analysis of the methods used to create this database.
Batrachochytrium dendrobatidis real time Taqman PCR assay

*Torrent tadpole mark-recapture*
Very little is known regarding dispersal or survival of torrent adapted tadpoles. The objectives of this mark recapture study were to 1) gain a greater understanding of site fidelity and dispersal of *L. nannotis* within the stream and 2) determine if widespread

mortality occurs in association with *B. dendrobatidis* infection and associated mouthpart loss. On May 15, 2007, a 5-meter stretch of torrent habitat (50-55 meter mark) was exhaustively sampled until the rate of capture was very low, indicating most of the tadpoles had been collected. Two researchers simultaneously conducted 7, 15-minute dip net sessions (protocol described previously). Each sampling session was separated by a 5-minute break. Linear regression between number of tadpoles collected and cumulative search effort can be used to calculate population size. Upon capture, tadpoles were allocated to individual bags, measured, weighed, swabbed and mouthpart loss was recorded as previously described. In addition, tadpoles were marked via visible implant elastomer (VIE; Northwest Marine Technology Inc.). Elastomers are a colored, silicone based polymer that is injected under the skin as a liquid and then cures as a biocompatible solid following placement. VIE has been used effectively to mark a range of taxa including adult and larval amphibians (Anholt et al. 1998, Nauwelaerts et al. 2000). A 0.3 cc syringe was used to apply a small volume of elastomer beneath the skin on the dorsal side at the base of the tail. To reduce possible transmission of disease, the syringe tip was immersed in 70% ethanol between each tadpole (Speare et al. 2004). Tadpoles below 8 mm in body size were excluded as previous trials indicated marking tadpoles of this size was very difficult (Cashins and Windmiller, unpublished). Following marking, all tadpoles were observed to ensure their condition was not noticeably affected. Out of 132 captured *L. nannotis* tadpoles, one died following dipnetting, and following marking one died and one was observed to be in poor condition. This tadpole was considered fine for release. All remaining tadpoles appeared of normal health and were released at the upstream section of the five meter site of capture.

On August 16, 2007, three months post-release, the exhaustive sampling protocol within the same 5 meter stretch of stream was repeated and the recapture rate and population estimate calculated. Tadpoles were also sampled above, below and within the 5 meter stretch 1 day and 3 weeks post release. These samplings were conducted with non standardized sampling efforts but provide useful information.

## Results

### Tadpole Behavior

*Litoria genimaculata* tadpoles left the substrate and entered the water column significantly more times than either *L. nannotis* or *L. rheocola* over a 5 minute period (ANOVA, $F(2,122)$ $p=0.0000$; Figure 45). *Litoria nannotis* tadpoles broke contact with the substrate, on average, 0.06 times over five minutes, *L. rheocola:* 0.2 times/5 minutes, and *L. genimaculata*: 11.4 times/5 minutes (Figure 45). Only 6% (3/50) of *L. nannotis* left the substrate and all three did so only briefly, one time. 12% (6/50) of *L. rheocola* moved from the substrate with only 3 individuals doing so more than once and none more than 3 times. All 25 *L. genimaculata* tadpoles left the substrate and did so frequently (between 5 and19 times).

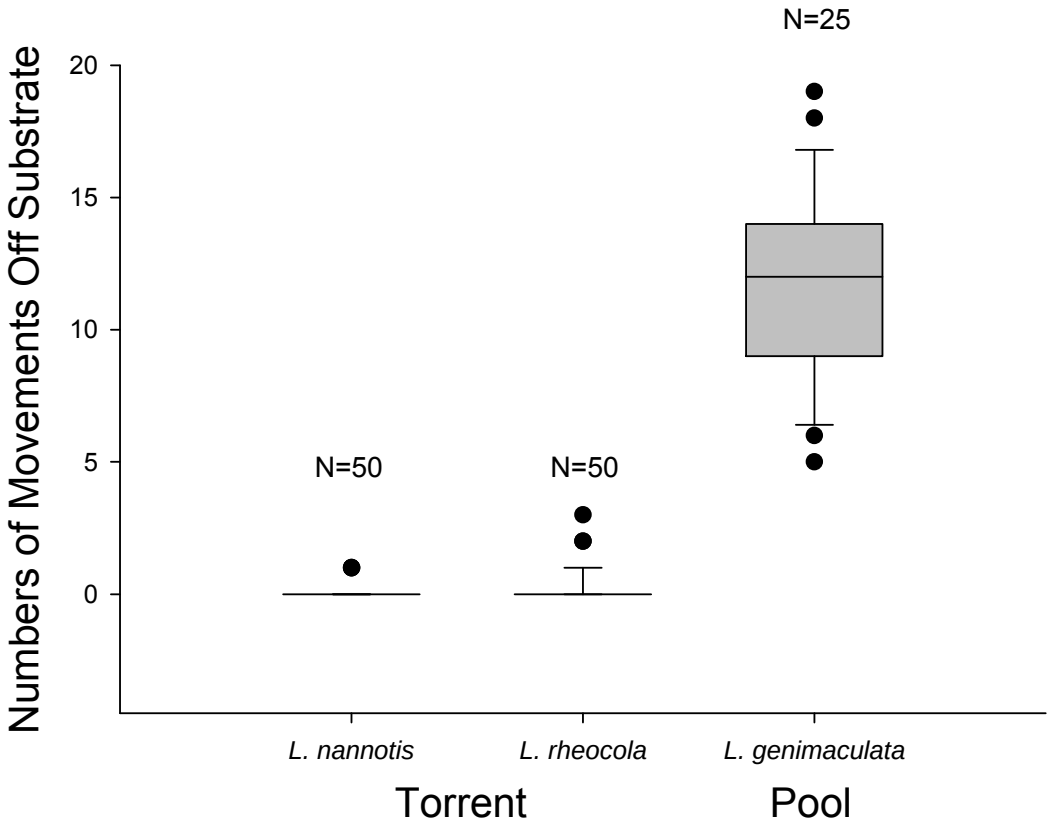


Figure 45. Boxplot of numbers of movements off the substrate of L. nannotis, L. rheocola and L. genimaculata over 5 minute observation periods. N = the number of tadpoles observed

Spatial Partitioning

Tadpoles in the functional group "pool" occupied habitat with significantly slower mean water flow than tadpoles in the "torrent" group (pool: 0.006 m/sec, torrent: 0.378 m/sec, df=2649, p=0.000; Figure 46). Between tadpoles of the different species, there is a significant difference in the mean water velocity of the habitat occupied (ANOVA; F(4, 2562), p=0.0000. Figure 47). Tukey's Unequal N HSD post hoc test shows tadpoles of all species occupied significantly different water velocity profiles except *Litoria genimaculata* and *L. xanthomera*. *Nyctimystes dayi* tadpoles were captured in the fastest flowing water (mean flow rate: 0.65 m/sec), followed by *L. nannotis* (0.40m/sec), *L. rheocola* (0.30 m/sec), *L. genimaculata* (0.01 m/sec) and *L. xanthomera* (0.00 m/sec).

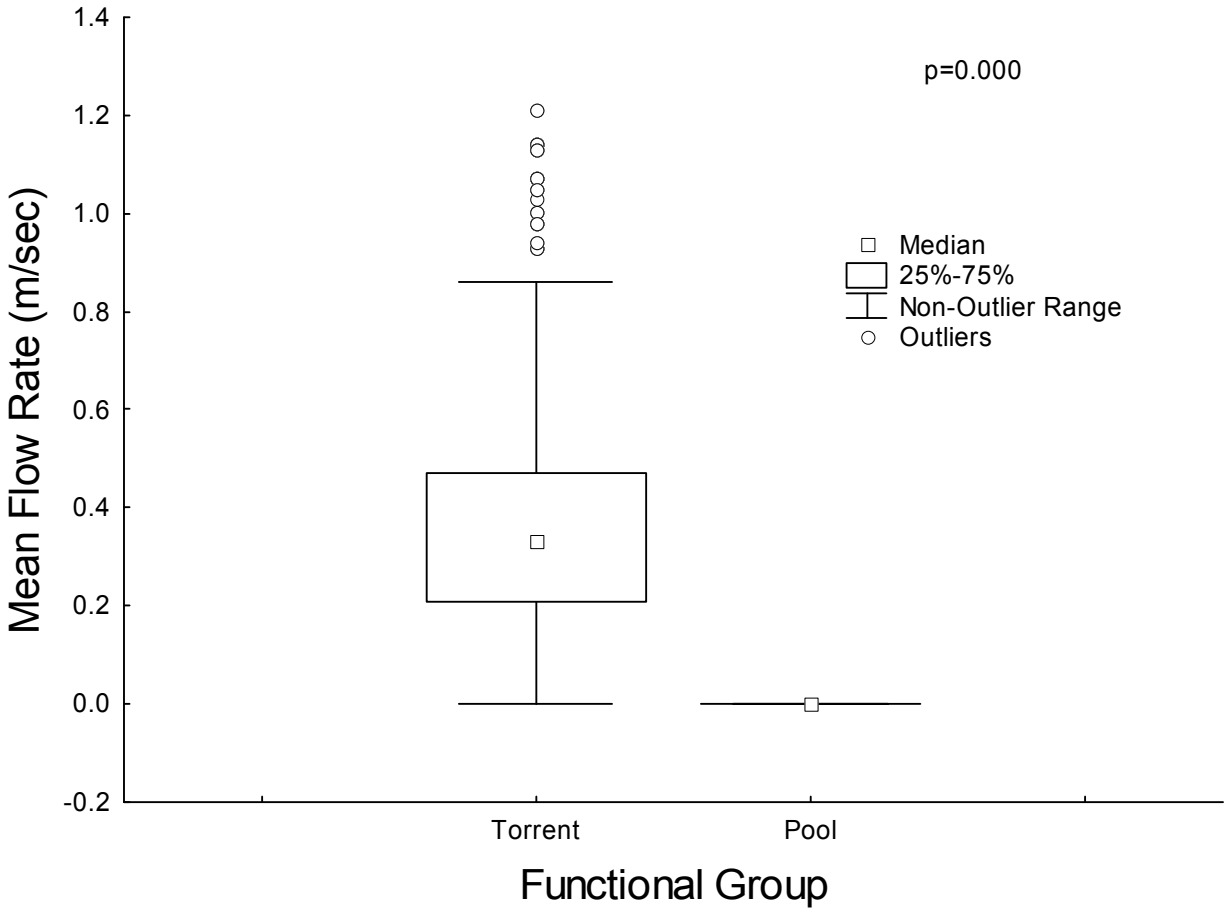


Figure 46. Boxplot of mean water flow rate by tadpole functional group, torrent and pool tadpoles at Tully Gorge National Park and Murray Upper National Park.

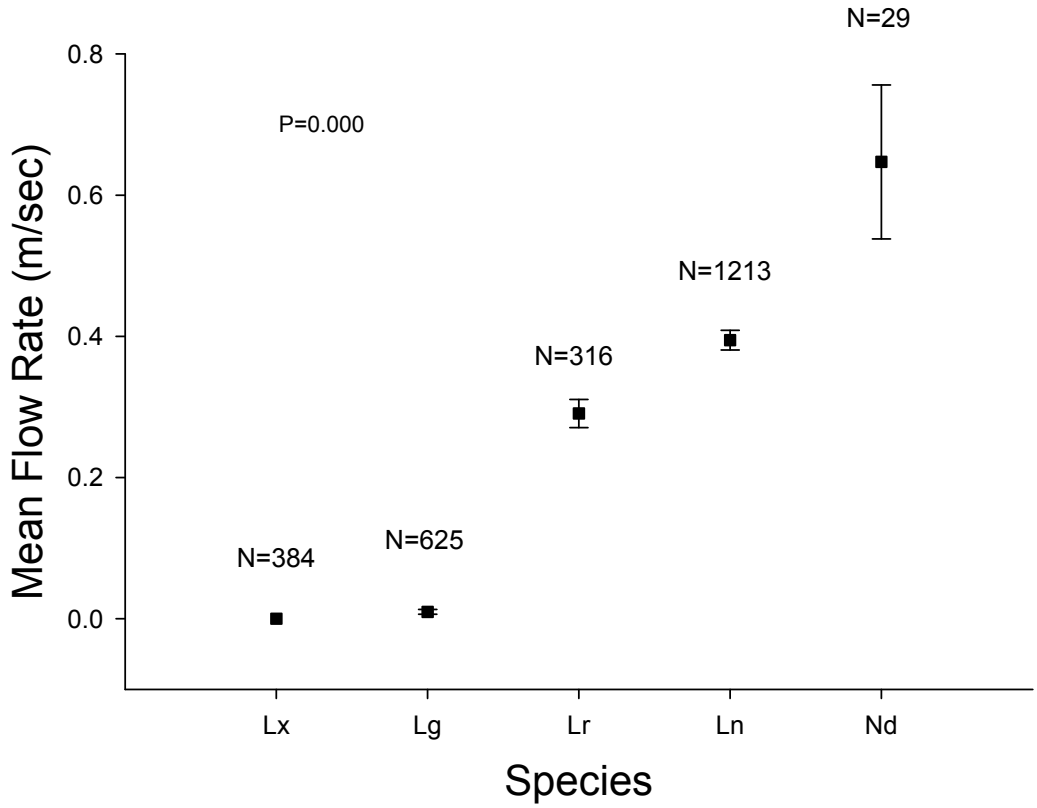


Figure 47. Mean water flow rate profile of tadpoles of each species at Tully Gorge National Park and Murray Upper National Park. Boxes represent the mean and whiskers represent the upper and lower 95% confidence intervals.

There was no difference in flow rate between sites (p=0.71), however, there was a significant species X site interaction (p=0.0003; Figure 48). *Litoria nannotis* tadpoles occupied a slightly slower water flow at Tully than at Murray Upper (Tully NP: 0.39 m/sec, Murray Upper NP: 0.45 m/sec, p=0.035). There was no significant site interaction with either *L. rheocola* (p=0.627) or *L. genimaculata* (p=0.999). *Nyctimystes dayi* was not included in the site interaction analysis as there were too few tadpoles captured at Murray Upper for a valid statistical comparison. There was no difference in the velocity occupied by *L.* genimaculata (F(5,186): p=0.776), L. *rheocola* (F(5,272); p=0.606) or *L. nannotis* (F97,1019; p=0.396; Figure 49) at Tully across size classes. Variability in the two smallest size classes is due to small sample sizes.

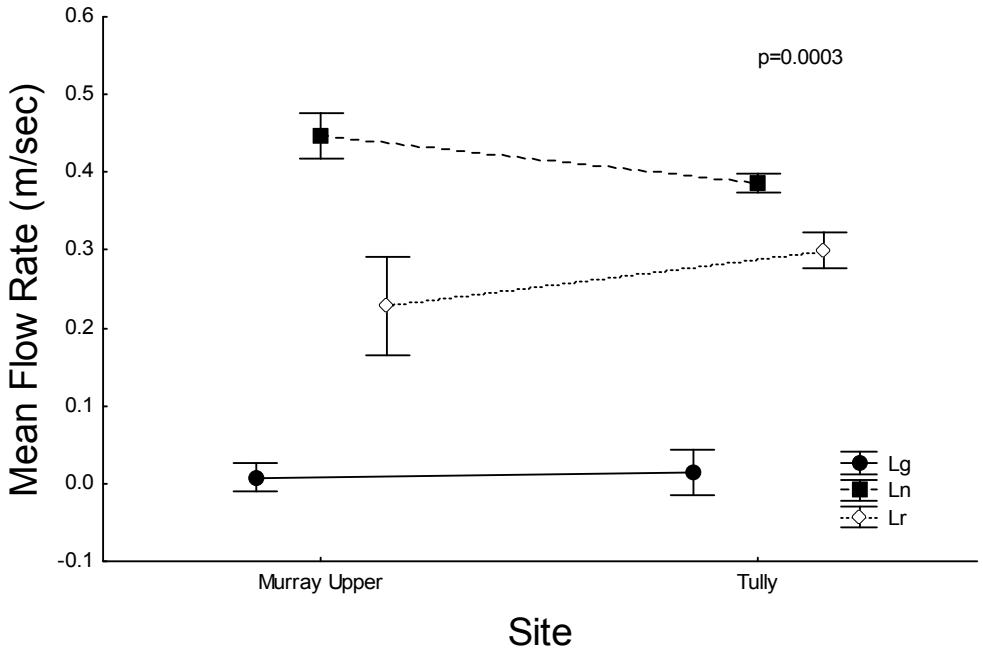


Figure 48. Mean water flow profile by site and species. Connective lines are a visual aid only. Symbols represent the mean and whiskers represent the upper and lower 95% confidence intervals.

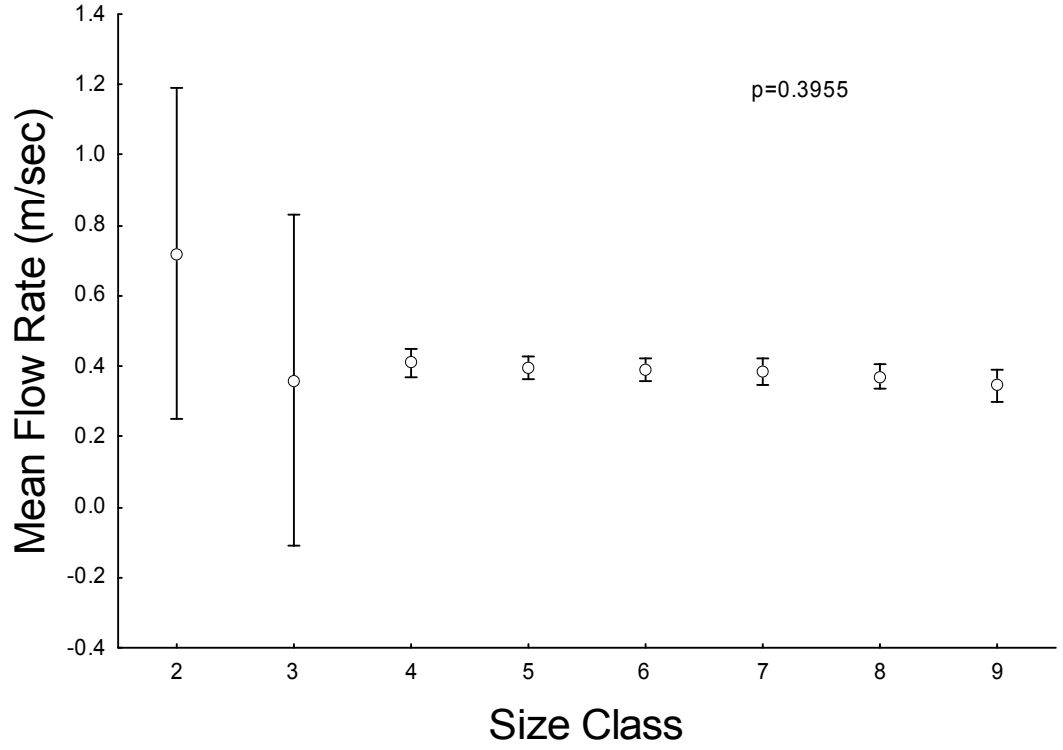


Figure 49. Mean water flow rate occupied by *Litoria nannotis* at Tully Gorge National Park across size classes. Circles represent the mean and whiskers represent 95% confidence intervals

Seasonal Size Structure

*Litoria nannotis*

Recruitment of small *Litoria nannotis* tadpoles into the population occurred throughout the year, with seasonal peaks over summer and early autumn; between the months of January and April (Figure 50 A). Large tadpoles were present throughout the year, with peaks in relative abundance over spring; between September and November (Figure 50 C). Metamorphosing individuals or recent metamorphs were observed exclusively between October and April of both years (Figure 50 C). Modal progression of size classes suggests tadpoles that hatched in early summer, grew and developed over the winter, and metamorphosed the following spring and summer, taking approximately 9 - 12 months to develop through metamorphosis. Large tadpoles, at an appropriate size for metamorphosis, but without emergent limbs were present following the end of the metamorphosis period in April. These large tadpoles which did not metamorphose would likely overwinter and presumably be among the first to metamorphose the following spring and summer. These overwintering individuals could therefore remain as larvae for 16 months or more. In June, in both 2006 and 2007 there was a slightly bimodal distribution of large and small size classes (Figure 51), possibly representing the larger tadpoles which failed to metamorphose and the recent recruitment of small tadpoles into the population.

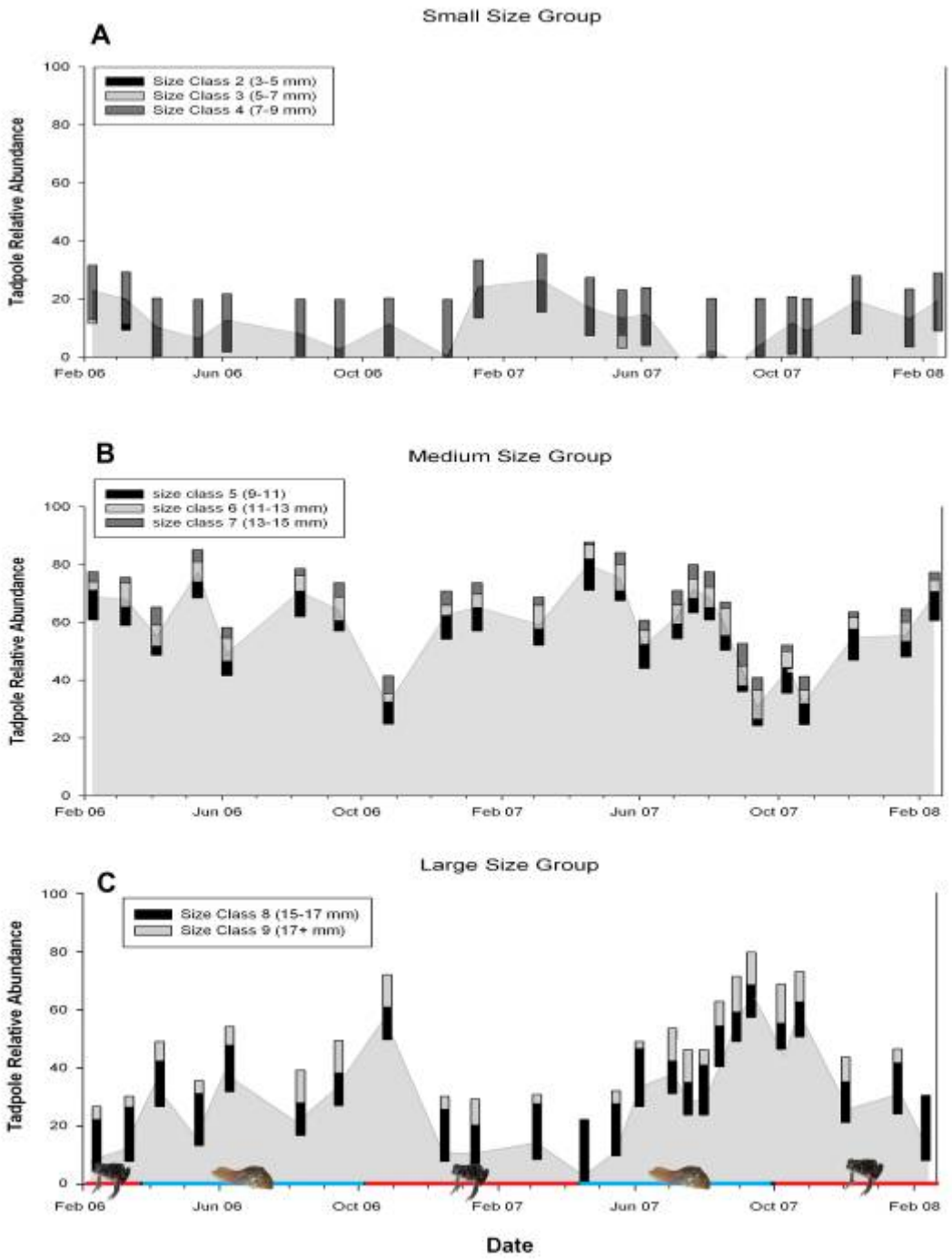


**Figure 50. Relative abundance of *Litoria nannotis* tadpoles at Tully Gorge National Park, February 2006 – February 2008. Shaded grey areas represent the relative abundance of each size group. The stacked bars represent the relative abundance of the individual size classes that comprise the size group. The horizontal red bars signify periods of tadpole growth without metamorphosis and the blue bars signify periods when metamorphosis occurs.**

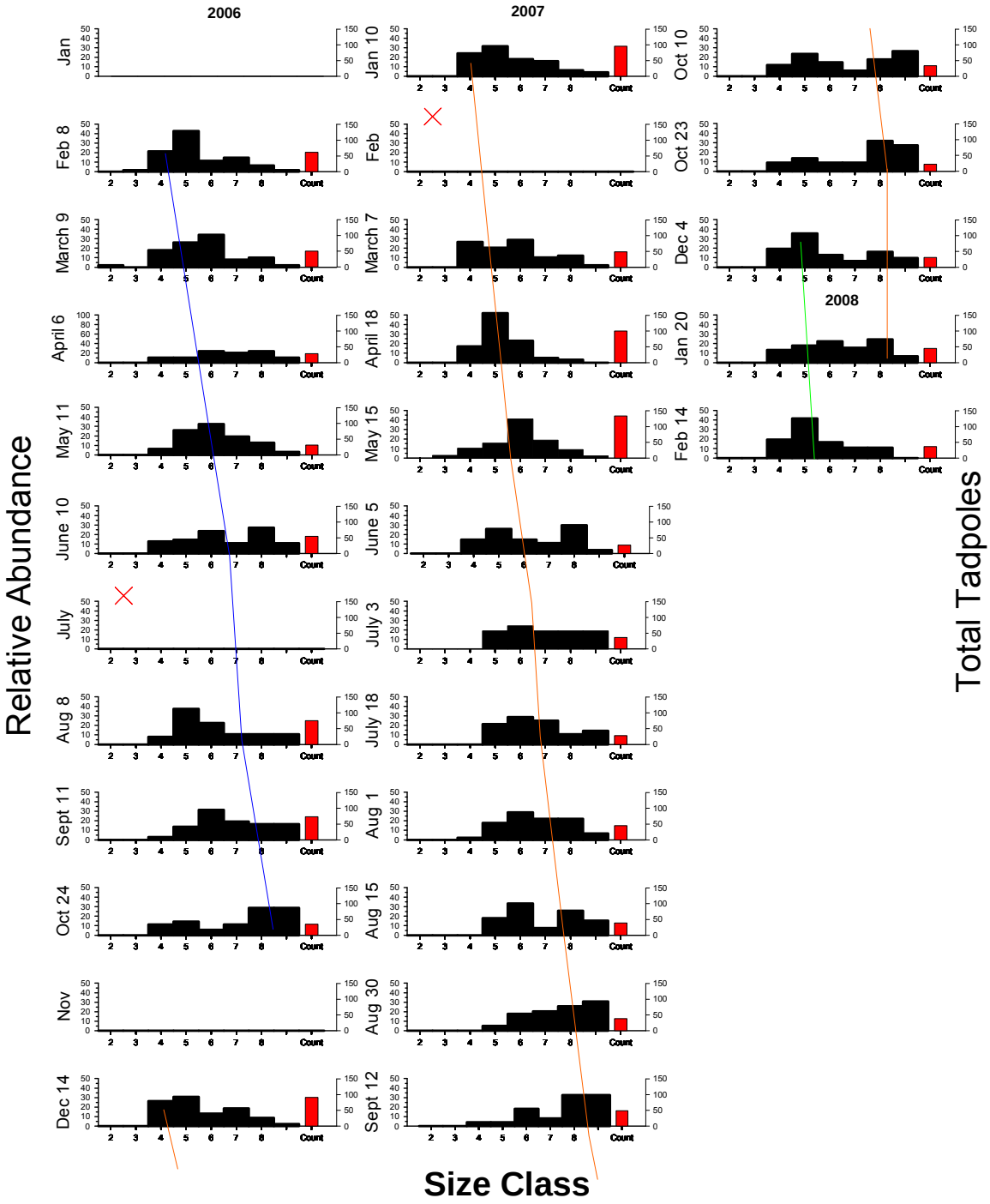


Figure 51. Black bars: Size class distribution of *Litoria nannotis* tadpoles at Tully Gorge National Park, Red bars: Total number of tadpoles captured. Black bars correspond to the left hand Y-axis. Red Bars correspond to the right hand Y-axis. Lines represent the modal progression of each years summer cohort.

*Litoria rheocola*

Tadpoles of *Litoria rheocola* were less abundant at all times over the two-year sampling period than *L. nannotis*. Similar to *L. nannotis, L. rheocola* tadpoles were present in a range of size classes throughout the year. However, the abundance of *L. rheocola* appears to be more seasonal than *L. nannotis*. Few *L. rheocola* tadpoles were present in the creek between February and April in both years. In May-June, the abundance

drastically increased with most captured tadpoles occurring in the small size group. This peak recruitment of small *L. rheocola* tadpoles occurred one to two months following the end of peak *L. nannotis* recruitment (January-April). Only four metamorphosing *L. rheocola* tadpoles were captured over the two-year study, however, similar to *L. nannotis*, these metamorphs occurred exclusively between late October and February, coinciding with the period when tadpoles were at their largest. This data combined with the modal progression of size classes suggest *L. rheocola* tadpoles hatch in late autumn, grow over winter, and metamorphose the following spring – summer, taking between 5-9 months to metamorphose. Thus, like *L. nannotis* it appears that most of *L. rheocola* growth and development occurs over winter. *Litoria rheocola* tadpoles, however, achieve a significantly smaller body size than *L. nannotis* (Figures 52-53).

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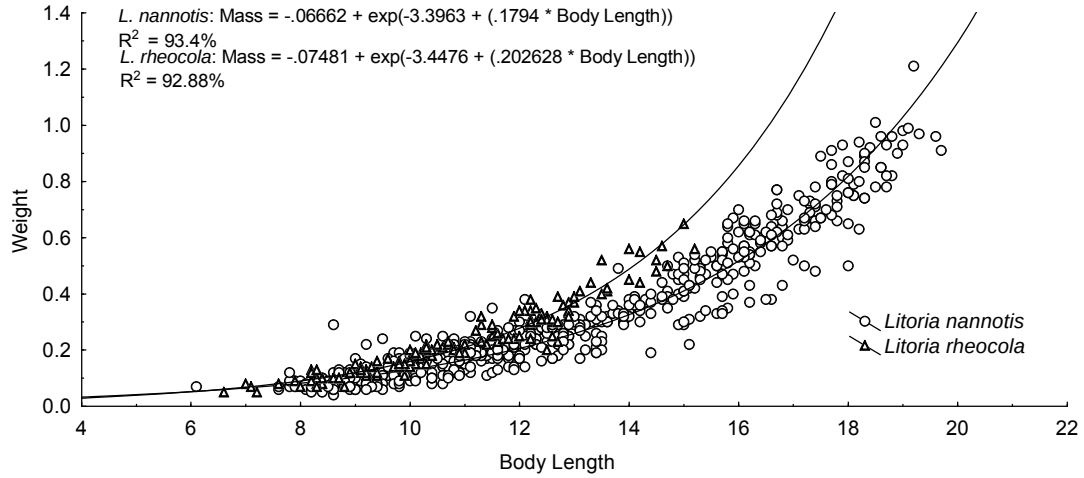


Figure 52.. Length vs mass best fit exponential regression using quasi-newton estimation methods. *Litoria nannotis* and *Litoria rheocola* tadpoles Tully Gorge National Park

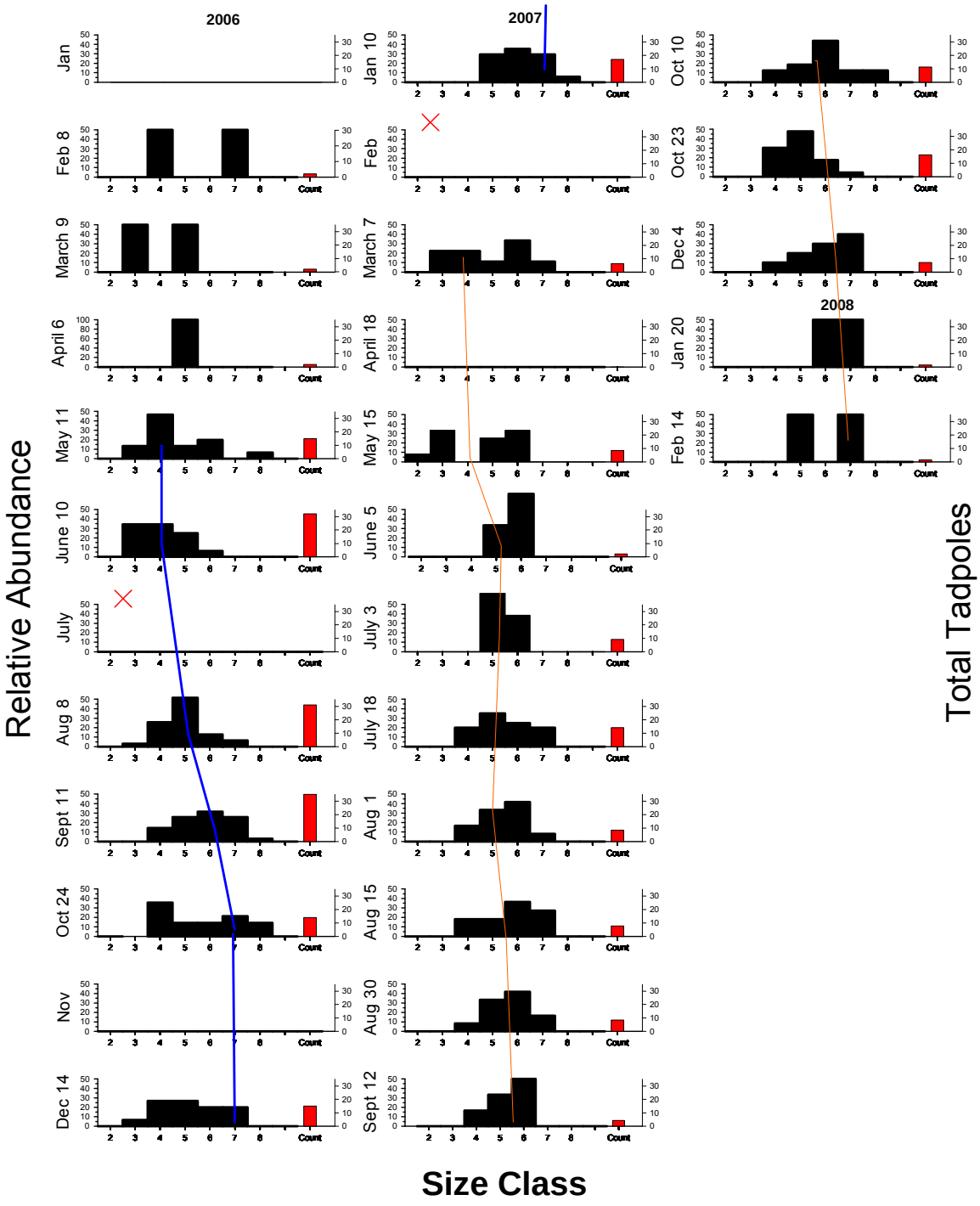


Figure 53. Black bars: Size class distribution of *Litoria rheocola* tadpoles at Tully Gorge National Park. Red bars: Total number of tadpoles captured. Black bars correspond to the left Y-axis. Red bars correspond to the right Y-axis. Lines indicate modal progression of tadpoles from from each late summers cohort

*Litoria xanthomera*

Tadpoles of *L. xanthomera* were present throughout the year except for a two - three month period in winter when the stream-side isolated pools were dry (July-September; Figure 54). The modal progression shows very little overlap in size classes indicating parental driven partitioning through selective egg deposition. In total, four distinct size cohorts utilized the habitat over the course of the year. It is not known if cohorts were from single or multiple egg clutches. Developmental time of *L. xanthomera* tadpoles appears to be approximately 8-12 weeks.

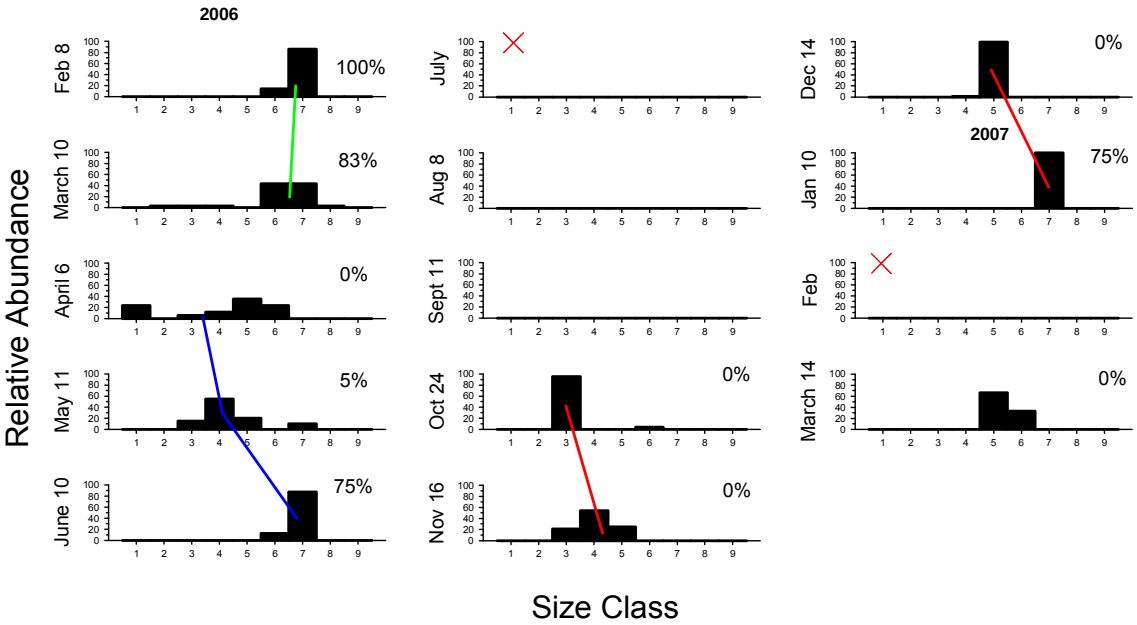


Figure 54. Size class distribution of *Litoria xanthomera* tadpoles at Tully Gorge National Park. Percentages in the upper right corner of each graph represent the percentage of tadpoles with emergent limbs. Colored lines indicate developmental cohorts. Red crosses represent sampling periods when data was unable to be collected.

*Litoria genimaculata*

Tadpoles of *Litoria genimaculata* were present throughout the year, however there were no clear patterns in seasonal size structure (Figures 55-56).

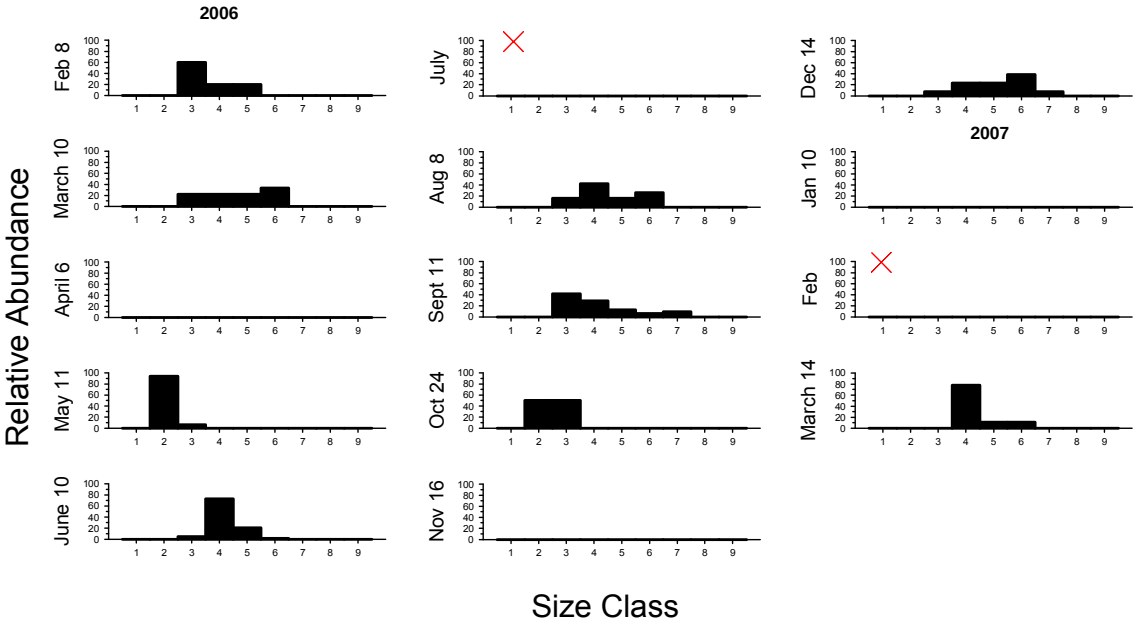


Figure 55. Size class distribution of *Litoria genimaculata* in Tully Gorge National Park. Red crosses represent sampling periods when data was unable to be collected.

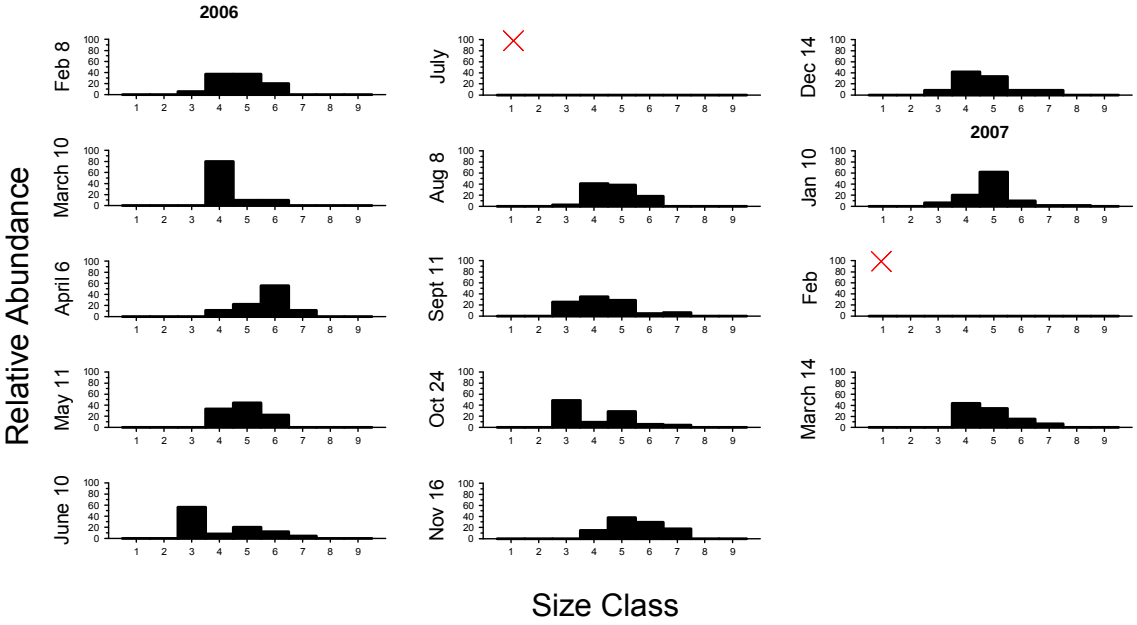


Figure 56. Size class distribution of *Litoria genimaculata* in Murray Upper National Park. Red Crosses represent sampling periods when data could not be collected.

Mark recapture

There was little change in the population size estimates between May 15, 2007 (184 tadpoles) and August 16, 2007 (190 tadpoles). The recapture rate of torrent tadpoles three months post release was 2.2% (4/180).

24 hours post-release, 7/32 tadpoles (21.9%) collected within the original sampling stretch (50-55 meters) were recaptures. In comparison, 1/26 (3.9%) tadpoles captured upstream (55-60 meters) and 0/6 tadpoles captured downstream (45-50 meters) were marked. Three weeks post release, 3/105 tadpoles (2.9%) within 45-60 meters were

marked. Interpreting the 24 hour and 3 week results is difficult due to the fact that the recapture sampling was performed differently and was not time standardized. However, it indicates that soon after release tadpoles were more likely to remain within the original area. Within 3 weeks, however, tadpoles had dispersed from the area and recapture rates were below 3%.

Discussion

The tadpole community within these rainforest streams was found to partition spatially by habitat type (isolated pools, connected pools and torrents) and within habitat type by water velocity (torrents) and parental choice (isolated pools). Tadpoles within torrents also exhibited temporal partitioning as *Litoria rheocola* were found to hatch approximately two months following *L. nannotis*. Development in *L.* nannotis was seasonal with a discernable cohort that hatched in summer/early fall and developed over the ensuing year, metamorphosing the following summer. Torrent tadpoles (*L. nannotis*, *L. rheocola* and presumably *N. dayi*) maintain close contact with the stream substrate almost continusously while *L. genimaculata* were found to be more active in the water column. Dispersal in *L.nannotis* was high indicating a population with a high level of mixing.

The suctorial tadpole morphology represents an interesting adaptation to exploit the limited resources present in high energy streams. The mechanism of adhesion has not been studied in tadpoles present at these sites, however, work on the functionally similar, suctorial tadpoles of the North American *Ascaphus truei* lend insight. In these tadpoles the water trapped between the oral disc and substrate is sucked through the buccal cavity and out the spiracle, creating a negative pressure that draws the oral disc tightly to the substrate surface (Gradwell 1971, Cannatella 1999). Tooth rows, particularly the two outermost rows appear to aid in adhesion (Altig & Johnston 1989). Once adhered to the rock, an oral valve seals the mouth from within, permitting respiration through the nostrils and movement ("mouth-hitching") across the substrate without compromising the partial vacuum (Gradwell 1971). Mouth-hitching across rocks is accomplished by a reduction in suction by partially opening the oral valve, combined with a forward movement of the upper and lower labia (Gradwell 1971, Cannatella 1999). I have even observed *L. nannotis* tadpoles advancing up rocks of a small waterfall under a thin film of water (Figure 42 A).

Hitching is also a primary method of feeding as the labial tooth rows and jaw sheath scrape against the substrate during movement. Following movement, the transfer of water from the oral disc and substrate interface, through the buccal cavity to reestablish the partial vacuum also acts to draw the newly dislodged material across the filter apparatus and then into the stomach for digestion (Viertel & Richter 1999). *Litoria nannotis* and *L. rheocola* tadpoles are frequently observed hitching in place, repeatedly scraping a particular section of the substrate, apparently feeding (pers obs.). This behavior was also observed in the laboratory, while tadpoles grazed on glass feeding slides covered in algae. The ability to move and feed without disrupting adhesion allows torrent tadpoles to rarely break contact with the rock surface (Figure 45). To move larger distances tadpoles will release suction and enter the current before reattaching to substrate downstream or slide across the rock surface before reengaging. In slower flows they may use their strong tails to swim in short bursts (pers. obs.).

Richards (2002) proposed that pool tadpoles were excluded from torrents by the inability to maintain position in turbulent or fast water. Interestingly, there appears to also be a partitioning within torrents. The three torrent adapted tadpoles studied here, occupied habitat with distinct mean flow rates (Figure 46). *Nyctimystes dayi* occupied the fastest flowing water, *L. rheocola* the slowest flowing riffles, and *L. nannotis* was in between. Morphological differences between these tadpoles suggest the ability to maintain position is the factor limiting entry into higher velocity environments. A suite of morphological characteristics that assist in avoiding and withstanding water flow are expressed across a gradient in the three torrent tadpoles. *Litoria rheocola* have the least hydrodynamic, flattened body shape, smallest oral disc and least developed tail musculature. *Nyctimystes dayi* tadpoles have a more flattened body shape than *L. rheocola*, a very large oral disc that occupies up to 50% of their body (Trenerry 1988), and the most developed tail musculature with the most shallow fins of the three species. *Litoria nannotis* also have a highly depressed body form, but an intermediate size oral disc and intermediately developed tail musculature. While these morphological adaptations may limit entry into higher velocity water, it is not known why *L. nannotis* and *N. dayi* generally avoid slower flowing habitats. Competitive interactions or physiological requirements are possible explanations.

Although torrent tadpoles may remain within a preferred velocity profile, they are able to disperse readily. The recapture rate of marked *L. nannotis* 24 hours post release was high (~22%), but soon dropped below 3% three weeks post release and did not markedly change after 3 months. During this time the size of the population did not decrease significantly, indicating widespread mortality did not occur.

A pulse of small tadpoles indicating egg hatching was observed in summer. First in *L. nannotis* and then approximately 2 months later in *L. rheocola*. Tadpoles grew and developed over winter until metamorphosis, which was apparent by a spike and then drop in large tadpole abundance, the following summer. Despite this apparent seasonality, a wide range of size classes was observed throughout the year, indicating hatching was not restricted to one time period. However, there was a distinct period of metamorphosis between October and April, outside of which, emergent limb buds or metamorphosing tadpoles were not seen. This indicates that tadpoles that were not of an appropriate size to metamorphose during this time frame would overwinter and metamorphose the following summer. Metamorphosis during the summer months ensures froglets emerge in a moist terrestrial environment at a time when invertebrate prey are most abundant (Frith & Frith 1985, Frith & Frith 1990, Richards & Alford 2005)

*Litoria genimaculata* are members of the "clasping" ecomorphological guild (Altig & Johnston 1989) and were found in connected pools and backwaters with accumulated detritus and slow water flow (<0.05 m/sec; Figure 46). In experimental flow tanks small *L. genimaculata* tadpoles could not physically maintain position in 0.25 m/sec flow and no tadpoles of any size could withstand 0.50 m/sec flow or turbulence at any speed (Richards 2002). *Litoria genimaculata* have ventrally positioned mouthparts that are partially concealed by the upper and lower labia, and a rounded body form, weak tail muscle, and tail fins of medium depth. They feed by rasping and picking at the substrate, including leaf pack (Iwai et al. 2008) and likely by filtering particulates from the water column (Alford 1999). During timed observations, *L. genimaculata* tadpoles frequently left the substrate to swim to nearby locations or to remain suspended in the

water column, presumably filter feeding (Figure 45). Dispersal in *L. genimaculata* is low. Individuals were found to remain within a single pool until metamorphosis, death or possible flushing during heavy rains (Trenerry 1988).

*Litoria xanthomera* deposit eggs in ponds and isolated pools in clutches of 800-1500 eggs (McDonald 1998). At the Tully transect the stream-side isolated pools were small (less than $1m^2$) and often had a high density of tadpoles. There was very little overlap of size classes suggesting that females only deposit eggs when the previous clutch was nearing or had completed metamorphosis. On the one occasion when there appeared to be two distinct size cohorts (April 6, 2006), two larger tadpoles were observed cannibalizing, apparently live, conspecific larvae of a smaller size class. Cannibalistic behavior has not been reported in tadpoles of this species and could explain the reluctance of gravid females to deposit eggs in an occupied pool. Crump (1983) proposed that cannibalism in larval anurans may occur more often in ephemeral pools where larval density can be high, resources low and time available to metamorphose is short. *Litoria xanthomera* in isolated pools matches each of these criteria. Alford (1999) reviewed cannibalism in anuran larvae and identified 30 species which exhibit cannibalism in some form. Twelve of these, similar to *L.* xanthomera, were unspecialized tadpoles that consumed conspecific tadpoles. Cannibalism in ephemeral, high density environments is likely to be beneficial for the cannibal as it provides a high protein food source, which promotes faster growth (Kupferberg 1997), reduces density and competition for resources, and, if the prey are from a separate clutch, reduces genetic competition as well (Alford 1999).

This study describes the tadpole communities of lowland rainforest streams in Queensland and discusses their behaviour, seasonality and partitioning of resources. Flow rate is a principle factor driving the partitioning of these tadpoles, even within torrents. The size structure of *L. nannotis* and *L. rheocola* were seasonal, with peaks in hatching and metamorphosis occurring in summer. This data on tadpole ecology will be used to better understand disease processes within tadpoles and the environment.

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***Project 4.5 Epidemiology of Batrachochytrium dendrobatidis in Rainforest Stream Tadpoles***

Scott D. Cashins

Introduction

Many diseases of wildlife are highly seasonal and respond to changes in temperature, rainfall, and resource availability. This seasonality can impact on host-pathogen interactions through a range of pathways, including changes in host behavior which affect pathogen transmission, variation in contact rate with infective agents in the environment, pulses in host birth and death rates and changes in host immune defenses (Altizer et al. 2006). The effects of seasonality can be seen in changes of prevalence of infection and intensity of infection in hosts over time. Infection is the successful colonization of a host by a pathogen and intensity is the subsequent buildup in numbers of the pathogen post colonization. These processes and their underlying mechanisms represent the initial steps in development of disease and determine the severity and scope of the pathogen's effect on a population and community. Understanding these processes is crucial for species conservation and effective management, particularly when the pathogen is extremely virulent and poses a global threat, as does the fungal pathogen of amphibians, *Batrachochytrium dendrobatidis* (*Bd*). This study investigates the epidemiology of enzootic *Bd* dynamics in tadpoles in order to build a foundation from which to develop a more complete understanding of *Bd* disease processes in rainforest streams.

Despite efforts to identify alternate hosts, adult and larval amphibians are currently the only known carriers of *Bd* (Rowley et al. 2007). While nearly all amphibians can become infected, not all are equally susceptible to disease or decline in the wild (Lips et al. 2003). *Bd* is a flagellated aquatic fungus that requires moisture, ceases growth in culture at 28 C and is not known to enter a resting stage (Longcore et al. 1999; Piotrowski et al. 2004). Amphibians with a strong stream association are at a greater risk of decline (Bielby et al. 2008; Brem and Lips 2008; Hero et al. 2005; Lips et al. 2003; Mahony 1996; Williams and Hero 1998) and transmission of *Bd* has been shown to occur readily via water (Berger et al. 1998; Rachowicz and Vredenburg 2004). In Panama, riparian species declined most severely following *Bd* emergence (Lips et al. 2006). The evidence identifies water bodies; streams in particular, as the "central artery" of *Bd* transmission in rainforests. In the Wet Tropics of Australia, non-terrestrial species are significantly more likely to have declined if their tadpoles develop in streams, as opposed to stand alone ponds or ephemeral pools (Hero et al. 2005). Larvae of many species, therefore, reside continuously within the central artery of transmission, are a major known *Bd* host, and are associated with an increased risk of species decline.

Transmission during an epizootic in the amphibian rich neotropics appears to be extremely rapid. During an epizootic in Panama, prevalence increased from 0% to >50% in three months as amphibian populations crashed (Brem and Lips 2008). While declines were most severe in riparian species, pond and terrestrial species were impacted as well indicating transmission during epizootics extends well beyond the stream. Following a dieoff, amphibian abundance can be reduced by 70% (Lips et al. 2006). As *Bd* becomes enzootic, transmission appears to contract closer to the water

bodies and of the species that remain, prevalence is higher in non-terrestrial frogs (Brem and Lips 2008).

These facts indicate that larvae, particularly following an epizootic, are likely to play an important role in pathogen persistence, amplification, transmission, host recruitment, population recovery and possibly even evolution of resistance. Data based models predict that increased numbers of larvae increase the likelihood of species extinction by increasing the rate of transmission (Briggs et al. 2005; Mitchell et al. 2008). Infection in the larval stage, however, remains largely unstudied.

Apart from the likely important role of tadpoles in disease dynamics, the study of larvae also offers an epidemiological understanding that the study of terrestrial adults cannot provide. Many terrestrial habitats are too warm or too dry for *Bd* survival (Ron 2005). In habitats where *Bd* is present, seasonal or even daily temperature fluctuations can affect prevalence and intensity of infection (Kriger and Hero 2007; Woodhams and Alford 2005). Ambient air temperatures may act on the survival of *Bd* directly (Berger et al. 2004; Rowley 2006; Woodhams et al. 2003), or indirectly by altering host immune defenses (Andre et al. 2008). Some species may even display behavioral fever and actively seek warmer temperatures to clear infection (Richards 2008). In contrast, tropical stream larvae reside within a typically cooler and more thermally consistent environment. Infection dynamics in these tadpoles should be less variable than adults, and provide a less complicated measure of environmental pathogen dynamics.

Similar to adults, tadpoles of different species often have very different behaviors, ecological niches, and interactions with the microbial community, including pathogens. In limited sampling, tadpoles of different species within the Wet Tropics were found to have significantly different prevalence of infection (Woodhams and Alford 2005). Understanding such variance in prevalence and intensity of infection over time can offer insight into a number of currently unknown aspects of *Bd*, including methods of aquatic transmission, seasonal infection patterns within streams, and resistance to infection within larvae.

Here, I describe the epidemiology of enzootic chytridiomycosis in tadpoles. I monitored host population and *Bd* dynamics in tadpoles of five species in two rainforest streams over two years. I then consider the findings of this epidemiological study together with experimental results and data from published studies to generate a conceptual model of the factors affecting health and prevalence and intensity of *Bd* in tadpoles. This model can then be used to inform future research and management actions for conservation.

## Materials and Methods

### Field Sampling and Measurements

### Swabbing and Real Time Taqman Quantitative PCR

Infection of tadpoles occurs exclusively within the mouthparts. Currently the only way to non-destructively sample tadpoles for *Bd* infection is by swabbing the mouthparts and using PCR to identify the presence of *Bd* (Retallick et al. 2006).

Following capture by dip-net, tadpoles were placed in a sorting tray and then quickly transferred to individual zip lock bags, avoiding any direct handling (Fig 43). Each tadpole was handled with well-rinsed vinyl gloves to prevent mortality and gloves were changed between each individuals to prevent disease transmission (Cashins et al. 2008). For swabbing for *B. dendrobatidis*, tadpoles were poured from the zip lock bag into the palm of the hand and secured ventral surface up between the forefinger and thumb. A fine tipped swab (Medical Wire & Equipment Co. MW 100–100) was then gently passed over the mouthparts; 8 times horizontally across the upper and lower tooth rows and jaw sheath and 8 times vertically across all rows for a total of 24 strokes. Over the first year of sampling, tadpole body length was recorded for each individual. Beginning in the second year, tadpole body mass and a score for mouthpart and jaw loss were also recorded, as described elsewhere. Following sampling, tadpoles were returned to the individual bags and held for at least fifteen minutes to confirm that they were unaffected by handling before release.

Swabs were processed with a real-time PCR TaqMan assay following the procedure developed by Boyle et al. (2004) with minor modifications. Swab samples were kept below 25°C while in the field and at 4°C or below in the lab until processing. Storage at these temperatures does not affect recovery of *B. dendrobatidis* DNA (Hyatt et al. 2007). For DNA extraction, swab tips were immersed in 50 µl PrepMan Ultra (Applied Biosystems) and bead-beaten with 30 – 40 mg of 0.5 mm diameter zirconium/silica beads (Biospec Products) twice for 45 seconds, followed each time with centrifugation for 1 minute at 16.1 x $10^3$ RCF. Extraction tubes were incubated at 100°C for 10 minutes, cooled at room temperature (23°C) for 2 minutes and then centrifuged at 16.1 x $10^3$ RCF for 1 minute to remove condensation. Approximately 30 µl of homogenate, including negative control, was recovered and 10 µl diluted 1:10 in Molecular Grade Water (Sigma-Aldrich). Stock homogenate and 1:10 dilution were stored at -80°C until the PCR assay, unless the PCR assay was run immediately. Extraction dilutions were processed through a real-time TaqMan® (Applied Biosystems) quantitative PCR assay. The analysis was performed on the Rotor-Gene™ 6000 (Corbett Research) using Gene-Disc 100 tubes. Triplicate analyses were performed for each sample, negative control and no-template control, and quadruplicate analyses were undertaken for each standard (100, 10, 1, 0.1 zoospore equivalents). A stock solution to create standard dilutions was provided by the Australian Animal Health Laboratory (AAHL) in Geelong, VIC. Negative samples were rerun with an internal positive control to confirm the sample was not negative due to inhibition. No tadpole swabs were negative as a result of inhibition.

Statistical Analyses

Tadpoles were considered positive if at least two of the three replicate wells returned a positive PCR reaction. Zoospore equivalents as determined by quantitative PCR were Log transformed prior to analysis as data ranged from 1 to 36,000. For intensity of infection analyses, only infected tadpoles were included. Statistical analyses and graphs were performed using Statistica 7 (StatSoft), SigmaPlot 10 (Systat) and SPSS (SPSS Inc.). Prevalence values were compared using chi square. The continuity correction was used in cases of 2x2 tables. Correlations between intensity and prevalence or density of infected tadpoles were determined using time series cross correlation. *Nyctimystes dayi* and *L. xanthomera* were both only found at Tully Gorge National Park, therefore they could not be included in comparisons between sites.

Logistic Regression Model (Infection status)

Following analysis of the intensity and prevalence of infection, and environmental and ecological factors and consideration of published studies, a set of independent variables that were considered most likely to affect infection status were considered in constructing a binomial logistic regression model using SPSS (version 16, SPSS Inc.). The dichotomous dependent variable was infection status. A tadpole was considered infected if 2 or more wells returned a positive PCR reaction. Models were constructed following recommendations by Hosmer and Lemeshow (2000). Categorical variables included site, species and body size class. Continuous variables included air temperature (1.data collected on site and 2. interpolated values), water temperature, rainfall, water flow rate, solar radiation, evaporation, vapor pressure, evapotranspiration and relative humidity (1. at max temperature and 2. at min temperature). All continuous variable data (except flow rate) were averaged over the previous 7, 14, 21 and 28 days prior to sampling. The air and water temperature data were averaged over these time periods by: 1) all data 2) daily minimum temperature 3) daily maximum temperature. Flow rate was analyzed by the mean, minimum and maximum values. All 14 variables with 66 total iterations were examined in separate univariate analyses. Any variable with a p-value $< 0.25$ was considered for inclusion in the multivariate analysis. For variables with multiple iterations, the one with the lowest Wald statistic, or best predictive value, was selected. The following variables were selected: site, species, body size class, 7 day mean air temp., 7 day mean water temp, 28 day mean rainfall and mean flow rate. All variables were examined for collinearity. It was found that air and water temperature were highly correlated and rainfall and water flow were moderately correlated. Tadpoles are aquatic organisms, therefore air temperature was eliminated. Flow rate was a direct measurement and likely to be more accurate of stream-level conditions, compared with rainfall data which was interpolated from area weather stations. Rainfall was therefore eliminated.

The remaining five variables were fit to the model. Any variable that did not significantly predict infection status was removed from the model. Each pair-wise interaction was then added to the model individually and tested for significance ($p<.05$). All significant interactions were then added to the main-effects model and any variables or interactions that no longer significantly improved the model were removed. Overall model significance was measured with a model chi-square test. the Hosmer and Lemeshow goodness-of-fit test was used to measure whether the models predicted data fit the observed values and Cox and Snell $R^2$ and Nagelkerke $R^2$ were calculated to estimate the percentage of variance explained by the model.

Standard Multiple Regression Model (Intensity of Infection)

Due to small numbers of infected *L. genimaculata*, *L. xanthomera* and *N. dayi*, the linear regression model was restricted to *L. nannotis*, the tadpole with the largest available sample size. Only infected ($\geq 2$ PCR positive wells) tadpoles were considered for the analysis. Variables were selected a priori to analysis based on current published literature and previous data analysis.

Variables were checked for outliers, normality, linearity, and homoscedasticity. Outliers were assessed using Mahalanobis distances. Normality was assessed visually via histogram and was accepted if skewness was between –0.8 to 0.8 and kurtosis was between –3 to 3. Linearity and homoscedasticity were assessed via visual inspection of

individual scatterplots of the residuals and via the Normal Probability Plot (P-P) of the Regression Standardized Residual.

The dependent variable (zoospore equivalents) was Log transformed as the data were strongly positively skewed (4.65) and kurtosic (26.76). Following transformation, values of skewness and kurtosis were -.326 and -.784 respectively. A priori selected independent variables included water temperature, air temperature, body length, water flow rate, solar radiation, rainfall, and site. These variables were selected based on the results from the previously described logistic regression analysis on infection status and results from published studies. Rainfall was positively skewed and was thus Log transformed to achieve normality.

A univariate regression analysis was performed on each iteration of all selected variables. ANOVA was used to test for univariate significance ($p<.05$). The statistically significant iteration with the highest $R^2$ value from each variable was included in the multivariate analysis. Following the univariate analyses, air temperature was found to be a non-significant predictor and was eliminated from further consideration. Mean 28-day rainfall ($R^2$: 7.9%), mean 28-day minimum water temperature ($R^2$: 5.2%), site ($R^2$: 5.0%), body length ($R^2$: 4.5%), mean 28-day solar radiation ($R^2$: 2.6%) and mean water flow rate ($R^2$: 1.1%) were all significant univariate predictors. These variables were fit to the model and any non-significant factors were removed and the model re-fit. Mean flow rate was no longer significant and was removed. The final suite of independent variables were checked for bivariate correlation using Pearson product-moment correlation and multicollinearity using the Tolerance statistic (T) and the Variance inflation factor (VIF). Variables were considered correlated if $r > .7$ and to display multicollinearity if $T < .1$ and $VIF > 10$. A total of 806 cases were available for analysis.

Overall model significance was determined via ANOVA and overall predictive ability via the $R^2$ statistic. The contribution of each of the independent variables was assessed via the Standardised Coefficient beta values.

Results

Prevalence

Yearly mean prevalence of infection differed between tadpoles of each species ($df=4$, $n=1776$, $x^2=475$, $p<0.0005$, Figure 57) with torrent-adapted tadpoles having a higher prevalence of infection than pool-adapted tadpoles (Table 18). Within the torrent-adapted group, *N. dayi* had a lower prevalence than either *L. nannotis* or *L. rheocola*. Mean prevalence did not differ within *L. genimaculata* or *L. rheocola* between sites, however, *L. nannotis* had a higher prevalence at Murray Upper National Park (Table 18, Figure 58).

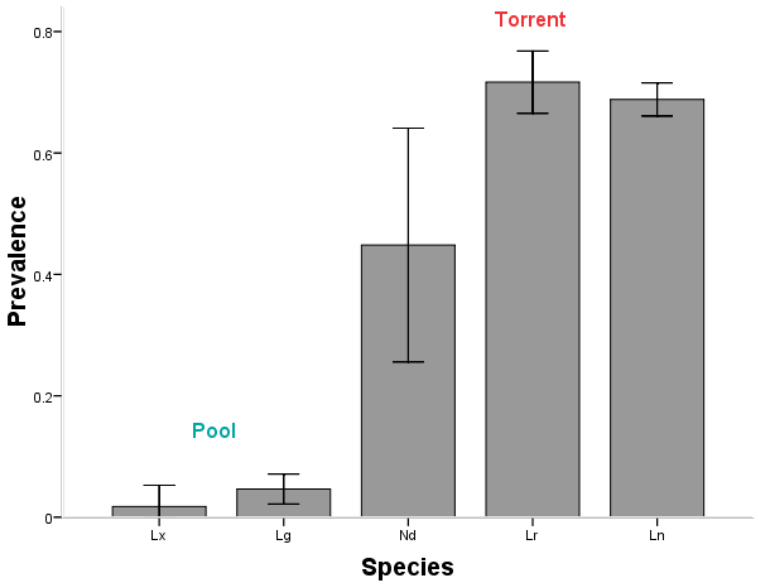


Figure 57 Mean prevalence of *B. dendrobatidis* in tadpoles of five species at Tully Gorge NP and Murray Upper NP (2006-2008). Whiskers represent 95% confidence intervals for the mean.

Table 18. Chi square comparison of prevalence between tadpoles of each species and between sites (Tully Gorge NP and Murray Upper NP) within species (2006-2008).

| *Comparison* | *Tad 1* | *Tad 2* | *df* | *n* | $X^2$ | *p* |
|---|---|---|---|---|---|---|
| **x Species** | ***Lg*** | ***Lx*** | 1 | 337 | 0.992 | 0.527 |
| | | ***Ln*** | 1 | 1390 | 373.7 | <0.0005* |
| | | ***Lr*** | 1 | 580 | 272.7 | <0.0005* |
| | | ***Nd*** | 1 | 309 | 55.065 | <0.0005* |
| | ***Lx*** | ***Ln*** | 1 | 1167 | 108.0 | <0.0005* |
| | | ***Lr*** | 1 | 357 | 97.971 | <0.0005* |
| | | ***Nd*** | 1 | 86 | 26.165 | <0.0005* |
| | ***Ln*** | ***Lr*** | 1 | 1410 | 0.767 | 0.381 |
| | | ***Nd*** | 1 | 1139 | 6.443 | 0.011* |
| | ***Lr*** | ***Nd*** | 1 | 329 | 7.736 | 0.005* |
| **x Sites** | ***Lg*** | ***Lg*** | 1 | 280 | 0.696 | 0.404 |
| | ***Ln*** | ***Ln*** | 1 | 541 | 5.926 | 0.015* |
| | ***Lr*** | ***Lr*** | 1 | 167 | 0.000 | 0.992 |

* Indicates significant difference in prevalence

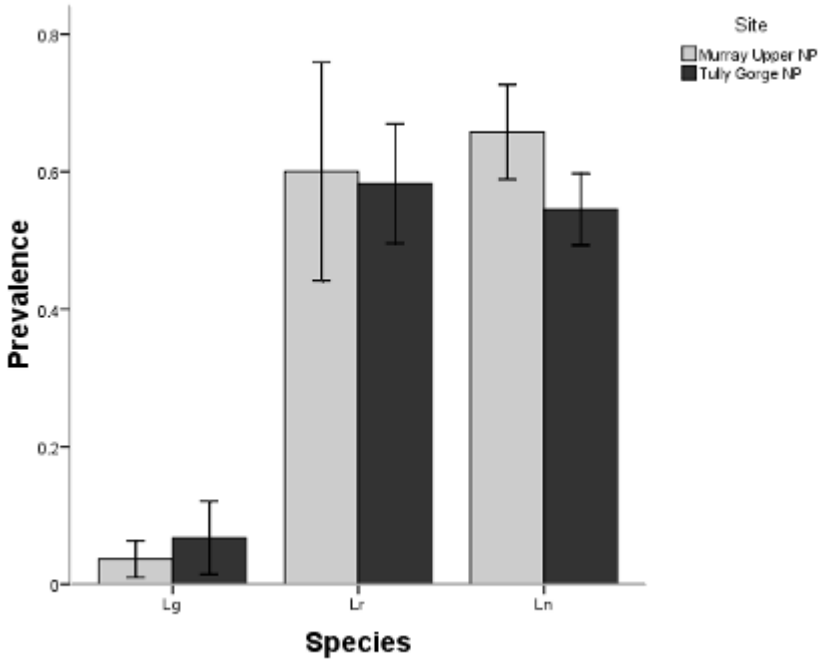


Figure 58. Mean prevalence of infection in tadpoles at Tully Gorge NP and Murray Upper NP (2006-2008). Whiskers represent 95% confidence intervals of the mean.

Prevalence by Size Class

Prevalence in both *L. nannotis* and *L. rheocola* increased with size of tadpole across seasons (Figure 59). Prevalence was low for the smallest of tadpoles, then increased significantly in the mid-range size classes before leveling off at high prevalence in the largest tadpoles. The change in prevalence between classes decreased with increasing size, indicating transmission occurs primarily in the early stage classes and tadpoles are unlikely to lose infection. Prevalence increased in the larger size classes of *L. genimaculata* tadpoles (Figure 60), and infected tadpoles on average had significntly higher prevalence than smaller tadpoles (independent samples t-test: t=-4.580, df=278, p=<.0005, Figure 61). Prevalence in *L. nannotis* tadpoles increased with increasing body size n both the winter and summer seasons (Figure 62), though the increase was lower at interediate body sizes in summer. Prevalence in *L. xanthomera* remained very low across all size classes.

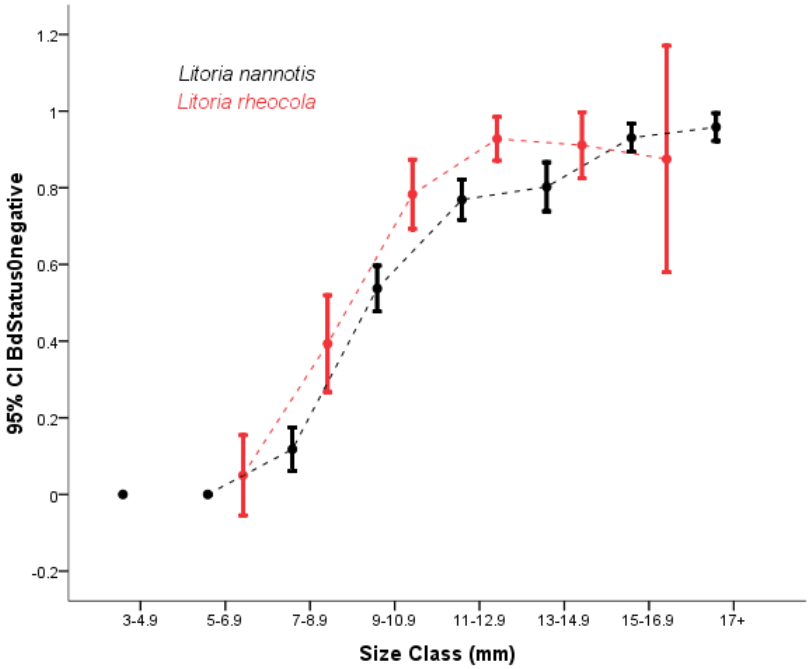


Figure 59. Mean prevalence of *B. dendrobatidis* in *L. nannotis* and *L. rheocola* by size class. Tully Gorge NP and Murray Upper NP (2006-2008). Connecting lines are for visualization only.

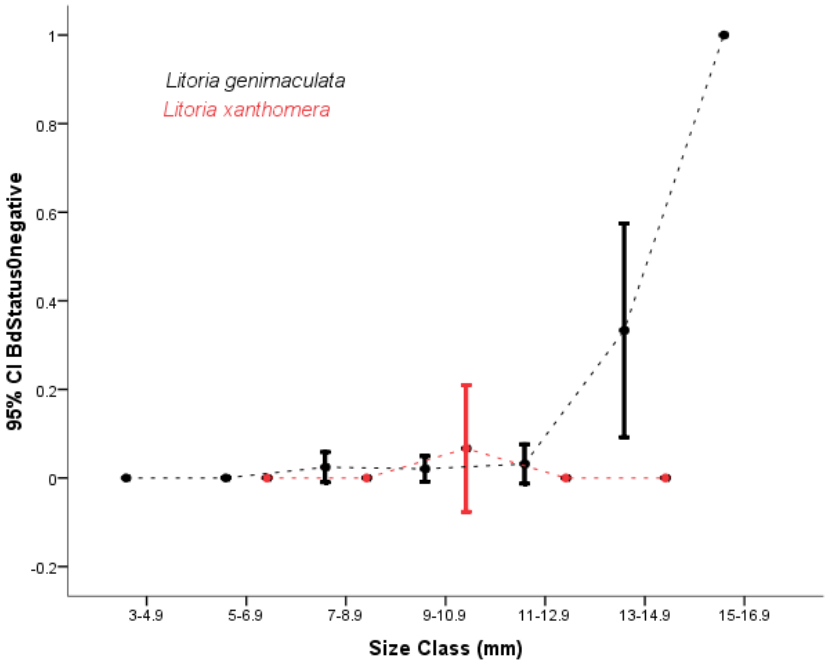


Figure 60. Mean prevalence of *B. dendrobatidis* in *L. genimaculata* and *L. xanthomera* by size class. Tully Gorge and Murray Upper NP (2006-2008). Connecting lines are for visualization only.

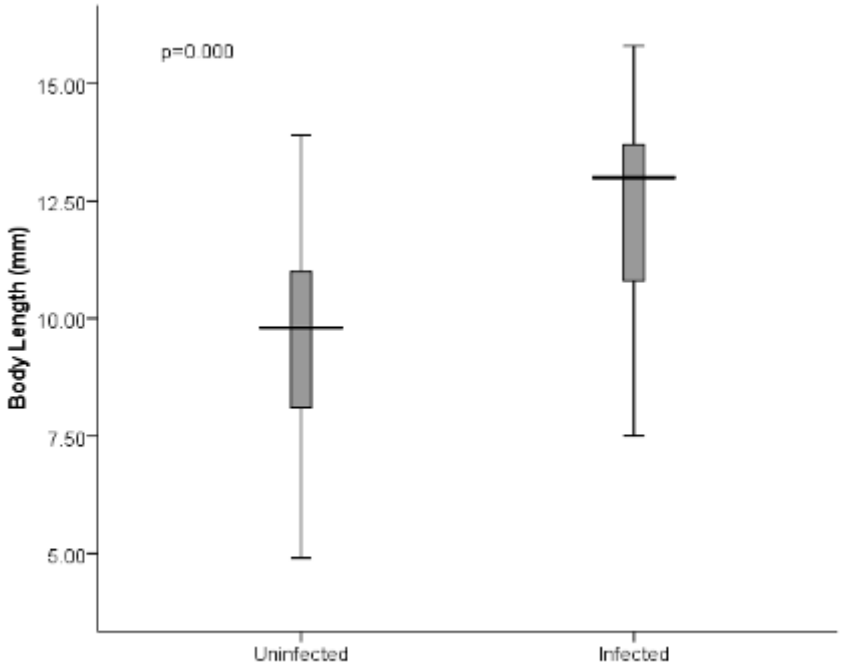


Figure 61. Mean size of infected and uninfected *L. genimaculata* tadpoles at Tully Gorge NP and Murray Upper NP (2006-2008).

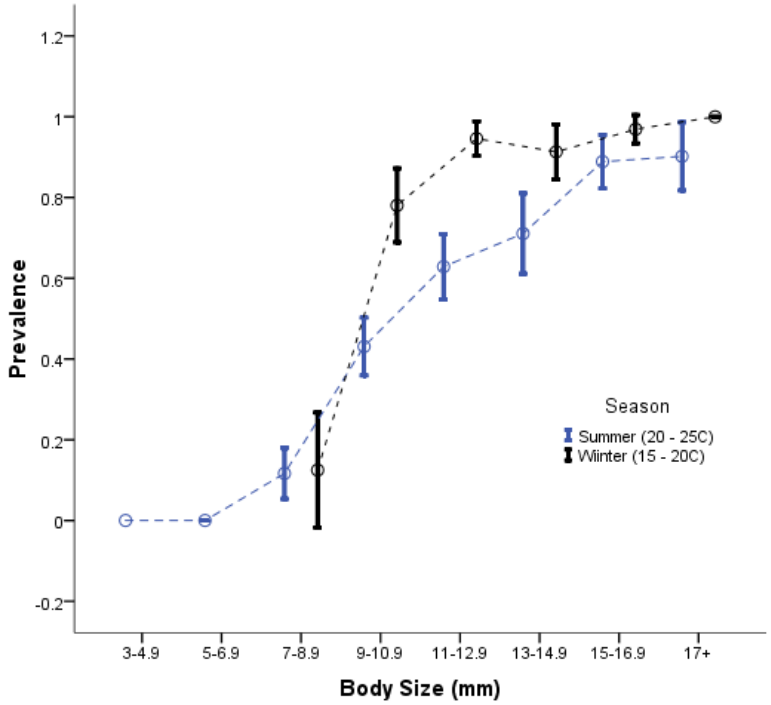


Figure 62. Mean prevalence of *B. dendrobatidis* in *L. nannotis* at Tully Gorge NP and Murray Upper NP by size class and season (2006-2008). Connecting lines are for visualization only. Whiskers represent the 95% confidence interval.

**Prevalence by Water Temperature**

Yearly water temperature extremes ranged between approximately 15 and 25 C (Figures 63, 64). For this analysis, I considered "winter" the time of the year when mean temperature was below 20 C (May1 - September 30) and "summer" when mean water temperature was above 20 C (October 1 - April 30).

No tadpoles smaller than 7 mm were captured in winter and there was no difference in prevalence in tadpoles sized 7-8.9 mm between winter and summer (Figure 62). The low prevalence in small (7-8.9 mm) tadpoles across seasons indicates that recently hatched tadpoles are unlikely to be infected at any time, likely due to their limited duration of exposure to the risk of infection. However, prevalence was significantly higher in winter than in summer in tadpoles larger than 9 mm, except size class 15-16.9 mm which was near significant (Figure 62, Table 19). The difference between seasons in the larger size classes may largely be explained by seasonal breeding and development; many *L. nannotis* tadpoles hatch in early summer, grow more slowly over winter and metamorphose the following spring/summer. Therefore, many tadpoles captured during winter would have been in the stream and exposed to *Bd* for a relatively longer period of time than tadpoles of the same body size captured in summer. It is also possible that the consistently lower prevalence in summer across all larger size classes (particularly the largest tadpoles), indicates that higher temperatures exert a relatively small, but significant downward pressure on infection either through a decreased rate of transmission or clearing of an existing infection both of which are likely to be host mediated.

Table 19. Chi-squared 2 X 2 contingency tests for each size class examining the null hypothesis that prevalence of infection is related to the temperature of the water in which individuals were found (because water temperature changes seasonally, apparet effects of water temperature may be caused by other seasonal factors). Data for *Litoria nannotis* at Tully Gorge NP and Murray Upper NP (2006-2007).

| | Prevalence | | | | |
|---|---|---|---|---|---|
| ***Size Class (mm)*** | ***>20 C*** | ***<20 C*** | ***df*** | ***$X^2$*** | ***p*** |
| **3 - 4.9** | **0** *(0/1)* | | | | |
| **5 - 6.9** | **0** *(0/2)* | | | | |
| **7 - 8.9** | **11.7** *(12/103)* | **12.5** *(3/24)* | **1** | **0.0** | **1.000** |
| **9 - 10.9** | **43.1** *(81/188)* | **78.0** *(64/82)* | **1** | **26.7** | **<0.0005*** |
| **11 - 12.9** | **62.9** *(88/140)* | **94.6** *(105/111)* | **1** | **33.3** | **<0.0005*** |
| **13 - 14.9** | **71.1** *(59/83)* | **91.3** *(63/69)* | **1** | **8.5** | **0.004*** |
| **15 - 16.9** | **88.9** *(80/90)* | **96.9** *(94/97)* | **1** | **3.5** | **0.062** |
| **17 +** | **90.2** *(46/51)* | **100** *(69/69)* | **1** | **4.8** | **0.028*** |

* Indicates significant difference in prevalence

The pattern of higher prevalence occurring with cooler temperatures was also observed in the other torrent tadpoles (Figure 63). *Litoria genimaculata*, on the other hand, were more likely to be infected in warmer water. This may be because warmer temperatures coincide with larger tadpoles that have spent a longer time in the water. This is consistent with the hypothesis that it is duration of exposure to the risk of infection that is the principle determinant of prevalence rather than temperature. Tadpoles of the different species were exposed to similar water temperatures (Figures 63, 64).

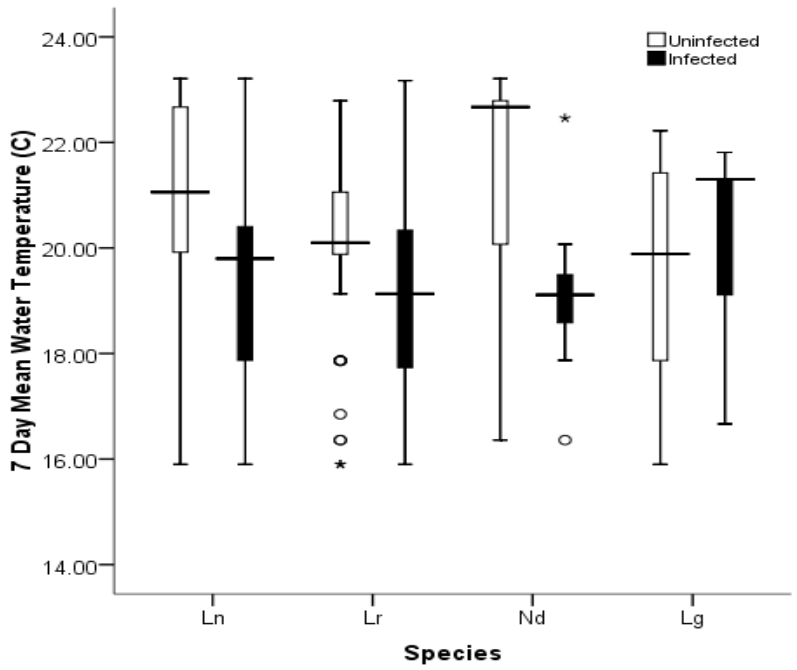


Figure 63. Seven-day mean water temperature of stream habitat for *B. dendrobatidis* infected and uninfected tadpoles of L. nannotis, L. rheocola, N. dayi and L. genimaculata prior to capture in Tully Gorge NP and Murray Upper NP (2006-2008).

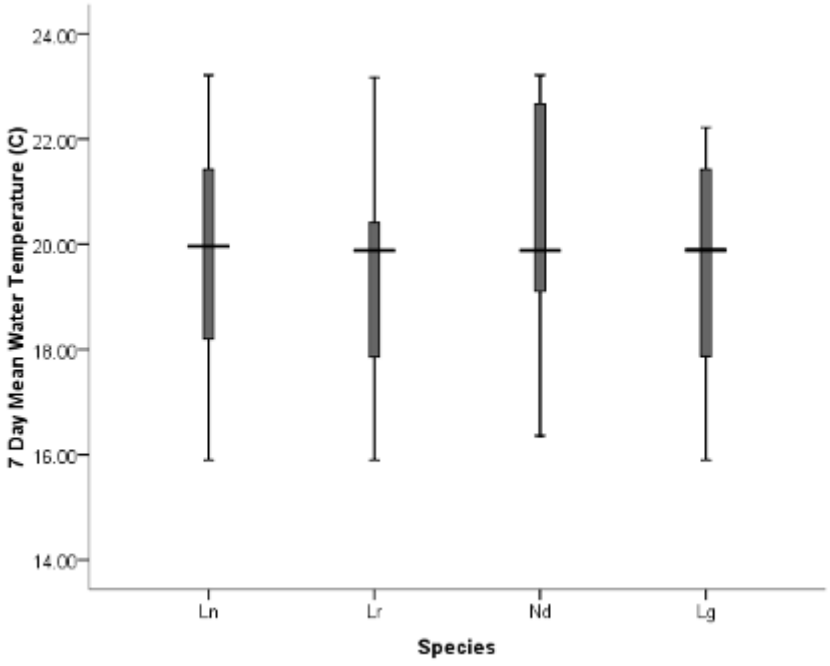


Figure 64. Seven-day mean water temperature of stream habitat for combined uninfected and infected *L. nannotis*, *L. rheocola*, *N. dayi*, and *L. genimaculata* prior to capture in Tully Gorge NP and Murray Upper NP (2006-2008).

Seasonal Prevalence

Overall prevalence is very seasonal in torrent-adapted tadpoles but not in pool-adapted tadpoles. Prevalence in pool tadpoles is low throughout the year with occasional

increases in prevalence (Figures 65, 66). Periods of increased prevalence in pool tadpoles is explained by the presence of larger size class *L. genimaculata* tadpoles (Figure 65). As a result of frequent rain events that turned connected pools into torrents and flushed pool-adapted tadpoles out of their habitat there was no clear developmental progression in *L. genimaculata*. Prevalence in torrent tadpoles tends to be lowest during the summer months, then increases over the ensuing year, reaching 80 -100% prevalence before decreasing again the following summer (Figures 65, 66). This effect of seasonality however, is primarily seen in the medium size-group tadpoles (Figure 67). Small size-group tadpoles remain at low prevalence throughout the year and large size-group tadpoles remain at high prevalence throughout the year, with the notable exception of a decrease in prevalence in summer between January and March of each year. Therefore, transmission rates are highest in the medium size-group tadpoles, as predicted by figure 6.3. The seasonal decrease in prevalence occurs during the period when large tadpoles metamorphose and exit the population and new, small tadpoles hatch and enter the population (Figure 67). This demographic change is seen as fluctuations in abundance of tadpoles in this size class in figure 67 where abundance decreases dramatically and then recovers. The decrease in prevalence within the medium size-group is therefore caused by the largest, infected tadpoles exiting into the large size-group and new, uninfected tadpoles entering from the small size-group (Figure 67).

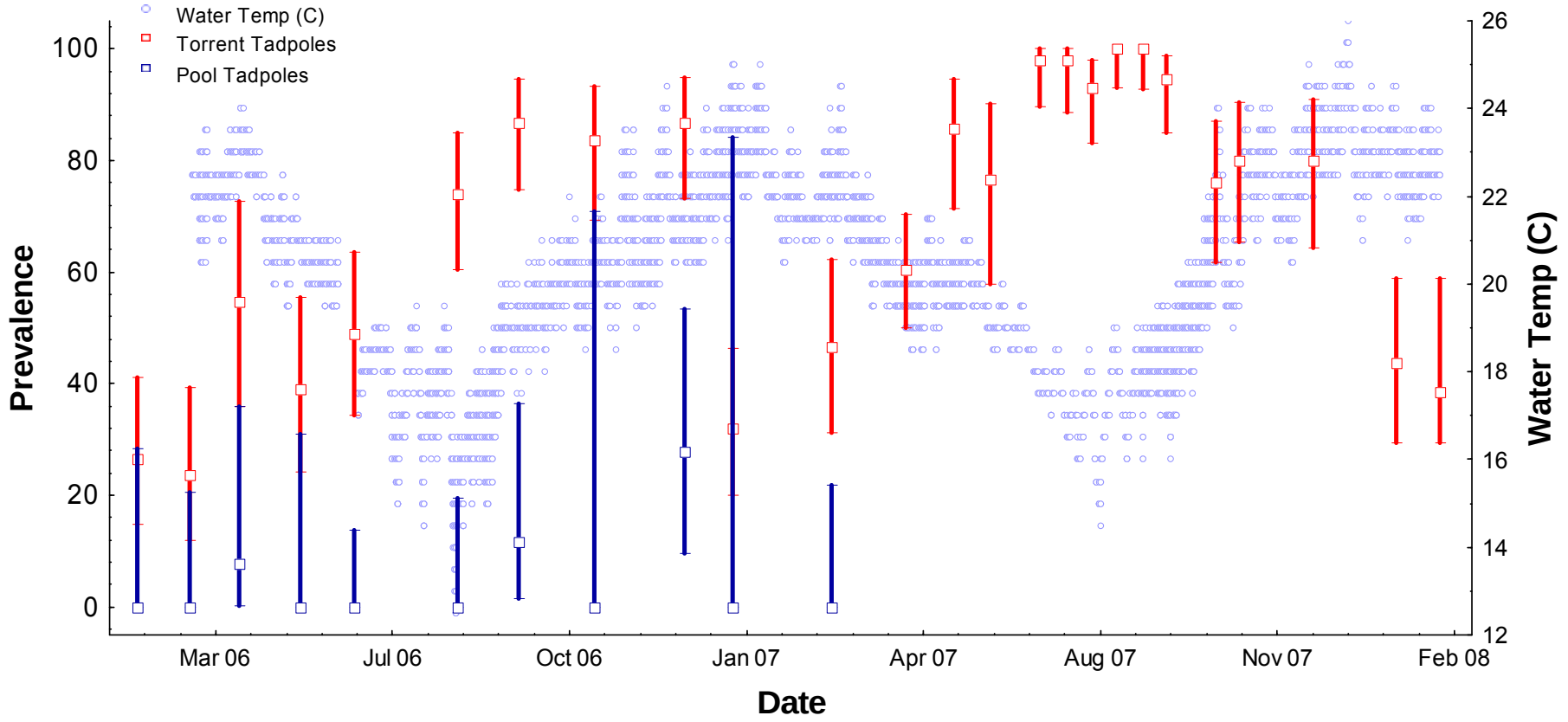


Figure 65. Seasonal prevalence of *Batrachochytrium dendrobatidis* in torrent-adapted (*L. nannotis*, *L. rheocola*, *N. dayi*) and pool-adapted (*L. genimaculata* and *L. xanthomera*) tadpoles at Tully Gorge National Park, 2006 – 2008. Whiskers represent 95% confidence intervals. Blue dots represent the range in water temperature.

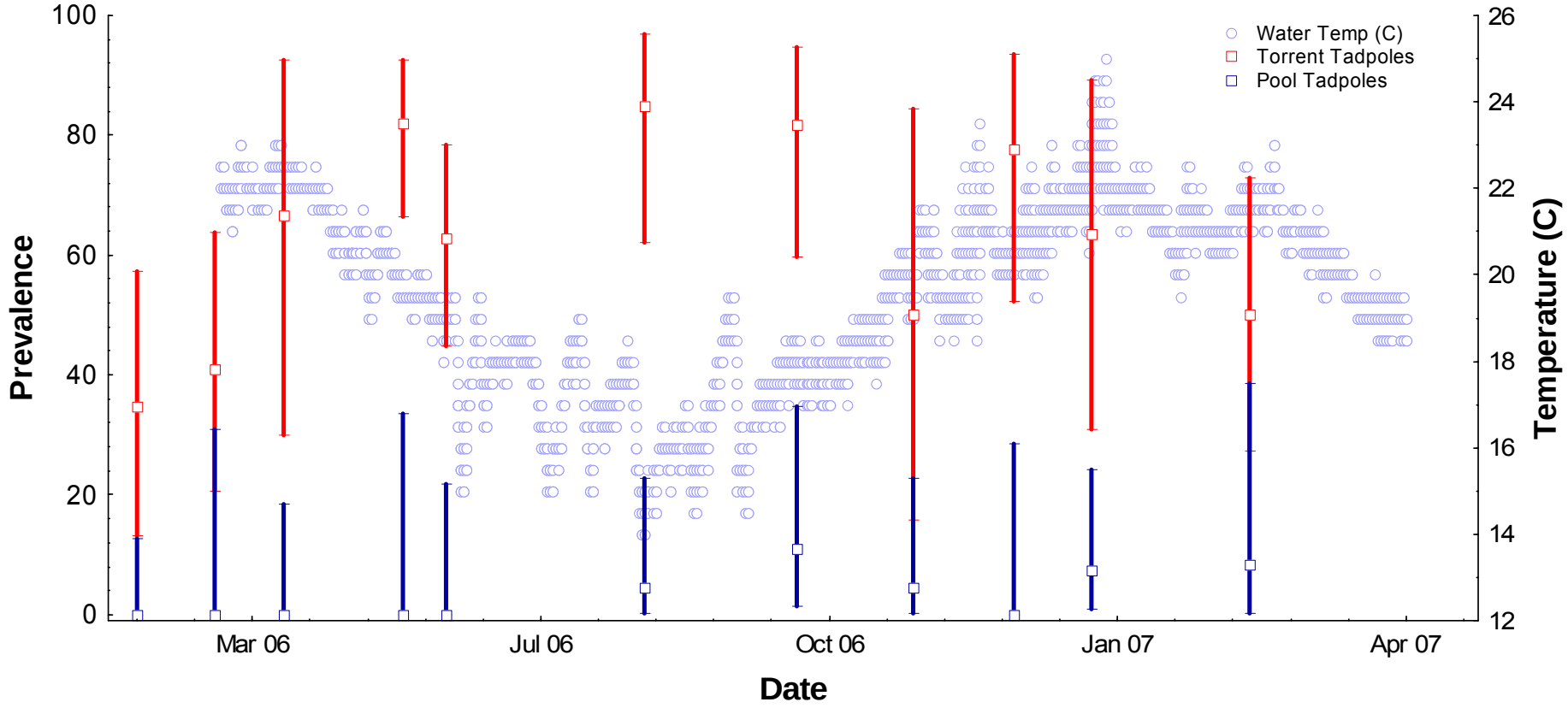


Figure 66. Seasonal prevalence of *Batrachochytrium dendrobatidis* in torrent-adapted (*L. nannotis*, *L. rheocola*, *N. dayi*) and pool-adapted (*L. genimaculata*) tadpoles at Murray Upper National Park, 2006 – 2007. Whiskers represent 95% confidence intervals. Blue dots represent the range in water temperature.

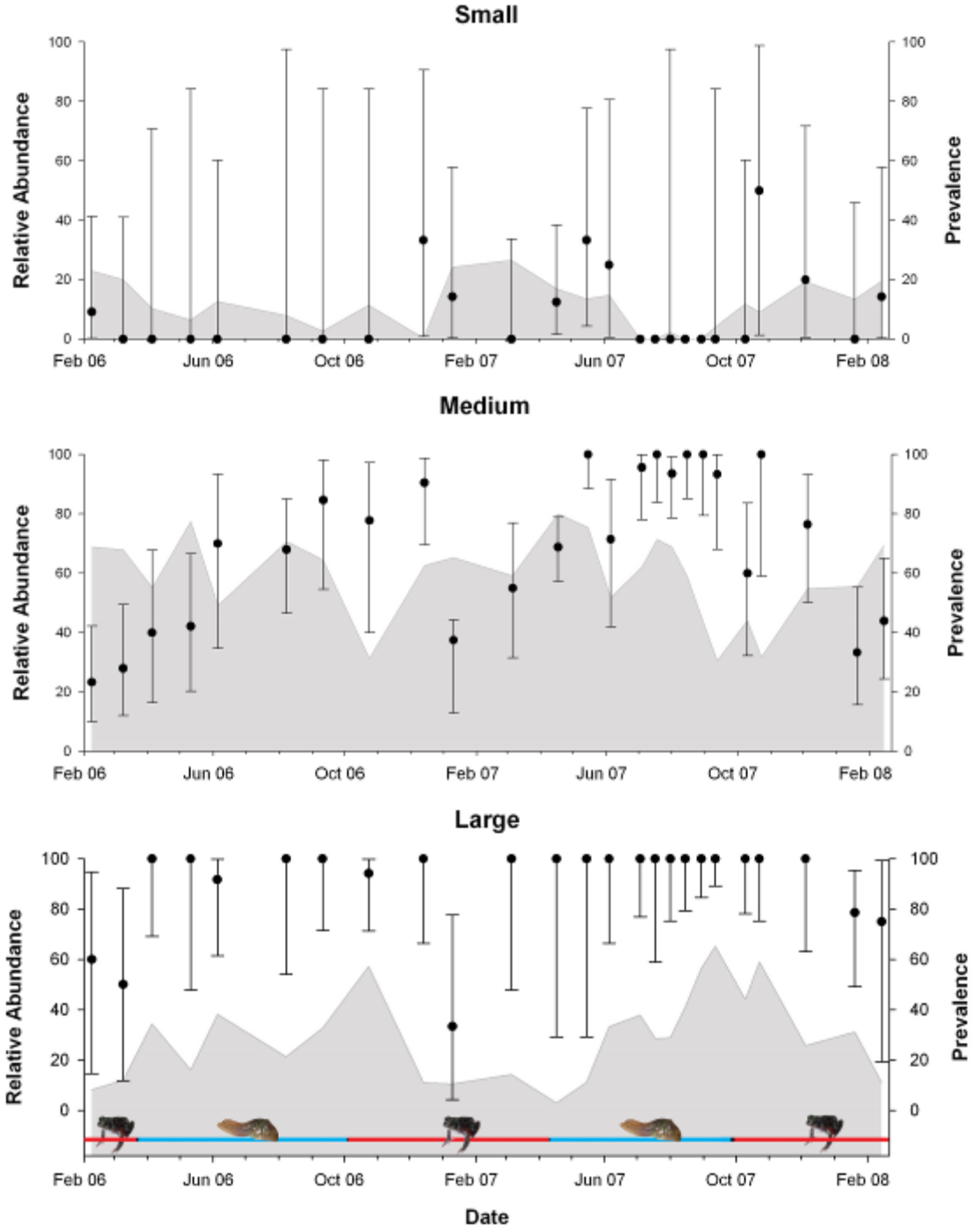


Figure 67. Prevalence and abundance of *L. nannotis* tadpoles at Tully Gorge National Park (2006-2008). Each panel presents data from small, medium and large size group tadpoles. The shaded gray areas represent the relative abundance of each size group. Whiskers represent the 95% confidence interval. The bar at the bottom depicts the periods of metamorphosis in red for the large size group and increased transition of tadpoles from the medium to large size group and recruitment of tadpoles to the small size group.

Binary Logistic Regression Model

The significant variables in the logistic regression model included 7-day mean water temperature, species and size class (Table 20). Site and flow rate were non-significant and were removed. Flow rate only became non-significant following inclusion of the interactions. The interaction of flow rate by species was the only statistically significant interaction and was included in the final model.

The odds ratio indicates that for every 1° C decrease in water temperature the chances of being infected increase by a factor of 1.4, all else being equal. Species was important with *Nyctimystes dayi* (16.7 x), *L. nannotis* (67.58 x) and *L. rheocola* (191.48 x) being more likely than *L. genimaculata* to be infected. Size class was the best predictor of infection status. Every increase in size class yielded a higher odds ratio for infection status. The largest size class was 1098 times as likely to be infected as the smallest size class. The significant interaction indicates that torrent adapted tadpoles have an increased likelihood of infection when water flow rates are slower.

The Nagelkerke $R^2$, indicated 64.6% of variation was explained by the model. Cases were accurately predicted 85.8% of the time using the model, compared with 60.5% using the null model. The model was significant (model chi-square test, $x^2_{14} = 868.767$, $p<0.0005$) and showed no evidence of lack of fit (Hosmer and Lemeshow goodness-of-fit test, $x^2_8 = 10.359$, $p = 0.241$).

Table 20. Logistic regression model. [s. a] Model Chi Square for Univariate Model and Whole Model

| *Variable* | *Coefficient* | *SE* | *Wald* | *df* | *p* | *Odds ratio* | *Lower 95% CI* | *Upper 95% CI* |
|---|---|---|---|---|---|---|---|---|
| Water Temperature (7 day mean) | -0.36 | 0.04 | 70.58 | 1 | 0.00 | 0.70 | 0.64 | 0.76 |
| Species | | | 170.52 | 4 | 0.00 | | | |
| *Litoria xanthomera* | 0.05 | 1.08 | 0.00 | 1 | 0.96 | 1.05 | 0.13 | 8.79 |
| *Litoria nannotis* | 4.21 | 0.37 | 128.55 | 1 | 0.00 | 67.58 | 32.62 | 139.99 |
| *Litoria rheocola* | 5.26 | 0.48 | 120.98 | 1 | 0.00 | 191.48 | 75.07 | 488.41 |
| *Nyctimystes dayi* | 2.81 | 1.53 | 3.39 | 1 | 0.07 | 16.65 | 0.84 | 331.70 |
| Size Class (mm) | | | 188.89 | 6 | 0.00 | | | |
| 7-8.9 | 2.33 | 1.07 | 4.78 | 1 | 0.03 | 10.30 | 1.27 | 83.34 |
| 9-10.9 | 4.00 | 1.06 | 14.17 | 1 | 0.00 | 54.44 | 6.79 | 436.42 |
| 11-12.9 | 4.88 | 1.07 | 20.91 | 1 | 0.00 | 131.54 | 16.25 | 1065.00 |
| 13-14.9 | 5.71 | 1.08 | 27.83 | 1 | 0.00 | 301.57 | 36.17 | 2515.00 |
| 15-16.9 | 6.55 | 1.11 | 34.80 | 1 | 0.00 | 702.15 | 79.57 | 6196.00 |
| 17+ | 7.00 | 1.22 | 33.15 | 1 | 0.00 | 1098.00 | 101.29 | 11910.00 |
| Intercept | -0.13 | 1.36 | | | | | | |
| Univariate model | | | 965.55 | 8 | <.0005 | | | |
| Flow rate (mean) x Species | | | 13.75 | 3 | 0.00 | | | |
| *Litoria nannotis* | -1.17 | 0.42 | 7.76 | 1 | 0.01 | 0.31 | 0.14 | 0.71 |
| *Litoria rheocola* | -2.35 | 0.96 | 5.94 | 1 | 0.02 | 0.10 | 0.01 | 0.63 |
| *Nyctimystes dayi* | -1.08 | 2.40 | 0.20 | 1 | 0.65 | 0.34 | 0.00 | 37.30 |
| Whole Model | | | 868.77 | 14 | <.0005 | | | |

Intensity of Infection

Infected tadpoles of the different species had significantly different mean intensities of infection (ANOVA, F(4,1050), p=0.0003; Figure 68). *Litoria nannotis* had a higher intensity than *L. genimaculata* and *L. rheocola*. There was no difference in intensity between any other tadpoles. Only two *L. xanthomera* were found infected. However, the intensity in one of them was very high (33,000 zoospore equivalents). Although clearly an outlier, this individual was included in the analyses as there was no indication the value was false. Intensity was significantly different among size classes in *L. nannotis* (ANOVA, F(5, 341), p=0.0343; Figure 69) and tended to increase with increasing size, however Tukeys post hoc test did not identify any groups that were different from each other. Intensity did not differ with size class in *L. rheocola* (ANOVA, F(4,95), p=0.0674), *N. dayi* (ANOVA, F(2,9), p=0.1463) or *L. genimaculata* (ANOVA, F(4,8), p=.5803), however sample sizes were small in the latter two. When all torrent adapted tadpoles from both sites are combined, intensity differs with size class (ANOVA, (F(6,453), p=0.0005; Figure 70). Tadpoles in the 15-16.9 mm size class have higher infections compared with tadpoles in the 9-10.9 mm and 11-12.9 mm size classes and tadpoles in the 13-14.9 mm size class have higher intensity than size class 11-12.9 mm.

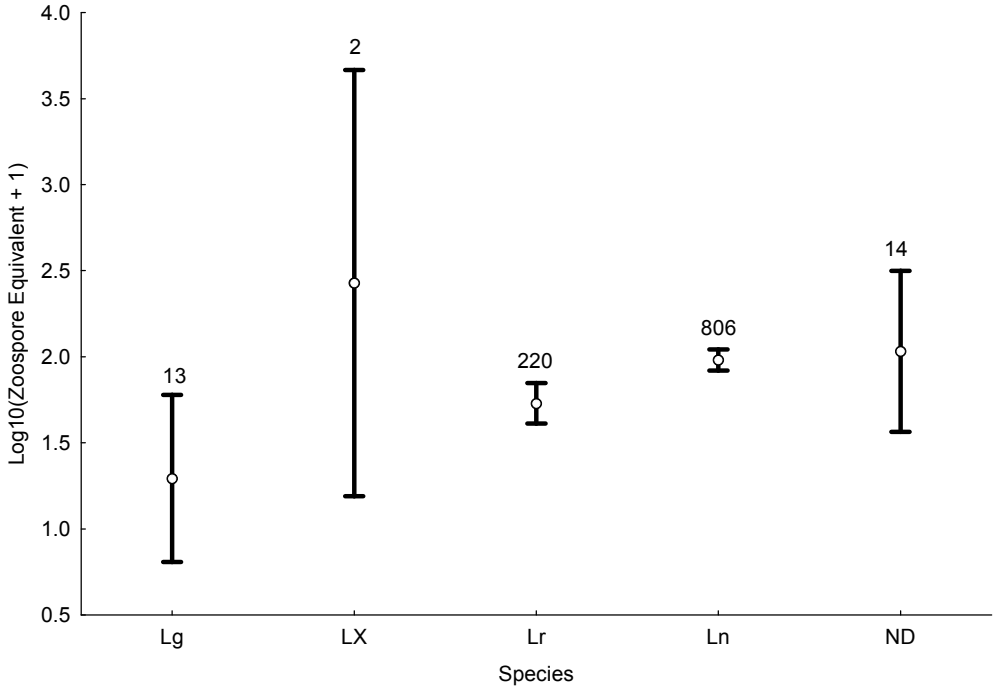


Figure 68. Mean intensity of infection in tadpoles of each species. Symbols represent the mean, whiskers represent the 95% confidence interval. Numbers above are the sample sizes.

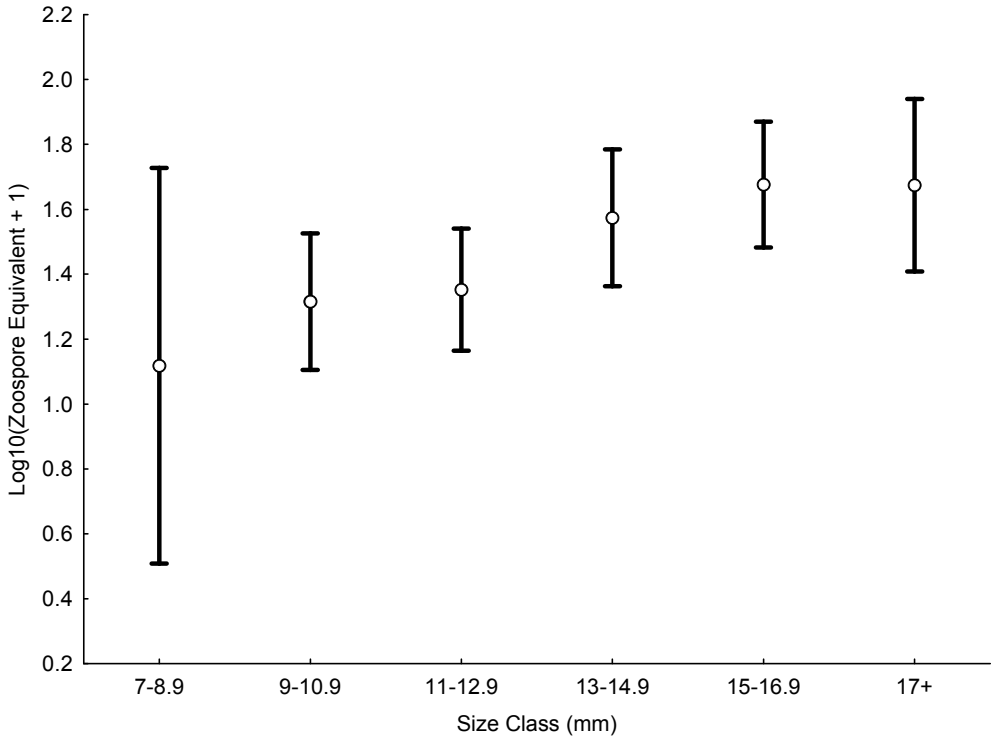


Figure 69. Intensity of infection by size class in infected *L. nannotis*. Tully Gorge and Murray Upper National Park. Symbols represent the mean, whiskers represent the 95% confidence intervals

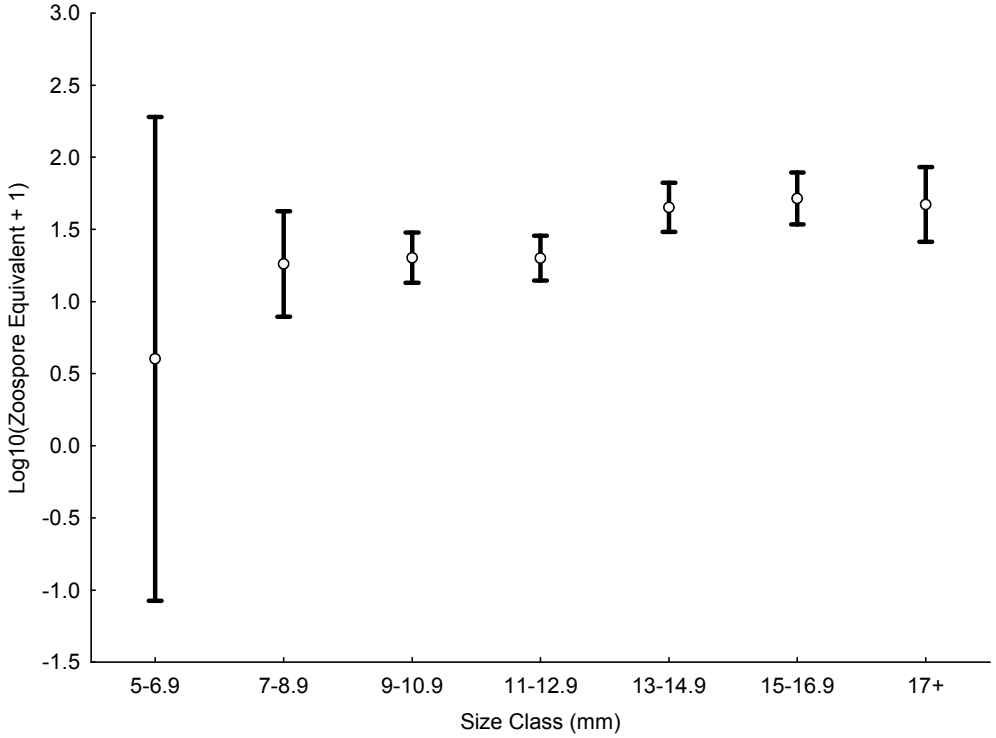


Figure 70. Intensity of infection in all infected torrent adapted tadpoles by size class. Symbols represent the mean, whiskers represent the 95% confidence intervals.

Site Differences

There was no effect of site on mean intensity of infection within tadpoles of *L. genimaculata*, *L. nannotis* or *L. rheocola* between February 2006 and February 2007 (ANOVA, F(2,455), p=0.9042; Figure 71). Due to the low prevalence of infection in *L. genimaculata*, infected tadpoles were too few for the site x size class and site x sampling period comparisons. *Nyctimystes dayi* and *L. xanthomera* were only found at Tully Gorge National Park and therefore were not compared between sites.

*Size Class*

Within *L. nannotis* there was a significant interaction between site and size class (ANOVA , F(5,335), p=0.00155; Figure 72). However, this difference was driven entirely by higher prevalence at Murray Upper in size class 9-10.9 mm (Tukeys HSD, p=0.0102). The remaining size classes were not significantly different between sites. There was no significant difference in intensity of infection in *L. rheocola* within size class between sites (ANOVA, F(4,90), p=0.1148; Figure 73).

*Sampling Period*

Mean intensity in *L. nannotis* (ANOVA, F(10,325), p=0.0000) and *L. rheocola* differed within sampling periods between the two sites. However, in both cases this was due entirely to a difference in intensity in August 2006 when Murray Upper had a significantly higher intensity of infection than Tully Gorge (Tukeys HSD; *L. nannotis,* p= 0.0000; Figure 74 and *L. rheocola,* p=0.0003; Figure 75). The remaining sampling periods were not significantly different.

Overall, there was a high level of agreement between the two sites, within both size class and sampling period in *L. nannotis*, and *L. rheocola*.

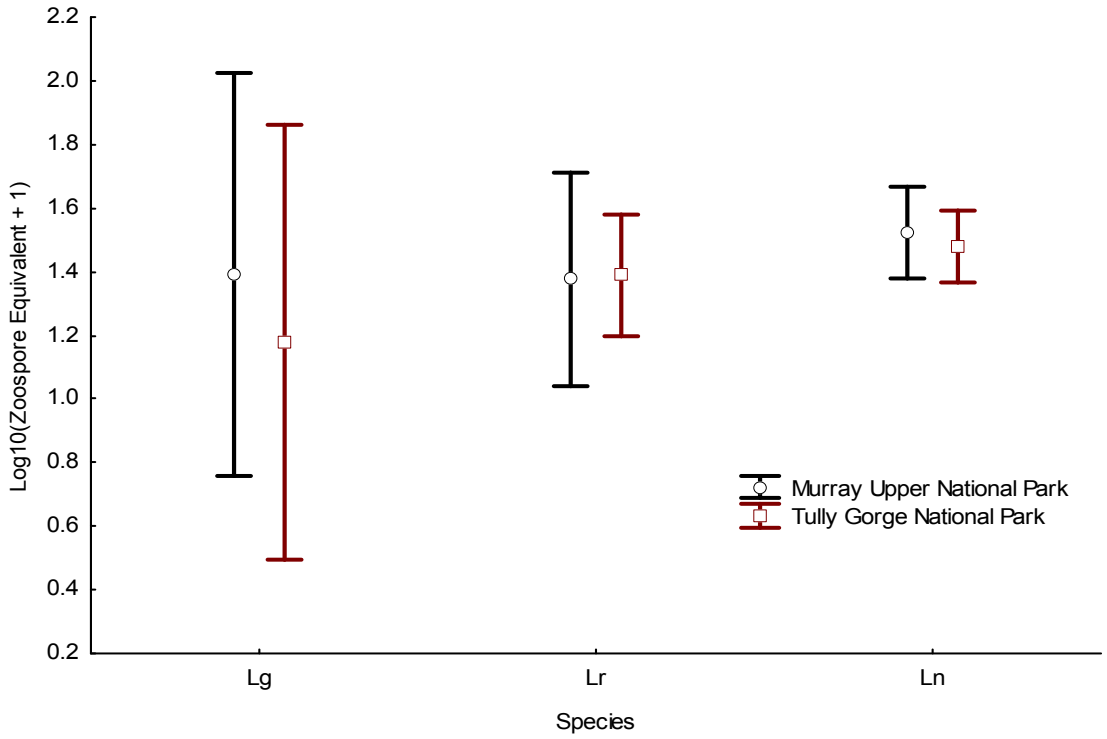


**Figure 71. Intensity of infection by species and site. Feb. 2006 - 2007. Symbols represent the mean, whiskers represent the 95% confidence interval**

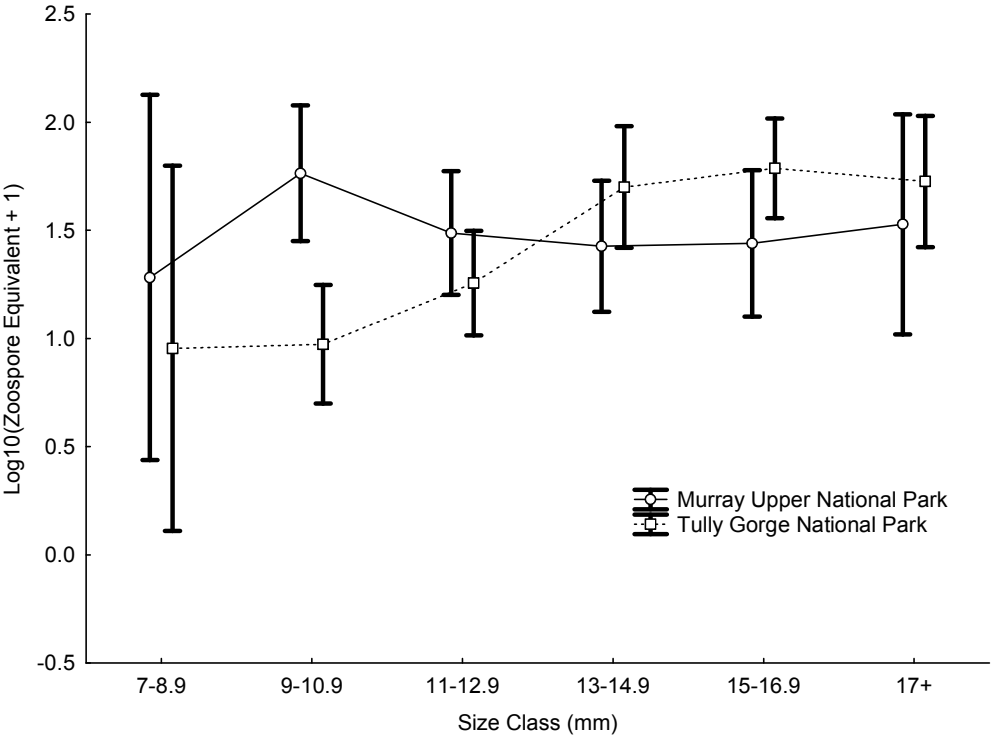


Figure 72. Intensity of infection in *Litoria nannotis* by size class and site. Symbols represent the mean, whiskers represent the 95% confidence interval. Connecting lines are a visual aid only.

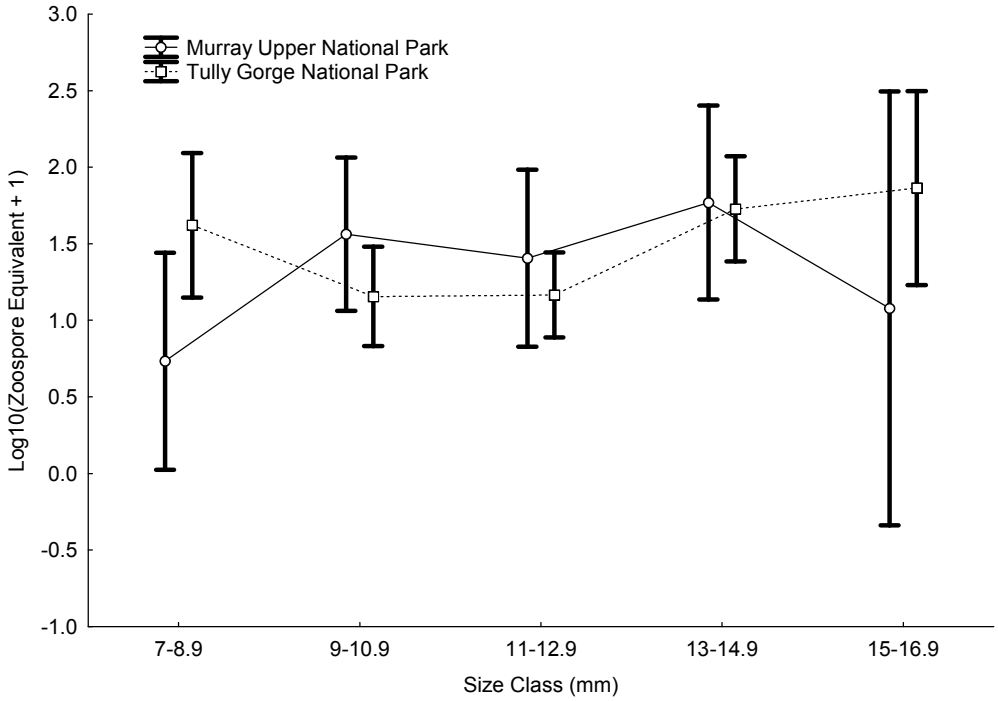


Figure 73. Intensity of infection in *Litoria rheocola* by size class and site. Symbols represent the mean, whiskers represent the 95% confidence interval. Connecting lines are a visual aid only.

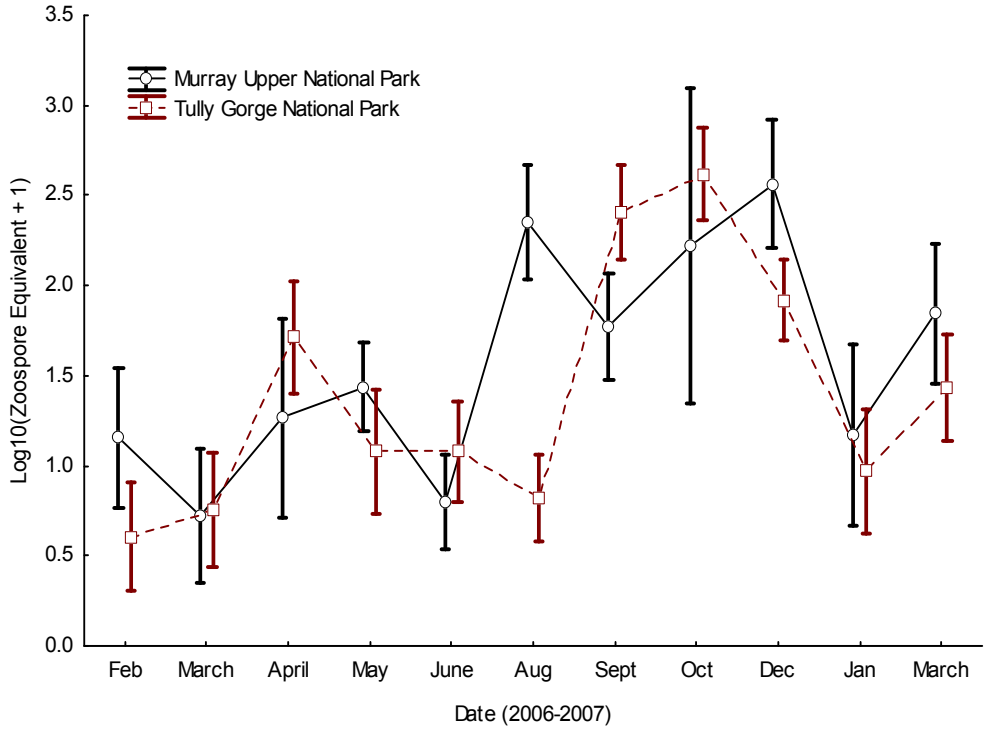


Figure 74. Intensity of infection of *Litoria nannotis* by sampling period and site. Symbols represent the mean, whiskers represent the 95% confidence interval. Connecting lines are a visual aid only.

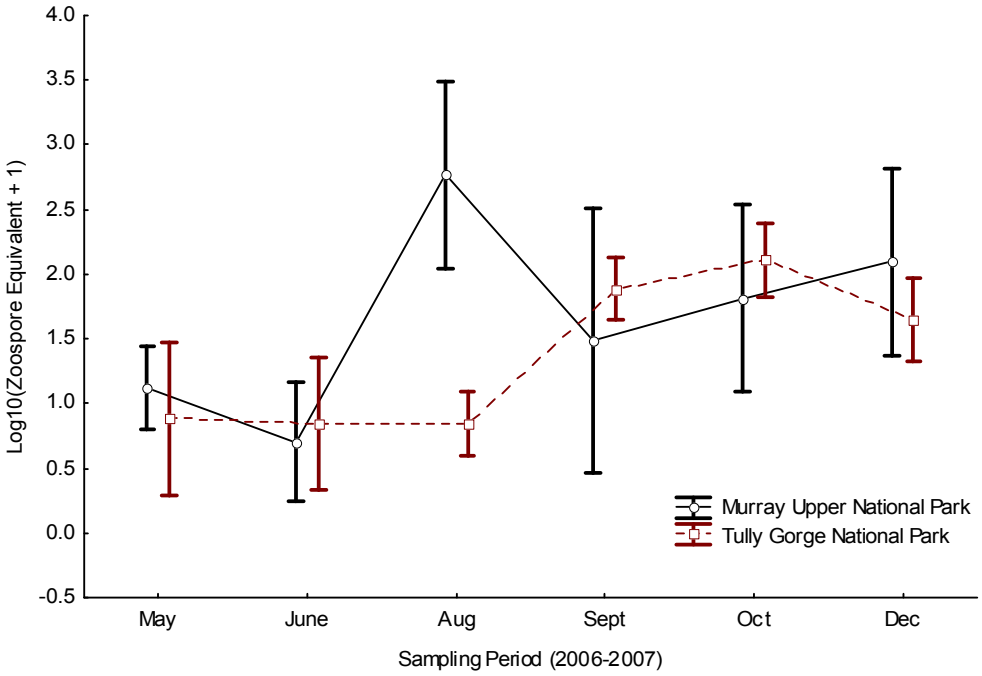


Figure 75. Intensity of infection of *Litoria rheocola* by sampling period and site. Symbols represent the mean, whiskers represent the 95% confidence interval. Connecting lines are a visual aid only.

*Seasonality*

Intensity of infection was highly seasonal and differed throughout the year (ANOVA, F (33, 1632), p=0.0000; Figure 76). Peak intensity occurred in spring of 2006 and winter of 2007. The mean intensity of infection in *L. rheocola* was strongly positively correlated with infection intensity of *L. nannotis* by sampling period (Time series cross-correlation r=.865; Figure 76) indicating that factors that determine a large proportion of the variance in infection intensity equally affect tadpoles of both species, with species differences only resulting in a consistently higher mean intensity in *L. nannotis*.

*Prevalence and Intensity*

Although *Litoria rheocola* and *L. nannotis* are strongly correlated, *L. rheocola* was excluded from the following analysis because *L. rheocola* were not captured during all sampling periods and could therefore skew mean intensity. Intensity of infection in infected *L. nannotis* was significantly positively correlated with mean prevalence over the two year sampling period (Time series cross-correlation r=.713; Figure 77). The strength of the correlation, however, decreases after the onset of mouthpart loss. Between the beginning of February (when prevalence and intensity are lowest) and the first indication of mouthpart loss, prevalence and intensity increase in near perfect synchrony (Time series cross-correlation r=.991). From the first sign of mouthpart loss until the following February, when prevalence and intensity bottom out, the correlation between the two is far weaker (Time series cross correlation r=.471). Following the first indication of mouthpart loss, intensity suddenly decreases. However, prevalence continues to increase, although at an apparently slower rate than before. When mouthpart loss is most severe, intensity bottoms out. Mouthparts then begin to recover.

As they do, intensity increases until reaching a mean maximum of 630 ($10^{2.8}$) zoospore equivalents. Even as intensity increases above or near pre-mouthpart loss levels, tadpoles maintain their full complement of teeth. Within 4-8 weeks tadpoles begin to show signs of metamorphosis and new recruits enter the population. Mean intensity and prevalence begins to decrease.

Standard Multiple Regression Model (Intensity of Infection)

The significant contributors to the multiple regression model of *L. nannotis* infection intensity were, in descending importance, the preceding 28 day mean rainfall, bodylength, site and solar radiation (Table 20). The whole model explained 20.1% of total variance ($F_{4, 801}$, $p<0.0005$).

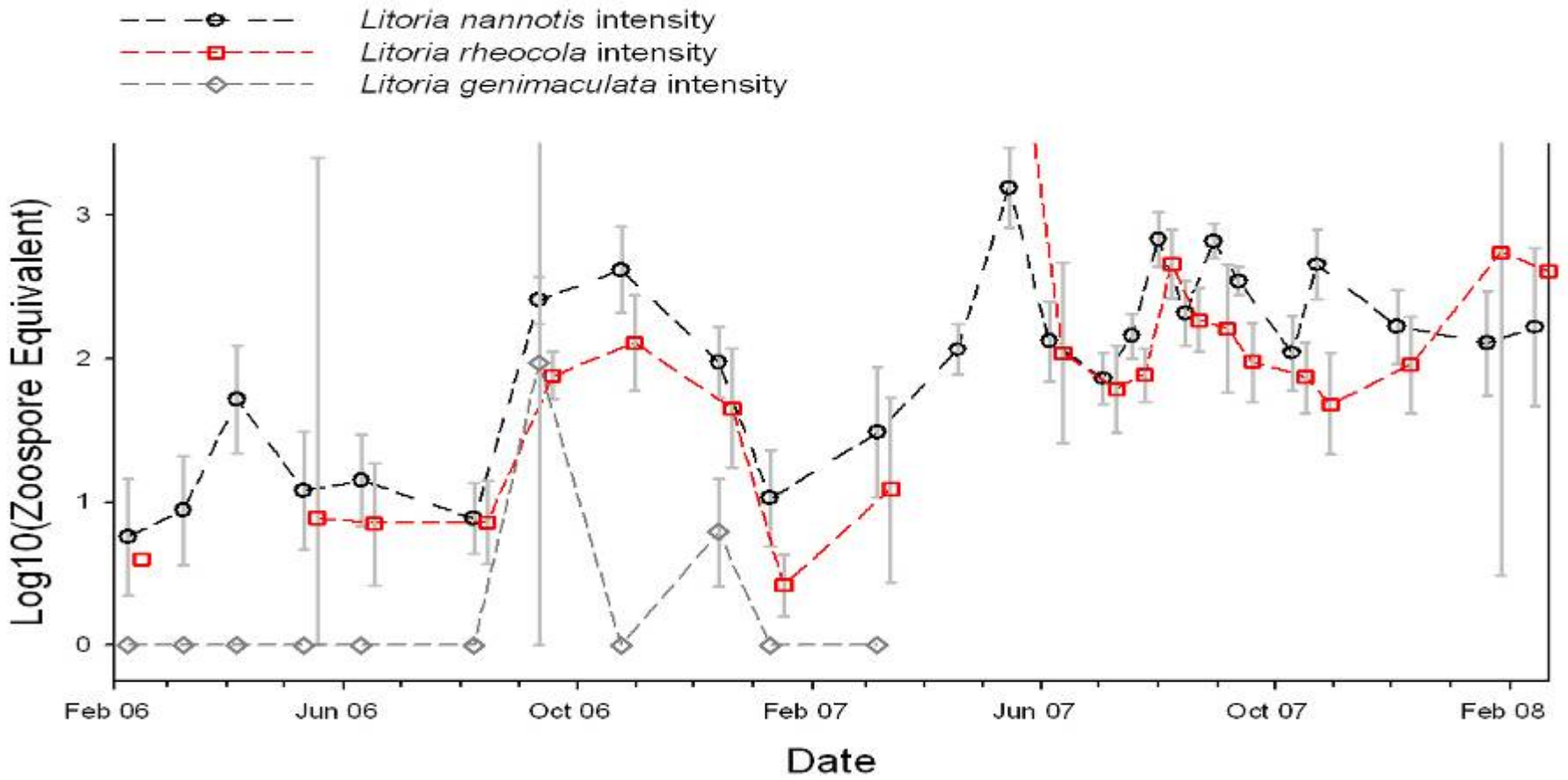


Figure 76. Intensity in infected *Litoria nannotis, L. rheocola* and *L. genimaculata* over time at Tully Gorge National Park (2006-2008). Values of 0 were added to indicate tadpoles were captured but were uninfected. Missing values indicate tadpoles were not found.

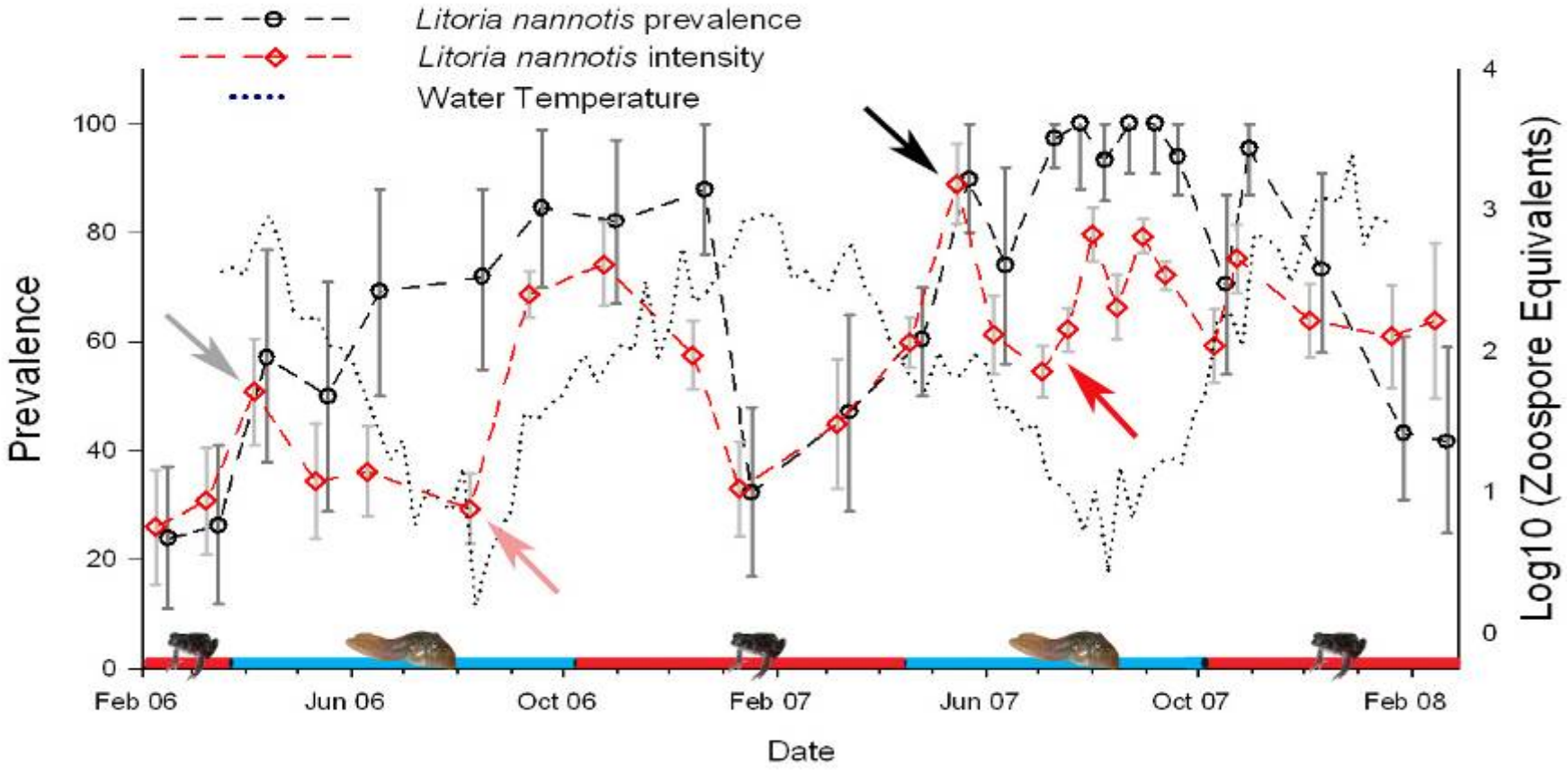


Figure 77. Prevalence and intensity of infection over time in *Litoria nannotis* at Tully Gorge National Park (2006-2008). Blue dots represent mean weekly water temperature and are provided as an indication of season. The black arrow indicates the observed start of mouthpart loss. The red arrow indicates the observed peak of mouthpart loss. The lighter colour arrows indicate the estimated timing of the mouthpart loss events based on field notes and intensity data. The red bar illustrates the period of growth and the blue bar illustrates the period of metamorphosis of large tadpoles and recruitment of small tadpoles.

**Table 21.** Standard multiple regression model of intensity of infection in *L. nannotis* tadpoles

| *Variable* | *Unstandardized Coefficient* | | *Standardized Coefficient* | *Sig.* | $R^2$ | *SE* | *df 1* | *df 2* |
|---|---|---|---|---|---|---|---|---|
| | *B* | *SE* | *B* | | | | | |
| Rainfall (28 day mean) | -0.831 | 0.083 | -0.319 | 0.000 | | | | |
| Body Length | 0.052 | 0.010 | 0.161 | 0.000 | | | | |
| Site | 0.384 | 0.080 | 0.156 | 0.000 | | | | |
| Solar Radiation (28 day mean) | 0.028 | 0.009 | 0.102 | 0.002 | | | | |
| Intercept | 1.030 | 0.226 | | | | | | |
| ***Model Summary*** | | | | | 0.201 | 0.827 | 4 | 801 |

Effects of Density
Transmission

For the first year, the tadpole sampling design allowed the total number of tadpoles captured to be used as an estimate of host abundance. If transmission was strongly density dependent, then prevalence of infection should increase more rapidly with host abundance. This analysis was restricted to the prevalence of Bd within *L. nannotis* within the medium size group from Tully National Park as the dependent variable. The two sites could not be combined because host abundance differed between sites and the medium size-group demographic was selected because the majority of new infections occurred within this group (Fig 67). Host density data was inclusive of torrent-adapted tadpoles from all size groups. There was no evidence of a correlation between prevalence in medium sized *L. nannotis* and torrent tadpole abundance (Spearmans rho, $r_{11} = .282$, $p = .40$) nor a relationship with rate of increase in prevalence and abundance. Densities of tadpoles of the individual species were tested as well, however, none were significant.

Intensity

If intensity within an individual increases due to self re-infection then intensity levels should vary primarily due to individual differences such as host immunity or duration of infection. On the other extreme, if intensity increases primarily due to external re-infection (ie. from other infected hosts) then intensity levels should vary in response to the intensity of the infection of infected tadpoles (assumes that higher intensities will release more zoospores) and the density of infected tadpoles (which combined with intensity is a measure of the density of Bd within the stream and therefore the force of infection). The timed sampling method employed between 2006 - 2007 allows the number of tadpoles collected to be used as an estimate of population density. To control for the effect that size has on intensity of infection (Figure 67), only infected *L. nannotis*

tadpoles within the large size-group (>= 15 mm) were considered. The overall density of torrent tadpoles (*L. nannotis, L. rheocola* and *N. dayi*) was multiplied by mean overall prevalence to estimate the relative density of infected tadpoles. A time series cross-correlation (r = .563; Figure 79) revealed that intensity of infection in large size-group *L. nannotis* was moderately positively correlated with density of infected torrent tadpoles.

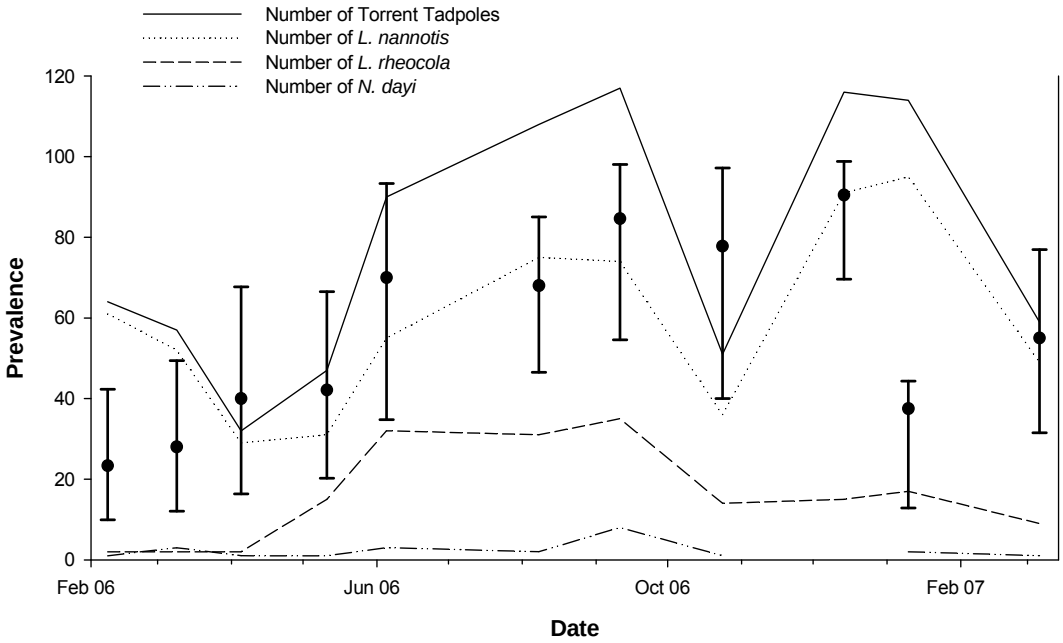


Figure 78. Prevalence of infection in medium size group *Litoria nannotis* and host density estimates. Tully Gorge National Park, 2006-2007. Squares represent mean prevalence and whiskers are the 95% confidence intervals. Lines indicate host density patterns.

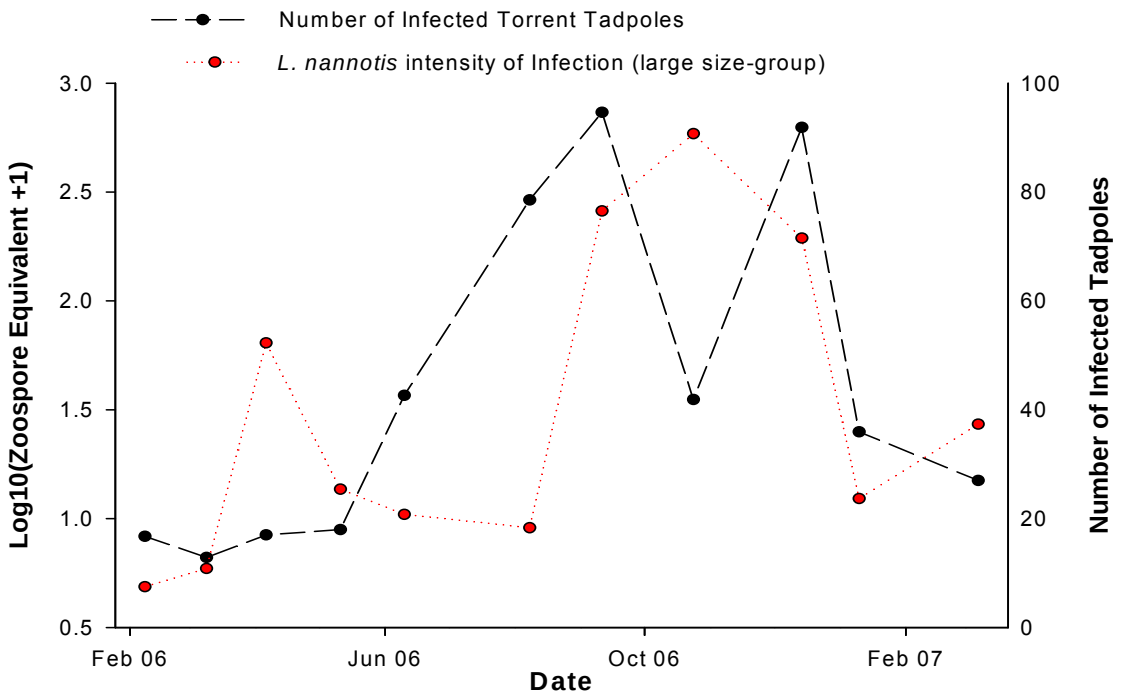


Figure 79. Intensity of large size-group *Litoria nannotis* tadpoles and relative density of infected torrent adapted tadpoles, Tully Gorge National Park (2006-2007).

Discussion

I found that risk of infection varies greatly between tadpoles of different species sharing a single stream habitat. Tadpoles that are adapted to fast flowing water were far more likely to become infected than tadpoles that inhabit pools. The variables; species, body size, temperature and water velocity were able to accurately identify the infection status of 85.8% of tadpoles. The determinants of infection intensity are more complex and appear to include a combination of species, rainfall, body size, density of infected hosts and mouthpart loss. Interestingly, *L. nannotis* tadpoles show evidence of acquired immunity and/or tolerance to infection following a period of severe mouthpart loss. This resistance and/or tolerance allows tadpoles to regrow mouthparts, feed and acquire nutrients and metamorphose. Resistance developed in the larval stage could contribute to resistance in adults following metamorphosis (Rollins-Smith 1998).

Tadpoles within Tully Gorge National Park and Murray Upper NP had very similar prevalence and intensity patterns over time and by size class indicating that the patterns discussed here are not specific to one site but are likely to have broad applicability throughout the Wet Tropics.

Transmission

Torrent adapted tadpoles had significantly higher prevalence and intensity of infection than pool adapted tadpoles and *N. dayi* had lower prevalence than either *L. nannotis* or *L. rheocola* (Table 18). The differences in prevalence within these stream communities are

best explained by (model parameters in parentheses) an interaction of host behavior (species), flow rate (flow rate x species) and exposure time (size class).
Host behavior (species)

*Litoria xanthomera* develop in small stream-side rock depressions that form when high water levels recede following heavy rain (isolated pools). For transmission in isolated pools to occur, *Bd* would have to either be in the water when the pool formed or be introduced after the pool formed, possibly by a visiting infected frog. Only 2 *L. xanthomera* were found infected suggesting either these tadpoles are resistant to infection or *Bd* is not commonly present in this habitat.

Prevalence and intensity of infection in *L. genimaculata* did not differ significantly from those measured in *L. xanthomera*. *Litoria genimaculata* occupy gently flowing pools that are connected to the main stream (connected pools). Occasionally extended rainless periods cause some pools to become temporarily isolated. Water temperature did not significantly differ from the main stream during this time (Cashins, unpublished data). Larger *L. genimaculata* tadpoles had a higher prevalence but not intensity of infection, indicating that risk of infection increases with exposure time. This is consistent with results from other studies (Smith et al. 2007; Symonds et al. 2007). Experimental work using field enclosures reveals single *L. genimaculata* tadpoles infrequently acquire infection from stream water alone, but transmission rate increases dramatically when sharing an enclosure with an infected individual (S. Cashins, unpublished data). This indicates the low prevalence found in the field is likely due to infrequent environmental transmission, but predicts prevalence in the wild may increase if host density was high or if the larval stage were longer, increasing exposure time.

Torrent adapted tadpoles have significantly higher prevalence and intensity of infection than both *L. xanthomera* and *L.* genimaculata. Torrent tadpoles adhere to rocks in fast flowing water almost continuously. They acquire infection at a small size in the wild and in field enclosures, transmission occurs quickly (within 2 weeks) in *L. nannotis* kept alone (Cashins, unpublished data) indicating transmission is widespread and occurs readily via the environment. The significantly higher prevalence and apparently higher transmission rate in torrent adapted tadpoles indicates their behavior may bring them into closer contact with *Bd* and/or zoospores may be more abundant in torrents.

In torrent tadpoles there are two primary avenues for *Bd* zoospores to enter the oral disc; 1) zoospores may be drawn in through the spiracle from the water column and then into the oral cavity or 2) zoospores on rock surfaces are extracted by the tooth rows and jaw sheath during grazing.

Zoospores entering through the spiracle from the water column is unlikely. In the North American torrent tadpole *Ascaphus truei*, filtration of respiratory currents was shown to be inconsequential for collecting particles from the environment. Instead, food is collected and ingested almost entirely by the scraping of substrate surfaces. (Altig and Brodie Jr 1972). Observation of feeding behavior in the field and lab indicate torrent tadpoles of Australia are no different and data presented here indicate that contact with

zoospores on the stream substrate in torrents is the primary avenue of food acquisition and disease transmission. These results support recent observations that adult frogs in frequent contact with moist rock surfaces either underwater or in splash zones may be more at risk of *Bd* transmission and decline (Rowley and Alford 2007; Ryan et al. 2008)

All submerged surfaces within a stream have a thin layer (up to 5 mm) of reduced water velocity (0-90% of main stream flow) called a boundary layer (Allan and Castillo 2007). Within this layer forms a complex biofilm consisting of algae, protozoa, bacteria, fungi and invertebrates. Turbulent water delivers nutrients and microscopic organisms to this benthic community but retains particulate matter in suspension (Sigee 2005). However, increasing water velocity decreases the size of the boundary layer causing settlement rates to decrease as a result of either a reduction in settlement or an increase in resuspension (Stevenson et al. 1996). Like any microorganism, *Bd* zoospores are expected to settle within the boundary layer on rocks in torrents, and, the settlement rate is predicted to decrease with increasing velocity. The data supports these predictions, however controlled experiments are needed. The odds ratio of infection risk in torrent tadpoles (Table 19) is inversely related to the velocity of their microhabitat. *Litoria rheocola* occupy slower velocity currents and have the highest risk of infection and *N. dayi* occupy the fastest currents and have lowest risk of infection. Further, within species, infection risk was higher in torrent tadpoles captured in slower flowing water (controlled for temperature).Infection intensities were also higher following drier months when flow rates were low (Table 20 and 21).

*Bd* can survive by growing and reproducing for up to 7 weeks in sterile pond water (Johnson and Speare 2003). Therefore, zoospores settling out of the current and within the boundary layer could survive and potentially develop into zoosporangia to release additional zoospores for an extended period of time assuming they are not out competed by other microorganisms. Models predict increased zoospore survival will increase transmission rates and risk of species extinction (Mitchell et al. 2008). Determining the duration of zoospore viability and whether growth and reproduction occurs in different stream microhabitats is essential for future modeling. I successfully detected low levels of *Bd* directly on rocks within torrents using filtration and PCR, however this technique needs improvement for more detailed analyses. Analogous to the properties of the community of microflora on amphibian skin (Harris et al. 2006), the composition of microorganisms within biofilm can differ between sites, seasons and even rock faces (Lear et al. 2008). Bacteria within biolfilms have been shown to both enhance (Joint et al. 2000) and inhibit (Holmstrom et al. 1996) the settlement rate of algal spores and could have a significant impact on settlement and survival time of *Bd* within streams. Other factors that are known to affect zoospore settlement include negative phototaxis, chemotaxis, surface chemistry and topography (Patel et al. 2003).

If increased velocity is predicted to decrease zoospore settlement rate, then larger numbers of zoospores are expected to gather in slow flowing pools. However, the accumulation of particulates, leaf litter, detritus and other microorganisms may lead to increased competition and decreased zoospore survival time. In vitro, *Bd* is often overrun and killed by bacteria isolated from the field (James 2007; Woodhams et al. 2007). I

found zoospores survived longer (based on observed motility) in increasingly dilute water samples containing stream collected detritus (Cashins, unpublished data). Therefore, despite an increased rate of settlement, *Bd* survival time may be lower in pools and could help explain why *L. genimaculata* have a lower prevalence of infection.

Seasonality

Prevalence

*Batrachochytrium dendrobatidis* is the only Chytridiomycete fungus known to infect a vertebrate (Berger et al. 1998). Many other Chytridiomycetes (chytrids) are parasites of plankton, and are often host specific (Bruning et al. 1992; Canter and Jaworski 1982; Holfeld 1998). Epidemics in these chytrids have been partly explained by factors including light, temperature, nutrients, pH, turbulence and grazing by zooplankton (Kagami et al. 2007). In some cases, changes in these environmental factors cause shifts in plankton dominance patterns resulting in periodic, or even seasonal, bloom events (Alster and Zohary 2007). The plankton bloom is soon followed by a parasitic chytrid bloom. Prevalence of infection can be over 90% (Ibelings et al. 2004). Both host and parasite go bust, typically within weeks, following host death, and the cycle is repeated when host densities are again high enough (Ibelings et al. 2004).

Similar to other chytrids, the prevalence of *Bd* in torrent tadpoles is very seasonal and achieves a high prevalence of infection before decreasing. However, there was no evidence of a *Bd* bloom similar to that seen in chytrid parasites of plankton. In *Bd*, prevalence increases steadily, then quickly decreases. The process occurs over the course of a year, instead of a few weeks. This is a result of the very different amphibian host life cycle and the response of larvae to infection. Seasonality in prevalence was driven primarily by tadpole population dynamics; namely hatching, development and metamorphosis, with a relatively small, but significant influence from higher water temperatures that appears to reduce the rate of new infections and may even clear a small percentage of existing infections in large size-group tadpoles. Interestingly, the highest 7-day mean maximum water temperature over the two year study was 24.07 C, which is within the optimum temperature range for *Bd* growth in culture (Piotrowski et al. 2004). This indicates that increased temperature is likely to be having an indirect effect on zoospore survival, perhaps via microbial competition or increased host defenses. Infection did not appear to cause high tadpole mortality and water temperatures were not warm enough for a widespread clearing of infection. Consequently a *Bd* "bust" in prevalence only occurred when tadpoles left the population via metamorphosis.

Importantly, tadpole infection dynamics operate independently of adult infection dynamics. This indicates that adults may be minor contributors to within-stream *Bd*, at levels or in locations that are infectious to tadpoles. It is not clear if the reverse is true and zoospores produced by tadpoles are a minor source of infection for adults. However, adults of these species, are found on wet rock surfaces at night and in cracks or underwater in riffles during the day (Scott Cashins, unpublished data). Therefore, they are often in direct contact with tadpole habitat.

Descriptive Prevalence Model

A pulse of egg hatching occurs during the summer of each year (Figure 67). Fewer small, recently hatched tadpoles are found outside of summer, yet infection prevalence in this size group remains low throughout the year indicating they acquire infections after hatching. Following summer, tadpoles grow over the cooler, drier winter. No metamorphosis takes place during this time, and fewer eggs hatch. As the existing larvae graze rock surfaces in search of food, the cumulative risk of infection appears to increase steadily, even as water temperatures reach their lowest point and then begin to increase again into summer (this is largely because temperatures remain within the favourable range for Bd *in vitro*). The combination of reduced immigration and emigration and the increasing cumulative risk of infection in growing tadpoles causes a seasonal increase in prevalence. As early summer approaches, the proportion of large tadpoles in the population increases until approximately 60% of all tadpoles are of adequate size to metamorphose. Prevalence in these large tadpoles is at or near 100%. Individuals soon begin to show signs of metamorphosis such as the formation of rear limb buds and the emergence of hind limbs. As these large infected tadpoles leave the population via metamorphosis, an influx of new, uninfected tadpoles hatches from eggs. This combination of small uninfected tadpoles entering and large infected tadpoles leaving causes a seasonal drop in overall prevalence.

Interestingly, the prevalence in large size group tadpoles tends to decrease below 90% around February of each year and then quickly increase again. This may be due to infected tadpoles metamorphosing sooner to escape the stream environment, leaving behind a higher percentage of uninfected tadpoles to continue increasing body mass before emergence. Alternatively, this may indicate some infected tadpoles have cleared *Bd* infection. The intensity of infection is at its lowest around February (Figure 74) indicating a population wide decrease in pathogen load that may allow some individuals to shed infection completely. This coincides with maximum temperatures that can assist the host to fight Bd infection as has been demonstrated in adults (Woodhams et al 2003; Berger et al 2004).

Intensity

Variation in intensity of infection and density of hosts will indicate when *Bd* zoospore numbers will be high and transmission is likely to be maximal. Understanding the causes behind increased intensity may lead to direct management strategies to lower intensity within the stream in an attempt to reduce transmission within tadpoles and adults during periods of high risk. This form of management may prove useful for the conservation of critically endangered species at select sites and during the reintroduction of captive bred populations (Australian Government 2006; Gascon et al. 2007)

Four variables in a multiple regression model explained 20.1% of variation in *Bd* intensity in *L. nannotis*; 1) 28-day mean rainfall was inversely related to intensity. 2) body length, and 3) 28-day mean solar radiation were positively related to intensity. 4) Site: Tadpoles from Tully National Park tended to have higher intensity.

Decreased rainfall is associated with slower water flows which may increase settlement of zoospores and the rate of external reinfection within torrents (see discussion above). Similarly, water velocity was found to be an important predictor of infection status (Table 20 and discussion above).

Larger body lengths indicate both a larger mouthpart surface area and a longer exposure time that plausibly leads to increased infection intensity (swabbing does not adjust for mouthpart size). *L. rheocola* tadpoles are smaller than *L. nannotis* tadpoles. This size difference may explain why intensity in *L. rheocola*, although strongly correlated was consistently lower than *L. nannotis.* However, intensity did not differ with body size in tadpoles other than *L. nannotis*.

It is not clear why increased solar radiation may be associated with increased intensity. Among many possibilities, solar radiation may affect tadpole behavior, biofilm composition or survival time of zoospores. Alternatively the association may be a type 1 error or confounded with an unknown determinant.

In theory, individual infection intensity will be determined by the difference between the rate of re-infection (self-reinfection or external-reinfection) and the rate at which zoospores are shed. Tadpoles that shed a large percentage of their zoospores will reduce self re-infection and maintain an overall low intensity in the absence of external re-infection. In tadpoles with a high rate of self re-infection, changes in the density of infected tadpoles should have minimal effect on intensity which will increase quickly to very high levels in the absence of host defenses.

Hosts can theoretically display both resistance (the ability to limit parasite burden) and tolerance (the ability to limit the severity of disease caused by a burden) to pathogens (Raberg et al. 2007). These two defenses are believed to be independent but not mutually exclusive and may be based on immune systems, changes to cell surfaces to prevent infection, changes to behavior or changes in life-history strategies (Restif and Koella 2004). Most empirical evidence of tradeoffs between resistance and tolerance is from plants. In plants, the relative contribution of tolerance and prevalence within a species may change with environmental conditions (ref) and, according to theory, the relative contribution of each may evolve with the parasite. For example, hosts exposed to parasites with a high transmission rate and low virulence may favor evolution of tolerance as a "biological weapon" against competitors. Increased tolerance may, in turn, allow increased virulence of the parasite (Restif and Koella 2003).

I consider a theoretical framework of factors affecting intensity, including the significant variables from the multiple regression model, the theoretical relationship between re-infection vs. shedding, the possible presence of host defenses and the observed variation in *L. nannotis* prevalence to interpret causes affecting intensity of *Bd* in *L. nannotis* tadpoles over time.

Starting in February when the number of susceptible hosts was high, there was an initial rapid epidemic growth of prevalence and intensity that increased in step with the other, indicating a period of unimpeded pathogen replication and transmission. This exponential growth phase, however, appears disrupted by the onset of mouthpart loss. As mouthpart condition worsens, intensity declines. However, prevalence continues to increase at an apparently slower rate, up to 100% (in 2007). Mouthpart loss reaches a peak, after which the oral structures recover and intensity begins to rapidly increase again. Mouthparts, however, continue to fully recover despite the increasing intensity. This recovery of tooth functionality while infected strongly indicates a tolerance to infection has developed. Intensity also appears to now be moderated by something apart from mouthpart loss. Intensity levels off at an approximate peak intensity of 630 ($10^{2.8}$) zoospore equivalents despite apparently abundant available substrate (keratinized mouthparts). This suggests tadpoles may be more resistant to infection than previously in the epidemic cycle.

If you consider: 1) torrent tadpole survival depends on functional teeth to extract nutrients from rock surfaces 2) infection with *Bd* causes a loss of mouthparts 3) transmission rate of *Bd* to torrent tadpoles approaches 100% and 4) individuals that die as tadpoles can not reproduce. It follows that there would be a strong selective pressure on torrent tadpoles that could cope with infection and preserve the ability to gather nutrients in order to metamorphose. In non-torrent tadpoles that can continue feeding despite a loss in mouthparts this selective pressure is predicted to be far less or non-existant.

Because tadpoles can be a significant contributor of zoospores within a water body, selection in tadpoles for tolerance and/or resistance to infection could have significant effects on both the levels and virulence of *Bd* in water bodies. For instance, high levels of larval tolerance could promote an increase in pathogen virulence as spoorangia that encyst and produce more zoospores at a faster rate would have a competitive advantage. In contrast, an increase in larval resistance could result in a decrease of zoospore numbers.

Metamorphosis is a period of immune system reorganization where one set of tadpole lymphocytes is replaced by another set of frog lymphocytes (Rollins-Smith 1998). This immunologically deficient transition period is believed to contribute to the high susceptibility of many metamorphs to infection and mortality due to *Bd*. However, studies indicate that some lymphocytes generated in the tadpole life stage persist through metamorphosis (Rollins-Smith 1998). This suggests that if tadpoles do mount an effective immune response it could help prepare them for challenges from the same pathogen as adults and metamorphs. These interactions are likely very complex however, as complications arising from infection, including premature metamorphosis could result in a significant decline of lymphocytes at metamorphosis (Rollins-Smith 1998). Regardless, the presence of tolerance and/or resistance in the tadpole stage, could have significant effects on the evolution of both the host and the pathogen. A fact that has not been considered previously.

Descriptive Intensity Model

At the beginning of the year in summer (January – February) the intensity in infected individuals is low. There has just been recruitment of uninfected tadpoles into the population and many of the infected tadpoles are recent recruits that have not been in the stream long and thus have low intensities. The water starts becoming cooler and the last of the metamorphs exit the stream, leaving the remaining tadpoles to grow and develop over winter. From here, the intensity of infection increases exponentially and in lockstep with prevalence. This exponential growth is halted around May as tadpoles begin to lose mouthparts presumably as a result of infection. It is unclear, however, why intensity progressed to a higher level before mouthpart loss began during the second year. It is possible there was a higher tolerance to infection or that something other than intensity triggers mouthpart loss. As the keratinized teeth and jaw sheath are shed following infection, infection intensity decreases likely as a direct result of shedding infected cells and also due to a decrease in suitable substrate for infection. Prevalence, however, continues to increase despite the overall decrease in intensity in already infected tadpoles, although at an apparently slower rate. Mouthpart condition and intensity of infection decline for about two months before bottoming out around July. Mouthparts then begin to regrow and infection intensity increases. Despite the increasing intensity, tadpole mouthparts remain in good condition, indicating tolerance. However, intensity does not continue on a path of exponential increase, suggesting resistance. Intensity appears to now have an upper ceiling and reaches a maximum of approximately 630 zoospore equivalents.

This pattern suggests: 1) tadpoles through the course of infection offer some measure of resistance that keeps the infection in check and/or 2) tadpoles can develop a tolerance that prevents loss of mouthparts despite elevated intensities.

Following mouthpart recovery intensity remains relatively stable before decreasing as tadpoles exit the stream upon reaching the minimum size for metamorphosis. This drop in mean intensity is caused by a combination of large infected tadpoles leaving the population and a decrease in intensity (Figure 74). This drop in intensity may result from a decrease in external reinfection. The intensity of large infected *L. nannotis* was moderately correlated with the density of infected torrent tadpoles (r=.563). This relationship suggests that external re-infection from environmental zoospores may contribute to intensity. Self re-infection may be reduced in torrent tadpoles compared to frogs because as they feed they pass water over their mouthparts, through the vent and back out into the current behind them. Therefore, zoospores exiting zoosporangia may be actively flushed away before being able to encyst. This may disperse Bd effectively within the stream, however it may reduce self re-infection.

Conclusions

Pathogens can be powerful selective forces of wildlife. Some populations that have survived *Bd* epidemics are persisting in an apparent host-pathogen equilibrium (Retallick et al. 2004). Other affected populations have rebounded to some degree accompanied by

a decrease in prevalence but have not returned to pre-decline levels (McDonald et al. 2005) suggesting that selection for increased resistance or decreased virulence is occurring. Previously it was considered that infection had no effect on larvae. However, I have shown that torrent tadpoles lose the ability to feed as a result of infection. This strong negative effect on fitness means that selection for tolerance/resistance in the larval stage is likely and the patterns of prevalence and intensity of infection and mouthpart loss support this. Greater attention to the larval phase of the biphasic amphibian life cycle is necessary to fully understand this disease.

Future Directions

The results reported here suggest a number of potentially rewarding areas of future research. Understanding the apparent tolerance and/or resistance to *Bd* in larval *L. nannotis* and whether this contributes to changing pathogen virulence or increased resistance following metamorphosis may provide insight into how some anurans recover long-term following a chytridiomycosis epizootic. Histological examination of preserved tadpole mouthparts collected before, during and after mouthpart recovery may reveal changes in pathology, such as altered infection sites or cellular responses to infection and will be important to understand this process.

Research on the interaction of *Bd* with stream microbiota will greatly further the currently very limited understanding of spatial variation in zoospore survival. In particular, biofilm composition may exert a strong influence both spatially and seasonally on *Bd* survival and transmission. Finally, experimental investigation of the contributions of self re-infection vs external re-infection to intensity of infection in tadpoles will also significantly help in understanding how *Bd* levels in the stream vary. If intensity of infection is primarily a result of repeated external re-infection then actions such as removing an appropriate number of tadpoles from the stream for captive rearing could reduce transmission rates and lower intensity of infection across the population. This reprieve from high transmission could theoretically increase the odds of survival and provide more time for adults to mount an acquired immune response. This form of active management may only be feasible in habitats with low amphibian diversity or during a reintroduction program when densities are low.

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***Project 5. Experimental studies on acquired immunity and the evolution of resistance.***

Project 5 addresses **Objective 1** by examining how prior exposure and antibody response of hosts affect their survival. It addresses **Objective 4** by determining whether frogs that have survived infection are less susceptible than naïve animals in experimental infections, looking for evidence of antibody production using Western Blotting. It addresses **Objective 6** by examining the details of the development of acquired immunity in species known to be vulnerable to chytridiomycosis. Understanding how the immune system responds is a critical step in determining how the resistance of species at risk might be increased.

This project investigates aspects of acquired and innate immunity in frogs with chytridiomycosis, excluding skin peptides. This project was modified in light of information that became available after the proposal was written, and some delay was caused by Lee Berger's maternity leave (Sep 2005-Aug 2006). Preliminary work on this project was carried out in 2005 by Lee Berger and Rebecca Webb, a technician hired using funds from Tender 42-04, in collaboration with Dr. Natkunam Ketheesan, an immunologist at JCU. They showed that Bd does stimulate immune activity in vitro, using homogenised Bd in lymphocyte proliferation tests. The reason for lack of inflammation in tissues is therefore not due to suppression by Bd; this suggests that we may find effects of prior exposure on host response to infection. We advertised for a Ph.D. student in early 2005 but had relatively few responses, probably because of the specialised nature of work on amphibian immunology.

A Ph.D. student, Sam Young, attached to Richard Speare's group, is carrying out work on acquired immunity. Sam Young's PhD project is largely funded by a separate grant from DEH (Emerging amphibian diseases and disease surveillance). Her work is now collecting a large amount of data on amphibian immunity that is synergistic with Project 5, and is progressing very well. Sam is currently collecting base-line immunology data for healthy *L. caerulea* and *L. infrafrenata.* Methods for evaluating the immune function in frogs have been established. Sam has collected dry season blood samples from *L. caerulea* for white blood cell counts.

We completed pilot work on the cellular immune response in infected frogs. Using blood samples obtained from the *L. caerulea* used experimentally for project 7, total white cell counts, red cell counts and manual differential cell counts were done on infected and uninfected frogs. Red cell counts remained stable but white cells decreased. Frogs in terminal stages of chytridiomycosis had a severe reduction in lymphocytes. However, lymphocytes also decreased in the uninfected and mildly infected frogs. There was an increase in globulin levels in severely infected frogs that may indicate production of inflammatory proteins.

***Project 6. Determining the proximate causes of morbidity and mortality due to chytridiomycosis.***

Project 6 addresses **Objective 1** by determining the actual means by which chytridiomycosis produces morbidity and mortality in hosts. It directly addresses **Objective 3** by using experimental infections and monitoring of tissue enzymes, electrolytes, heart function, blood oxygen and ultrastructural changes to determine cause of death.

An initial study was completed in 2005 on the biochemical changes that occur in ill frogs with chytridiomycosis. For this pilot study we used green tree frogs from an outbreak that occurred after frogs became infected during transport to Townsville. Blood was collected from 12 anaesthetized frogs within 3 weeks of arrival, when there were no clinical signs. Haematology and biochemistry, on an in- house blood gas machine, were performed. Frogs were tested for Bd by PCR every 10 days. When 7 frogs showed clinical signs and heavy infections with Bd between 53 and 72 days after arrival, a larger blood sample was collected before euthanasia for these tests as well as for shipping frozen to a veterinary laboratory in Brisbane for more comprehensive testing of tissue enzymes. Two uninfected frogs and three infected frogs that remained healthy during this time were also bled before euthanasia at 73 and 74 days. Our study showed that a large decrease in electrolytes occurred in frogs in terminal stages of disease. This result was the same as obtained by Jamie Voyles with 2 American toad species during her masters project in Colorado. Fortunately she commenced her PhD with us in March 2006 and continued her work on pathogenesis. Her work has showed that chytridiomycosis leads to osmotic imbalance and eventually death due to heart failure. It has been publisned in "Electrolyte depletion and osmotic imbalance in amphibians with chytridiomycosis" (Voyles et al. 2007, Project 6.4 below), which is the first study in the world to definitively demonstrate that in the late stages of chytridiomycosis, electrolyte balances are sufficiently perturbed to lead to mortality, and in "Pathogenesis of chytridiomycosis, a cause of catastrophic amphibian declines" (Voyles et a.l 2009, Science 326:582-585, and Project 6.5 below).

***Project 6.1 Review of virulence and pathogenesis as related to amphibian chytridiomycosis***.

Chapter 2 in Voyles, J. 2009. Virulence and pathogenesis of chytridiomycosis: A lethal disease of amphibians. Ph.D. thesis, James Cook University

Jamie Voyles.

Part I. The concept of virulence
Introduction

The word virulence is derived from the Latin word "virulentus", meaning "full of poison" (Glare 1982). A general search for a contemporary explanation of the term virulence provides the following definitions:
1. the relative ability of a microbe to cause disease (Stedman 2000)
2. the degree of pathogenicity of a given pathogen (Vaughan & Morrow 1989)
3. the degree to which a disease-causing organism can affect the organism it attacks (Science 1997).
In various academic fields the term means different things. Microbiologists often refer to virulence as a composite product of two features: the ability to colonize a host (infectivity) and the severity of the disease produced (Read & Harvey 1994). Epidemiological modellers have quantified virulence as the mathematical rate of parasite-induced mortality, represented in mathematical disease models by the Greek small letter for alpha (Anderson & May 1978, May & Anderson 1978, Anderson & May 1979, May & Anderson 1979). Ecologists often focus on parasite-mediated effects on host fitness (including morbidity and the ability to reproduce), rather than specifically on mortality. Because many of the definitions of virulence appear to be focused on attributes of the pathogen, they could lead to the assumption that virulence is strictly a microbial property. However, this does not take into account the fact that disease development is also highly dependent on the host (Casadevall & Pirofski 1999, 2001). That is, disease is as much a consequence of host susceptibility or resistance (Read & Harvey 1994) as it is of the characteristics of the pathogen. A microbe that is highly virulent in an immunocompromised host may not cause disease in a healthy host. For example, the ubiquitous bacterium *Aeromonas hydrophila* causes disease in immunocompromised hosts (Wolff et al. 1980, Cailleaux et al. 1993, Simmaco et al. 1998). Therefore, virulence is best considered a dynamic variable that is dependent on host-microbe interactions and the effects of the environment on these interactions (Read & Harvey 1994, Levin 1996, Casadevall & Pirofski 1999, 2001).

The importance of clarifying the definition and concept of virulence is not merely academic. Understanding that virulence is a property of the host-pathogen system, rather than simply of the pathogen alone, strongly changes the focus of research aimed at answering questions such as: Why are some pathogens more virulent than others? Why do some diseases have greater impacts in certain geographical regions? Under what circumstances might we be able to intervene and diminish the impacts of disease on human and animal populations? The answers to such questions are important for a wide

range of basic and applied biological issues (Levin 1996, Dybdahl & Storfer 2003). Resolving the evolutionary principles driving disease should provide insight into its role in generating biological diversity (Bull 1994, Dybdahl & Storfer 2003). It is also critical information in a variety of fields including medicine, agriculture and conservation management (Read & Harvey 1994, Ebert & Bull 2003).

**A historical view of virulence- the conventional wisdom**

It was historically believed that pathogens should evolve towards benignness or avirulence (in the sense of reduced pathogenicity, not reduced transmission) in any host species (Ewald 1983, Bull 1994, Nowak & May 1994, Ewald 1994). The logic was simply that damage to the host would be harmful to the pathogen: if the host dies, the pathogen dies too. This theory assumes pathogens that minimize their pathogenic effects will maximize their transmission rates by maintaining mobile, living hosts that provide opportunities for transmission. This idea has become so ingrained in medicine and the study of parasitology that it is referred to as the "conventional wisdom" and is sometimes used as the sole basis for hypotheses about host-pathogen biology today (Bull 1994, Levin 1996, Galvani 2003, Ewald 1994).

While the characteristics of some diseases are consistent with this hypothesis, many others are not, and there are likely many cases in which its assumptions are incorrect (Anderson & May 1978, 1979, May & Anderson 1978, 1979, Levin 1996, Ewald 1994). There is empirical evidence indicating evolution toward avirulent commensalisms is not universal. For example, some very old parasites are highly virulent. Herre (1993) suggested that Panamanian nematodes, which have an ancient association with fig wasps, have evolved to be more virulent based on the population structures, and the corresponding transmission dynamics, of the host fig wasp species. By some estimates malaria has been plaguing hominids since the evolutionary split of humans and chimpanzees (Pennisi 2001). Yet today, some 8 million years later, malaria persists in multiple strains and with variable degrees of virulence, which, in some cases, are highly lethal (Snow et al. 2005). Furthermore, some diseases caused by one parasite may differ in severity based on geographical rather than temporal parameters. The parasite *Helicobactyer pylori* isolated from human populations in different geographic areas are genetically indistinguishable, but the severity of gastric disease caused by the parasite appears to vary by region (Chattopadhyay et al. 2002).

Proponents of conventional wisdom have defended its inconsistencies (Levin 1996). They have argued that perhaps 8 million years isn't long enough for the malarial pathogen to evolve toward benignness or that geographical variation in virulence may be the result of confounding variables (Levin 1996). Such defences of the conventional wisdom worked for a long time and few individuals took issue with the many contradictions. Paul Ewald (1994) suggested that in the absence of alternative hypotheses, the conventional wisdom was widely accepted because it was "too appealing to be rejected". However, evolutionary biologists have largely moved away from historical views of virulence.

**An alternative model of virulence- the trade-off hypothesis**

The field of evolutionary epidemiology applies the principles of evolution and natural selection to host-pathogen systems (Galvani 2003, Ewald 1994). This synthesis occurred only in the later part of the 20th century despite its importance for human health (Levin 1996, Ewald 1994, Stearns & Koella 2007). Roy Anderson and Robert May (1978, 1979) laid the groundwork for mathematical and theoretical modelling and thus spurred the formation of evolutionary epidemiology. With attention on the potential applications for human interests (Bull 1994, Ebert & Bull 2003, Stearns & Koella 2007), the study of the evolution of virulence has advanced from a neglected topic to theoretical discussions to modelling of disease systems (Galvani 2003). Consequently, multiple alternative models of virulence have been generated and discussed as evolutionary biologists consider and test the evolutionary underpinnings of disease (Bull 1994).

A key advance in the study of virulence was the careful consideration of the relationship between parasite-mediated mortality and between-host transmission. Simply stated, the "trade-off" model (sometimes called the optimal virulence hypothesis) recognizes a necessary balance between virulence and transmission (Anderson & May 1982, Ewald 2004). This hypothesis implies that natural selection can maintain virulence over time. The optimal level of virulence will depend on the ecology of the host-parasite interaction and, more specifically, factors such as host density, host and parasite life spans and mode of transmission among others (Levin 1996). This model involves explicit assumptions: host mortality (virulence) is directly related to parasite reproduction and also, higher concentrations of the parasite increase the probability of transmission (Bull 1994, Ebert & Bull 2003).

A commonly cited example is the myxoma virus, which infects the European rabbit (*Oryctolagus cuniculus*) (Fenner & Ratcliffe 1965). A highly virulent parasite, the myxoma virus initially caused high mortality rates (~99%) in rabbit populations in Australia. In subsequent experimental infections in the laboratory, the recovered virus (from wild, surviving rabbits) had attenuated and did not cause comparable levels of mortality (down to ~70%). The results of this study were taken as evidence in support of an extricable link between transmission and virulence (Fenner 1959, Anderson & May 1982) and offered the promising prospective of virulence management (Ebert & Sokolova 2001, Ebert & Bull 2003). For example, Ewald (1994) discusses the importance of managing transmission rates for the virulence of human immunodeficiency virus (HIV) and human T-cell lymphotropic viruses (HTLVs) (i.e., preventative measures towards reducing sexual transmission should not only reduce prevalence, but also have an evolutionary effect on viral virulence). However, the interpretations of the myxoma virus studies have been challenged and similar tests of the trade-off model in other host-pathogen systems have been less convincing (Ebert & Bull 2003). The resulting controversy has led to a characterisation of the model as "too simplistic" (Ebert & Bull 2003), and perhaps only applicable in single host-pathogen systems where the parasite

cannot exist in the environment. Nevertheless, the trade-off hypothesis remains the fundamental basis for the study of virulence evolution (Day 2003).

**Current views of virulence**

Perhaps the principal shortcoming in previous models of virulence was addressing how natural selection operates for parasites. Previously, studies focused on host mortality rather than examining traits more directly related to parasite fitness (Bull 1994, Ebert & Bull 2003). Natural selection should optimise fitness, the measure of an organism's success at passing genetic information on to future generations (Stearns 1976, Roff 1992), for both the host and the parasite as independent organisms (Ewald 1994). For example, a host must balance the energetic cost of mounting an immune response against the risk of infection (optimal immune defence), as demonstrated in studies of foraging energetics and immune responses to parasitic infections in eider ducks (Hanssen et al. 2004, Houston et al. 2007). However, until recently factors influencing parasite evolution were overlooked, misunderstood or difficult to study (Bull 1994, Ebert & Bull 2003, Ewald 1994).

Parasite evolution should function under the same principles of natural selection governing the evolution of any organism (Bull 1994, Nowak & May 1994, Ebert & Bull 2003, Ewald 2004). These include the possibility of competition between parasite genotypes within a single host (Bull 1994, Ebert & Bull 2003). A basic assumption is that parasite fitness should be maximised by striking an optimal balance between the parasite's own within-host growth and reproduction (fecundity) and between-host transmission (persistence) (Day 2003, Dybdahl & Storfer 2003, Galvani 2003). The distinction from previous hypotheses is the recognition of the importance of within-host evolutionary dynamics (Levine et al. 1970, Bull 1994, Ebert 1998). For example, in an oversimplified system such as direct transmission within a single host species, theoretical models predict a wide variety of possible virulence outcomes based on parasite population dynamics (Bull 1994). Competition among parasite strains within a single host should select for higher parasite reproductive rates, but this may or may not be directly linked to virulence when explicitly defined as host morbidity or mortality (Bull 1994) depending on inherent characteristics of the parasite (e.g., mutations rates of microparasites such as RNA viruses are very different from those of macroparasites such as parasitic worms).

The evolutionary dynamics of parasites and their adaptations within hosts have been investigated using serial passage experiments in which parasites are propagated under defined conditions (Ebert 1998). Serial passage experiments (SPEs) have been used for *in vitro* and *in vivo* investigations of a wide variety of parasites including viruses (Sabin & Schlesinger 1945, Zuckerman et al. 1994), bacteria (Cushion & Walzer 1984, Maisnier-Patin et al. 2002, Somerville et al. 2002), protozoa (Lecomte et al. 1992), fungi (Levine et al. 1970), and others (Ebert 1998). They have also been applied for vaccine development (e.g., yellow fever and polio which used attenuated strains of the parasite (Kew et al. 1981)). The advantage of SPEs is that alterations in parasite genotype,

phenotype and often virulence can be tracked in real time (Ebert 1998). During *in vitro* experiments parasites are propagated by transfer to a new host or to a new artificial environment (such as culture medium) at a specific point in time or life phase (Ebert 1998, Ford et al. 2002). It is commonly observed that parasites maintained in culture become "attenuated" (i.e., become less virulent in hosts). It is usually thought that this occurs because as they become adapted to growth in culture, their ability to grow within hosts decreases (Ebert 1998, Ford et al. 2002). Evidence suggests that the degree of attenuation in culture is influenced by the timing of passage in the parasite's life history and maintenance practices (da Silva & Sacks 1987, Wozencraft & Blackwell 1987, Rey et al. 1990) and attenuated parasite strains can rapidly revert to higher virulent forms when re-exposed to naïve hosts (Cann et al. 1984, Macadam et al. 1989, Minor 1993, Nielsen et al. 2001). This makes it clear that virulence can be altered by within-host evolutionary dynamics. In nature, it must also be affected by between-host evolutionary factors including (but not limited to) heterogeneous host populations (Regoes et al. 2000, Dobson 2004, Keesing et al. 2006), spatially (geographically) confined host adaptation (Boots 1999, Dybdahl & Storfer 2003, Galvani 2003) and various modes of parasite transmission (Day 2001).

In summary, virulence is an extremely complex product of the host, the pathogen and the environment that we are just beginning to understand. Although concepts regarding virulence have advanced significantly in recent decades, a comprehensive understanding of host-pathogen dynamics and "virulence management" remains a challenge for future research. It is now commonly accepted that virulence is a dynamic product of the interactions among hosts, pathogens and their environments and that evolution does not necessarily lead to a benign association between host and parasite. Implicit in all developing views of virulence is an appreciation for the complexities of host-parasite dynamics. It is clear that the level of virulence, from benign commensalism to high lethality, will greatly depend on the biology of the host and the parasite and on their shared environment. Current advances in the fields of evolutionary epidemiology, disease ecology, molecular biology and microbiology should enhance our understanding of virulence and disease.

## Part II. Amphibian Chytridiomycosis

### Worldwide amphibian declines

At the World Congress of Herpetology in 1989, herpetologists discussed unusual and alarming patterns of disappearances of amphibian populations in protected areas (Barinaga 1990, Wake & Morowitz 1991, Collins & Storfer 2003). Due to a lack of high-quality, long-term census data, it was difficult to determine if anecdotal observations reflected normal amphibian population fluctuations or true declines (Pechmann & Wilbur 1994). However, evidence of genuine declines continued to mount and Conservation International's Center for Applied Biodiversity Science in completing a global synthesis of the declines concluded that amphibians are far more threatened than birds or mammals (Stuart et al. 2004). In their report Stuart et al. (2004) noted that many amphibian species were suffering declines due to typical causes such as habitat

destruction and overexploitation (such as harvesting for human consumption). However, the report also acknowledged that many dramatic population declines were taking place in pristine locations where no obvious cause was apparent (La Marca & Reinthaler 1991, Tyler 1991, Crump et al. 1992, Carey 1993, Fellers & Drost 1993, Ingram & McDonald 1993, Richards et al. 1993, Vial et al. 1993, Blaustein et al. 1994, Pounds & Crump 1994, Stebbins & Cohen 1995, Drost & Fellers 1996, Laurance et al. 1996, Lips 1998, 1999, Alford et al. 2001, Young et al. 2001, La Marca et al. 2005)

The various hypotheses on the causes of amphibian declines were collated and included: overexploitation, land use change, UV radiation, contaminants, global climate change, and emerging infectious diseases (Alford & Richards 1999, Collins & Storfer 2003). Although it was clear that many amphibian declines are related to factors such as habitat loss, it was suggested that many dramatic declines and disappearances in apparently undisturbed habitats were due to disease (Carey 1993, Laurance et al. 1996, 1997, Berger et al. 1998, Berger et al. 1999a, Daszak et al. 1999, Daszak et al. 2003). However, there was no general consensus on the most plausible cause of frog declines in protected areas and these losses were termed "enigmatic" by Stuart et al. (2004).

**Amphibian declines and disease**

In Australia, where amphibian population disappearances were reported for multiple species in the 1990s (Tyler 1991, Ingram & McDonald 1993, Richards et al. 1993) researchers proposed that an introduced pathogen may be responsible for the declines (Laurance et al. 1996). This suggestion was met with scepticism mostly because the available data were considered insufficient to support the hypothesis (Alford & Richards 1997, Hero & Gillespie 1997, McCallum 2005) and also because traditionally disease was not viewed as capable of causing permanent declines or extinction events (Scott 1988, Anderson & May 1991, Hudson et al. 2002).

Proving causation in wild amphibian population declines was a challenge (Carey & Bryant 1995, Carey et al. 2001, Daszak et al. 2003). Declines that were detected occurred rapidly (Laurance et al. 1996, Lips 1998, 1999, Briggs et al. 2005, Lips et al. 2006, Woodhams et al. 2008b) and often there was little available information on populations prior to declines (Alford & Richards 1997, Lips et al. 2008). In order to definitively link diseases with amphibian declines, Daszak et al. (2003) identified four critical requirements for demonstrating the causal relationship. First, Koch's postulates needed to be fulfilled for the putative pathogenic agent. Koch's postulates, named after Dr. Robert Koch, are multi-step assessments that involve isolating and purifying a pathogen from a diseased animal, exposing otherwise healthy hosts, reproducing the disease and re-isolating the same pathogen (Koch 1891). Koch's postulates, formulated in the 19$^{th}$ century, cannot be applied in all host-pathogen systems (Fredricks & Relman 1996) and have since been refined and updated (Evans 1976) but they remain the basis for principles in identifying causative agents of disease. Second, the pathogenic agent needed to be associated with mortality events that led to an overall decline in the population. Third, an explanation for the cause of death, or the mechanism of

pathogenesis, needed to be provided and supported by pathological evidence. Fourth, the evidence that the mortalities caused population declines needed to be unequivocal. Daszak et al. (2003) proposed that these criteria should be met in order to sufficiently demonstrate a link between disease and amphibian declines.

### *Batrachochytrium dendrobatidis* and chytridiomycosis

In 1990's, researchers identified an organism associated with wild frog mass-mortality events in Australia and Central America (Berger et al. 1998) and in captive frogs in North America (Pessier et al. 1999, Longcore et al. 1999). A fungus, *Batrachochytrium dendrobatidis (Bd)*, was identified in histological sections taken from the skin of dead and dying frogs (Berger et al. 1998, Berger 2001), satisfying the second criterion of Daszak et al. (2003). The taxonomy of the organism was initially unknown and required the creation of a monotypic genus in Chytridiomycota, a phylum of fungi not previously known as pathogens of vertebrates (Longcore et al. 1999). This microbe appeared to cause disease in otherwise healthy amphibians when exposed to infected pieces of shed skin and the biology of the aquatic pathogen seemed to explain the patterns of declines (Berger et al. 1998, Berger 2001). Furthermore, once isolated from the skin of an infected frog, *Bd* was pathogenic and lethal to amphibians in subsequent exposures (Longcore et al. 1999, Nichols et al. 2001), demonstrating the ability of the organism to cause disease, fulfilling Koch's postulates and the first criterion proposed by Daszak et al. (2003). Following this discovery, reports associating *Bd* and amphibian declines came from nearly every continent where amphibians occur (Berger et al. 1998, Muths et al. 2002, Briggs & Burgin 2003, Briggs et al. 2005, Weldon 2005, Bosch & Martínez-Solano 2006, Lips et al. 2006, Kusrini et al. 2008). Retrospective studies of museum specimens showed *Bd* was present during past amphibian die-offs (Aplin & Kirkpatrick 2000, Ouellet et al. 2005, Puschendorf et al. 2006b). Although the patterns of *Bd* emergence were not always clear (Ouellet et al. 2005, Puschendorf et al. 2006b) and infections have been detected with limited or no evidence of amphibian die-offs (Beard & O'Neill 2005, Longcore et al. 2007, Frías-Alvarez et al. 2008), additional proof to meet the second and forth criteria came from more recent, well-documented declines (Briggs et al. 2005, Lips et al. 2006, Woodhams et al. 2008b).

Amphibian declines due to *Bd* continue to be reported (Muths et al. 2003, Woodhams et al. 2008b, Kusrini et al. 2008). Today *Bd* is widely recognised for its to ability to spread rapidly though amphibian populations, infect numerous, phylogenetically distant species, cause high mortality and persist even at low host densities (Berger et al. 1998, Retallick et al. 2004, Woodhams & Alford 2005, Lips et al. 2006, Skerratt et al. 2007). These disease characteristics render population recovery from chytridiomycosis especially difficult and present strong empirical evidence for disease-induced extinctions (Schloegel et al. 2006, Mitchell et al. 2008). However, the third criterion of Daszak et al. (2003) is still unfulfilled. A fundamental question remains unanswered: how does *Bd* kill amphibians? Resolving the mechanism of pathogenesis is an imperative for explaining how a superficial skin fungus could be responsible for the loss of amphibian biodiversity worldwide.

## Hypotheses regarding pathogenesis

Even with a growing body of literature on chytridiomycosis, the mechanism of mortality remains an important unanswered question (Gascon et al. 2007, Mitchell et al. 2008, Rosenblum et al. 2008, Wake & Vredenburg 2008). This gap in our understanding of chytridiomycosis exists for several reasons. First, cutaneous fungal infections are not commonly fatal to terrestrial vertebrates unless acting opportunistically with other predisposing factors, so there were no similar systems to which chytridiomycosis could be compared. Second, the location of the fungus in the most superficial layers of skin (Berger et al. 1998, Berger et al. 1999b, Berger 2001, Berger et al. 2005c, Puschendorf & Bolaños 2006, North & Alford 2008) and the minimal host reaction to infection (Berger et al. 2005c, Woodhams et al. 2007a) are unusual for fatal infections in vertebrates. Third, because there are no consistent pathological changes in internal organs of diseased amphibians detectable at the level of light microscopy (Berger et al. 1998, Berger 2001), traditional methods used to understand pathogenesis have proved ineffective.

Two hypotheses for the cause of mortality in amphibians infected with *Bd* have been suggested. The first is that *Bd* disrupts osmoregulation in the skin of infected amphibians (Berger et al. 1998, Berger 2001). This hypothesis was proposed because 1) the skin of amphibians is a physiological organ that tightly regulates the exchange of respiratory gases, water and electrolytes (Jorgensen et al. 1954, Alvarado & Kirschner 1963, Deyrup 1964, Moore & Lofts 1964, Mullen & Alvarado 1976, Fischbarg & Whittembury 1977, Shoemaker 1977, Larsen et al. 1987, Boutilier et al. 1992, Erspamer et al. 1994, Jorgensen 1994a, b, 1997, Wright et al. 2001, Word & Hillman 2005) and 2) *Bd* is predominantly found in the ventral integument which is particularly important to physiological processes (Berger et al. 1998, Berger et al. 1999b, Berger 2001, Berger et al. 2005c, Puschendorf & Bolaños 2006a, North & Alford 2008). The second hypothesis concerning pathogenesis is that *Bd* produces a toxin that affects organs (Berger et al. 1998, Blaustein et al. 2005). The two hypotheses are not necessarily mutually exclusive and to date the available evidence is insufficient to determine if either or both are correct.

## Host characteristics- amphibian epidermis

*Anatomy of amphibian epidermis*— The epidermis, which is superficial to the dermis, consists of multiple (5-7) layers of epithelial cells and specialized cell types (Farquhar & Palade 1965, Erspamer et al. 1994). Epidermal layers include (from deep to superficial): *stratum germinitivum, stratum spinosum, stratum granulosum,* and *stratum corneum*. The *stratum germinativum* is the basal layer and is composed of cuboidal or columnar cells (Farquhar & Palade 1965). Cellular division at this layer generates new cells that migrate superficially (Erspamer et al. 1994) and differentiate into various cell types. The cells of the *stratum corneum* are differentiated as keratinocytes, meaning they contain the cytoskeletal protein keratin (Farquhar & Palade 1965, Erspamer et al. 1994), and specialized cells including horny beak cells, goblet cells, mitochondria-rich cells, flask

cells, Merkel cells, Leydig cells, Langerhans cells, cement gland cells, melanocytes and others (Erspamer et al. 1994). The dermis contains multiple types of glands (Lillywhite 1971, Blaylock et al. 1976, Erspamer et al. 1994, Clarke 1997, Bowie et al. 1999, Lenzi-Mattos et al. 2005).

Physiology of amphibian epidermis— Amphibian skin is unique among terrestrial vertebrates because it is highly physiologically active; the skin is permeable to water and a site of regulated transport for ions (electrolytes) and respiratory gases (Jorgensen et al. 1954, Alvarado & Kirschner 1963, Deyrup 1964, Moore & Lofts 1964, Fischbarg & Whittembury 1977, Shoemaker 1977, Larsen et al. 1987, Boutilier et al. 1992, Erspamer et al. 1994, Jorgensen 1994a, b, 1997, Wright et al. 2001, Word & Hillman 2005). In order to maintain osmotic balance, amphibians must maintain a hyperosmotic internal environment relative to the hypoosmotic external environment. This is accomplished by active regulation of transport of multiple electrolytes (including sodium, magnesium, potassium, chloride) across the surface of the skin (Deyrup 1964, Moore & Lofts 1964, Boutilier et al. 1992, Erspamer et al. 1994, Jorgensen 1997, Wright et al. 2001). A steady inward flux of sodium must be maintained despite its movement against an electrochemical gradient. This is accomplished via a cyclic AMP regulated pathway, the sodium potassium pump, which exchanges potassium ions for sodium ions, thereby regulating intracellular and extracellular concentrations. Water flow results when an osmotic gradient is established by electrical currents, induced by an exchange of these solutes (Larsen 1970, Kirschner 1983, Jorgensen 1997).

The selective barrier properties of frog skin are primarily determined by electrolyte transport in the flask-shaped mitochondrial-rich cells (MR cells) of *stratum granulosum* (Masoni & Garcia-Romeu 1979, Brown et al. 1981, Larsen et al. 1987, Larsen et al. 1996, Ehrenfeld 1997). However, all epidermal cells "work" together. As a multi-layered composition of many cell types, amphibian epidermal cells probably act as "functional syncytium" to maintain required concentrations of electrolytes and water balance (Larsen 1991). The permeability of frog skin varies over the body surface of an individual and also among species (Moore & Lofts 1964, Erspamer et al. 1994). In some species, for example in bufonids, osmotic permeability is greatest in an area of ventral integument commonly referred to as the pelvic patch (Czopek 1965, Baldwin 1974, Word & Hillman 2005), where there is dense cutaneous vasculature (Czopek 1965). Despite some variation in permeability, amphibian skin, across all species, is a central organ in maintaining water and electrolyte equilibrium.

Healthy amphibians regularly moult by shedding their outer keratinised layer, as a replacement *stratum corneum* forms (Ewer 1951, Jorgensen & Larsen 1961, Budtz & Larsen 1973, 1975, Larsen 1976, Budtz 1977, Masoni & Garcia-Romeu 1979, Budtz 1985b, a, 1988, Jorgensen 1988). The process is dependent on physiology and behaviour (Bouwer et al. 1953, Castanho & De Luca 2001). The frequency of moulting varies by species and can vary from a few days to several weeks depending on many factors including temperature, age, size and sex (Larsen 1976). Alterations in water permeability and electrolyte transport occur during a normal moulting event (Jorgensen 1949, Larsen

1970). A temporary increase in sodium permeability as well as an increase in sodium excretion lead to a net sodium loss (Jorgensen 1949).

The unique properties of amphibian skin have made it a model for studying epithelial transport (Ussing & Zerahn 1951, Koefoed-Johnsen et al. 1952, Kirschner 1983), which is now the foundation for a wide range of medical research (Larsen 2002). However, the importance of these cutaneous functions in maintaining homeostasis makes amphibians especially vulnerable to the effects of epidermal infections (Wright et al. 2001).

Epidermal pathology of chytridiomycosis— In infected amphibians Bd sporangia are found in cells of the statum corneum and the stratum granulosum of the epidermis (Berger et al. 1998, Berger et al. 1999b, Pessier et al. 1999, Berger 2001, Berger et al. 2005a, Berger et al. 2005c). Primary pathological abnormalities include cell hyperplasia and hyperkeratosis or "thickening" of the stratum corneum (Berger et al. 1998, Berger et al. 1999b, Pessier et al. 1999, Berger 2001, Berger et al. 2005a, Berger et al. 2005c). Other pathological changes in the epidermis include cytoplasmic degeneration and vacuolation in scattered cells in the stratum granulosum (Berger 2001, Berger et al 2005a). These cytopathic changes are not severe in most cases, but can result in sloughing of the stratum corneum and erosions (Berger 2001). Almost all subsequent reports of amphibian mortality in association with Bd have consistently described the same epidermal clinical signs of infection, especially irregular skin sloughing, epidermal hyperplasia and hyperkeratosis (Nichols et al. 2001, Mazzoni et al. 2003, Daszak et al. 2004, Carey et al. 2006, Puschendorf & Bolaños 2006, Kriger et al. 2007)**.**

The significance of irregular skin sloughing is unclear. In one study, salamanders (*Ambystoma tigrinum*) sustained *Bd* infections more than 60 days after experimental exposure, seemingly without adverse affects, but with a notable increase in the sloughing of skin (Davidson et al. 2003). Because *Bd* was found in sloughed skin, it was hypothesised that the increase in sloughing might assist amphibians in shedding the infection (Davidson et al. 2003). In support of this hypothesis Berger et al. (2004) suggested that increased moult frequency may be a reason frogs can clear infection at higher temperatures (Berger et al. 2004). One study monitored moult patterns of toads (*Bufo boreas*) by noting the disappearance of a lipstick mark on the dorsal integument (Bendsen 1956) and quantifying skin shedding before and after *Bd* infection (Voyles et al. 2005). Toads were randomly assigned to exposure and control groups, inoculated by immersion in *Bd* and control solutions, and molt patterns were tracked over the course of infection. Control toads typically had a 2-6 day intermoult cycle, producing 1-2 large pieces of sloughed skin. In contrast, infected frogs that developed severe clinical signs of disease did not have a full body moult, but continually shed more than 100 small, tattered pieces of skin. It was suggested that the change in moult pattern is a maladaptive response to *Bd* infection in this species (Voyles et al. 2005).

Parasite Characteristics- *Batrachochytrium dendrobatidis*
Morphology, growth and development— Species in the phylum Chytridiomycota are identified by ultrastructural morphology of the zoospore, especially the flagellar

apparatus (Mueller et al. 2004, James 2007). *Batrachochytrium dendrobatidis* was originally isolated from, and named for, a blue poison dart frog (Dendrobates azureus) (Longcore et al. 1999). Multiple Bd isolates from various amphibian species have been isolated into pure culture (personal communication, Joyce Longcore). Once in culture, Bd develops through multiple life stages. The earliest life stage is the infectious, motile zoospore, utilising a posterior flagellum (Berger et al. 1998, Longcore et al. 1999, Berger 2001, Berger et al. 2005a). The zoospore encysts, absorbs the flagellum and develops rhizoids (Berger et al. 2005a). The maturing thallus develops a zoosporangium (i.e. container for zoospores) in which the contents cleave and develop into flagellated zoospores. In this mature life stage, a discharge tube forms and the zoospores are released into the external environment to continue the life cycle (Berger et al. 1998, Longcore et al. 1999, Berger 2001, Berger et al. 2005a).

To date our understanding of *Bd* nutrient utilization is incomplete (Longcore et al. 1999, James 2007, Piotrowski et al. 2004, Berger et al. 2005a, Rosenblum et al. 2008, Symonds et al. 2008). This is a critical question for two reasons. First, it is unclear whether *Bd* can persist in the environment as a saprobe, utilizing non-amphibian organic materials, which has important implications for the evolution of virulence and the biology of chytridiomycosis (James 2007, Mitchell et al. 2008). Second, determining what amphibian nutrient sources are utilised by *Bd* may explain a key step in pathogenesis (Rosenblum et al. 2008, Symonds et al. 2008).

It is a misconception that *Bd* only uses keratin as a nutrient source (Altig 2007). Although *Bd* is found in keratinising epidermal cells and tadpole mouthparts (Berger et al. 1998, Longcore et al. 1999, Berger 2001, Fellers et al. 2001, Rachowicz & Vredenburg 2004, Berger et al. 2005a, Blaustein et al. 2005, Woodhams & Alford 2005, Knapp & Morgan 2006, Smith et al. 2007), *Bd* is also found in cells containing prekeratin (Berger et al. 2005a) and it does not grow in pure keratin cultures (Piotrowski 2004, Rosenblum et al. 2008). *In vitro Bd* grows on various agar preparations. Mixtures of various concentrations have included peptonized milk, typtone, gelatin hydrolysate, lactose, glucose, asparagine, yeast extract, malt extract, peptone, sucrose, maltose, sorbitol, glycerol, sterilized snakeskin, ground feather meal, sloughed amphibian skin (Longcore et al. 1999, Piotrowski et al. 2004, Symonds et al. 2008) and aquatic insects (Cashins et al. in prep). Growth and development rates in culture can vary among nutrient conditions. Even within cultures with standardised nutrients, growth rates can be inconsistent (Symonds et al. 2008, Cashins et al. in prep). Currently *Bd* isolates are most commonly cultured on tryptone/gelatin hydrolysate/lactose (TGhL) media (Longcore et al. 1999). Serial culturing influences growth and development (and infectivity and virulence) of many pathogens and some researchers have expressed concern regarding selective pressures on *Bd* in culture (Retallick & Miera 2007, Symonds et al. 2008).

Rates of growth and development of *Bd* are also influenced by temperature. The optimal temperature range appears to be approximately 17-23ºC, though *Bd* can grow at lower temperatures (Berger 2001, Piotrowski et al. 2004, Woodhams et al. 2008a). Cultures of *Bd* incubated at 30ºC and higher died (Piotrowski 2004). Woodhams et al. (2008a) observed and modeled how reproductive life-history traits were adjusted depending on

temperature. At lower temperatures (7-10ºC) motile *Bd* zoospores took longer to encyst, mature and produce propagules than *Bd* zoospores maintained in warmer (17-23ºC) temperatures. It was concluded that this mechanism enables *Bd* to maintain a relatively high long-term growth rate across a range of temperatures (Woodhams et al. 2008a). These results may help to explain how pathogenicity is maintained at lower temperatures (Carey et al. 2006) and the population level effects of *Bd* in low temperature environments (Woodhams et al. 2003, Berger et al. 2004, Woodhams & Alford 2005, Pounds et al. 2006, Kriger & Hero 2007b, a, 2008, Muths et al. 2008, Skerratt et al. 2008).

Batrachochytrium dendrobatidis *and disease*— The reproductive life cycle of *Bd* is presumed to be the same in frog skin where the thalli live and mature within the *stratum granulosum* and *stratum corneum* (Longcore et al. 1999, Pessier et al. 1999, Berger 2001, Berger et al. 2005a). Zoospores colonise cells deeper in the epidermis and develop endogenously (i.e. within the epidermal cells) (Longcore et al. 1999, Berger et al. 2005a). Although it is unclear how cell entry is achieved, Longcore et al. (1999) hypothesised that a zoospore might encyst on the exterior surface of an epidermal cell and insert *Bd* nucleic material using a germ tube. Following cell entry, *Bd* sporangia develop within the epidermal cells, completely filling the cells, and move from deep to more superficial, coinciding with the normal directional movement of epidermal cells. Berger et al. (2005a) observed that the rate of development of *Bd* was timed such that the final stages of development, formation of discharge tube, occurred when infected epidermal cells were most superficial. Also, discharge tubes were oriented toward the external environment, suggesting that *Bd* is well adapted to frog skin (Berger et al. 2005a).

Some evidence suggests that *Bd* enzymatic activity directly influences pathogenesis. The initial penetration of *Bd* into amphibian epidermal cells likely requires digestive enzymes and dissolution of cellular cytoplasm has been observed (Berger et al. 2005a). In culture *Bd* secretes extracellular proteases that degrade casein and gelatin (Piotrowski et al. 2004) and proteolytic enzyme activity has been detected for multiple *Bd* isolates (Piotrowski et al. 2004, Symonds et al. 2008, Fisher et al. 2009). At the molecular level, genomic research into *Bd* is revealing differential expression patterns in genes such as serine protease and fungalysin metallopeptidase, two enzymes involved in pathogenesis in multiple fungal pathogens (Rosenblum et al. 2008). These enzymes may have toxic effects on amphibians. In a test of susceptibility of multiple amphibian species at the larval stage, Blaustein et al. (2005) observed rapid mortality and suggested the possibility that *Bd* produced a "toxic substance" that killed the tadpoles.

In addition to enzymatic activity, the reproductive biology of *Bd* is likely to be an important determinant of pathogenicity. When maintained in *in vitro* high-nutrient conditions, exponential growth and a peak in zoospore production is followed by a decrease in zoospore production and activity, presumably due to exhaustion of nutrient resources (Woodhams et al. 2008a) or possibly a buildup of inhibitory metabolites. In frog skin, however, growth limitations due to nutrient exhaustion are unlikely (Woodhams et al. 2008a), and local re-infection on an individual host (Berger et al. 2005a) probably leads to an exponential increase in *Bd* load and thus, development of

severe infections and mortality (Carey et al. 2006). In infection experiments on susceptible frogs, clinical signs of disease develop in amphibians with the highest numbers of zoospore equivalents, suggesting that reproduction and zoospore production and/or intensity of infection are important in pathogenesis.

The influence of temperature on *Bd* growth and development is probably one of the most important determinants of chytridiomycosis outbreaks in wild amphibian populations (Bradley et al. 2002, Woodhams et al. 2003, Berger et al. 2004, Retallick et al. 2004, Woodhams & Alford 2005, Carey et al. 2006, Drew et al. 2006, Kriger & Hero 2006, Pounds et al. 2006, Rachowicz et al. 2006, Kriger & Hero 2007a, b, Rowley & Alford 2007, Kriger & Hero 2008, Muths et al. 2008, Skerratt et al. 2008). Severe amphibian declines attributed to chytridiomycosis occur in upland sites in tropical areas where temperatures are generally cool (Berger et al. 2004, Lips et al. 2006, Skerratt et al. 2007, Brem & Lips 2008, Skerratt et al. 2008) and low temperature is significantly related to high prevalence (Woodhams & Alford 2005, Drew et al. 2006, Kriger & Hero 2007a, b, Muths et al. 2008). Furthermore, niche modelling of *Bd* distributions predicts that *Bd* should occur where temperatures are <27ºC (Ron 2005, Skerratt et al. 2008, Puschendorf et al. in press). Recognition of the importance of temperature led to the hypothesis that human-induced global climate change could be driving outbreaks of chytridiomycosis if changing climatic conditions create a thermal optimum for *Bd* (Pounds et al. 2006). This hypothesis is controversial (Alexander & Eischeid 2001, Alford et al. 2007, Bosch et al. 2007, Di Rosa et al. 2007, Fisher 2007, Skerratt et al. 2007, Laurance 2008, Lips et al. 2008) and largely untested, but it highlights the need to better understand the role of temperature in the impact of chytridiomycosis and the potential influence of changing climatic conditions.

To date genetic work has shown that little variation exists among isolates worldwide and suggests that *Bd* is a pandemic clone (Morehouse et al. 2003, Morgan et al. 2007). Experimental evidence suggests that *Bd* virulence may differ among isolates (Berger et al. 2005b, Retallick & Miera 2007, Fisher et al. 2009), but the underlying reasons for the variation are unknown. Reduced mortality has been observed in amphibian populations after surviving the initial emergence of chytridiomycosis (Morgan et al. 2007), and different populations, even within species, can exhibit different outcomes to infection (Briggs et al. 2005), suggesting the possibility of evolving *Bd* virulence. Differential virulence among isolates is a topic that remains to be explored and perhaps more importantly, offers promise of insight into putative co-evolution between *Bd* and amphibians (Retallick et al. 2004).

## Conclusions and direction

Despite a decade of research and increased attention on chytridiomycosis, critical gaps remain in our understanding of the disease (Skerratt et al. 2007, Gascon et al. 2007, Wake & Vredenburg 2008). This study investigated the virulence and pathogenicity of chytridiomycosis because these are some of the most important and least understood aspects of the disease. First, even within a single host species, virulence of *Bd* isolates

appears to vary (Berger et al. 2005b, Briggs et al. 2005, Morgan et al. 2007, Retallick & Miera 2007, Fisher et al. 2009). The cause of this variability has not been determined. To characterise differences among isolates this study examined growth and development *in vitro* for three *Bd* isolates and manipulated nutrient and temperature conditions for a single isolate over multiple generations. Finally, it is unknown why and how amphibians die from chytridiomycosis.

***Project 6.2 Life history adjustments of an amphibian pathogen: Differential growth of Batrachochytrium dendrobatidis***

**Chapter 4 in Voyles, J. 2009. Virulence and pathogenesis of chytridiomycosis: A lethal disease of amphibians. Ph.D. thesis, James Cook University**

**Paper in preparation. Authors: Jamie Voyles, Scott D. Cashins, Ross A. Alford, Lee Berger, Lee F. Skerratt, Rebecca Webb, Rick Speare**

Abstract

Life-history adjustments have been investigated in a wide variety of organisms but the application of life history theory to host-pathogen systems is relatively new. Evolutionary pressures act on both the host and the pathogen to optimize fitness. Pathogen life-history evolution is important because microbial characteristics, such as rates of maturation or reproduction, often play a central role in disease development. The fungal pathogen *Batrachochytrium dendrobatidis (Bd)* causes the lethal amphibian disease chytridiomycosis and although *Bd* virulence is not well understood, disease development appears to be closely linked to pathogen load. We investigated life-history adjustments of a single isolate of *Bd* by quantifying infectious zoospores in different temperature and nutrient conditions for multiple generations. Cultures maintained at 4ºC for 17 passages had an earlier release of zoospores and longer period of high zoospore densities than cultures maintained at 23ºC for the same number of passages, suggesting an adaptive response to lower temperatures. Cultures passaged in 0.2% tryptone TGhL broth for 24 passages had earlier zoospore release and a faster life cycle turnover, but lower zoospore densities, than cultures serially passaged in 1.6% tryptone TGhL. This pattern was reversed when cultures maintained in 0.2% tryptone TGhL were passed back into 1.6% tryptone TGhL, demonstrating phenotypic plasticity in response to nutrient concentrations after 24 passages. These findings may help to explain why the impact of chytridiomycosis has been severe in wild amphibian populations in a wide range of environments. Investigating the response of *Bd* to biotic and abiotic factors, as well as the possible evolution of this lethal pathogen in response to selective pressures, may be critical for understanding virulence. A better understanding of these factors also provides the potential to predict future outbreaks and target management actions to minimise impacts.

## Introduction

Evolution of life histories, and variation in life-history adjustments, contributes to the enormous complexity and diversity of the natural world (Stearns 1976, Roff 1992). A basic assumption of life-history theory is fitness (i.e., success at passing genetic information on to future generations) is maximised within a set of constraints by attaining an optimal balance of the costs and benefits of life-history trade-offs (Roff 1992). For example, reproduction comes with a cost for growth and survival. Trade-offs between fecundity and rate of maturation have been investigated in a wide range of plants and animals. Parasite evolution operates under the same principles of natural selection as it does in any other organism (Bull 1994, Nowak & May 1994, Ebert & Bull 2003, Ewald 2004). Parasite fitness is maximised by striking an optimal balance between reproduction and persistence (Day 2003, Dybdahl & Storfer 2003, Galvani 2003) and epidemiological models predict that parasite life-history strategies are selected to maximise lifetime transmission (Galvani 2003). Within this framework, life-history adjustments that balance within-host reproduction and between host transmission will likely influence the incidence of disease (Day 2003, Woodhams et al. 2008a), but a wide range of factors, including environmental conditions such as temperature and nutrient availability, could alter the balance of the host-pathogen dynamic.

One host-pathogen system that has been used to investigate these theoretical principles is a fungal disease of amphibians called chytridiomycosis. The pathogen that causes chytridiomycosis, *Batrachochytrium dendrobatidis* (*Bd*), is lethal to many species of amphibians in laboratory experiments (Berger et al. 1998, 2005b, Longcore et al. 1999, Berger 2001, Nichols et al. 2001, Daszak et al. 2004, Woodhams et al. 2004, Carey et al. 2006, Lips et al. 2006). Outbreaks of chytridiomycosis have dramatically reduced the abundance of amphibian species in naïve populations (Berger et al. 1998, Lips 1998, 1999, Lips et al. 2006, Schloegel et al. 2006, Skerratt et al. 2007) but *Bd* is endemic in surviving amphibian populations (Woodhams & Alford 2003, Retallick et al. 2004, Kriger & Hero 2006, 2007a, b, 2008, Brem & Lips 2008). The presence of *Bd* in extant populations offers an opportunity to examine differential selection pressures on *Bd* in a variety of environments. Adaptations of either the host or the pathogen that affect the rate of reproduction of *Bd* may influence the virulence of *Bd* and probability of chytridiomycosis outbreaks.

In culture, *Bd* develops through multiple life stages (Longcore et al. 1999, Berger et al 2005a). The earliest life stage is the infectious motile zoospore, with a posteriorly located flagellum (Longcore et al. 1999). Zoospores encyst, absorb the flagellum and develop a thallus with rhizoids. The maturing thallus produces zoosporangium in which the contents cleave and develop into flagellated zoospores (Longcore et al. 1999, Berger et al 2005a). At maturity, the zoosporangium forms a discharge tube and the zoospores are released into the environment (or possibly within skin layers of a host) to continue the life cycle. When maintained in high-nutrient conditions *in vitro*, exponential growth and a peak in zoospore production is followed by a decrease in zoospore production and activity, presumably due to exhaustion of nutrient resources (Woodhams et al. 2008) or the build up of inhibitory metabolites. In frog skin, however, growth limitations due to

nutrient exhaustion are unlikely (Woodhams et al. 2008a). Local re-infection on an individual host can lead to an exponential increase in *Bd* load and thus development of severe infections (Carey et al. 2006). It was also suggested that infections above a species-specific threshold may lead to mortality (Carey et al. 2006). Build-up of infection could be slowed in some amphibians by shedding and cell turnover (Berger 2001). In infection experiments on susceptible frogs, clinical signs of disease develop in amphibians with the highest numbers of zoospore equivalents, supporting the suggestion that rate of reproduction and zoospore densities are important in pathogenesis. In contrast resistant amphibian species manage to suppress their sporangial burden, and can remain infected but otherwise healthy (Daszak et al. 2004, Berger et al. 2009).

**Temperature and *Batrachochytrium dendrobatidis***

Patterns of chytridiomycosis outbreaks and subsequent endemic prevalence in wild amphibian populations suggest that environmental conditions such as temperature (Bradley et al. 2002, Woodhams et al. 2003, Berger et al. 2004, Retallick et al. 2004, Woodhams & Alford 2005, Carey et al. 2006, Drew et al. 2006, Kriger & Hero 2006, Pounds et al. 2006, Rachowicz et al. 2006, Kriger & Hero 2007a, b, Rowley & Alford 2007, Kriger & Hero 2008, Muths et al. 2008, Skerratt et al. 2008), and humidity (Puschendorf et al. 2009) are important determinants of disease. Amphibian declines in tropical areas that are attributed to chytridiomycosis have mainly occurred in upland sites with lower temperatures (McDonald and Alford 1999, Lips et al. 2006, Skerratt et al. 2007) and prevalence and mortality rates are greater at these locations during cooler months (Berger et al. 2004, Drew et al. 2006, Kriger & Hero 2007a, b). Niche modelling predicts *Bd* occurrence where temperatures are < 27ºC (Ron 2005, Skerratt et al. 2008, Puschendorf et al. 2009). The link between disease and temperature is further supported by laboratory experiments. Maximal growth of an isolate of *Bd* in culture (Piotrowski et al 2004) occurred within a temperature range of approximately 17-25ºC and high mortality of experimentally inoculated frogs occurs at < 25ºC (Berger 2001, Woodhams et al. 2003, Berger et al. 2004, Carey et al 2006). Furthermore, the mounting evidence of the importance of temperature led to the hypothesis that human-induced global climate change could be driving outbreaks of chytridiomycosis if changing climatic conditions create a thermal optimum for *Bd* (Pounds et al. 2006). This hypothesis is controversial (Alexander & Eischeid 2001, Alford et al. 2007, Bosch et al. 2007, Di Rosa et al. 2007, Fisher 2007, Skerratt et al. 2007, Laurance 2008, Lips et al. 2008), but it highlights the need to better understand the role of temperature in controlling the impact of chytridiomycosis.

**Nutrient availability and *Batrachochytrium dendrobatidis***

In addition to temperature, the ability to respond to nutrient availability may be a critical factor in disease development (James 2007). Some evidence suggests *Bd* may persist independent of amphibian hosts either in an alternative life stage (Di Rosa et al. 2007) and/or by saprobic growth (Johnson & Speare 2003, Mitchell et al. 2008) using non-

amphibian organic materials as nutrient resources. Saprobic growth may help to explain the devastating impact of the disease because models demonstrate that amphibian-independent persistence of *Bd* should amplify the impact on populations (Mitchell et al. 2008). There is limited evidence of *Bd* in the environment; it has not been isolated but DNA has been detected in water bodies (Kirshtein et al. 2007, Walker et al. 2007) and on rocks (Lips et al 2006). However, *Bd* can be cultured on various sterile natural substrates and on simple artificial media, and therefore does not appear to be an obligate intracellular parasite. In the laboratory *Bd* can alter its growth and reproduction in response to differences in media concentration (James 2007), on substrates such as snakeskin (Symonds et al. 2008, Cashins et al. in prep) and aquatic insects (Cashins et al. in prep). These changes have all been characterised during short-term experiments; no experiments to date have investigated long-term responses of *Bd* isolates to differing environmental conditions. Long-term experiments on *Bd* are important to determine phenotypically plastic and/or adaptive responses of *Bd* to environmental conditions. The presence of phenotypic plasticity in response to variation in environmental conditions might decrease the effects of natural selection, if the plasticity is such that a near-optimal response is produced in all environments encountered. However, it has also been suggested that plasticity itself may have various limits and costs (DeWitt et al. 1998, Relyea 2002). To determine whether *Bd* shows responses to differences in environmental conditions in the long-term, and the nature of any response, we investigated whether *Bd* adapts to differences in temperature and nutrient conditions over multiple generations.

## Methods

### Experiment 1- Temperature

*Culture of* Batrachochytrium dendrobatidis— The isolate, GibboRiver-L.lesueuri-00-LB-1, was originally obtained from a diseased juvenile *Litoria lesueuri*, cultured on tryptone/gelatin hydrolysate/lactose (TGhL) agar with antibiotics (Longcore et al. 1999) and then cryoarchived (Boyle et al. 2003). An aliquot of the cryoarchived culture was revived (Boyle et al. 2003) and then passaged into liquid TGhL broth (16 g tryptone, 4 g gelatin hydrolysate, 2 g lactose, 1000 ml distilled water) (Longcore et al. 1999, Boyle et al. 2003). Two 25-$cm^2$ cell culture flasks containing "Low Temperature History" culture (LTH) were moved into 4°C and passaged every 2 to 3 months by transferring 2ml of actively growing culture into 8 ml of new TGhL liquid medium. After 38 months, a second aliquot of the same cryoarchived isolate, GibboRiver-L.lesueuri-00-LB-1, was revived and passaged into liquid TGhL broth using identical procedures. These "High Temperature History" cultures (HTH) were maintained at 23ºC and passaged every 4-6 days, when zoospore density was near maximum levels (determined by visual inspection). When LTH and HTH cultures were matched in passage history (17 passages), a series of plate experiments were conducted.

*Preparation of inocula*— LTH and HTH cultures were filtered to remove sporangia using a sterile filter paper (Whatman filters, Number 3). Cultures were centrifuged (500 g x 10 minutes), removing the supernatant and resuspending the zoospores in sterile dilute salt solution (in mMol: $KH_2PO_4$ (1), $CaCl_2.H_2O$ (0.2), $MgCl_2.2H2O$ (0.1)). Zoospore

concentrations were determined using a haemocytometer (Improved Neubauer Bright-line) and adjusted as needed by addition of dilute salt solution to a concentration of 120 x $10^4$ zoospores per ml. The LTH and HTH zoospore inocula (50 µL) were each pipetted into 25 wells of six sterile 96 well plates (see below). Two aliquots of the LTH and HTH zoospores were heat killed by holding in a 90ºC water bath for 20 minutes and then each were pipetted into 5 wells for a negative control. Six plates were organised with two sections (for LTH and HTH inocula) of wells each containing 50 µL TGhL medium, and a perimeter of 36 wells with 100 µL sterile water to avoid evaporation of the wells containing *Bd* inocula.

*Reciprocal transplant experiment*— Six identically organised plates were created as outlined above, each containing both LTH and HTH cultures. Three plates were incubated at 23ºC and three plates at 4ºC to allow examination of the responses of the cultures to two thermal conditions. Plates were inspected by light microscopy to monitor zoospore encystment and maturation of the zoosporangia. Once the maturing zoosporangia released the first zoospores, zoospore densities were quantified by randomly selecting 10 wells from the LTH and HTH sections of each plate, drawing off 30 µL of supernatant and counting zoospore numbers using a haemocytometer. This procedure was repeated daily (10 wells per day) for plates at 23ºC because they contained faster-growing cultures. The procedure was repeated every 7 d (10 wells per day) for slower-growing cultures held at 4ºC.

**Experiment 2- Nutrient availability**

An aliquot of the same cryopreserved isolate, GibboRiver-L.lesueuri-00-LB-1, was revived and passaged into flasks containing 1.6% tryptone TGhL liquid broth with "High nutrient history" (HNH) and 0.2% tryptone TGhL "Low nutrient history" (LNH). These cultures were incubated at 23ºC and were passaged every 4-6 days, which was the time required to complete the *Bd* lifecycle and reach peak zoospore densities in the higher concentration cultures. After 24 passages in these two media, inocula of each culture were prepared as described above, however, the plates were organized into two sections for each of the inocula (HNH and LNH). In one section the 50 µL of the media (1.6% and 0.2% tryptone TGhL broths) in which the cultures had been passaged were added to the wells. In the second section, 1.6% TGhL was added to wells. With this plate design we could quantify zoospore densities of HNH and LNH in their original media and could also determine whether cultures maintained in lower media concentrations (LNH) would lose the ability to respond to higher media concentrations. Plates were incubated at 23ºC, monitored daily and zoospore densities were quantified in 5 wells per culture per day as described above.

*Statistics*— All data were analysed using R, version 2.7.1. We quantified days to zoospore release and zoospore densities on the first day of release and day of maximum zoospore densities. Zoospore densities are reported as mean ± s.d. per ml ($x10^4$).

## Results
### Experiment 1. Temperature

*Encystment and maturation*—Rate of encystment was not quantified, but zoospores maintained at 23ºC had all encysted within 24 h when examined microscopically. In contrast, most zoospores maintained at 4ºC were still active (although moving slowly) up to 4 d after plate inoculations. Encysted zoospores maintained at 23ºC matured and produced new zoospores within 3 d whereas, at 4ºC, new zoospores were not observed until 24 d after encystment (Figure 4.1c).

*Zoospore densities*— There was a highly significant group effect for zoospore densities of HTH cultures and LTH cultures at 23ºC (repeated measures ANOVA, $P < 0.001$; Figure 80a) and at 4ºC (repeated measures ANOVA, $P < 0.001$; Figure 80b). Cultures maintained at 23ºC had greater numbers of zoospores on the first day of zoospore release than cultures maintained at 4ºC (HTH (at 23ºC day 4): 80.5 ± 28.59, (4ºC day 35): 5.6 ± 4.55; Student's T test, $P < 0.001$; LTH (at 23ºC day 3): 125 ± 43.95, (at 4ºC day 27): 7.6 ± 6.02; Student's T test, $P < 0.001$: Figure 80c). The period of time that zoospores were active was longer at 4ºC (HTH: 43 d, LTH: 49 d) than at 23ºC (HTH: 6 LTH: 7 d). It is unclear if cultures maintained at 4ºC had a greater total number of zoospores (as measured by the cumulative number of active zoospores per day), or if the protracted period of high zoospore density occurred because sporangia matured more slowly and individual zoospores were active for longer periods of time. Regardless, the period of high zoospore density was substantially greater in cultures held at 4ºC (Figure 80c).

*Differential response between HTH and LTH cultures*—Cultures maintained in high temperatures (23ºC) and low temperatures (4ºC) for multiple generations differed in their response to the temperature treatments. Both cultures had similar numbers of zoospores on the day of maximal zoospore density at 23ºC (HTH: 165.2 ± 34.96, LTH: 168 ± 59.51; Student's T test, $P = 0.89$; Figure 80a), and also at 4ºC (HTH: 64.1 ± 25.21, LTH: 54.2 ± 26.8; Student's T test, $P = 0.45$; Figure 80b), but the timing of zoospore release and the densities of zoospores on the first day of release were different. At 23ºC LTH cultures released zoospores on day 3 whereas HTH cultures did not release zoospores until day 4 (Figure 80a). On their respective first days of zoospore release, zoospore densities were significantly higher in LTH cultures at 23ºC (LTH day 3: 125 ± 43.95, HTH day 4: 80.5 ± 28.59; Student's T Test, $P = 0.016$, Figure 80a). At 4ºC LTH cultures released zoospores on day 27 whereas HTH cultures released zoospores on day 35 (Figure 80b). In both temperature conditions, LTH cultures had a longer period of high zoospore densities. It is not clear whether LTH cultures produced a greater total number of zoospores or if the zoospores they produced were simply active for a longer period of time. This is more clearly seen in a composite image of the results from each culture history at each temperature (Figure 80c).

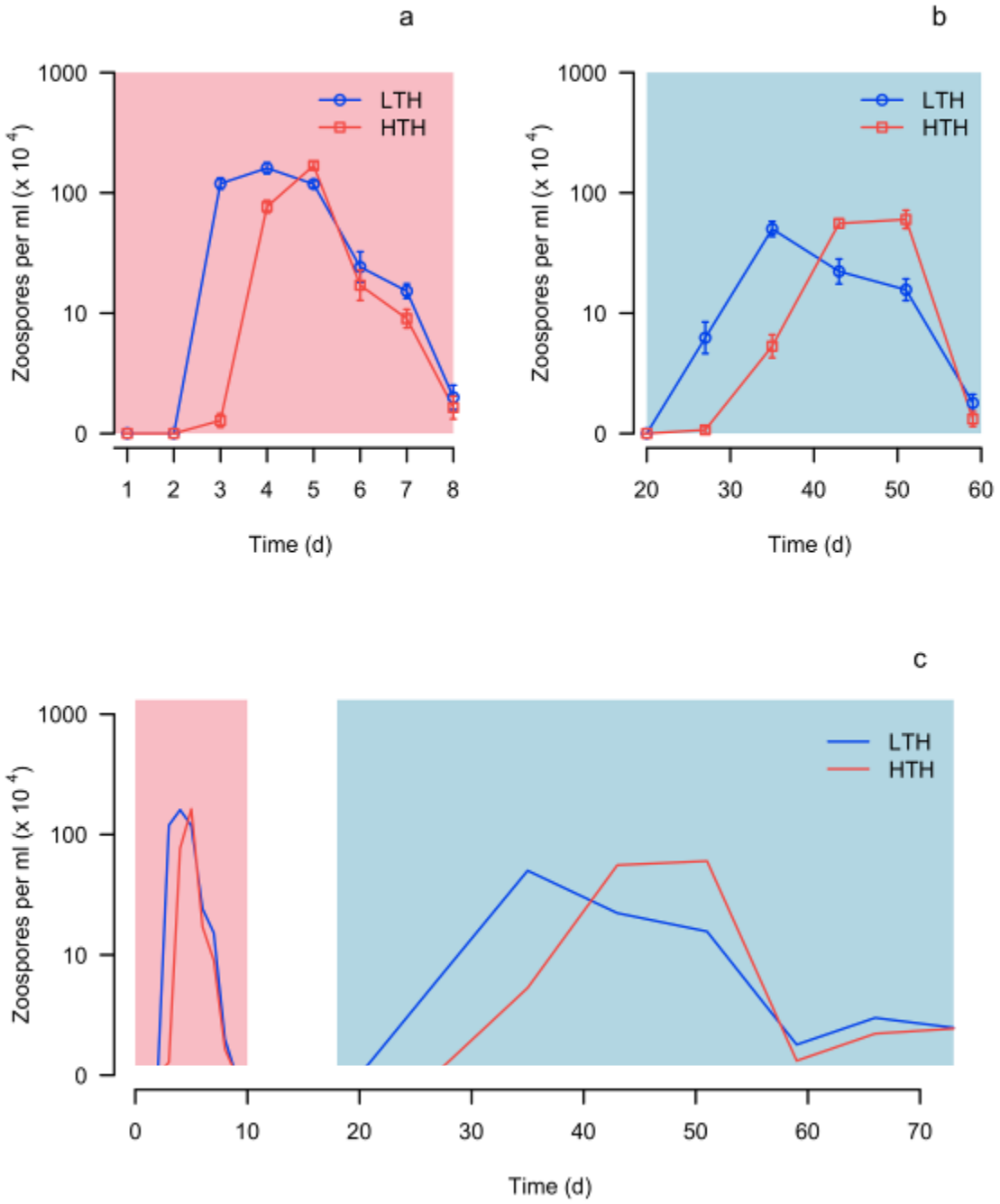


**Figure 80.** Density of zoospores at (a) 23ºC, (b) 4 ºC and (c) both 23ºC and 4ºC (on same time scale) in cultures of an isolate of *Batrachochytrium dendrobatidis* with two thermal histories. Red line indicates cultures previously maintained at 23ºC, "High Temperature History" (HTH). Blue line indicates cultures previously maintained at 4ºC, "Low Temperature History" (LTH).

**Experiment 2. Nutrient availability**

*Encystment and maturation*— Cultures in low nutrient concentrations (0.2% tryptone TGhL) encysted, matured and released zoospores sooner than cultures in high nutrient conditions (1.6% tryptone TGhL) (Figure 81a). Rate of encystment was not quantified, but cultures in 0.2% tryptone TGhL encysted within 24 h whereas most zoospores from cultures in 1.6% tryptone TGhL were still motile up to day 2 when viewed by microscopic examination. Cultures in 0.2% tryptone TGhL matured faster than those in 1.6% tryptone TGhL and had new zoospores within 2 d of inoculation. In contrast, zoospores were not observed in high nutrient cultures until day 3 (Figure 81a).

*Zoospore densities*— There was a highly significant group effect for zoospore densities when LNH cultures were in 0.2% tryptone TGhL and HNH cultures were in 1.6% tryptone TGhL (repeated measures ANOVA, $P < 0.001$; Figure 81a). Cultures previously maintained in 1.6% tryptone TGhL had a greater number of zoospores than cultures previously maintained at low nutrient concentrations on the first day of zoospore release (LNH in 0.2% day 2: 1.2 ± 0.84, HNH in 1.6% day 3, 48.2 ± 14.34; Student's T test, $P = 0.002$; Figure 4.2a) and on the day of maximal zoospore density (LNH in 0.2% day 3: 20 ± 7.03, HNH in 1.6% day 4: 406.2 ± 58.68; Student's T test, $P < 0.001$; Figure 4.2a). The period of zoospore activity was shorter in low nutrient conditions with no motile zoospores after day 7 (Figure 81a).

There was also a significant group effect for zoospore densities when LNH cultures and HNH cultures were in 1.6% tryptone TGhL (repeated measures ANOVA, $P < 0.001$; Figure 81b). On the first day of zoospore release LNH cultures had significantly higher densities of zoospores in 1.6% typtone TGhL than in 0.2% tryptone TGhL (LNH in 0.2% day 2: 1.2 ± 0.84, LNH in 1.6% day 3: 45.38 ± 11.67; Student's T test, $P < 0.001$) but still lower densities than HNH cultures (LNH in 1.6% day 3: 45.38 ± 11.67, HNH in 1.6% day 3: 76.75 ± 27.63; Student's T test, $P = 0.015$; Figure 81b).

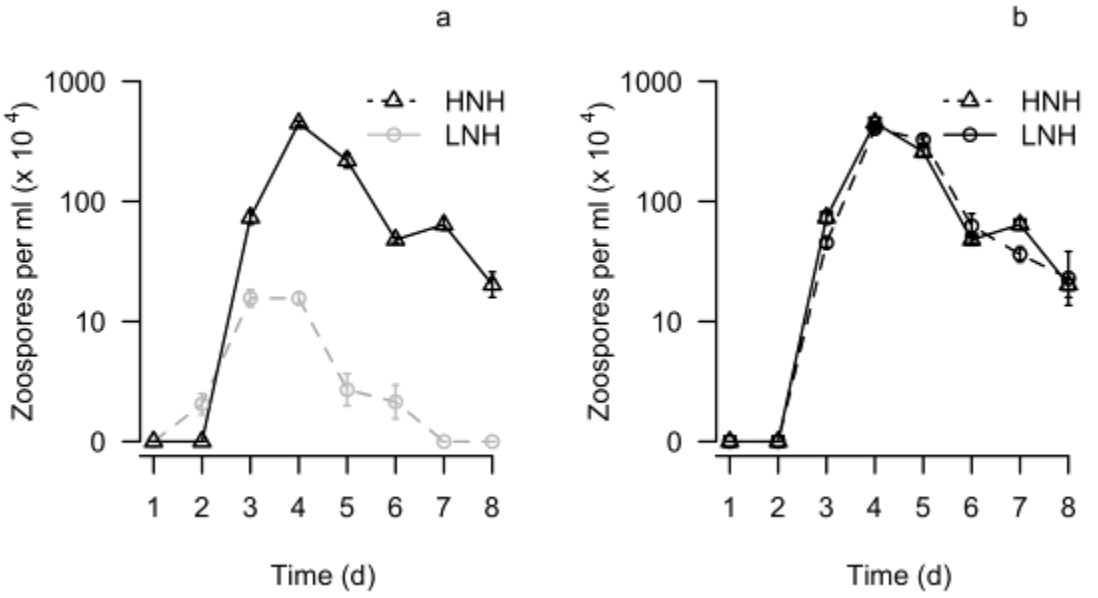


**Figure 81.** Zoospore densities of *Batrachochytrium dendrobatidis* from a single isolate previously maintained in 1.6% and 0.2% tryptone broth nutrient concentrations for 24 passages when cultured in (a) media they had been maintained in and (b) 1.6% TGhL. The grey dashed line (a) indicates "Low Nutrient History" (LNH) cultures in low (0.2% tryptone broth) nutrient conditions. The black dashed line (b) indicates "Low Nutrient History" (LNH) in 1.6% TGhL (b). The solid black line (a and b) indicates "High Nutrient History" (HNH) cultures in 1.6% TGhL.

**Discussion**

Life-history adjustments should maximise fitness for all organisms (Stearns 1976, Roff 1992), including pathogens (Day et al. 2003). Changes in pathogen rates of growth, maturation and reproduction may influence the incidence of disease (Day et al. 2003, Woodhams et al. 2008a). We used *in vitro* serial passage experiments to investigate rates of growth and development of *Bd* in two temperatures and two nutrient concentrations over multiple generations in culture. Changes in the patterns of zoospore densities observed through time indicate that *Bd* responds to changes in biotic and abiotic factors in ways that should lead to relatively high fitness across a wide range of environmental conditions. Our results demonstrate that variability may exist within a single *Bd* isolate and characteristics may be selected for (*in vitro*) after a relatively small number of life cycles. *Bd* could potentially respond to long-term culturing in particular environments and therefore influence virulence and the impact of chytridiomycosis.

In Experiment 1 *Bd* cultures had longer periods of high zoospore densities in colder temperatures. These results agree with the findings of Woodhams et al. (2008a) who suggested that *Bd* may trade increased fecundity for extended zoospore activity in colder

temperatures and thus influence the transmission dynamics of chytridiomycosis. By maintaining *Bd* at 4ºC for multiple generations we aimed to examine the response of *Bd* to low temperatures for a longer period of time. However, due to the response of *Bd* to the cold temperatures, it was difficult to determine the point of maximum zoospore densities for passaging. We cannot rule out the possibility that the results reflect timing of passage practices if passaging occurred after a peak in zoospore densities. Our results suggest, however, that *Bd* cultures maintained at 4ºC over multiple generations may have had an adaptive response to temperature. Adaptations to shift zoospore release earlier in the life history under cold conditions may have also led to earlier zoospore release in warmer temperatures: "Low Temperature History" (LTH) cultures had zoospore densities for a longer period than "High Temperature History" (HTH) cultures. This shift in the norm of reaction was seen at both 23°C and at 4°C (LTH cultures had longer periods of high zoospore densities than HTH cultures), but at 4°C peaks in zoospore densities differed by 16 days compared with 1 day at 23°C.

Although *Bd* growth rate decreases at low temperatures, adjustments to the *Bd* lifecycle, such as prolonged period of high zoospore densities, may lead to enhanced transmission. If long-term exposure to low temperatures alters the response of *Bd* so that zoospore densities are high for longer periods when introduced to warmer temperatures, amphibians may face a greater risk the end of cold periods or when the pathogen is introduced from cooler regions into warmer ones. Such an adaptive response could lead to higher infection rates and a greater impact on amphibian populations. If this hypothesis is true, selection for *Bd* growth characteristics due to different climates is likely to occur as *Bd* becomes endemic to host populations or spreads into new regions. Additional research is needed to further understand the adaptive response of *Bd* in a wide variety of temperature conditions. Future experiments should focus on the response of *Bd* at a range of temperatures experienced by amphibians in the wild. Also, under the chytrid-thermal-optimum hypothesis Pounds et al. (2006) proposed that *Bd* should "flourish" in areas with less variable temperature conditions, where cloudiness makes microhabitats more homeothermic. By manipulating the temperature regimes for more and less variable thermal conditions *in vitro*, their hypothesis could be explicitly tested to better understand *Bd*'s response to varying climate conditions.

In Experiment 2 *Bd* maintained in low nutrient conditions released zoospores earlier than *Bd* in high nutrient conditions. This agrees with the results of James (2007). In low nutrient conditions developing sporangia matured faster and released new zoospores earlier than those in high nutrient conditions, suggesting *Bd* can be a virulent pathogen in a wide range of nutrient environments. The *Bd* cultures in low nutrient conditions appeared to have faster life cycle turn over with no motile zoospores after day 7. This may have been due to higher zoospore mortality and/or zoospores encysting more rapidly. When zoospores with a "Low Nutrient History" were passed back into high nutrient conditions the timing of maturation and development closely matched that of cultures maintained in high nutrient conditions, although zoospore densities were statistically different. These results suggest *Bd* is phenotypically plastic in its response to changes in nutrient conditions but there may be little adaptive variation available as the norms of reaction remained fixed under a wide range of nutrient regimes.

It is important to note that these experiments examined *Bd* growth and development but *Bd* virulence was not tested. It is therefore difficult to say if differential zoospore densities would have corresponded with differential virulence. An exposure experiment on a susceptible frog species testing tests two cultures passaged under different nutrient and/or temperature regimes could resolve this question. Our results have implications for *Bd* maintenance following isolation as they indicate very strongly that new isolates should always be cryo-archived so they can later be studied without the possible effects of long-term evolution in culture. Small variations in laboratory practices may have important effects on *Bd*, which has relatively short generation times. By passaging *Bd* at periods near maximal zoospore densities (approximately day 4) we may have selected for *Bd* with an earlier release of zoospores and greater zoospore densities but these results might have been drastically different if *Bd* cultures were passaged after peak zoospore densities (approximately day 7-9). An additional experiment could test this possibility by reviving aliquots of the same isolate and using serial passage experiments for both cultures: (at 23ºC) one culture could be passaged at peak zoospore densities (days 4-6), the other at a point past peak zoospore densities (days 7-9). Any change in the rates of zoospore production could also be measured by quantifying zoospore densities in the two cultures in a time series (for example, at passage 10, 20, 30 and so on).

There have been several key studies on the physiology of *Bd* (Piotrowski et al. 2004, Symonds et al. 2008), but background information about isolate origins or passage history is sometimes incomplete. The same is true for laboratory infection experiments aimed at understanding host-pathogen dynamics. This information could be extremely important to the interpretation of experimental outcomes and should be carefully considered by researchers working with *Bd*. Furthermore, the importance of cryo-archiving isolates for future virulence research cannot be understated. By sourcing cryo-archived isolates to compare with more recently isolated strains, our methods could be used to characterise a possible evolution in *Bd* virulence and may be important for long-term amphibian conservation efforts.

## *Project 6.3. Differential growth and virulence of* Batrachochytrium dendrobatidis

**Chapter 5 in Voyles, J. 2009. Virulence and pathogenesis of chytridiomycosis: A lethal disease of amphibians. Ph.D. thesis, James Cook University**

**Paper in preparation. Authors: Jamie Voyles, Scott D. Cashins, Ross A. Alford, Lee Berger, Lee F. Skerratt, Rick Speare**


### Abstract

Virulence of infectious parasites can be unstable and can evolve rapidly depending on the evolutionary dynamics of the organism. One potential consequence of altered parasite virulence is disease outbreaks that can have devastating effects on host populations. Serial passage experiments (SPEs) aim to characterise parasite biology and dynamics, often with the underlying objective of understanding virulence. We used SPEs to investigate differential growth and virulence of *Batrachochytrium dendrobatidis*, a fungal pathogen of amphibians that causes a highly lethal disease known as chytridiomycosis. Zoospore densities among three isolates of *Bd* are significantly different. Two cultures that were originally derived from the same cryo-archived isolate were passaged 50 and 10 times near the point of peak zoospore densities. These two cultures ("P50" and "P10") had significantly different zoospore densities on the first day of zoospore release and on the day of maximum zoospore densities. These patterns of zoospore densities *in vitro* corresponded with differences in prevalence and intensities of infection in exposed *Litoria caerulea*. However, the differences in response variables *in vivo* (prevalence and intensities of infection in exposed *L. caerulea*) were not significant and no mortality occurred in any experimental group. These results demonstrate that variation can exist within a single isolate of *Bd* and have important implications for practical laboratory maintenance of *Bd* isolates as well as for understanding *Bd* virulence.


### Introduction

Infectious diseases can alter host population densities, community dynamics and potentially entire ecosystems (Scott 1988, Daszak et al. 2000, Hudson et al. 2002, de Castro & Bolker 2005, Whiles et al. 2006). In many cases the biology, life histories and evolutionary dynamics of the pathogens are not well understood despite their significant threats to wildlife diversity and human health (Daszak et al. 2003). Insight into parasite evolution should provide a better understanding of the evolution of virulence and the mechanisms driving disease epidemics (Bull 1994, Ebert 1998, Ebert & Bull 2003). The evolutionary dynamics of parasites and by extension, virulence, have been investigated using serial passage experiments in which parasites are propagated under constant environmental conditions (Ebert 1998). Serial passage experiments (SPEs) have been used for *in vitro* and *in vivo* investigations of a wide variety of parasites including viruses (Sabin & Schlesinger 1945, Zuckerman et al. 1994), bacteria (Cushion & Walzer 1984, Maisnier-Patin et al. 2002, Somerville et al. 2002), protozoa (Lecompte et al. 1992),

fungi (Levin et al. 1970), and others (Ebert 1998). The advantage of SPEs is that alterations in parasite genotype, phenotype and often virulence can be tracked in real time (Ebert 1998). During SPEs parasites are propagated by transfer to a naïve host or to a new artificial environment (such as culture media) at a specific point in time or life phase of the parasite (Ebert 1998, Ford et al. 2002).

Although it is commonly thought that parasite virulence attenuates in culture (Ebert 1998, Ford et al. 2002), evidence suggests shifts in virulence are influenced by passage timing, the point of the parasite's life cycle at which it is propagated (da Silva & Sacks 1987, Wozencraft & Blackwell 1987, Rey et al. 1990). Attenuated parasite strains often can rapidly revert to a virulent form when re-exposed to a naïve host (Cann et al. 1984, Macadam et al. 1989, Minor 1993, Nielsen et al. 2001). Thus it is increasingly clear that virulence can be greatly affected by how parasites are maintained in culture. Understanding how parasites evolve in culture, including the evolution of attenuation, may aid in understanding the evolution of virulence, and may also have practical applications. For example, the ability to reliably infect hosts in controlled conditions is critical to the study of disease (Ford et al. 2002). Because *in vitro* serial culturing puts selective pressures on parasites that may lead to attenuation, the culture history of a parasite may distort the outcome of experiments that are aimed at understanding host-pathogen interactions in nature, where hosts encounter strains of the pathogen with very different histories. This makes it necessary to understand how laboratory culture practices can influence parasite growth, development and virulence.

We used SPEs to investigate growth and virulence of a lethal amphibian pathogen. The fungus *Batrachochytrium dendrobatidis* (*Bd*) causes the disease chytridiomycosis and is highly virulent to multiple species of amphibians in laboratory infection experiments (Berger et al. 1998, 2005b, Woodhams et al. 2003, Daszak et al. 2004, Carey et al. 2006, Lips et al. 2006). However, not all species of amphibians succumb to disease in exposure experiments (Berger 2001, Daszak et al. 2004, Retallick & Miera 2007, Carey et al. in prep). In wild amphibian populations outbreaks of chytridiomycosis have dramatically reduced the abundance of amphibian species with local and global extinctions reported (Lips et al. 2006, Schloegel et al. 2006, Woodhams et al. 2008b) but some species and populations of species that have survived initial declines now persist in the wild with a wide range of levels of infection (Retallick et al. 2004, Woodhams & Alford 2005, Kriger & Hero 2007a,b). These apparent changes in the effects of *Bd* infection on individuals and populations is likely due to environmental conditions influencing disease dynamics but also, it suggests the possibility of differences in host susceptibility, differences in parasite virulence or both. Although many studies have focused on the roles of innate immunity (Rollins-Smith et al. 2002a, Rollins-Smith et al. 2002b, Woodhams et al. 2004, Rollins-Smith et al. 2005, Woodhams et al. 2006a, Woodhams et al. 2006b, Woodhams et al. 2007b) and behaviour (Rowley 2006, Rowley & Alford 2007) in host susceptibility, the possibility of differential virulence has not been thoroughly examined (Retallick & Meira 2007, Fisher et al. 2009).

Many investigators have observed that growth patterns differ among *Bd* isolates in culture (Symonds et al. 2008, Fisher et al. 2009, James et al. in prep, personal

communication R. James, R. Webb, S. Bell, N. Kenyon, E. Davidson and D. Woodhams) and preliminary data suggests that *Bd* virulence may differ among isolates when susceptible species are exposed to them in infection experiments (Berger et al. 2005, Retallick & Miera, 2007, Fisher et al. 2009, personal communication D. Woodhams, C. Briggs, N. Kenyon). Reduced mortality has been observed in populations after surviving initial emergence (Morgan et al. 2007) and different populations, even within species, can experience a range of the effects following initial outbreaks (Woodhams & Alford 2005, Briggs et al. 2005). Temperature has been consistently shown to be an important factor in determining the population effects of chytridiomycosis (Berger 2001, Bradley et al. 2002, Woodhams et al. 2003, Berger et al. 2004, Retallick et al. 2004, Woodhams & Alford 2005, Carey et al. 2006, Drew et al. 2006, Kriger & Hero 2006, Pounds et al. 2006, Rachowicz et al. 2006, Kriger & Hero 2007a, b, Rowley & Alford 2007, Kriger & Hero 2008, Muths et al. 2008, Skerratt et al. 2008), however, the other determinants of this variation within species are unknown. Because amphibians often become morbid and die from chytridiomycosis when intensities of infection are high, the rate production of infectious *Bd* zoospores is likely to be an important factor in pathogenesis and *Bd* virulence (Carey et al. 2006). Therefore, we quantified zoospore production among *Bd* isolates and within replicates of a single isolate with differing passage histories. We also measured the virulence of *Bd* cultures that had experienced different passage histories using a frog exposure experiment.

## Methods

### *Experiment 1- Comparing three* Batrachochytrium dendrobatidis *isolates*

Isolates GibboRiver-L.lesueuri-00-LB, TullyRiver-L.rheocola-07-LB and Rockhampton-L.caerulea-99-LB, were originally obtained from diseased *Litoria lesueuri*, *Litoria rheocola* and *Litoria caerulea* respectively. All isolates were purified and cultured on tryptone/gelatin hydrolysate/lactose (TGhL) agar with antibiotics (Longcore et al. 1999) and then passaged into liquid TGhL broth (tryptone, gelatin hydrolysate, lactose) (Longcore et al. 1999) into 25-$cm^2$ cell culture flasks. Cultures were maintained at James Cook University in TGhL broth at 4°C and passaged every 2 to 3 months.

The *Bd* cultures were filtered to remove sporangia using sterile filter paper (Whatman, 3). Cultures were washed by centrifuging (500 g for 10 minutes), after removing the supernatant the zoospores were resuspended in sterile dilute salt solution (in mMol: $KH_2PO_4$ (1), $CaCl_2.H_2O$ (0.2), $MgCl_2.2H2O$ (0.1). Zoospore concentrations were determined using a haemocytometer (Improved Neubauer Bright-line) and adjusted as needed by addition of dilute salt solution to a concentration of 90 x $10^3$ zoospores $ml^{-1}$. Experiments were done using sterile 96 well plates (Tissue culture test plates-96, TPP) that were organized with three sections for each isolate. Zoospore inocula (50 µL) were each pipetted into 20 wells containing 50 µL TGhL media. The plate had a perimeter of 36 wells with 100 µL sterile water to avoid evaporation. Plates were inspected microscopically each day to monitor zoospore encystment, development and maturation of the zoosporangia. Once the maturing zoosporangia produced the first zoospores, zoospore density was quantified daily by randomly selecting 10 wells containing each of

the three isolates, drawing off 30 µL of supernatant and counting zoospore numbers using a haemocytometer.

***Experiment 2- Multiple passages with one* Batrachochytrium dendrobatidis *isolate***

The isolate, GibboRiver-L.lesueuri-00-LB-1, was originally obtained from a diseased juvenile *L. lesueuri*, cultured on tryptone/gelatin hydrolysate/lactose (TGhL) agar with antibiotics (Longcore et al. 1999) and then cryoarchived (Boyle et al. 2003). An aliquot of the cryoarchived culture was revived (Boyle et al. 2003) and then passaged into liquid TGhL broth (Longcore et al. 1999) in 25-$cm^2$ cell culture flasks. Flasks were visually inspected daily to monitor zoospore encystment and maturation of the zoosporangia. Cultures were passaged into new media when zoospore density was near its maximum, for 50 passages. This culture with a higher number of passages will be referred to as "P50". A second aliquot of the same isolate was revived 250 d later and was treated identically for 10 passages. This culture with a lower number of passages will be referred to as "P10". Zoospore concentrations were determined using a haemocytometer (Improved Neubauer Bright-line) and adjusted as needed by addition of dilute salt solution to a concentration of 93 x $10^4$ zoospores $ml^{-1}$. The two isolates were inoculated into 96-well plates and zoospore production was quantified daily (10 wells per culture per day) as described above.

Experiment 3- Experimental exposures of *Litoria caerulea*

Adult common green tree frogs (*Litoria caerulea*; $n = 30$, mean mass: 21.34 ± 5.64 s.d.) were collected in January and February, 2008 from residential areas of Townsville, Queensland. Each frog was collected using a new plastic bag and then transferred to an individual plastic container (200x240x330 $mm^3$) that was maintained in temperature (18-23ºC) and light (12L/12D) controlled facilities at James Cook University, Townsville, Australia. Frogs were fed vitamin-dusted crickets (medium-sized, Pisces Inc.) *ad libitum* twice per week. Tap water (250 ml) was changed twice a week until experimental exposures began and was then replaced by 20% Holtfretter's solution (in mMol: NaCl (6), KCL (0.06), $CaCl_2$ (0.09), $NaCO_3$ (0.24), pH 6.5, 250 ml). Containers were maintained in a level position so water covered the bottom but frogs were able to climb up the dry walls. To confirm that frogs were not infected with *Bd* prior to inoculation, samples for testing for *Bd* were obtained by rubbing a sterile cotton swab over the ventral surfaces and digits (Hyatt et al. 2007). Diagnosis of *Bd* infection status was performed using a Taqman real-time polymerase chain reaction (PCR) assay (Boyle et al. 2004). All samples were analyzed in triplicate and compared with Australian Animal Health Laboratory zoospore standards to determine zoospore equivalents.

Zoospores from isolates with high (P50) and low (P10) passage histories were filtered to remove sporangia (described above). Zoospore concentrations were determined using a

haemocytometer (Improved Neubauer Bright-line) and adjusted as needed to reach 93 x $10^4$ zoospores $ml^{-1}$ by diluting with dilute salt solution. Frogs were randomly assigned to exposure (P50 and P10 isolate treatments) and control groups. Frogs were exposed to *Bd* in plastic containers via shallow immersion in a bath of Holtfretter's solution containing zoospores. Uninfected control frogs were held in a bath of with added the same volume of TGhL but without zoospores. After 24 h frogs were moved to fresh containers with 20% Holtfretter's solution (pH 6.5).

Frogs were randomly assigned to exposure (P50 and P10 isolate treatments) and control groups. Exposure solutions (P50 and P10 isolate treatments) consisted of *Bd* zoospores in a dilute salt solution and 20 % Holtfretter's solution (Wright et al. 2001). Frogs were exposed to *Bd* zoospores in plastic containers via shallow immersion in a bath of exposure solutions. Uninfected control frogs were held in a bath containing equal volumes (equal to volumes in exposure solutions) of sterile TGhL with no *Bd* zoospores and Holtfretter's solution. After 24 h frogs were moved to fresh containers with 20% Holtfretter's solution (pH 6.5). Following exposure to *Bd*, frogs were swabbed again at 21, 58 and 108 d post exposure.

Results

***Experiment 1- Comparing three* Batrachochytrium dendrobatidis *isolates***

We hypothesized that zoospore densities might differ among isolates. To test this hypothesis we recorded time to zoospore release (number of days to first observed zoospores) and quantified zoospore densities on the first day of zoospore release and on the day of maximum zoospore density of three *Bd* isolates. Microscopic inspection of these cultures suggested that their rates of maturation and morphological characteristics were not substantially different (Figure 82), but zoospore densities among the three isolates were significantly different (repeated measures ANOVA, $P < 0.001$; Figure 83). New zoospores were visible and active on day 3 for all three isolates. Zoospore densities in isolates GibboRiver-L.lesueuri-00-LB (155.3 ± 50.82) and TullyRiver-L.rheocola-07-LB (171.5 ± 25.17) did not differ on the first day of zoospore release (ANOVA with Tukey HSD test $P = 0.545$) but were significantly different than zoospore densities in Rockhampton-L.caerulea-99-LB (5.25 ± 2.71: ANOVA with Tukey HSD test $P < 0.001$; Figure 83). On the respective days of peak zoospore densities, the GibboRiver-L.lesueuri-00-LB isolate (day 4: 213.6 ± 33.87) had significantly more zoospores than TullyRiver-L.rheocola-07-LB (day 3: 171.5 ± 25.18) and Rockhampton-L.caerulea-99-LB (day 4: 38.13 ± 8.20 ANOVA $P < 0.001$). For all three isolates the period of maximum zoospore densities was completed by day six but zoospores were active to ten days following initial inoculations.

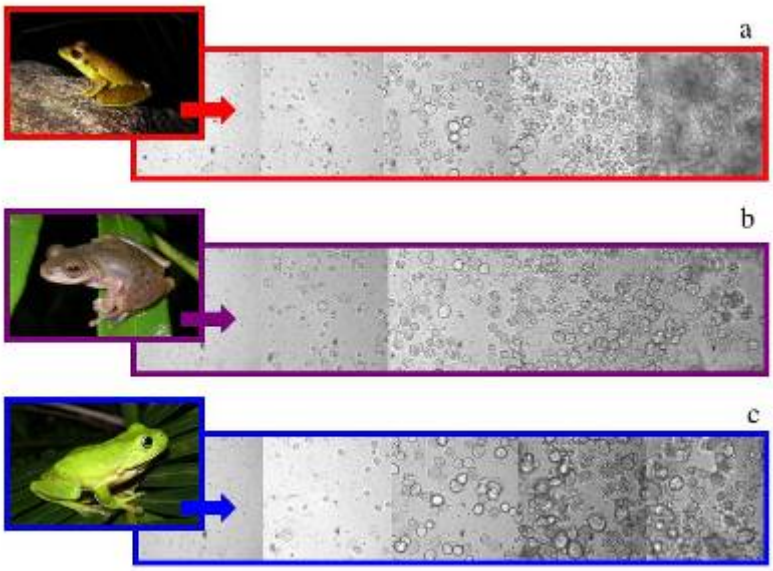


**Figure 82.** Light microscopy images of three isolates of *Batrachochytrium dendrobatidis*: (a) GibboRiver-L.lesueuri-00-LB, (b) TullyRiver-L.rheocola-07-LB, (c) Rockhampton-L.caerulea-99-LB. Images show successive developmental stages of *Bd* from active zoospores (far left) to mature sporangia (far right). The images of frogs (left side) are representative images of the frog species from which each isolate was originally cultured. The colors match the lines in Figure 5.2 showing zoospore production for each isolate.

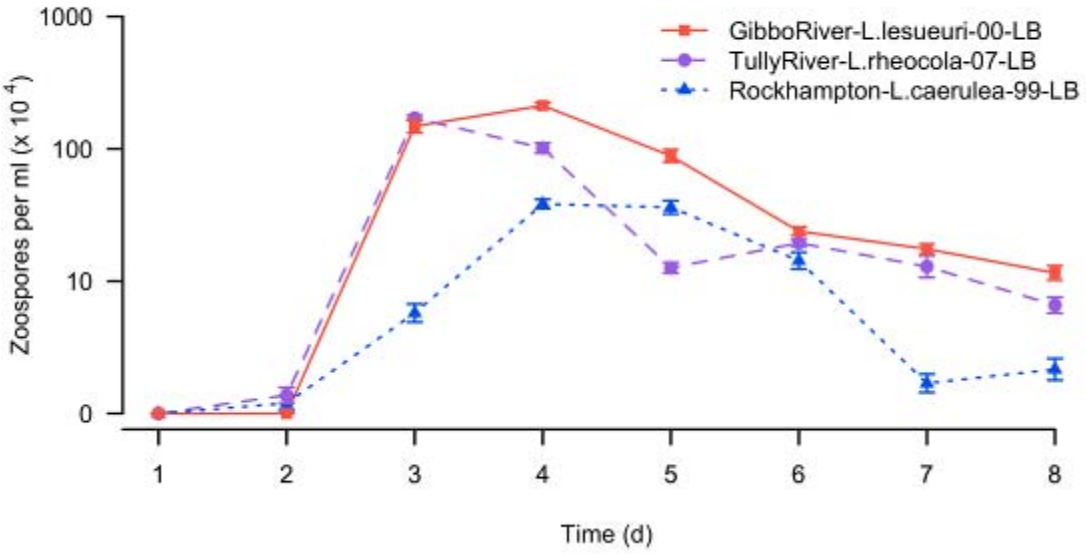


**Figure 83.** Zoospore density over time in three isolates of *Batrachochytrium dendrobatidis*, red: GibboRiver-L.lesueuri-00-LB-P24, purple: TullyRiver-L.rheocola-07-LB-P23, blue: Rockhampton-L.caerulea-99-LB-P24.

***Experiment 2- Multiple passages with one* Batrachochytrium dendrobatidis *isolate***

By serially passaging the GibboRiver-L.lesueuri-00-LB isolate at the peak of its zoospore density, we eliminated the contribution of zoospores produced after this peak in each generation. This produced strong selection for early zoospore release and maximum zoospore densities. We predicted that 50 passages with this selective pressure should produce a greater response than 10 passages. We found significant differences between the high and low passage isolates patterns of zoospore density over time (repeated measures ANOVA, $P < 0.001$). New zoospores were observed in cultures from both histories three days after inoculations. The culture with the higher number of passages (P50), contained significantly more zoospores (127.2 ± 33.38) on the first day of zoospore release, day 4, than the culture with a lower number of passages (P10) (1.8 ± 1.2: Student's T test, $P < 0.001$; Figure 5.3). The P50 culture also had higher zoospore densities on the day of maximum zoospore densities than the P10 culture (P50, day 6, 58.8 ± 9.38, P10, day 5, 135.1 ± 36.06; Student's T test, $P < 0.001$; Figure 84). In both cultures the period of maximum zoospore density was completed by day 7 however, active zoospores were seen up to 14 days following initial inoculations.

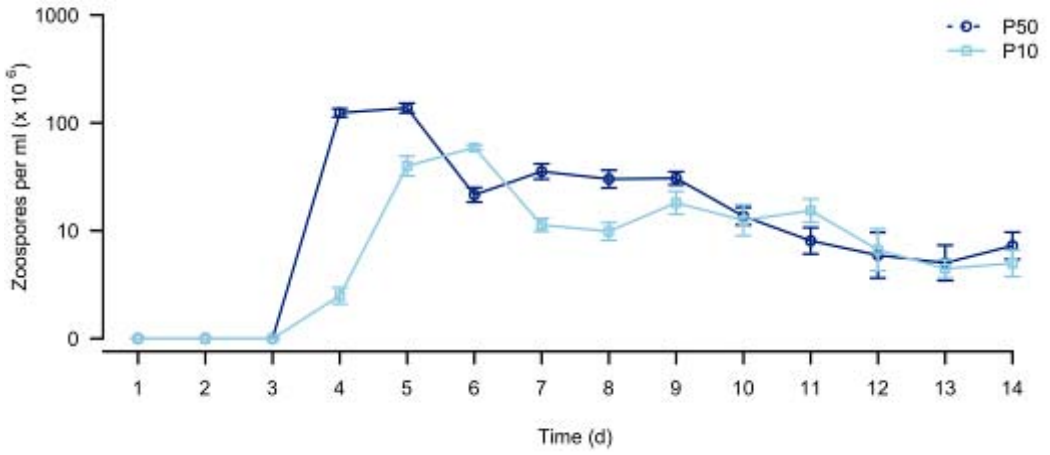


**Figure 84.** Zoospore densities over 14 days in culture of an isolate of *Batrachochytrium dendrobatidis*, GibboRiver-L.lesueuri-00-LB, that had two different passage histories. The dark blue line shows zoospores produced in an isolate that had been passaged 50 times (P50). The light blue line shows zoospores produced in an isolate that had been passaged 10 times (P10).

Experiment 3- Experimental exposures of *Litoria caerulea*

The same cultures as in experiment 2, P50 and P10, were used in an exposure experiment on adult frogs of *Litoria caerulea*. Although the results of experiment 2 indicated that selection had led to earlier zoospore release and higher zoospore densities in the P50 culture, this might not have produced increased virulence in infected frogs; if it reflected adaptation to conditions *in vitro*, virulence could have decreased. Unexpectedly, there was no mortality in any group. Mild clinical signs of infection including lethargy, inappetence and slight discoloration were recorded for two frogs in the P50 group and 2

frogs in the P10 group. More severe clinical signs of infection did not develop in any group. Frogs with mild clinical signs seemed to recover, regaining normal color, activity and appetite, by the termination of the experiment. There was a significant decrease in mass (paired T test, $P = 0.003$) in the P50 group whereas there was no significant change in the P10 group (paired T test, $P = 0.6641$). However, the control group also lost weight (paired T test, $P = 0.03$). The change in mass (final minus initial weight) was greater for the P50 group. However this is not significantly different from the P10 and control groups (ANOVA, $P = 0.48$ Figure 85).

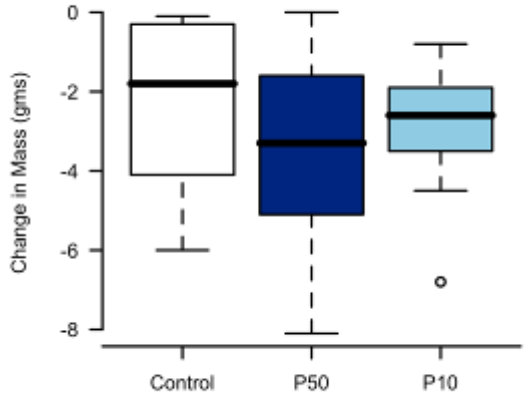


**Figure 85.** Change in mass (final weight minus initial weight) in *Litoria caerulea* experimentally exposed to two cultures (dark blue bar: P50 and light blue bar: P10) of the GibboRiver-L.lesueuri-00-LB isolate with different passage histories and in control frogs exposed to sterile medium.

Using PCR analysis on swab samples, we found that there was no significant difference in prevalence (Fisher's exact test, $P = 0.63$) in *L. caerulea* exposed to the P50 zoospores, 80% (8/10) became infected during the experiment. In comparison, 60% (6/10) frogs became infected in the group that was exposed to P10 zoospores. The frogs that became infected with the P50 zoospores had slightly higher intensities of infection than the frogs exposed to P10 zoospores, but there was no significant group effect (repeated measures ANOVA, $P = 0.156$; Figure 86).

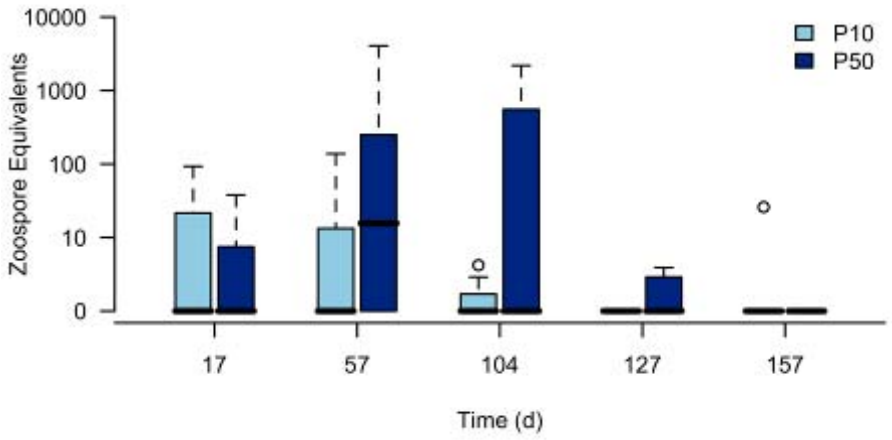


**Figure 86.** Intensity of infection in *Litoria caerulea* infected with one isolate of *Batrachochytrium dendrobatidis* with 2 different passage histories. The frogs exposed to zoospores from a higher passage culture (P50- dark blue) had greater intensities of infection than frogs exposed to zoospores from a lower passage culture (P10-light blue).

Discussion

Differential growth and virulence of *Batrachochytrium dendrobatidis* has been reported in several studies. However, it is unclear why this variation exists (Berger et al. 2005, Retallick & Miera 2007, Fisher et al. 2009). We quantified zoospore densities among three isolates originally cultured from different amphibian species and found that they had significantly different zoospore densities. Two of the isolates tested were known to be differentially virulent to *Litoria caerulea* in controlled infection experiments (Berger et al. 2005b). However, long-term maintenance practices for these isolates were not identical and it is therefore impossible to know whether it was their different origins or differences in their passage histories that affected their virulence.

By reviving two aliquots of a single isolate of *Bd*, GibboRiver-L.lesueuri-00-LB, and subjecting them to different passage histories, we demonstrated significant effects of passage history on zoospore densities. Zoospore densities of the P50 culture was not quantified early after revival, so the differences we observed could conceivably have been caused by differences in the aliquots that were revived to start the P50 and P10 cultures. However, both were samples from the same culture, and the P10 was frozen in liquid nitrogen until it was revived, so sample differences or effects of history during preservation seem very unlikely. Our results in experiment 2 were consistent with our predictions; by passaging at the point of peak zoospore density, we were selecting for earlier reproduction and because passaging involves subsampling, we were also selecting for the production of greater number of zoospores. The P50 culture reproduced earlier and produced more zoospores than the P10 culture with a shorter history of selection. Thus serial culturing practice appears to have affected the rate and temporal pattern of

zoospore production. The mechanisms underlying these effects are unknown. However, this study demonstrates that patterns of zoospore production can differ within a single isolate of *Bd*. These suggest that *Bd* may rapidly adjust and adapt to changes in conditions, and that the phenotypes of serially maintained cultures in the laboratory may diverge from those of *Bd* in nature.

Although quantifying patterns of zoospore density in culture is valuable for understanding rates of population growth of *Bd*, the importance of zoospore numbers for *Bd* virulence is less clear. In experiment 3 we found that the P50 culture that produced more zoospores and we predicted that the P50 might be more virulent. The patterns observed in our measured response variables, prevalence and intensity of infection, support this possibility. However, these differences were not significant and furthermore, there was no mortality in any group of *Litoria caerulea* exposed to P50 and P10 cultures. This result was unexpected because this isolate, GibboRiver-L.lesueuri-00-LB, was originally chosen for this investigation because it was highly virulent in other infection experiments. This isolate caused 90% mortality of *Litoria caerulea* in a separate experiment using virtually identical methods. Differences between the other experiment and the present study include the length of time frogs were held in captivity, the seasonal timing of exposure, and isolate maintenance practices (cultures for the previous infection experiment had comparable passage history, but were held at 4°C rather than 23°C). Therefore, it is difficult to explain the lack of mortality, and additional exposure experiments with similar methods are needed to resolve this question.

Our results do demonstrate that the P50 culture had earlier and higher zoospore densities and suggest that it may have been more virulent following serial culturing practices. This contradicts the general assumption that pathogens tend to attenuate in culture. Attenuation is presumably the result of pathogens becoming adapted to the conditions of culture and losing some of their ability to exploit hosts as resources. However, evidence from other systems suggests that attenuation should be highly dependent on the biology of the pathogen and the culturing practices (Rey et al. 1990, Wozencraft et al. 1987, DaSilva & Sacks 1987). Our results support that hypothesis; our culture system and medium may have been similar enough to host tissues in features that are important to the growth and proliferation of *Bd* that becoming adapted to rapid reproduction in culture also increased the reproductive rate of *Bd* in infected amphibian hosts.

Due to short generation times and high proliferation rates, pathogens can evolve rapidly (Cann et al. 1984, Macadam et al. 1989, Minor 1993, Ebert 1998, Nielsen et al. 2001). For disease research, stability of virulence properties presents a challenge (Michel & Garcia 2003) as virulence can evolve rapidly in both directions (Ebert & Bull 2003, Stearns & Koella 2007). However, changes in virulence also represent an opportunity to better understand the mechanisms of disease. For example, evolution of virulence that is manipulated towards attenuation but then reverts to high virulence may reveal factors that determine the optimal virulence for a particular host-pathogen dynamic. We found significant differences in zoospore densities, lifespan of zoospores and period of zoospore activity in *Bd* isolates maintained in cold (4°C) conditions for multiple generations. We also found that timing of pathogen propagation may influence growth and zoospore

production of *Bd*. If zoospore production plays a role in pathogenesis of chytridiomycosis, then serial culturing practices may impact the evolution of *Bd* virulence.

Understanding mechanisms of virulence and factors driving its evolution are important given the threat of infectious disease to wildlife and human health. Understanding the factors that influence pathogenicity will lead to a greater understanding of the disease and thus, direct more effective conservation action for threatened amphibians. With advancements in basic virulence and disease research we may be able to distinguish novel pathogens from pathogens that have increased in virulence (Daszak et al., 2000, Rachowicz et al., 2005), assess potential disease risk (Kuiken et al. 2005), track pathogen movements (Kuiken et al. 2005, Kang et al. 2006), use attenuated strains as vaccines and incorporate disease resistance into breeding programs (Schlaepfer 2007). Management of infectious diseases will remain a challenge for continuing research until we can define the mechanisms of virulence and understand the evolutionary dynamics of infectious pathogens.

***Project 6.4 Electrolyte depletion and osmotic imbalance in amphibians with chytridiomycosis***

**Chapter 6 in Voyles, J. 2009. Virulence and pathogenesis of chytridiomycosis: A lethal disease of amphibians. Ph.D. thesis, James Cook University**

Published as: Voyles J, Berger L, Webb R, Young S, Speare R, Werner J, Rudd D, Campbell R, Skerratt LF (2007). Electrolyte disruption and osmotic imbalance in amphibians with chytridiomycosis. Diseases of Aquatic Organisms 77, 113-118.

## Abstract

Mounting evidence implicates the disease chytridiomycosis, caused by the fungus *Batrachochytrium dendrobatidis* (*Bd*), in global amphibian declines and extinctions. While the virulence of this disease has been clearly demonstrated, there is, as yet, no mechanistic explanation for how *Bd* kills amphibians. To investigate the pathology of chytridiomycosis blood samples were collected from uninfected, aclinically infected and clinically diseased amphibians and analyzed for a wide range of biochemical and hematological parameters. Here we show that green tree frogs (*Litoria caerulea*) with severe chytridiomycosis had reduced plasma osmolality, sodium, potassium, magnesium and chloride concentrations. Stable plasma albumin, haematocrit and urea levels indicated that hydration status was unaffected, signifying depletion of electrolytes from circulation rather than dilution due to increased water uptake. These results suggest that *Bd* kills amphibians by disrupting normal epidermal functioning leading to osmotic imbalance through loss of electrolytes. Determining how *Bd* kills amphibians is fundamental to understanding the host-pathogen relationship and thus the population declines attributed to *Bd*. Understanding the mechanisms of mortality may also explain interspecific variation in susceptibility to chytridiomycosis.

## Introduction

Amphibians are currently undergoing the fastest rate of extinction of any vertebrate group (Stuart et al. 2004). While habitat destruction or overexploitation are obvious primary causes in some declines, determining why amphibians are experiencing catastrophic declines in protected areas has been more challenging. Amphibian mass-mortalities and declines have coincided with the appearance of the fungal pathogen *Batrachochytrium dendrobatidis* in wild amphibian communities (Briggs et al. 2005, Lips et al. 2006, Woodhams et al. 2008b). This fungus is lethal to many species of amphibians (Longcore et al. 1999, Nichols et al. 2001, Daszak et al. 2004, Lips et al. 2006, Berger et al. 1998, 2005b, Carey et al. 2006), yet the mechanism by which it causes death is unknown (Gascon et al. 2007, Mitchell et al. 2008, Rosenblum et al. 2008, Wake & Vredenburg 2008). *Batrachochytrium dendrobatidis* is confined to the superficial layers of the epidermis and causes no consistent pathological changes in internal organs (Berger et al.

1998, Pessier et al. 1999). Two hypotheses as to the cause of mortality in amphibians infected with *Bd* have been suggested. The first is that *Bd* disrupts osmoregulation in the skin of infected amphibians and the second is that *Bd* produces a toxin that affects organs (Berger et al. 1998, Berger 2001, Blaustein et al. 2005). These hypotheses are not necessarily mutually exclusive. However, no study has provided data to determine if either or both hypotheses are correct.

Amphibian skin is well studied due to its unique functions. The integument is a site of regulated transport for water, ions (electrolytes) and respiratory gases (Jorgensen et al. 1954, Alvarado & Kirschner 1963, Deyrup 1964, Moore & Lofts 1964, Fischbarg & Whittembury 1977, Shoemaker 1977, Larsen et al. 1987, Boutilier et al. 1992, Erspamer et al. 1994, Jorgensen 1994a, b, 1997, Wright et al. 2001, Word & Hillman 2005). Permeability of frog skin varies over the body surface of an individual and also among species (Deyrup 1964, Heatwole & Barthalmus 1994). In some species osmotic permeability is greatest in an area of ventral integument commonly referred to as the pelvic patch (Baldwin 1974, Czopek 1965, Word et al. 2005), where there is dense cutaneous vasculature (Czopek 1965). Concomitantly, *Bd* occurs more commonly and at higher density in the ventral integument of infected frogs (Berger et al. 2005c, Puschendorf & Bolaños 2006). *Batrachochytrium dendrobatidis* grows within the keratinized cells of the superficial epidermis and causes irregular skin sloughing, hyperplasia and hyperkeratosis (Berger et al. 1998, 1999, 2005 a,b,c Pessier et al. 1999). Other pathological changes including cytoplasmic degeneration and vacuolation in scattered cells have been observed by light and electron microscopy, but these changes are not usually severe (Berger 2001). Thus it is unclear how a superficial skin infection kills frogs.

The aim of this research was to investigate pathogenesis in amphibians with chytridiomycosis. We evaluated changes in physiological parameters after infection with *Bd* in the common green tree frog (*Litoria caerulea*), a species known to be susceptible to infection (Berger et al. 2005b). We found severely diseased frogs had reduced blood plasma osmolality and electrolyte concentrations indicating osmotic imbalance. Other biochemical and hematological parameters including plasma proteins, tissue enzymes and haematocrit were measured as markers of general health and did not vary significantly.

## Methods

Blood samples were collected twice over the course of infection during an outbreak in captive-bred *Litoria caerulea*. This outbreak was monitored by swabbing frogs every ten days for 73 days for *Bd* zoospore equivalents, determined by real-time PCR analysis (Boyle et al. 2004). Together with clinical signs of disease, most of which are apparent only in the few days before death (Berger 2001), these PCR results were used as indicators of severity of infection (Figure 4.1). Blood was collected for haematology and plasma biochemistry before and after frogs became diseased.

Frogs were housed individually at 18-23ºC and fed vitamin-dusted crickets. Ten *L. caerulea* became infected and two were uninfected. Within 16 days after suspected exposure, blood (< 1% frog weight) was collected with a heparinized syringe and needle via the ventral abdominal vein or heart after anesthesia by shallow immersion in a solution bath of 0.1% MS222 (tricaine methanesulfonate, Sigma Chemical). MS222 does not kill *Bd* (Webb et al. 2005). A sample of whole blood was analyzed (Bayer 865 rapidlab blood gas analyzer) for electrolyte concentrations, pH and carbon dioxide. Haemoglobin was measured using the modified Drabkin's method. Total red cells were counted using a modified Neubauer haemocytometer with Nat-Herrick's solution as the diluent. Plasma osmolality was measured using a Knauer automatic osmometer with 400 mMol $l^{-1}$ standards.

*Litoria caerulea* were monitored daily for clinical signs of chytridiomycosis including lethargy, inappetence, decreased respiration rate, cutaneous erythema, irregular skin sloughing and abnormal posture (legs splayed out posteriorly). A second blood sample was collected immediately before euthanasia for seven infected *L. caerulea* when they showed obvious clinical signs between 53 and 73 days post exposure (days 53, 56, 59. 59, 59, 60, 72) and for three infected and two uninfected frogs that were clinically normal (days 72 to 74). Samples were tested by blood gas analysis as previously described. A large range of blood biochemical parameters was measured to evaluate functioning of, or damage to, organs including liver, kidney muscle and pancreas. For this, plasma was analyzed for 16 biochemical parameters (amylase, lactate, aspartate, aminotransferase, creatine kinase, glutamate dehydrogenase, hemoglobin, platelets, haematocrit, albumin, bile acids, cholesterol, glucose, globulin, total protein and urea; Olympus AU400 at 37C at IDEXX Laboratories, Brisbane).

These methods were performed with James Cook University Animal Ethics Committee approval (Permit No. A593). All data were analyzed using SPSS statistics, version 12.0. Data sets were tested for normality and homogeneity of variance (homoscedasticity) and non-parametric instead of parametric tests were used when violations of assumptions occurred. Asterisks (*) in graphs and table indicate significance when $p < 0.05$.

**Results**

Skin swab samples collected on the date of death in diseased frogs ($n = 7$) and at termination of the experiment in aclinically-infected amphibians ($n = 3$), indicated *Bd* zoospore equivalents were greater in severely diseased amphibians (Mann-Whitney Test, $P = 0.017$; Figure 87). Swabs from uninfected amphibians ($n = 2$) were negative.

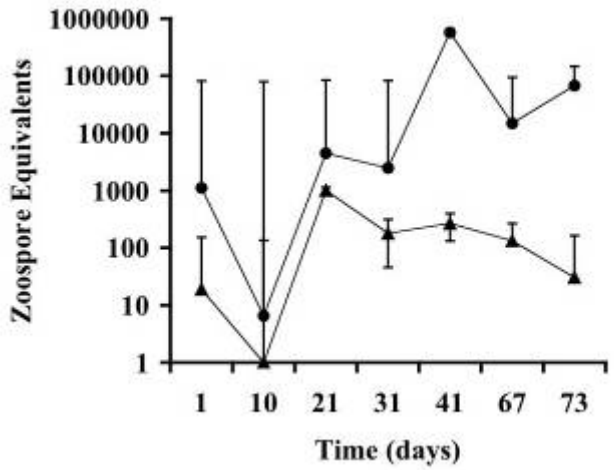


**Figure 87.** Zoospore equivalents over time in severely diseased (circles: $n = 7$) and aclinical (triangles: $n = 3$) *Litoria caerulea* infected with *Batrachochytrium dendrobatidis*. Uninfected amphibians (with zero zoospores) are not shown. Results are from real-time PCR on skin swabs.

There were no significant differences in blood test results between frogs that were uninfected and frogs that were infected but aclinical. However, amphibians with severe clinical signs of disease had significantly reduced plasma osmolality compared with both uninfected and aclinical groups combined (Table 22). Further, severely diseased frogs had significant reductions in plasma sodium, potassium, chloride and magnesium concentrations (Table 22). None of the other 16 biochemical parameters (listed in Methods) varied significantly (Table 22).

| Infection Status | Uninfectd/Aclinical | | | Clinical | | | |
|---|---|---|---|---|---|---|---|
| **Measured Variables** | **Mean** | **S.D.** | **N** | **Mean** | **S.D.** | **N** | **p-value** |
| Mass (gms) | 50.52 | 16.1 | 5 | 35.7 | 5.3 | 7 | p = 0.109 |
| **Electrolyte Concentrations** | | | | | | | |
| Osmolarity (mMol $l^{-1}$) | 217.2 | 28.5 | 5 | 153.5 | 46.6 | 6 | p = 0.026 * |
| Sodium (mMol $l^{-1}$) | 102.8 | 7.9 | 5 | 74.4 | 9.5 | 7 | p < 0.001 * |
| Potassium (mMol $l^{-1}$) | 2.74 | 0.3 | 5 | 1.5 | 0.8 | 7 | p = 0.016 * |
| Magnesium (mMol $l^{-1}$) | 0.98 | 0.4 | 5 | 0.3 | 0.2 | 7 | p = 0.027 * |
| Chloride(mMol $l^{-1}$) | 79 | 7.5 | 5 | 47.3 | 9.4 | 7 | p < 0.001 * |
| Sodium: Potassium Ratio | 32.52 | 3.5 | 5 | 30.2 | 6.6 | 6 | p = 0.491 |
| **Blood Gases** | | | | | | | |
| CO2 (pCO2 mmHg) | 35.54 | 12.2 | 5 | 25.3 | 9.7 | 6 | p = 0.153 |
| pH | 7.31 | 0.1 | 5 | 7.2 | 0.2 | 7 | p = 0.369 |
| **Enzymes/Markers** | | | | | | | |
| Amylase (IU $l^{-1}$) | 3100.6 | 948 | 5 | 3482.4 | 1517.5 | 5 | p = 0.646 |
| AST (IU $l^{-1}$) | 368 | 194.5 | 5 | 1003.3 | 895.2 | 7 | p = 0.154 |
| GLDH (IU $l^{-1}$) | 12 | 4.8 | 5 | 31.7 | 30 | 7 | p = 0.181 |
| Creatine Kinase (IU $l^{-1}$) | 1627.8 | 895.6 | 5 | 2782.4 | 2524.1 | 7 | p = 0.356 |
| **Hematology** | | | | | | | |
| RBC (x $10^{12}$ $l^{-1}$) | 0.42 | 0.96 | 5 | 0.52 | 0.17 | 6 | p = 0.291 |
| Hemoglobin (gms $l^{-1}$) | 0.163 | 0 | 5 | 0.2 | 0.1 | 6 | p = 0.704 |
| Platelets | 8680.2 | 5220.2 | 5 | 6523 | 3158.703 | 6 | p = 0.418 |
| PCV (%) | 22.8 | 5.6 | 5 | 26.5 | 7.1 | 6 | p = 0.371 |
| **Other Blood Constituents** | | | | | | | |
| Albumin (gms $l^{-1}$) | 26.6 | 5.4 | 5 | 30.1 | 3.9 | 7 | p = 0.212 |
| Bile Acids (µMol $l^{-1}$) | 7 | 8.5 | 5 | 35.7 | 41.1 | 7 | p = 0.160 |
| Cholesterol (mMol $l^{-1}$) | 1.4 | 0.9 | 5 | 2 | 2.3 | 5 | p = 0.630 |
| Glucose (mMol $l^{-1}$) | 3.62 | 1.1 | 5 | 3.6 | 2.1 | 3 | p = 0.964 |
| Globulin (gms $l^{-1}$) | 17.2 | 3.7 | 5 | 26.7 | 9.8 | 7 | p = 0.068 |
| Total Protein (gms $l^{-1}$) | 43.8 | 8.7 | 5 | 56.9 | 12.9 | 7 | p = 0.079 |
| Urea (mMol $l^{-1}$) | 9.02 | 2.4 | 5 | 11.5 | 8.1 | 7 | p = 0.531 |

**Table 22.** Biochemical and hematological parameters in *Litoria caerulea* infected with *Batrachochytrium dendrobatidis*. Designation of aclinically infected/uninfected or severely infected was determined by clinical signs and by measuring the number of zoospore equivalents by real-time PCR. Uninfected frogs were grouped with the aclinical frogs because there were no significant differences between the two groups.

In addition to comparing results among groups of amphibians with different severities of disease, we evaluated the changes in a range of biochemical parameters within individual *L. caerulea* as infections progressed. Significant biochemical changes occurred solely in amphibians that developed severe disease. Plasma concentrations of sodium, potassium, chloride and blood pH in severely diseased amphibians were significantly decreased in final blood samples compared with initial blood samples (Figure 88). None of these changes were detected in aclinically infected or uninfected amphibians (Figure 88). Furthermore, no other measured parameter (carbon dioxide, glucose, red cell count, haematocrit and hemoglobin) varied significantly in any group over time.

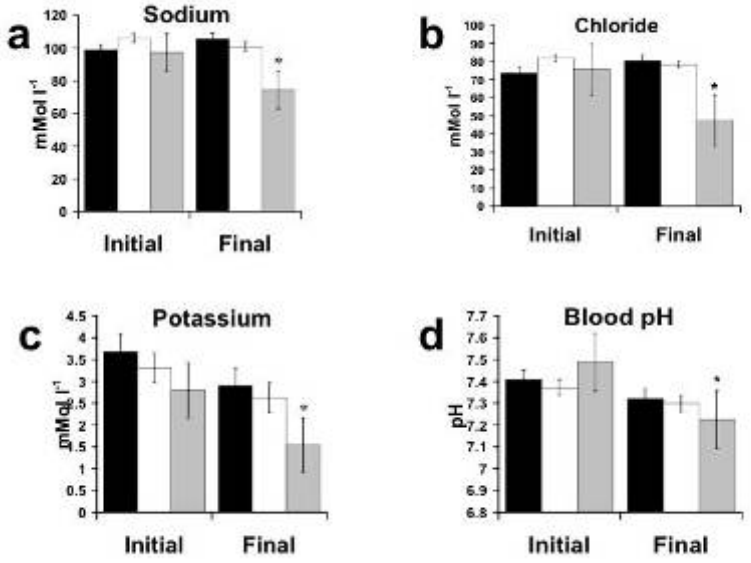


**Figure 88.** Changes in electrolyte concentrations and blood pH between initial and final blood samples in *Litoria caerulea* infected with *Batrachochytrium dendrobatidis*. The bar graphs show mean values (± s.e.m.). For this graph uninfected individuals are separated from aclinically-infected individuals. a, Plasma sodium concentrations were reduced in final blood samples from severely diseased *Litoria caerulea* (grey bars, $n = 7$, Paired T test, $P < 0.001$) but not in aclinically infected (white bars, $n = 3$) or uninfected (black bars, $n = 2$). b, Plasma chloride concentrations were reduced in final blood samples from the same severely diseased *Litoria caerulea* (Paired t test, $P < 0.001$) but not in aclinically infected or uninfected amphibians. c, Plasma potassium concentrations were reduced in final blood samples from the same severely diseased *Litoria caerulea* (Paired T test, $P = 0.036$) but not in aclinically infected or uninfected amphibians. Blood pH was reduced in final blood samples from the same severely diseased *Litoria caerulea* (Paired T test, $P = 0.006$) but not in aclinically infected or uninfected amphibians.

## Discussion

We provide evidence that severe chytridiomycosis causes decreases in blood pH and plasma osmolality sodium, potassium, magnesium and chloride concentrations. Reduction in electrolyte concentrations could occur if electrolytes were diluted due to increased water uptake or if there was an overall loss from the blood. We found no significant change from initial to final measurements in body mass, haematocrit, albumin or urea, indicating that hydration status is probably unaffected in severely diseased frogs. Therefore, it appears that the reductions in electrolyte concentrations were due to depletion from circulation rather than water uptake. The disproportionate loss of electrolytes compared with water signifies an imbalance in osmotic homeostasis.

Osmotic balance in amphibians is complex because there are multiple sites of regulation (Deyrup 1964, Heatwole & Barthalmus 1994, Jorgensen 1997). Amphibian skin is one site of regulation and is critical to water and electrolyte homeostasis (Deyrup 1964, Heatwole & Barthalmus 1994, Jorgensen 1997). Water flow results when an osmotic gradient is established by an exchange of electrolytes across the integument (Ussing & Zerahn 1951, Deyrup 1964, Erspamer et al. 1994, Jorgensen 1997). This tightly regulated transport is influenced not only by the salinity of the external aquatic environment, but also by plasma osmolality (Parsons et al. 1990) and skin circulation (Erspamer et al. 1994). Thus cutaneous osmoregulation and plasma osmolality are linked. Further, damage to amphibian skin can lead to fatal electrolyte imbalances (Wright et al. 2001). We suggest that *Bd* disrupts normal cutaneous transport, possibly through the alteration of electrolyte channels, leading to electrolyte loss and osmotic imbalance in diseased amphibians.

Pathogens are known to compromise function in other epithelia such as mammalian trachea and intestine (Kunzelmann et al. 2000, Berkes et al. 2003). Electrolyte transport is disrupted by pathogen adhesion to protein-based receptors (Cameron & Douglas 1996, Kunzelmann et al. 2000, Berkes et al. 2003) or by pathogen-secreted toxins that alter cell function (Kunzelmann et al. 2000, Berkes et al. 2003). For example, sodium transport and fluid balance were disrupted when a viral pathogen was introduced to mouse tracheal epithelium (Kunzelmann et al. 2000). This resulted from a down-regulation of amiloride-sensitive sodium channels in the apical membranes of the epithelium (Kunzelmann et al. 2000). It is possible that *Bd* disrupts sodium channels by a comparable mechanism. The selective barrier properties of frog skin are primarily determined by electrolyte transport in the mitochondrial-rich cells of stratum granulosum (Masoni & Garcia-Romeu 1979, Brown et al. 1981, Larsen et al. 1987, Larsen et al. 1996, Ehrenfeld 1997), where *Bd* is found in severe infections (Berger et al. 1998, Berger et al. 1999b, Berger 2001, Berger et al. 2005c, Puschendorf & Bolanos 2006, North & Alford 2008). This hypothesis is further supported by Ussing chamber tests (Ussing & Zerahn 1951) using skin samples from infected *Bufo woodhousii,* which showed that active sodium transport was reduced in diseased toads under short-circuit conditions (Voyles et al. 2005). However, these results did not clarify if the loss of sodium and other electrolytes could occur via the skin or other excretion pathways. More specific tests investigating disruption of electrolyte transport are underway.

Although our results do not detail the exact mechanism by which *Bd* disrupts epidermal functioning, the severe reduction in plasma electrolytes is a plausible cause of mortality. Reduced plasma osmolality and reduced plasma electrolyte concentrations, particularly hyponatremia (low sodium) and hypokalemia (low potassium), are potentially life-threatening conditions because these electrolytes are crucial in cell membrane function. In addition, sodium and potassium facilitate action potential conduction in smooth and cardiac muscle and are important in multiple physiological processes.

The three *L. caerulea* that appeared healthy despite infection had no significant changes in plasma osmolality or electrolyte concentrations. These results suggest that electrolyte reductions occur only in terminal stages of infection and may account for the

neurological signs such as muscle tetany that precede death (Berger 2001). While most amphibians can tolerate changes in plasma electrolyte levels (Deyrup 1964), the observed decrease of approximately 30% plasma sodium and 50% plasma potassium concentrations in diseased frogs may be too extreme. Additional research is needed to resolve whether these conditions lead to death through cardiac arrhythmia, myocardial failure, organ failure, or a combination of these and other factors.

Determining how *Bd* kills amphibians is essential to understanding the biology of chytridiomycosis. Additionally, research on the mechanisms of pathogenesis may explain why there is interspecific variation in susceptibility to the disease, a key question for amphibian conservation. Resolving the pathophysiological effects will provide crucial information for researchers, wildlife managers and veterinary clinicians in treating captive frogs and facilitating the recovery of wild frog populations that are currently affected by the disease.

***Project 6.5 Pathogenesis of amphibian chytridiomycosis: a pathological perfect storm?***

**Chapter 7 in Voyles, J. 2009. Virulence and pathogenesis of chytridiomycosis: A lethal disease of amphibians. Ph.D. thesis, James Cook University**

Papers in preparation. Authors: Jamie Voyles, Sam Young, Lee Berger, Craig Campbell, Wyatt F. Voyles, Anuwat Dinudom, David Cook, Rebecca Webb, Ross A. Alford, Lee F. Skerratt, Virginia Boone, Rick Speare.

Published paper: Voyles, J, Young, S, Berger, L, Campbell, C, Voyles, WF, Dinudom, A, Cook, D, Webb, R, Alford, RA, Skerratt, LF, Speare, R. 2009. Pathogenesis of chytridiomycosis, a cause of catastrophic amphibian declines. ***Science*** 326:582-585.


Abstract

Fungi are rarely highly virulent as primary pathogens in terrestrial vertebrates and yet the fungal pathogen *Batrachochytrium dendrobatidis* (*Bd*), which causes the skin disease chytridiomycosis, is implicated in global amphibian declines and extinctions. How a superficial skin fungus could lead to catastrophic extirpations worldwide is perplexing. With few examples of disease-induced extinctions, chytridiomycosis is the subject of intensive investigations aimed at understanding disease emergence and its threat to global biodiversity. Central to these investigations is resolving the mechanism by which *Bd* causes death. Here we show a sequential alignment of pathophysiological changes associated with chytridiomycosis which are initially localized to the skin but progressively alter systemic functioning, leading to mortality. Experimentally infected green tree frogs (*Litoria caerulea*) had inhibited electrolyte transport in the skin and reduced plasma electrolyte concentrations with asystolic cardiac arrest as the terminal event. The unique importance of the skin in maintaining amphibian homeostasis, and the ability of *Bd* to disrupt critical cutaneous functions are two key factors that make a life-compromising pathophysiology and explain how mortality can occur in a wide range of amphibian species. The disease characteristics of chytridiomycosis, taken separately, might not be so devastating, but together create a "perfect storm" of concomitant variables that threaten amphibians worldwide. That these factors have converged, resulting in a global pandemic, provides insight into infectious diseases and the associated risks for the loss of biodiversity.


Introduction

The phrase “perfect storm” has been used to describe the convergence of multiple variables creating favourable conditions for unusual, but not unexplainable, events. The phenomenon of worldwide amphibian declines due to chytridiomycosis may well illustrate such a scenario. Initially there was a reluctance to accept disease as a direct driver of declines and extinctions (Alford & Richards 1997, Hero & Gillespie 1997, McCallum 2005). However, *Bd* is now recognized for its ability to spread rapidly though

amphibian populations, infect numerous, phylogenetically distant species, cause high mortality and persist even at low host densities (Berger et al. 1998, Retallick et al. 2004, Woodhams & Alford 2005, Lips et al. 2006, Skerratt et al. 2007). These disease characteristics not only render population recovery from chytridiomycosis especially difficult, but also present strong empirical evidence for disease-induced extinctions (De Castro & Bolker 2005, Mitchell et al. 2008). A "perfect storm" of severe disease can best be explained if all components, and their spatiotemporal alignment, are considered. In the case of chytridiomycosis, a gap remains in our understanding of all variables and a fundamental question remains unanswered: how does *Bd* kill amphibians? Resolving the mechanism of pathogenesis is imperative to demonstrate how a superficial skin fungus can cause devastating declines (Daszak et al 2003).

The details of the pahtogenesis of chytridiomycosis have evaded scientists for several reasons. First, cutaneous fungal infections are not commonly fatal without other predisposing factors (Schaechter et al. 1993, Mueller et al. 2004); so there were no similar systems for comparison. Second, the taxonomy of the organism was initially unknown and required creation of a monotypic genus in the Chytridiomycota, a phylum of fungi not previously known as pathogens of vertebrates (Longcore et al. 1999). Third, the location of the fungus in the most superficial layers of skin (Berger et al. 1998, Berger et al. 1999b 2005c) and the minimal host reaction to infection (Berger et al. 2005c, Woodhams et al. 2007) are unusual for fatal infections in vertebrates. Finally, there are no consistent pathological changes in internal organs of diseased amphibians detectable using light microscopy (Berger et al. 1998), rendering traditional methods used to understand pathogenesis ineffective. At the molecular level, differential expression of life-stage specific peptidase genes suggests *Bd* pathogenicity factors (Rosenblum et al. 2008). However, as in most diseases, determining the proximate cause of death in an infected host is inherently challenging because multiple physiological systems shut down prior to death. Given the cryptic mechanisms of disease development in chytridiomycosis, it was necessary to investigate the functional response to infection using pathophysiological techniques.

Amphibian skin is unique among terrestrial vertebrates because it is actively involved in the exchange of respiratory gases, water and electrolytes (Jorgensen et al. 1954, Alvarado & Kirschner 1963, Deyrup 1964, Moore & Lofts 1964, Fischbarg & Whittembury 1977, Shoemaker 1977, Larsen et al. 1987, Boutilier et al. 1992, Erspamer et al. 1994, Jorgensen 1994a, b, 1997, Wright et al. 2001, Word & Hillman 2005). In infected frogs, *Bd* is confined to the outer layers of the epidermis with higher intensities of infection in the ventral areas and the digits (Berger et al. 1998, Pessier et al. 1999, Puschendorf & Bolanos 2006). Due to the role of amphibian skin in maintaining osmotic balance, other studies have suggested that *Bd* might disrupt cutaneous osmoregulatory functioning (Berger et al. 1998, Voyles et al. 2005). To investigate this possibility, we performed multiple, linked infection experiments with green tree frogs (*Litoria caerulea*), which are susceptible to chytridiomycosis in laboratory infection experiments (Berger et al. 2005b).

***Methods: General***

*While tracking the development of infections, we measured epidermal electrolyte transport in isolated skin preparations with Ussing chambers, measured biochemical parameters in blood and urine, assessed changes in epidermal condition from biopsy samples, and followed cardiac electrical activity using implanted biotransmitters. To determine if the changes in biochemical parameters are specific to chytridiomycosis, an additional infection experiment was performed in which an electrolyte-enriched solution was administered to frogs that became severely diseased. Also, the laboratory results from severely diseased L. caerulea were compared with blood values from free-living, healthy L. caerulea and L. caerulea with other lethal conditions.*

*Animal care*— Adult common green tree frogs (*Litoria caerulea*; n = 66, mean mass ± s.e.m: 62.46 ± 2.4 g) were collected in January and February 2007 and 2008 from residential areas of Townsville, Queensland. Each frog was collected with a new plastic bag and then transferred to individual plastic containers (200x240x330 $mm^3$) in temperature- (18-23ºC) and light- (12L/12D) controlled facilities at James Cook University, Townsville, Australia. Frogs were fed vitamin-dusted crickets (medium-sized, Pisces Inc., Kenmore, Australia) *ad libitum* twice per week. Tap water (250 ml) was changed twice a week until experimental exposures began then tap water was replaced by 20% Holtfretter's solution (in mM: NaCl (6), KCL (0.06), $CaCl_2$ (0.09), $NaCO_3$ (0.24), pH 6.5, 250 ml). Containers were maintained in a level position so water covered the bottom but frogs were able to climb up the dry walls.

*Diagnosis of* Batrachochytrium dendrobatidis *infections*— To confirm frogs were not infected with *Bd* prior to inoculation, swabs samples were collected from each individual frog by rubbing a sterile cotton swab over the ventral surfaces and digits (Hyatt et al. 2007) and polymerase chain reaction (PCR) for *Bd* was performed (Taqman real-time PCR assay, Boyle et al. 2004). Following exposure to *Bd*, frogs were swabbed at intervals of 6 to 12 days. All swab samples were analysed in triplicate and compared with Australian Animal Health Laboratory zoospore standards to determine zoospore equivalents.

*Culture of* Batrachochytrium dendrobatidis— Isolates were originally obtained from diseased frogs, cultured on tryptone/gelatin hydrolysate/lactose (TGhL) agar with antibiotics (Longcore et al. 1999) and then maintained in TGhL broth at 4°C and passaged every 2 to 3 months. The *Bd* isolate used for parts 1-5 was cultured from a wild adult *Litoria rheocola* from Tully River, Australia, identified as TullyRiver-L.rheocola-06-LB-1-p9 following the naming scheme proposed in Berger et al. (2005b). The isolate used for experiment 6 was from a juvenile *Litoria lesueuri*, GibboRiver-L.lesueuri-00-LB-1-p34. This isolate had been cryoarchived soon after isolation 7 years prior (Boyle et al. 2003). Zoospores for animal infections were harvested using dilute salt solution (in mMol: $KH_2PO_4$ (1), $CaCl_2.H_2O$ (0.2), $MgCl_2.2H2O$ (0.1)) after 4 d on agar plates maintained at 23°C and were counted in a haemocytometer (Boyle et al. 2004, Berger et al. 2005b).

*Animal infections*— Frogs were randomly assigned to exposure and control groups and exposed to *Bd* in plastic containers via shallow immersion in a bath of Holtfretter's

solution containing zoospores. Dose varied by experiment (details below). Uninfected control frogs were held in a bath of Holtfretter's solution with added dilute salt solution collected from agar plates without *Bd* cultures. After 24 h, frogs were moved to fresh containers with 20% Holtfretter's solution (pH 6.5) that was changed twice weekly for the rest of the experiment.

*Disease assessment*— Frogs were monitored daily for clinical signs of chytridiomycosis including lethargy, inappetence, cutaneous erythema, irregular skin sloughing, abnormal posture (hind legs abducted) and loss of righting reflex. Righting reflex tests was done by placing the frogs in dorsal recumbency and recording the time taken to reorient to a normal position. Clinical signs were scored to provide a semi-quantitative assessment of the severity of disease. Frogs with no clinical signs of infection scored a 0 and were designated as aclinical. Frogs scoring 1, 2, or 3 were designated as clinically diseased according to severity of clinical signs (1: lethargy and inappetence. 2: lethargy, inappetence, cutaneous erythema, and irregular skin sloughing. 3: lethargy, inappetence, cutaneous erythema, irregular skin sloughing, abnormal posture and loss of righting reflex).

Statistics— All data were analysed using R, version 2.7.1. Due to the high number of dependent variables, plasma and urine biochemical parameters were first analysed for dimension reduction using Principle Components Analysis. Four or more principle components were required to explain >75% of the data. Considering the high degree of independence among the variables, a general linear model was used with a Bonferroni correction technique: significance at $P = 0.004$ (plasma) and $P = 0.006$ (urine). A Bonferroni correction was also used for the Ussing chamber results, significance at $P = 0.012$. All data sets were tested for normality (Q-Q plots and Shapiro-Wilk's test), homogeneity of variance (Bartlett's test) and, where appropriate, sphericity (Maulchy's test). The results for PCR were log-transformed prior to statistical tests.

**Methods: Exposure experiments**

Blood, urine and skin biopsy samples were collected prior to exposure and over the course of infection to evaluate changes in physiological biochemistry and epidermal condition. Infected frogs reached terminal stages of infection asynchronously and therefore, to control for temporal sampling effects, we simultaneously tested a severely diseased frog with an uninfected frog and an infected but aclinical frog. Data were grouped by collection period: pre infection, post infection and late infection (following the first death, 60 d post infection). Due to the area of skin required for Ussing chamber (electrolyte transport) experiments, these tests could only be completed once, during late infection; sampling occurred when infected frogs showed clinical signs of severe disease.

For methods Parts 1-5, frogs in exposure groups were held in rectangular plastic containers (170x110x70 $mm^3$) that contained a 20% Holtfretter's bath solution (50 ml) inoculated with $5 \times 10^5$ *Bd* zoospores. Frogs in control groups were held in a solution inoculated with an equivalent volume of dilute salt solution with no *Bd* zoospores.

Part 1. Plasma Biochemistry

We measured a large range of plasma biochemical parameters to assess functioning of, or damage to, organs including liver, kidney, muscle and pancreas. Blood samples were collected 20 d prior to exposure, 30 d post infection and at late stages of infection when infected frogs showed obvious clinical signs of disease. Blood samples (< 1% frog weight) were collected with a heparinised syringe and needle via cardiac puncture after anaesthesia by shallow immersion in 0.1% MS222 solution (tricaine methanesulfonate, Sigma Chemical) buffered with sodium bicarbonate (0.4%, Sigma Chemical), which does not kill *Bd* (Webb et al. 2005). Although anaesthesia for blood sampling can be stressful for amphibians, it does not alter blood electrolyte concentrations (Andersen & Wang 2002). Blood samples were collected into 0.6 ml lithium heparin tubes (Microtainer, Becton-Dickson), immediately centrifuged (10,000 g for 10 m) and the supernatant was decanted and frozen at -80°C. Plasma was later analysed for 12 biochemical parameters: calcium, chloride, potassium, sodium, aspartate aminotransferase, creatine kinase, albumin, bile acids, globulin, glucose, phosphorus and total protein (VetScan VS2 Chemistry Analyzer, Abaxis Inc., Union City, CA 94587).

Part 2. Urine Biochemistry

To avoid interfering with osmotic conditions, urine samples were collected opportunistically, in clean plastic containers while swabbing for PCR, rather than by forced expression. Urine samples were analysed for six biochemical parameters (osmolality, sodium, potassium, magnesium, chloride, calcium) at Gribbles Pathology (Melbourne, Australia). Seven additional biochemical parameters (glucose, bilirubin, urobilinogen, ketones, protein, blood and pH were measured using Multistix Reagent Strips for Urinalysis (Bayer Diagnostics).

Part 3. Histology of skin biopsies

Small (1-2 $mm^2$) skin biopsies were collected from the ventral abdominal region (2-3 cm lateral to midline) of anaesthetized frogs following blood sample collections (20 d prior to exposure, 30 d post infection and in the late stages of infection). Biopsy incisions were closed with Vet Bond Tissue Adhesive (3M Animal Care Products). Skin samples were fixed in 10% buffered neutral formalin, embedded in paraffin, sectioned and stained with haematoxylin and eosin using routine histological techniques (Humason 1967, Berger et al. 2002, Olsen et al. 2004).

Part 4. Electrolyte transport in isolated skin samples

Following collection of final blood samples, frogs were transported to the University of Sydney (Sydney, Australia). Immediately after neural disruption, ventral skin samples (0.8 $cm^2$) were collected from the lower abdominal pelvic patch region and mounted in a modified Ussing chamber. Apical and basolateral surfaces of the monolayer were perfused with a modified frog Ringer's solution (in mM: NaCl (112), KCl (2.5), D-glucose (10), Na2HPO4 (2), CaCl2 (1), MgCl2 (1), Na+-HEPES (5), HEPES (5), pH 7.4) maintained at room temperature. The tissue was allowed to equilibrate prior to experimentation. All tests were carried out under open-circuit conditions (Ussing & Zerahn 1951, Kunzelmann et al. 2000). The transepithelial potential difference ($V_{te}$) was measured with reference to the blood side of the epithelium, and equivalent short-circuit current calculated according to Ohm's law using the Acquire and Analyse software (Physiologic Instruments, San Diego, CA). The rate of sodium absorption across the frog skin samples was assessed as the component of the short circuit current blocked by the addition of amiloride (10 µM, Sigma Chemical) to the apical solution. Amiloride is known inhibitor of epithelial sodium channels (Benos 1979, Benos et al. 1981, Sariban-Sohraby & Benos 1986). We also examined the responsiveness of the skin samples to carbachol (100 µM, Sigma Chemical), a muscarinic agonist that activates chloride secretion in frog skin (Alvarado et al. 1975), and noradrenaline (10 µM, Sigma Chemical), an adrenergic agonist that activates sodium absorption and chloride secretion (Castillo & Orce 1997).

In a test of the toxin hypothesis, we also used a variety of techniques aimed at filtering a possible toxin from *Bd* cultures. The responsiveness of skin functioning from control *L. caerulea* skin samples were subjected to following treatments: 1) culture supernatant that was filtered to remove *Bd* sporangia and zoospores using a 0.2 micron filter, 2) solution of *Bd* that had been washed to remove media, resuspended and bead-beaten for protein extraction, 3) water collected from a container with a clinically diseased (terminal) *L. caerulea* and filtered to remove *Bd*, and 4. a control dilute salt solution (in mMol: $KH_2PO_4$ (1), $CaCl_2.H_2O$ (0.2), $MgCl_2$.2H2O (0.1). These solutions were randomized and blindly introduced to the apical side of the epithelium following tissue equilibration.

Part 5. Cardiac biotelemetry

Approximately 30 d after collections, eight frogs were anesthetized by shallow immersion in 0.2% MS-222 (as above) to surgically implant cardiac biotransmitters. Procedures for ETA Surgical manual (Data Sciences International, 2001) for rats were followed with the following modifications.

Anaesthetised frogs were positioned in dorsal recumbency in a shallow oxygen- perfused pool of sterile 0.9% saline solution and were intermittently flushed with solution. A 35 mm skin and abdominal musculature incision was made along the ventral abdominal midline, caudal to the sternum. Implants (Chronic use TA10ETA-F20, Data Sciences International) were positioned in the abdominal cavity and leads were shortened to approximately 20 mm to accommodate the small body size. Biotransmitter leads were secured in the left and right sternal musculature with single interrupted non-absorbable

sutures (4-0 Prolene monofilament, Ethicon Inc.) and the overlying skin incision closed using the same suture pattern and reinforced with Vet Bond Tissue Adhesive. Biotransmitters were secured intracoelomically via incorporation into the abdominal musculature suture line; both abdominal musculature and skin incisions were closed using single interrupted non-absorbable sutures (4-0 Prolene monofilament, Ethicon Inc.). Frogs were monitored in a shallow pool of perfused saline for anaesthetic recovery. Antibiotics (50 mg/ml, 10 mg/kg enrofloxacin, Bayer Animal Health Australia Ltd.) and analgesics (1mg/ml, 1mg/kg, ketoprofen, Nature Vet Ltd.) were administered orally immediately following recovery from surgery. Frog patients were closely monitored for 24 hours and checked twice daily for one week following surgery. Enrofloxacin (5mg/kg) and ketoprofen (1 mg/kg) were applied topically on the dorsal skin surfaces daily for the following week.

Frogs were exposed to *Bd* four weeks following surgery. Frogs with cardiac biotransmitters were randomly assigned to control ($n = 3$) and exposure ($n = 5$) groups. Exposures to *Bd* occurred simultaneously with other frog groups and with identical procedures (described above). Cardiac electrograms were collected every 7 d and analysed for heart rate and signal amplitude. Once clinical signs became obvious in infected amphibians we continuously monitored and recorded all cardiac electrical activity.

Part 6. Electrolyte Supplementation

Frogs in exposure groups were held in round plastic containers (diameter- 12 cm x 6.5 cm) containing 35 ml of 20% Holtfretter's solution inoculated with 1 x $10^6$ zoospores. Frogs used to control for infection were held in the same volume of solution with no *Bd* zoospores. Infected frogs that became diseased were assigned to a treatment group (receiving electrolyte supplementation) or an untreated group (no electrolyte supplementation).

An electrolyte treatment solution was based on recommended osmolality values for maintaining electrolyte balance in diseased amphibians (12% Whitaker-Wright solution in mM: NaCl (242), $MgSO_47H_20$ (4.3), $CaCl_2$ (2.85), KCl (2.85): Wright et al. 2001). Given the damage to electrolyte transport processes in amphibian skin and that the gut is able to preferentially absorb electrolytes (Policies et al. 2005, Teramoto et al. 2005), we opted to administer electrolyte solutions orally (via a crop tube) rather than by bath. Intravenous electrolyte supplementation is risky without continuous blood tests (Rainger & Dart 2006). Based on previous results showing losses of approximately 30% plasma sodium and 50% plasma potassium in amphibian chytridiomycosis, a target concentration and dose per body weight was calculated based on formulae for treating hyponatremia (Adrogue & Madias 2000) and hypokalemia (Gennari 1998) in human patients with electrolyte imbalances.

Frogs in the treatment group ($n = 6$) received the oral supplement when they could no longer right themselves, or took longer than 10 s to reorient. Untreated frogs ($n = 9$) were

handled identically, the crop tube was inserted as for treated frogs, but no electrolyte was administered. Following treatment, frogs were returned to their containers with fresh Holtfretter's solution so skin shedding could be assessed at each treatment period. Treatments were re-administered every 4-6 h. Time from initial loss of righting reflex to death (once no cardiac activity could be detected) was recorded in hours.

### Part 7. Plasma biochemistry of free-living *Litoria caerulea*

Adult common green tree frogs (*Litoria caerulea*) were collected (as described above). Frogs were swabbed to confirm the animals were free of *Bd*, anaesthetised and blood samples were collected (as described above) within 48 hours of capture. Frogs were then released at the original collection point.

### Part 8. Plasma biochemistry in *Litoria caerulea* with other lethal conditions

Blood was collected from five diseased wild adults of *L. caerulea* between January 2007 and May 2008 following anaesthesia (as described above). Two frogs presented with severe generalized trauma, one with bacterial septicaemia, one with severe sparganosis and thigh muscle abscessation, and one with progressive weight loss, anorexia and lethargy of unknown aetiology.

Results

All *L. caerulea* were confirmed to be negative for *Bd* with PCR analysis prior to exposures. Following exposure, swabs were collected from control frogs ($n = 10$), from infected but aclinical frogs ($n = 8$) and from frogs that became severely diseased ($n = 15$). These sample sizes combine frogs from the cardiac experiment and blood biochemistry experiment because the experiments occurred simultaneously. Zoospores equivalents were significantly higher in frogs that became severely diseased (repeated measures ANOVA, $P < 0.001$; Figure 89).

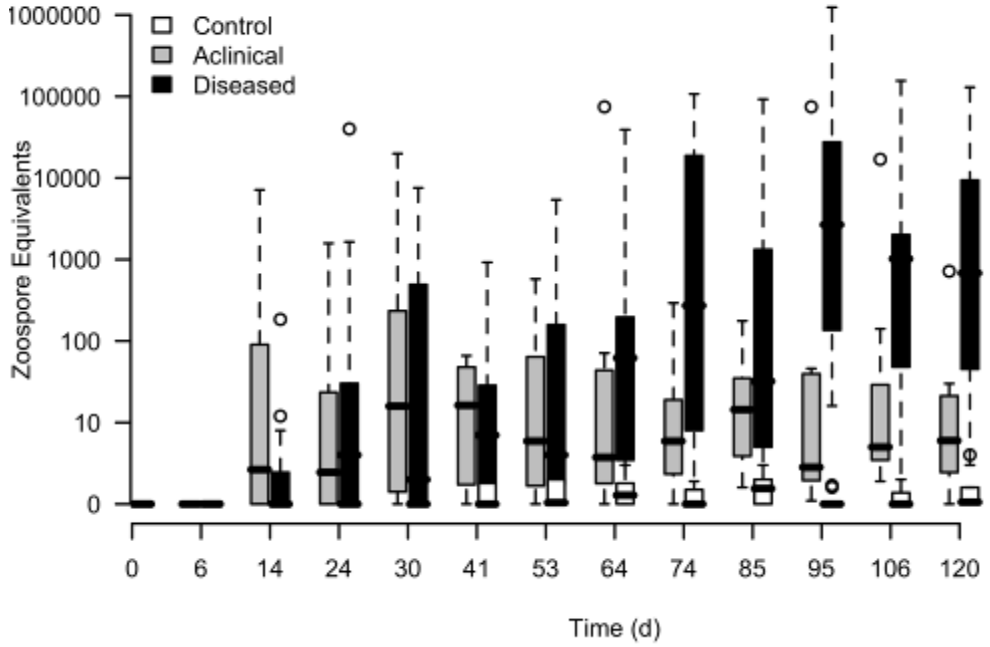


**Figure 89.** *Batrachochytrium dendrobatidis* load (zoospore equivalents) in experimentally infected *Litoria caerulea*. Results determined from real-time PCR on skin swabs collected over time (d). Frogs are grouped according to disease status: control ($n$ = 10), aclinical ($n$ = 8) and diseased ($n$ = 15).

Part 1. Blood biochemistry

Blood samples were collected from control ($n$ = 7), aclinical ($n$ = 7) and diseased ($n$ = 11) frogs, although sample sizes for each biochemical parameter varied depending on the analysis. Limited plasma volume restricted the number of tests that could be performed in some parameters, such as chloride. Plasma electrolyte concentrations were reduced in frogs with severe chytridiomycosis (Figure 90): plasma potassium (repeated measures ANOVA, $P$ = 0.028) and plasma sodium (repeated measures ANOVA, $P$ = 0.002*; Figure 90b). Of the 11 frogs that became diseased, all but one had plasma sodium concentrations that were below the detection limit of the available equipment (<100 mMol $l^{-1}$). For these frogs we used a conservative estimate of 99 mMol $l^{-1}$ although plasma sodium concentrations were likely to be lower based on results from previous work. Out of 10 additional blood biochemical parameters that were tested as markers of organ function and general health, none varied significantly (Table 23)

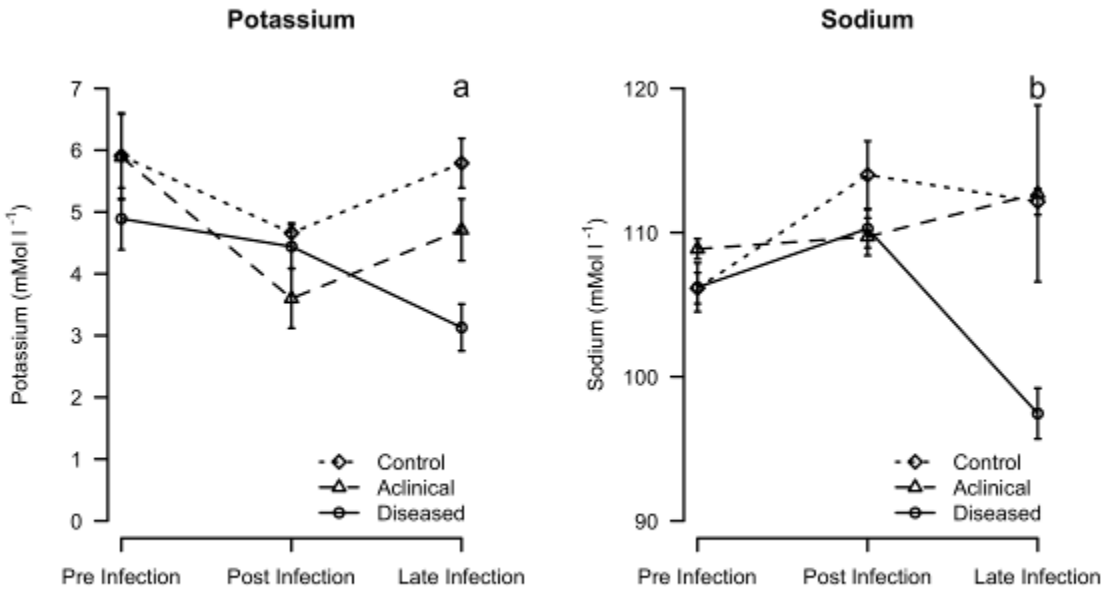


**Figure 90.** Blood plasma potassium and sodium concentrations in control and experimentally infected *Litoria caerulea* experimentally infected with *Batrachochytrium dendrobatidis* (dotted lines: control ($n$ = 7), dashed lines: aclinical ($n$ = 7), solid lines: diseased ($n$ = 11). Lines show mean (± s.e.m) from three sample collections over the course of experimental infection.

| Blood Biochemical Parameters | | | | | | |
|---|---|---|---|---|---|---|
| | Pre Infection | | Mid Infection | | Late Infection | |
| | Mean ± s.e.m | N | Mean ± s.e.m | N | Mean ± s.e.m | N |
| **CALCIUM (mMol l$^{-1}$):** f = 0.5802, p = 0.679 | | | | | | |
| Control | 2.34 ± 0.18 | 7 | 2.37 ± 0.14 | 7 | 2.43 ± 0.15 | 7 |
| Aclinical | 3.97 ± 1.09 | 7 | 4.08 ± 1.05 | 7 | 3.24 ± 0.13 | 7 |
| Diseased | 2.3 ± 0.16 | 10 | 2.33 ± 0.10 | 11 | 2.13 ± 0.15 | 10 |
| **CHLORIDE (mMol l$^{-1}$):** f = 0.3189, p = 0.861 | | | | | | |
| Control | 71.00 ± 1.23 | 6 | 86.25 ± 4.73 | 4 | 75.00 ± 4.26 | 4 |
| Aclinical | 73.00 ± 2.27 | 4 | 80.67 ± 6.25 | 6 | 77.40 ± 4.20 | 5 |
| Diseased | 70.10 ± 3.57 | 10 | 80.00 ± 3.01 | 6 | 67.00 ± 5.00 | 2 |
| **POTASSIUM (mMol l$^{-1}$):** f = 3.0174, p = 0.028 | | | | | | |
| Control | 5.91 ± 0.69 | 7 | 4.66 ± 0.16 | 7 | 5.79 ± 0.4 | 7 |
| Aclinical | 5.89 ± 0.69 | 7 | 3.6 ± 0.49 | 7 | 4.71 ± 0.50 | 7 |
| Diseased | 4.89 ± 0.50 | 10 | 4.44 ± 0.36 | 11 | 3.13 ± 0.39 | 10 |
| **SODIUM (mMol l$^{-1}$):** f = 5.0167, p = 0.002 | | | | | | |
| Control | 106.14 ± 1.08 | 7 | 114.85 ± 2.34 | 7 | 112.14 ± 0.91 | 7 |
| Aclinical | 108.86 ± 0.70 | 7 | 109.71 ± 1.30 | 7 | 112.71 ± 6.12 | 7 |
| Diseased | 106.20 ± 1.70 | 10 | 110.27 ± 1.32 | 11 | 97.45 ± 1.77 | 10 |
| **ASPARTATE AMINOTRANSFERASE (U l$^{-1}$):** f = 0.4591, p = 0.7652 | | | | | | |
| Control | 359.43 ± 50.37 | 7 | 292.66 ± 26.70 | 7 | 530.57 ± 169.83 | 7 |
| Aclinical | 218.87 ± 47.27 | 7 | 169.71 ± 31.56 | 7 | 494.57 ± 161.98 | 7 |
| Diseased | 275.1 ± 40.69 | 10 | 201.36 ± 22.92 | 11 | 637.8 ± 61.70 | 10 |
| **CREATINE KINASE (U l$^{-1}$):** f = 0.3307, p = 0.856 | | | | | | |
| Control | 2632.14 ± 734.87 | 7 | 2342.71 ± 399.71 | 7 | 2791.29 ± 447.9 | 7 |
| Aclinical | 1310.14 ± 218.56 | 7 | 1011.29 ± 259.10 | 7 | 2156.57 ± 428.48 | 7 |
| Diseased | 1778.9 ± 331.41 | 10 | 1416.09 ± 344.518 | 11 | 2834.38 ± 593.49 | 8 |
| **PHOSPHATES (mMol l$^{-1}$):** f = 1.6238, p = 0.187 | | | | | | |
| Control | 1.16 ± 0.15 | 6 | 1.23 ± 0.19 | 7 | 1.59 ± 0.11 | 7 |
| Aclinical | 1.41 ± 0.38 | 7 | 1.62 ± 0.2 | 7 | 1.55 ± 0.3 | 7 |
| Diseased | 0.84 ± 0.1 | 10 | 0.97 ± 0.07 | 11 | 1.72 ± 0.2 | 10 |
| **ALBUMIN (g l$^{-1}$),** f-value= 0.9014, p = 0.472 | | | | | | |
| Control | 19.29 ± 1.57 | 7 | 18.86 ± 1.78 | 7 | 22.57 ± 1.56 | 7 |
| Aclinical | 23.43 ± 1.88 | 7 | 21.57 ± 2.07 | 7 | 24.86 ± 2.77 | 7 |
| Diseased | 18.1 ± 2.29 | 10 | 18.27 ± 1.78 | 11 | 23.2 ± 1.55 | 10 |
| **URIC ACIDS (µMol l$^{-1}$):** f =2.101, p = 0.098 | | | | | | |
| Control | 5.33 ± 1.43 | 7 | 11.29 ± 4.00 | 7 | 16.29 ± 3.37 | 7 |
| Aclinical | 3.86 ± 1.34 | 7 | 8.0 ± 3.08 | 7 | 22.29 ± 6.09 | 7 |
| Diseased | 2.80 ± 1.76 | 10 | 8.18 ± 1.64 | 11 | 9.3 ± 2.08 | 10 |
| **GLUCOSE (mMol l$^{-1}$):** f = 2.1930, p = 0.087 | | | | | | |
| Control | 3.03 ± 0.31 | 7 | 2.5 ± 0.15 | 7 | 3.04 ± 0.22 | 7 |
| Aclinical | 2.63 ± 0.27 | 7 | 3.09 ± 0.27 | 7 | 3.11 ± 0.23 | 7 |
| Diseased | 2.58 ± 0.21 | 10 | 2.58 ± 0.13 | 10 | 2.18 ± 0.5 | 11 |
| **GLOBULIN (g l$^{-1}$):** f = 0.2100, p = 0.1177 | | | | | | |
| Control | 23.14 ± 2.04 | 7 | 22.4 ± 2.46 | 7 | 27.14 ± 1.7 | 7 |
| Aclinical | 28.71 ± 3.44 | 7 | 28.41 ± 2.41 | 7 | 37.1 ± 4.37 | 7 |
| Diseased | 26.7 ± 3.47 | 9 | 28.7 ± 3.6 | 9 | 38.4 ± 2.74 | 10 |
| **TOTAL PROTEIN (g l$^{-1}$):** f = 1.843, p = 0.1412 | | | | | | |
| Control | 42.57 ± 2 | 7 | 41.14 ± 3.84 | 7 | 49.71 ± 2 | 7 |
| Aclinical | 54 ± 4.09 | 7 | 53.57 ± 5.46 | 7 | 59 ± 6.67 | 7 |
| Diseased | 43.4 ± 4.9 | 10 | 45.73 ± 4.57 | 11 | 61.3 ± 3.85 | 10 |

**Table 23.** Plasma biochemical parameters from *Litoria caerulea* experimentally infected with *Batrachochytrium dendrobatidis*. Data sets tested with repeated measures ANOVA. Significant changes were detected in frogs that developed severe disease.

Hydration status in diseased frogs was not considerably altered. An increase of albumin and total protein with a decrease in body mass would indicate dehydration. In contrast overhydration is indicated by a decrease in total protein and albumin with an increase in body mass. Although there were slight increases in total protein and albumin, these changes occurred in all groups and were not statistically significant (Table 23). Body mass was slightly increased in the control group (in gms: Pre Infection: 46.32 ± 4.51, Post Infection: 45.25 ± 4.96, Late Infection 53.97 ± 5.53) but was unchanged in aclinical (in gms: Pre Infection: 42.21 ± 2.56, Post Infection: 40.1 ± 3.06, Late Infection: 43.7 ±1.56) and diseased groups (in gms: Pre Infection: 39.35 ± 3.75, Post Infection: 38.83 ± 2.59, Late Infection: 39.19 ± 3.29).

**Part 2. Urine biochemistry**

There were no significant differences in urine electrolytes, osmolality or pH among urine samples collected over the course of infection (Table 24). However, sample sizes were limited due to opportunistic collection of urine samples. Also, there was wide variation in urine parameters among individuals and the majority of data sets were not normally distributed. Therefore, these results should be interpreted with caution.

| Urine Biochemical Parameters | | | | | | |
|---|---|---|---|---|---|---|
| | Pre Infection | | Mid Infection | | Late Infection | |
| | Mean ± s.e.m | N | Mean ± s.e.m | N | Mean ± s.e.m | N |
| **CALCIUM** | | | | | | |
| Control | 1.84 ± NA | 1 | 0.51 ± 0.15 | 4 | 0.14 ± 0.05 | 4 |
| Aclinical | 0.6 ± NA | 1 | 0.08 ± NA | 1 | 1.63 ± NA | 1 |
| Diseased | 0.32 ± 0.16 | 2 | 0.13 ± 0.05 | 7 | 0.24 ± 0.1 | 4 |
| **CHLORIDE:** f = 3.0757, p = 0.1204 | | | | | | |
| Control | 17 ± NA | 1 | 21 ± 1.7 | 4 | 13.5 ± 1.66 | 4 |
| Aclinical | 25.00 ± NA | 1 | NA ± NA | 0 | 47.00 ± NA | 1 |
| Diseased | 21.00 ± 5.00 | 2 | 17.57 ± 1.09 | 7 | 23.50 ± 3.80 | 4 |
| **POTASSIUM:** f = 7.2897, p = 0.0425 | | | | | | |
| Control | 3.3 ± NA | 1 | 2.83 ± 0.53 | 4 | 2.93 ± 0.39 | 4 |
| Aclinical | 1.3 ± NA | 1 | 1.70 ± NA | 1 | 9.10 ± NA | 1 |
| Diseased | 4.7 ± 3.4 | 2 | 2.03 ± 0.23 | 7 | 3.00 ± 0.41 | 4 |
| **MAGNESIUM:** f = 0.3529, p = 0.7907 | | | | | | |
| Control | 0.5 ± NA | 1 | 0.28 ± 0.09 | 4 | 0.43 ± 0.14 | 4 |
| Aclinical | 0.20 ± NA | 1 | 0.20 ± NA | 1 | 0.50 ± NA | 1 |
| Diseased | 0.10 ± 0.10 | 2 | 0.24 ± 0.37 | 7 | 0.23 ± 0.06 | 4 |
| **SODIUM**: f = 3.0321, 0.1559 | | | | | | |
| Control | 15 ± NA | 1 | 23.25 ± 1.38 | 4 | 14.50 ± 2.50 | 4 |
| Aclinical | 29.00 ± NA | 1 | 27.00 ± NA | 1 | 32.00 ± NA | 1 |
| Diseased | 23.50 ± 14.5 | 2 | 19.14 ± 2.27 | 7 | 24.25 ± 3.42 | 4 |
| **OSMOLALITY,** f = 1.1418, p = 0.08 | | | | | | |
| Control | 20 ± 0 | 2 | 57.83 ± 15.49 | 6 | 35.50 ± 8.45 | 4 |
| Aclinical | NA ± NA | 0 | 37.50 ± 10.50 | 2 | 102.33 ± 61.36 | 3 |
| Diseased | 96.50 ± 10.5 | 2 | 46.75 ± 4.51 | 11 | 58.11 ± 6.22 | 9 |
| **pH**, f = 0.5575, p = 0.6982 | | | | | | |
| Control | 7.5 ± 0.5 | 2 | 7.83 ± 0.13 | 6 | 7.58 ± 0.05 | 5 |
| Aclinical | 7.5 ± NA | 1 | 6.85 ± 0.85 | 2 | 6.57 ± 0.56 | 3 |
| Diseased | 8 ± 0.5 | 2 | 7.51 ± 0.174 | 11 | 7.07 ± 0.27 | 10 |
| **HAEMOLYZED BLOOD**, f = 0.8963, p = 0.4959 | | | | | | |
| Control | 0 ± 0 | 2 | 0 ± 0 | 6 | 0 ± 0 | 5 |
| Aclinical | 0 ± NA | 1 | 0 ± 0 | 2 | 80 ± 0 | 4 |
| Diseased | 0 ± 0 | 2 | 2.92 ± 2.17 | 11 | 96 ± 29.93 | 10 |

**Table 24.** Urine biochemical parameters from *Litoria caerulea* experimentally infected with *Batrachochytrium dendrobatidis*. Data sets tested with ANOVA repeated measures. There were no positive reactions for the following urine parameters: glucose, bilirubin, ketones, protein, urobilinogen (data not shown).

Although none of these parameters varied significantly, one urine parameter, haemolysed blood, suggested a treatment effect. There were only positive reactions on the reagent strips for haemolysed blood in the urine of aclinical and diseased frogs whereas no blood was detected in control frogs (Table 24, Figure 91). These changes were not statistically significant but the pattern suggested a group effect and warranted additional investigation (Figure 91).

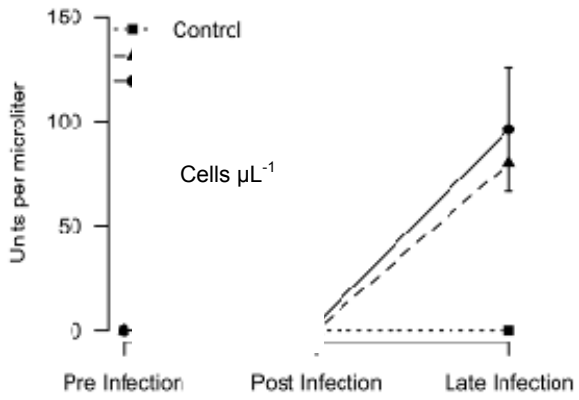


**Figure 91.** Haemolysed blood results in urine samples from control and experimentally infected *Litoria caerulea*. At late infection samples were collected from control (*n* = 5), aclinical (*n* = 4) and diseased (*n* = 10) frogs.

Negative results from commercial urinary sediment stains (Clay Adams Sedi-Stain, Becton Dickinson and Co.) in samples that tested positive for haemoglobin (*n* = 5) ruled out haematuria. In addition, kidney function appeared unaffected as there were no significant changes in plasma phosphorous levels (Table 23) and previous studies showed urea was not elevated in diseased frogs. Histological analysis of kidney sections suggested the possibility of haemoglobinuria (haemoglobin casts could be seen in the tubules), but after following up with a specific stain for haemoglobin, the histological results were inconclusive (personal communication Karrie Rose).

Because haemoglobinuria could have resulted from intravascular haemolysis of erythrocytes in hypoosmotic conditions, we tested osmotic fragility of cane toad (*Bufo marinus*) erythrocytes using routine techniques (Aldrich & Saunders 2001). A sample of packed toad erythrocytes (50 µL) was added to a range of concentrations of sodium chloride solution (prepared in distilled water at concentrations from 0.01 to 5% with distilled water as a positive control). The absorbance of the supernatants (read at 540 nm, Biorad spectrophotometer) indicated that erythrocyte haemolysis occurred when sodium chloride concentrations were 0.2% or less (<34 mM/L).

Part 3. Histology of skin biopsies

Sporangia of *Bd* were not seen on any pre infection samples and the epidermis appeared healthy. At 30 d post infection light, focal infections were seen in frogs that became diseased, associated with mild focal lesions such as erosions. Skin samples collected in late stages of infection had typical severe changes with spongiosis associated with hyperkeratosis, spongiosis of the *stratum granulosum*, irregular epidermal layers, erosions, and microscopic ulcerations where infected epidermis had sloughed.

**Part 4. Electrolyte transport in isolated skin samples**

Under resting conditions, the rate of net electrolyte transport across the skins, as assessed by the equivalent short circuit current, was significantly lower in infected ($n$ = 7) skin samples (27.11 ± 8.12 μA per $cm^2$) than in control ($n$ = 7) skin samples (65.44 ± 11.02 μA per $cm^2$; Student's T-test, $P$ = 0.009; Figure 92a). This decrease in electrolyte transport rate was accompanied by a reduction in the transepithelial resistance (infected: 400.95 ± 86.37 ohms per $cm^2$, control: 939.94 ± 109.17 ohms per $cm^2$; Student's T-test, $P$ = 0.0006; Figure 92b). This was due to marked inhibition of the rate of absorption of sodium (Figures 92 d and e), assessed as the component of the short circuit current that was blocked by amiloride (infected: 17.99 ± 9.80 μA per $cm^2$, control: 59.10 ± 14.72 μA per $cm^2$, Student's T-test, $P$ = 0.038: Figure 92d). The short circuit current in the presence of amiloride did not differ between infected (9.12 ± 1.32 μA per $cm^2$) and control (6.34 ± 0.75 μA per $cm^2$) skin samples (Student's T-test, $P$ = 0.079: Figure 92e). Additionally, we found that the responses to both carbachol (infected: 10.34 ± 5.22 μA per $cm^2$, uninfected: 44.84 ± 10.98 μA per $cm^2$, Student's T-test, $P$ = 0.015: Figure 92f) and noradrenaline (infected: 4.l8 ± 2.45 μA per $cm^2$, uninfected: 61.48 ± 13.74 μA per $cm^2$, Student's T-test, $P$ = 0.001: Figure 92g) were reduced in the skins of infected frogs. In our test of the toxin hypothesis, there was no detectable change in short-circuit current with any of the treatment solutions.

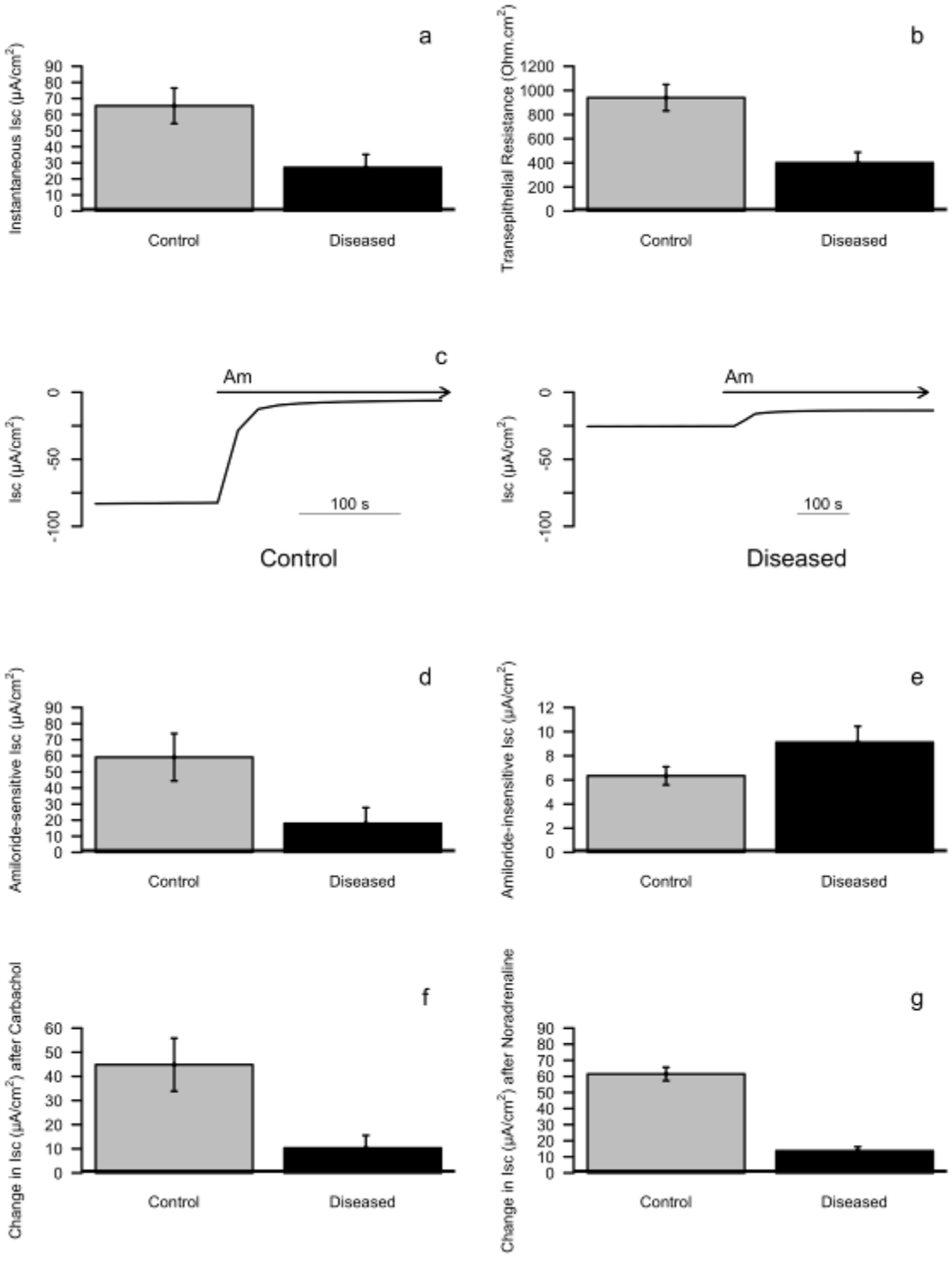


**Figure 92.** Electrolyte transport in ventral skin samples from *Litoria caerulea* experimentally infected with *Batrachochytrium dendrobatidis* in Ussing Chamber tests. Bar graphs show mean (± s.e.m) in skin samples from control (grey, $n = 7$) and diseased (black, $n = 7$) frogs in response to (d,e) amiloride, (f) carbachol and (g) noradrenaline. Tracings (c) show the response of control and infected skin samples to amiloride.

Part 5. Cardiac biotelemetry

Wet-field surgery was successful with no post-surgical complications. All eight frogs fully recovered within one hour after surgery and resumed normal behaviour. Cardiac implants were tested immediately following surgery. Ventricular depolarization was recorded but signal amplitudes were not sufficient to reliably determine other electrical activity such as depolarization of the atria. Electrical interference from non-cardiac muscles occurred in some frogs.

Of five frogs that became diseased and died, cardiac electrograms were collected from four individuals. Cardiac telemetry recordings demonstrated a consistent pattern of changes in each diseased frog. During the hours before death, heart rate slowed, ventricular depolarization amplitude decreased, and time of ventricular depolarization increased (Figure 93). These electrocardiographic changes progressed to cardiac standstill and asystolic arrest.

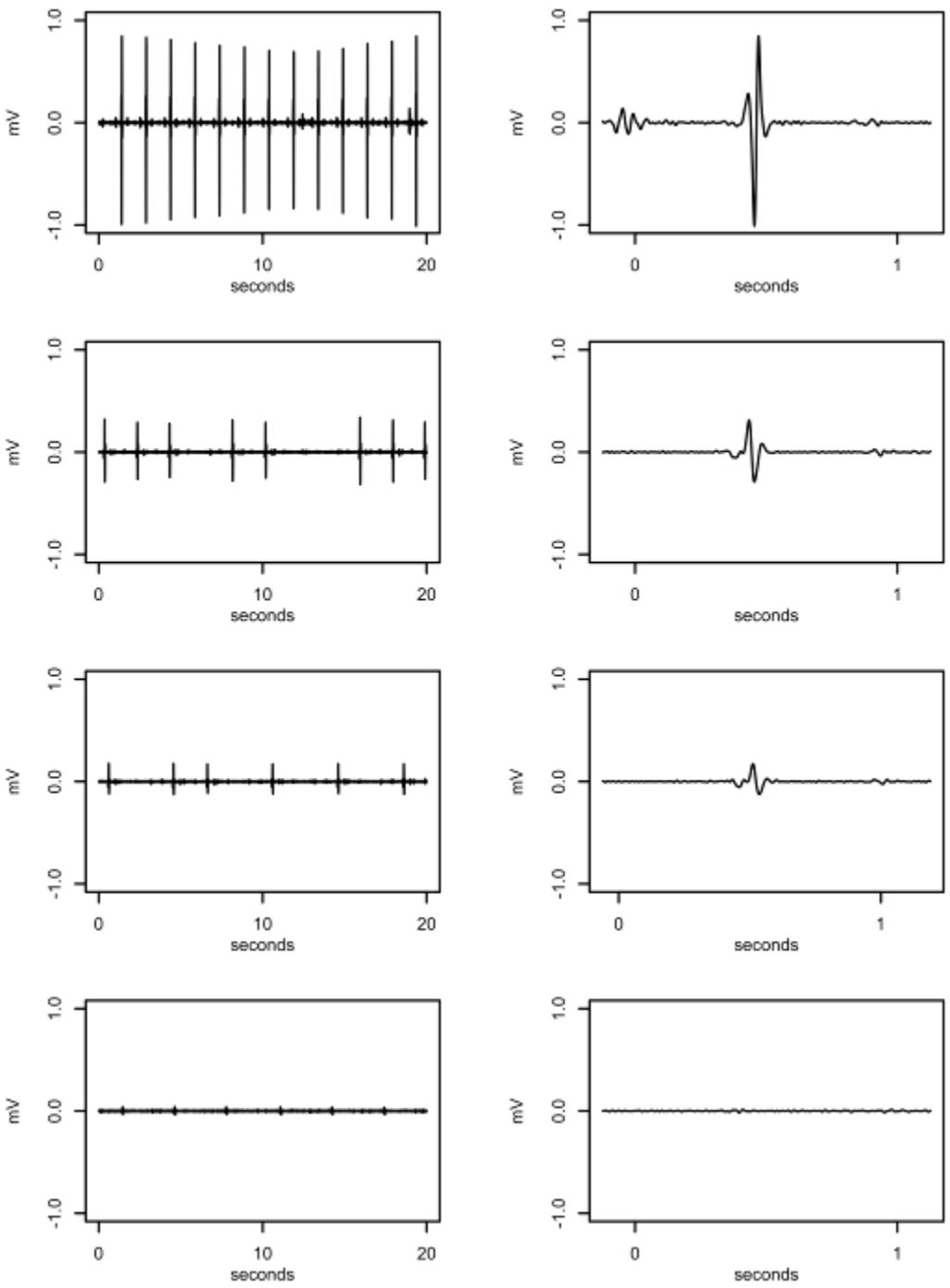


**Figure 93.** Original cardiac electrogram tracings from a *Litoria caerulea* with severe chytridiomycosis. Heart rate and signal amplitude were determined based on 20-second electrograms sweeps (left column). Cardiac electrical signal morphology was assessed using one-second sweeps (right column). Electrograms show cardiac electrical patterns consistent with asystolic cardiac arrest: slowing heart rate, decreasing ventricular depolarization amplitude and increasing time of ventricular depolarization from (first row) 18 to (second row) 3, (third row) 2 and (forth row) 0.5 hours prior to death.

Part 6. Electrolyte Supplementation

Of the 19 frogs exposed to *Bd*, 17 developed obvious clinical signs of disease between 25 and 58 d following exposure to *Bd*. Two frogs survived infection and were negative for *Bd* at the termination of the experiment and one frog died before treatment could be administered.

Following the initial supplementation, treatment frogs recovered normal posture and were considerably more active; one individual recovered sufficiently to climb out of the water on to the container walls and two individuals were able to jump to avoid capture. These signs of recovery were not seen in any untreated frogs. Treatment frogs ($n$ = 6) lived more than 20 hours longer than untreated frogs ($n$ = 9) (mean time post treatment: 32 ± 2.8 h, control: 10.7 ± 2.2 h, Student's T-test, $P < 0.001$). All diseased frogs continued to shed skin throughout the treatment period, regardless of supplementation. Although treatment continued every 4-6 hours and clinical signs were diminished in frogs with electrolyte supplementation, all frogs that became diseased eventually died.

**Parts 7 and 8. Plasma biochemistry of free-living *Litoria caerulea* and in *Litoria caerulea* with other lethal conditions**

We compared blood biochemical results from experimentally infected *L. caerulea* (in the late stages of infection) with those from free-living *L. caerulea* and *L. caerulea* with other lethal conditions. Sodium and potassium concentrations did not vary significantly among control, aclinical and free-living frog groups (Figure 94a and b). Plasma potassium concentrations in *L. caerulea* with severe chytridiomycosis are significantly different from those in control frogs (ANOVA Tukey post-hoc, $P$ = 0.009*; Figure 94a) and those in frogs with other lethal conditions (ANOVA Tukey post-hoc, $P$ = 0.04*; Figure 94a). Plasma sodium concentrations in *L. caerulea* with severe chytridiomycosis are significantly different from those in control frogs (ANOVA, Tukey Test, p = 0.01*; Figure 94b) and from those in free-living frogs (ANOVA, Tukey Test, p = 0.003*; Figure 94b). Some frogs with other lethal conditions had reduced sodium concentrations (Figure 94b) but the variance was not homogenous (Levene's test for homogeneity of variance, $P$ = 0.197)

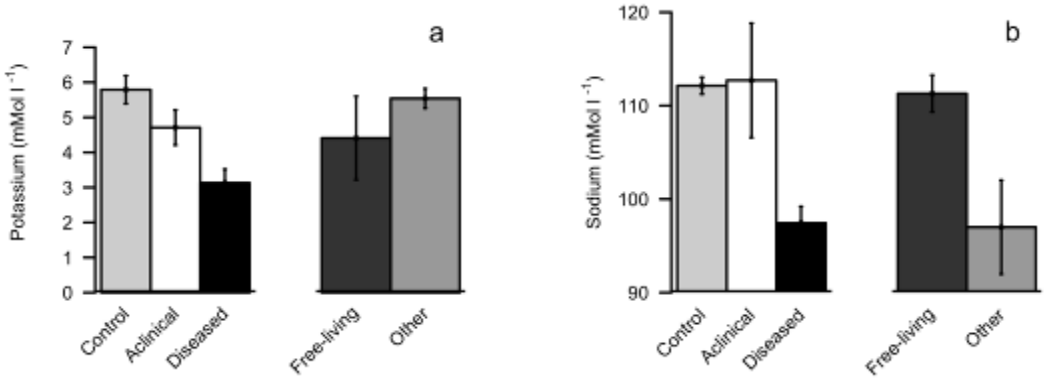


**Figure 94.** Blood plasma potassium (a) and sodium (b) concentrations in *Litoria caerulea* experimentally infected with *Batrachochytrium dendrobatidis*, free-living

"normal" *L. caerulea* ($n$ = 5) and *L. caerulea* ($n$ = 5) with other lethal conditions. Results show means (± s.e.m).

## Discussion

In this study severe clinical signs of disease appeared in *Litoria caerulea* with the highest burdens of *Bd*, concurrent with three critical changes in physiological systems: 1) inhibition of electrolyte transport across isolated ventral skin samples, 2) reductions in plasma electrolyte concentrations and 3) deterioration of cardiac electrical functioning. Our results show how disruption of normal osmoregulatory processes in amphibian skin and osmotic imbalance due to reduced electrolytes likely causes asystolic cardiac arrest in amphibians with severe chytridiomycosis.

First, the rate of net electrolyte transport and transepithelial resistance across the skin samples from infected frogs was significantly lower than those in uninfected skin samples (Figure 92), indicating that the effect of the infection was predominantly on sodium transport. These results demonstrate that *Bd* infection compromises electrolyte (sodium and chloride) transport and thus, osmoregulatory function in the skin of diseased frogs. The exact cellular mechanisms are unknown and additional Ussing chamber research is needed to reveal further details. An experiment that incorporates chemical treatments could test the active transport system by isolating of specific cellular channels and pumps. For example, applying nystatin, which permeabolizes cell membranes, to the apical bath could test the functioning of the ATPase (sodium-potassium pump). The selective barrier properties of frog skin are primarily determined by electrolyte transport in the flask-shaped mitochondrial-rich cells (MR cells) of *stratum granulosum* (Masoni & Garcia-Romeu 1979, Brown et al. 1981, Larsen et al. 1987, Larsen et al. 1996, Ehrenfeld 1997). Follow up studies to histologically examine the MR cells and/or the ATPase pump (Choe et al. 2004) could further characterise the disrepair to the epidermal transport system, which appears to be a key factor in the pathogenesis of chytridiomycosis.

Second, plasma potassium and sodium concentrations were reduced in frogs with severe chytridiomycosis. An additional 10 blood and 13 urine parameters were tested as markers of organ function and general health; none of these blood parameters varied significantly. As hydration status of diseased frogs was unchanged, reductions in plasma potassium and sodium concentrations probably resulted from a loss of electrolytes from circulation rather than electrolyte dilution due to water uptake. These results agree with previous findings where electrolyte reductions were found in diseased frogs during an outbreak of chytridiomycosis in captive *L. caerulea*. Electrolyte imbalance, low plasma potassium (hypokalemia) and low plasma sodium (hyponatremia), could result from depletion via the epidermis or the kidney (Moore & Lofts 1964, Boutilier et al. 1992). Follow up tests on the urine of diseased frogs suggested the possibility of haemoglobinuria, which can result from intravascular haemolysis of erythrocytes in hypoosmotic conditions. Erythrocyte osmotic fragility is lower in amphibians than other vertebrates (Aldrich et al. 2006), and our tests indicate that *Bufo marinus* erythrocytes are relatively resistant to changes in concentrations of sodium chloride. The sodium chloride

solutions probably did not adequately simulate the mixture of electrolytes normally present in the blood, and therefore additional work is needed to determine the importance of other plasma electrolytes (such as potassium and magnesium) in amphibian erythrocyte haemolysis. At this point we can draw no definitive conclusions regarding intravascular haemolysis and although we found no indication of renal damage with plasma biochemistry, electrolyte loss via the kidney could not be ruled out. In contrast, the skin, which regulates bi-directional flux and overall balance of sodium and potassium (Larsen 1991, Boutilier et al. 1992), was severely damaged when assessed with histology and Ussing chamber tests. Thus the most parsimonious explanation for pathophysiology of chytridiomycosis is that the skin is the primary organ involved in electrolyte shifts and consequent osmotic imbalance.

Third, when severely diseased frogs were within hours of death, their cardiac electrical activity closely resembled agonal patterns associated with cardiac standstill, also known as asystolic or bradyasystolic cardiac arrest (Paradis et al. 1996), which can result from shifts in electrolyte balance. Once initiated by a triggering event such as an electrical conduction abnormality, this kind of cardiac death results from a cycle of myocyte ischemia, further conduction dysfunction and inadequate blood flow to the heart and extremities. Disruption of electrical conduction leads to mechanical dysfunction and eventual circulatory collapse. Several conditions can initiate this cycle: hypothermia, dehydration, hypovolemia, hypoxia, acidosis and electrolyte disturbances including shifts in calcium and potassium (Paradis et al. 1996). However, most of these conditions can be ruled out in this study. Hypothermia can be discounted because there were no changes in ambient temperatures. As body mass, total protein and albumin were unaffected, dehydration and hypovolemia are unlikely. Blood oxygen saturation was measured in each frog three times over the course of infection (data not presented) using a pulse oximeter (TuffSat, Datex-Ohmeda Inc.) on the thigh of the hind limb. Although pulse oximeters are not ideal for use in amphibians because readings are influenced by skin pigmentation, we detected a drop of approximately 20% in peripheral blood oxygen saturation in one individual *after* changes in electrical functioning were observed. This suggests that hypoxia was not the initiating event for cardiac electrical dysfunction. Therefore shifts in electrolytes, particularly potassium, and/or acidosis as the result of progressive acidosis are the most likely causes of bradycardia and eventual cardiac standstill in frogs with severe chytridiomycosis.

To determine whether the extent of electrolyte loss is specific to chytridiomycosis, we collected blood samples from uninfected, free-living *L. caerulea* and from frogs with other lethal conditions such as severe sparganosis, bacterial septicaemia and trauma. Lower limits of plasma electrolyte concentrations for *L. caerulea* are unknown but sodium and potassium concentrations did not vary significantly among control, aclinical and free-living frog groups, suggesting that electrolyte concentrations in our captive frogs were normal. In contrast, frogs that developed severe chytridiomycosis had significantly reduced sodium concentrations compared to free-living frogs. Although some frogs with other lethal conditions had reduced sodium concentrations, the variance was not homogenous, indicating that sodium reduction is not an obligate terminal change.

Furthermore, plasma potassium levels in frogs with severe chytridiomycosis were significantly lower than concentrations in frogs with other lethal conditions.

In an additional infection experiment we administered an oral electrolyte supplement to *L. caerulea* in the terminal stages of infection. These frogs became more active and lived longer than diseased frogs that received no electrolyte treatment. Treated frogs continued to shed skin, a clinical sign of severe disease, and ultimately died. However, it is important to note that in this preliminary experiment the oral electrolyte supplement was administered in the terminal stages of infection, when the fatal cycle leading to asystolic cardiac arrest may have already been initiated. In understanding the mechanisms of death, our results support the hypothesis that electrolyte depletion is a crucial factor in pathogenesis, but additional work is needed to refine our understanding of the value of providing electrolytes as supportive care to amphibians with severe chytridiomycosis. We suggest multiple follow up experiments to build on these results: first, an experiment with a similar design, but provides electrolyte supplements earlier in the onset of clinical signs, second, an experiment including a range of dilutions of adjusted electrolyte solutions in the water baths (rather than an oral supplement) of infected amphibians and third, an experiment using electrolyte supplementation in combination with a fungicide known to be effective against *Bd* (such as itraconazole or chloramphenecol). In this last experiment, the elimination of *Bd* would be combined with the correction of electrolyte abnormalities. Experiments that identify the best way to treat amphibians with chytridiomycosis will be important because although administering hypertonic solutions has inherent risks, it may be the best way to manage chytridiomycosis in a clinical setting. Although electrolyte treatment did not lead to full recovery, clinical improvement and delayed time to death indicate that electrolyte depletion is an important cause of morbidity and mortality.

Amphibians can tolerate greater electrolyte fluctuations than other terrestrial vertebrates (Deyrup 1964), but our results support the epidermal dysfunction hypothesis, which suggests that the disruption to cutaneous functioning and the extent of electrolyte imbalance that occurs in severe chytridiomycosis are sufficient to cause mortality. This does not disqualify the so-called "toxin hypothesis". Convincing evidence of a systemic toxin has not been provided, but *Bd* could produce a "toxin" that causes localized epidermal damage. Although enzymatic activity is not normally described as "toxic", the term "toxin" is defined as a poisonous substance formed as a part of cells or tissues as an extracellular product (Steadman 2000). Enzymes produced by *Bd* enzymes may fit that definition. We did not detect any epidermal transport changes with solutions treated to filter a possible toxin, but this could be a matter of the dilution of the solutions used. The discovery of differential expression patterns in genes such as serine protease and fungalysin metallopeptidase, two enzymes involved in pathogenesis in multiple fungal pathogens (Rosenblum et al. 2008), suggests that enzymatic activity could be involved in *Bd* pathogenesis. However, the functional role of these genes remains unclear and their importance for pathogenicity has not been conclusively demonstrated. This is due to the challenges of working with the molecular profile of *Bd*, which, at best, is only distantly related to organisms with moderately understood genomes. As the genomes of other fungi are investigated and the functional roles of their genetic repertoire are better

understood, we may be able to generate a list of candidate proteins involved in chytridiomycosis pathogenesis (personal communication- Erica Rosenblum) and potentially establish the mechanism of destruction in key cell types of the amphibian epidermis.

The ability of *Bd* to compromise an essential organ is a critical attribute in a list of fatal pathogen characteristics that make *Bd* a formidable pathogen. Host-pathogen dynamics are driven by biotic and abiotic interactions at many levels, and the convergence of multiple variables (host susceptibility, pathogen virulence, population dynamics and environmental conditions) creates favourable conditions for disease outbreaks capable of causing population declines (Daszak et al. 2003, De Castro & Bolker 2005). Chytridiomycosis is an example of this possibility for several reasons. Compared to other terrestrial vertebrates, amphibians are particularly vulnerable to epidermal infections as their existence depends on physiological interactions of the skin with the external environment (Jorgensen et al. 1954, Alvarado & Kirschner 1963, Deyrup 1964, Moore & Lofts 1964, Fischbarg & Whittembury 1977, Shoemaker 1977, Larsen et al. 1987, Boutilier et al. 1992, Erspamer et al. 1994, Jorgensen 1994a, b, 1997, Wright et al. 2001, Word & Hillman 2005). Frog skin is a moist, nutritive substrate on which many microorganisms can flourish (Clark et al. 1997), but *Bd* has the added advantage of a temperature optimum for growth that matches the range of temperatures experienced by amphibian species (Piotrowski et al. 2004). Additionally, amphibian behavioural characteristics such as aggregating in retreat sites or during breeding season (Rowley & Alford 2007), and their association with water bodies (Lips et al. 2003, Skerratt et al. 2007, Brem & Lips 2008) increase opportunities for *Bd* transmission. The ability of *Bd* to persist in the environment, whether by an alternate life stage (Di Rosa at al. 2007) or by saprobic growth further amplifies the possibility of disease-induced extinctions (Mitchell et al. 2008), which had not previously been considered biologically possible (Anderson & May 1991, de Castro & Bolker 2005). Finally, anthropogenic spread of *Bd*, possibly via international frog trade, is a plausible explanation for its global emergence (Hanselmann et al. 2004). The *confluence* of all of these factors, including a life-compromising pathophysiology reported here, has created the disease equivalent of a "perfect storm" scenario such that amphibians are impacted over a broad host range and across the globe, resulting in an unparalleled pandemic

***Project 6.1-6.5 Bibliography and literature cited***

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***Project 6.6. Intensity of infection and sampling locality affect the distribution of Batrachochytrium dendrobatidis among body regions on green-eyed tree frogs (Litoria genimaculata)***

Published as North, S., and Alford, R. A. 2008. Intensity of infection and sampling locality affect the distribution of Batrachochytrium dendrobatidis among body regions on green-eyed tree frogs (Litoria genimaculata). Diseases of Aquatic Organisms 81:177-188.

ABSTRACT: *Batrachochytrium dendrobatidis* (*Bd*) causes chytridiomycosis, which has caused devastating amphibian population declines. Little is known about the biology of *Bd* on hosts, and techniques for diagnosing it on living and preserved animals are still evolving. We investigated the spatial distribution of *Bd* on the integument of naturally infected Australian hylid frogs, *Litoria genimaculata,* at four rain forest localities in northern Queensland, Australia. We collected 555 samples by swabbing 111 individuals on five regions of the body (back, abdomen, legs, forefeet and hindfeet). Numbers of zoospore equivalents on each body region were quantified using a Real-time Taqman PCR assay. The intensity of infection differed significantly among body regions; this pattern of differences differed among sampling localities. The lightest infections were centered on the abdomen, while heavier infections were most intense on the legs and feet. The back was always either lightly infected or uninfected. Many animals with light infections returned positive PCR results only for the abdomen or the legs. We compared swabbing legs and abdomen to detect infections, and found that swabbing either one would provide similar sensitivity, but that using both regions would lead to greater sensitivity than either region alone. Because swabbing may transfer zoospores from infected to uninfected regions within individuals, we suggest that the best procedure for all species is to employ separate swabs for each body region. If that cannot be done, swabbing patterns that minimize potential harm should be determined for each species, and possibly each class of individuals (e.g. males, females, juveniles) within species, by examining the distribution of infection among body parts in naturally infected individuals.
KEY WORDS: *Batrachochytrium dendrobatidis, Litoria genimaculata*, chytridiomycosis, disease develoment, diagnostic PCR.

INTRODUCTION

In 2004, the Global Amphibian Assessment estimated that of the 5,743 species of described amphibians , populations of 2,468 species were declining (Stuart et al. 2004). Habitat loss, climate change (temperature, rainfall, UV-B radiation), exotic species, exploitation, pollution and synergistic interactions between these factors have probably contributed to many amphibian declines (Alford & Richards 1999). Enigmatic declines (Stuart et al. 2004), occurring in areas with no known anthropogenic influence, however, have raised concerns of an alternative threat to amphibians. A strong candidate as the cause of many enigmatic declines is chytridiomycosis, an emerging infectious disease of amphibians caused by the chytrid fungus *Batrachochytrium dendrobatidis* (*Bd*; Berger et al. 1998; Longcore et al. 1999). Lips et al. (2006) demonstrated that chytridiomycosis

was the proximate cause of the decline of an entire amphibian assemblage, in a manner consistent with many of the enigmatic declines identified by Stuart et al. (2004).

The life cycle of *Bd* includes two stages (Longcore et al. 1999, Berger et al. 2000); no resting or sexual stages have been identified with certainty, although genetic recombination may occur (Morgan et al. 2007 PNAS), and Fagotti & Pascolini (2007) reported an unusual avirulent unicellular form in histological samples from *Rana lessonae*. The infectious stage is the flagellated zoospores (1-5 μm in diameter), which encyst on the skin surface and enter the cells of the stratum granulosum via germination tubes. They then form a thallus that produces a zoosporangium (10-40 μm in diameter). Immature zoosporangia occur within the deeper cells of the stratum granulosum, whereas mature zoosporangia occur in the stratum corneum (Berger et al. 2005b). As it matures, the zoosporangium forms at least one discharge tube, which eventually opens to the skin surface, releasing zoospores. These may reinfect the same host or disperse. Piotrowski (2004) found (*in vitro*) that more than 95% of zoospores stop moving within 24 hours, and on average zoospores swam less than 2 cm before encysting. This short-lived motile period may mean that many zoospores reinfect the host from which they originate.

In culture *Bd* thrives at 23˚C, and typically dies at temperatures greater than 29˚C (Longcore et al. 1999) or after one hour of desiccation (Berger 2001). In addition to growing in amphibian skin, Bd may also survive as a saprobe in moist soil (Longcore et al. 1999; Johnson & Speare 2005), or on bird feathers or other substrates (Johnson & Speare 2005). Johnson & Speare (2005) found that *Bd* cultures could be re-isolated for at least 3 months after inoculation into sterile, moist river sand with no added nutrients, suggesting that contact between hosts and substrates may serve as a route of infection after zoospores lose their motility. Chemotaxis and water flow are likely to be the main methods of dissemination (Johnson & Speare 2003), and release of zoospores from zoosporangia may not be possible without sufficient moisture (Berger et al. 2005b). The mechanism by which *Bd* infections cause host mortality has not been conclusively established. Carey et al. (2006) reported that the timing of death in experimentally infected *Bufo boreas* was consistent with the hypothesis that *Bd* populations grow exponentially following initial infection, with mortality occurring when a threshold population level is reached. Voyles et al (2007) demonstrated that frogs with very intense *Bd* infections develop ionic imbalances that are probably sufficient to cause mortality, and suggested that these appear to be caused by loss of the normal osmoregulatory functions of the skin.

The primary method of diagnosis of *Bd* infections from its discovery until 2004 was histological examination of thin skin sections (usually 5 μm), which leads to diagnoses being made based on a very small fraction of the total skin surface. The distribution of *Bd* on the body surface, particularly in early infections, is likely to be patchy (Puschendorf & Bolaños 2006). Nonlethal histological screening screening of frogs in the wild has usually relied on examination of toe clips (Berger et al.1998; Lips 1998; Woodhams & Alford 2005). The combination of these factors produces a high rate of false negatives (Hyatt et al 2007), at rates which could depend on the distribution of infection among body parts.

In a thorough histological examination of 24 apparently healthy *Eleutherodactylus fitzingeri* from Costa Rica, Puschendorf & Bolaños (2006) found that of 12 different body regions tested, the first digit (next to the thumb) and the pelvic patch

were the most reliable areas for locating *Bd*, and that the anterior abdomen and the gular area were often negative in animals that were positive elsewhere. In another histological study, the spatial distribution of *Bd* was examined on 10 heavily infected *Litoria caerulea* but no significant differences in the density of zoosporangia were found among the ventral sides of the head, legs and abdomen (Berger et al. 2005a). Marantelli et al (2004) found zoosporangia in the hind feet and tails metamorphosing *Mixophyes fasciolatus*. Longcore et al. (2007) histologically examined sections of skin from the toe webbing, tibial region, and pelvic region of road-killed frogs of seven species from Maine, USA, and found that in most species the toe webbing was more likely to be infected than the tibial or pelvic regions. They did not simultaneously examine distribution of the fungus among body regions and overall infection intensity.

Diagnostic PCR-based assays (Annis et al. 2004; Boyle et al. 2004) appear to be more sensitive means of detecting *Bd* infections. Collecting samples for PCR using sterile cotton swabs makes it possible to sample a much greater proportion of the animal's surface. This approach is therefore likely to be more sensitive than histology for detecting low-intensity infections. It also makes it possible to non-invasively examine the spatial distribution of *Bd* on naturally infected individuals in the wild, which may aid in improving diagnostic accuracy. Understanding the distribution of *Bd* on animals in nature could also provide insight into the biology of the disease, including how the fungus is transmitted and how the disease develops and spreads on individuals.

Our aims were: (1) to examine the spatial distribution of *Bd* on the integument of naturally infected frogs, and determine whether this distribution provides information on probable routes of infection and patterns of disease development, and (2) to contribute to the development of evidence-based methods for swabbing amphibians for the diagnosis of *Bd* infection by PCR analysis.

To address the first aim, we measured the numbers of zoospore equivalents detected on swab samples taken from five regions of the bodies of adult, male green-eyed tree frogs, *Litoria genimaculata*. Samples were taken from frogs in natural populations in upland rain forests in northeastern Australia during the cool, dry season in 2005. This species experienced short-term population declines during 1990-1994, while many other species declined to local extinction, and has since recovered in areas where sympatric species have not (Richards & Alford 2005). It is susceptible to chytridiomycosis, but currently occurs in good numbers at most sites in the Australian Wet Tropics (Woodhams & Alford 2005). We analyzed the data to compare intensities of infection on different body regions of individual frogs, and to determine whether patterns of differences in infection intensity among body regions varied with the geographical source of the host populations. We also classified infections into four rank groups, ranging from very light to heavy, and examined how patterns of differences in intensity among body regions were related to overall infection level. We tested five null hypotheses: (1) the intensity of chytrid infections is the same across body regions, (2) patterns of differences among body regions do not differ among frog populations, (3) the amount of Bd DNA detected is independent of surface area of the body region examined, (4) the overall intensity of infection does not affect patterns of differences of intensity of infection among body regions, (5) sampling only the legs or only the abdomen would not reduce the probability of detecting *Bd* DNA from an infected frog.

We addressed the second aim by proposing a standardized method for swabbing *L. genimaculata* to diagnose *Bd* infection using PCR. This method targets the regions of the integument most likely to be infected, while taking precautions to avoid facilitating reinfection or spreading the pathogen on the host. We also suggest an approach for the development and application of standardized methods for other species that will minimize possible harmful effects.

## METHODS

We sampled adult male *L. genimaculata* at two high (≥800 m) and two low (<400 m) elevation tropical rainforest streams in northern Queensland, Australia, during the dry season (late August to late September 2005), when the prevalence of *Bd* is likely to be high (Woodhams & Alford 2005). Site locations, elevations, and mean weather conditions at the times of the surveys are shown in Table 25.

We searched for frogs along 400 m stream transects on two consecutive nights at each site . At Kirrama Bridge 1, Kirrama Bridge 11, and Birthday Creek, less than half of the transect was sampled on the first night, and the second night's sampling commenced 20 meters past the point at which the first night's ceased, making it very unlikely than any frogs could be sampled more than once (*L. genimaculata* very rarely move more than a few meters between streamside perches on successive nights, Rowley & Alford 2007). At Frenchman Creek, only two frogs were located in 400 m on the first night. To avoid resampling these individuals, their capture locations were flagged, and individuals were not searched for within 5 meters of those flagged locations on the second night, when many more frogs were active. Frogs were captured by hand using plastic bags, then sexed, weighed, measured, and swabbed using five separate swabs, one for each designated body region. A new pair of latex gloves was worn to handle each individual frog. All equipment and footwear were disinfected between sites. Sampling followed the Hygiene Protocol for the Control of Diseases in Frogs (NSW National Parks and Wildlife Service 2000) to avoid cross-contamination of infected individuals or sites.

### Swabbing technique

To determine the distribution of the fungus among body regions, we sampled each individual with five separate swabs (MW100 - Medical Wire & Equipment Co (Bath) Ltd). An assistant held the frog during swabbing to ensure samples of different body regions were not cross-contaminated.

Swabbing was performed as similarly as possible on all individuals, to reduce variation in measured infection intensity that might be caused by differences in technique. Figure 95 is an outline of a male *L. genimaculata* and illustrates the locations and relative sizes of the areas sampled. Initially, the back was swabbed, moving in from the posterior terminus of the body towards the head as many times as required to cover the entire back. This was done twice to ensure an adequate sample. Next, the ventral surface of the abdomen was swabbed the same way until it entire area was covered twice. Third, the legs were sampled by swabbing the ventral surfaces of the thighs and calves in a proximal to distal direction enough times to cover their full ventral surfaces twice. Fourth, the ventral surfaces of both of the forefeet were swabbed twice using a motion that encouraged the frogs to grip the swab to ensure good contact. If the swab was not

well gripped during the first two passes, 1-2 additional passes were made. Finally, the ventral surfaces of both of the hind feet were swabbed twice each, by pressing them between the swab and the glove of the handler.

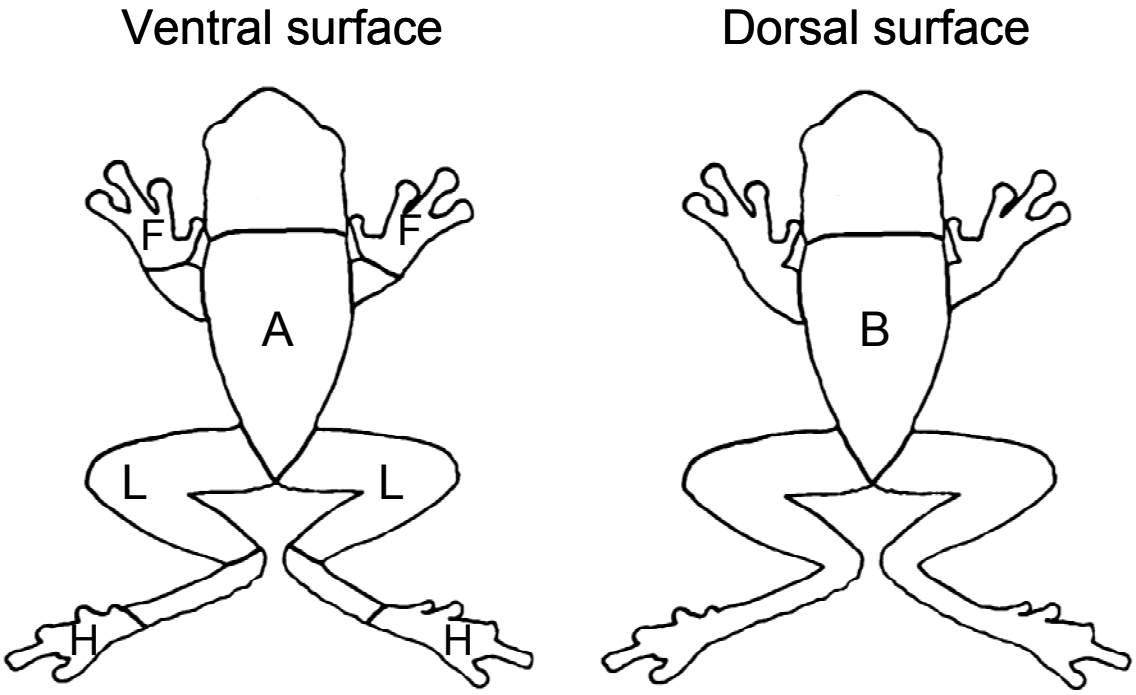


Figure 95. *Litoria genimaculata.* Outline indicating the extent and locations of ventral and dorsal body regions swabbed (A = abdomen, L = legs, H = hindfeet, F = forefeet, B = back).

Sample selection

Because each PCR assay is expensive, and many of the individuals sampled in the field might have been uninfected, we initially ran diagnostic PCR tests only on swabs from the legs and abdomen of each sampled individual to determine which were infected with *Bd*. The remaining three swabs from each individual that was positive in either region were tested separately. Finally, the samples from the forefeet and hindfeet of each individual that tested negative on both the legs and abdomen were combined during the extraction phase and tested together to determine whether any animals that had tested negative on both the legs and abdomen were infected in the feet. Samples from the backs of individuals that were negative on the legs and abdomen were not processed because the rates of back infection found on individuals positive in either of these areas made it apparent that this area has the lowest prevalence and intensity of infection, often remaining uninfected until all other areas are positive. We incorporated the possible implications of this when considering our results.

Real-time Taqman PCR assay

Extraction. PCR procedures for the extraction and amplification of *Batrachochytrium dendrobatidis* DNA were adapted from Boyle et al. (2004). PrepMan Ultra was used to prepare extractions of nucleic acids from swabs. Each swab was added to a 1.5 ml Eppendorf tube containing 30-40 mg 0.5 mm zirconium/silica beads (Daintree Scientific), to which 50 µl of PrepMan Ultra were added. One negative and one positive control swab were included per run. DNA from laboratory cultured *Bd* was used for the positive controls. Swabs were homogenized for 45 seconds using BeadBeater (Biospec) and were then centrifuged for 30 seconds at maximum speed to recover all material to the bottom of the tube. Homogenization and centrifugation were then repeated. The homogenized swabs were then heated in a dry heat block at 100˚C for 10 minutes, cooled for 2 minutes at room temperature, then centrifuged at maximum speed in a microfuge for 3 minutes. Between 20-40 µl of supernatant were collected and stored in columns of a microtitre plate (Sarstedt) at -80˚C.

PCR assay. The extract was diluted 1/10 in molecular biology grade water (Sigma) into even columns of the plate. Assays were conducted using a Corbett RotorGene 3000 model. A total of 25 µl (1 x Taqman Master Mix (Applied Biosystems), 900 nM PCR primer ITSI-3 Chytr and 900 nM PCR primer 5.8S Chytr, 250 nM ChytrMGB2 probe, and 5 µl of DNA (diluted $10^{-1}$ in water)) was added to each well and prepared in triplicate. Assay standards (prepared with dilutions of standard extract provided by Australian Animal Health Laboratory) were included in each run and used to create a standard curve. A control with distilled water (no DNA template) was also included and added after all samples and standards were made in triplicate.

Amplification conditions for the Corbett RotorGene 3000 are as follows: initial heating of 2 minutes at 50˚C, and 10 minutes at 95˚C, followed by 50 cycles of 15 seconds at 95˚C and 1 minute at 60˚C. The Ct values for each test and control reaction are determined using a ΔRn of 0.10.

Correcting for relative areas of body regions

The body sizes of individuals measured at the time of swabbing ranged from 34.6 to 46.0 mm snout-vent length (SVL). To examine intensity of infection in relation to the relative area of each of the regions of the body we sampled, we measured the SVLs of a series of preserved adult male *L. genimaculata* spanning this range of sizes. We then digitally photographed them against a light box to create silhouetted images. We used the program Image Tool (UTHSCA 1999) to measure the area of each body region in $cm^2$ and regressed log(area) of each region on log(SVL). All of the regressions had slopes were very close to 2.0, the slope expected if, within the limited size range of individuals we measured, all regions grew isometrically, and none differed significantly from 2.0. This indicates that the percentage of the total surface area contributed by eac body region remains constant over the range of body sizes we sampled. We therefore determined the percentage of the total mean surface area sampled attributable to each body region (27.1%, 26.5%, 28.2%, 8.0%, and 10.1% for the abdomen, legs, back, forefeet, and hindfeet, respectively), and compared the percentage of the total zoospore equivalents found on each body region to these percentages.

Statistical analyses

The data were entered and organized using Microsoft Excel 2003 and analyzed using SPSS version 12, except that Multiple Response Permutation Procedure and Multiple Response Blocked Procedure tests (MRPPs and MRBPs) were carried out using BLOSSOM version W2005.08.26 (Cade & Richards 2005). We log(n+1) transformed the PCR estimates of number of zoospore equivalents prior to analysis to reduce skewness and correlations between the mean and variance that were present in the untransformed data. To test the overall null hypothesis that there are no differences in intensity of infection among body regions, we calculated differences for each individual between the log-transformed intensities of infection for the legs and abdomen, abdomen and back, back and forefeet, and forefeet and hind feet, and tested these vectors of differences against the null hypothesis that on average they were equal to (0,0,0,0) using an MRBP test with hypothesis testing by 10,000 randomizations (Cade & Richards 2005); this is a distribution-free analogue of Hotelling's $T^2$ test for significant departure from a hypothesized mean vector. We performed a profile analysis (Morrison 1976) to test for differences in the distribution of intensity across body regions among sites. This analysis separately examined the total intensity of infection and patterns of differences in intensity of infection among body regions using MRPP tests with hypothesis testing by 10,000 randomizations (Cade & Richards 2005). Following significant overall tests, we performed separate profile analyses comparing each pair of sites using the same technique. We determined the significance of these tests using a Bonferroni-adjusted alpha of 0.0167 to preserve the overall experimentwise alpha level at 0.05. We tested the null hypothesis that all body parts are infected with equal intensity by using an MRBP test of the null hypothesis that the percentage of zoospore equivalents on each body region was equal to the percentage contribution of that body region to the total area sampled. We also used an MRPP test to determine whether patterns of intensity of infection among body areas differed among frogs assigned to four groups based on their total infection intensities.

RESULTS

Overall prevalence and intensity of infection in our samples

Weather conditions at each site at the times of sampling were within the range for adequate but not maximal *Bd* growth (Table 25). 58 of the 111 individuals sampled were positive for *Bd* on the legs or abdomen, and therefore all other swabs for these individuals were analyzed separately. We performed PCR analyses on the combined forefeet and hindfeet swabs from the 53 individuals that were negative for *Bd* on both the abdomen and the legs; these revealed two additional positive individuals that were very lightly infected (1 and 4 zoospore equivalents, from Kirrama Bridge 1 and Kirrama Bridge 11, respectively). These individuals were not included in detailed statistical analyses because we had combined the samples from the forefeet and hindfeet, making comparisons among body regions impossible. There was no evidence for any inhibitory activity against the positive controls incorporated into the TaqMan master mix (Hyatt et al. 2007).

Only 3 of 34 animals sampled at Bridge 1 in the Kirrama State Forest (9%) tested positive (Table 25). This low prevalence fits with patterns seen in other studies (e.g., Woodhams & Alford 2005), which have found that prevalence of Bd is usually lower at lowland sites. Data from this site were omitted from the detailed analyses of the

Table 25. *Litoria genimaculata*. Details of sampling localities, with environmental variables and infection prevalences and intensities. Prevalence and intensity data include results for all frogs sampled, including those that initially tested negative on the legs and abdomen.

| Site | Latitude/ Longitude | Elevation (m) | Air (°C) | Water (°C) | Relative Humidity (%) | Number of animals | | Percent positive | Number of zoozpore equivalents on positive individuals | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | sampled | positive | | Mean | S.D. | Minumum | Median | Maximum |
| Kirrama Bridge 1 | 18o12'11"S 145o53'00"E | 100 | 18 | 19 | 100 | 34 | 3 | 9 | 190.5 | 328.7 | 0.5 | 1.0 | 570.0 |
| Kirrama Bridge 11 | 18o12'55"S 145o47'48"E | 850 | 15 | 16.8 | 94 | 23 | 19 | 83 | 160.2 | 327.3 | 1.0 | 4.5 | 1073.0 |
| Frenchman Creek | 17o18'32"S 145o55'16"E | 40 | 19 | 19.8 | 95 | 17 | 15 | 88 | 22.6 | 38.5 | 1.0 | 7.0 | 135.0 |
| Birthday Creek | 18o58'54"S 146o10'02"E | 800 | 16.5 | 19.6 | 95 | 37 | 23 | 62 | 731.5 | 1694.4 | 1.5 | 221.5 | 8035.0 |

Table 26. *Litoria genimaculata*. Summary of positive PCR results by body region for the 14 individuals in each infection rank group.

| Infection Rank | Body Region | Number of Positives | Number that were also positive on: | | | | | Number Negative in All Other Regions |
|---|---|---|---|---|---|---|---|---|
| | | | Legs | Abdomen | Back | Forefeet | Hindfeet | |
| Very Light | Legs | 6 | -- | 1 | 1 | 3 | 2 | 3 |
| | Abdomen | 9 | 1 | -- | 1 | 2 | 2 | 6 |
| | Back | 1 | 1 | 1 | -- | 1 | 1 | 0 |
| | Forefeet | 4 | 3 | 2 | 1 | -- | 2 | 0 |
| | Hindfeet | 3 | 2 | 2 | 1 | 2 | -- | 0 |
| Light | Legs | 12 | -- | 11 | 7 | 9 | 10 | 0 |
| | Abdomen | 13 | 11 | -- | 8 | 10 | 10 | 1 |
| | Back | 8 | 7 | 8 | -- | 7 | 8 | 0 |
| | Forefeet | 10 | 9 | 10 | 7 | -- | 9 | 0 |
| | Hindfeet | 11 | 10 | 10 | 8 | 9 | -- | 0 |
| Moderate | Legs | 13 | -- | 10 | 10 | 11 | 12 | 0 |
| | Abdomen | 11 | 10 | -- | 9 | 10 | 11 | 0 |
| | Back | 11 | 10 | 9 | -- | 9 | 10 | 0 |
| | Forefeet | 12 | 11 | 10 | 9 | -- | 12 | 0 |
| | Hindfeet | 13 | 12 | 11 | 10 | 12 | -- | 0 |
| Heavy | Legs | 14 | -- | 14 | 12 | 14 | 14 | 0 |
| | Abdomen | 14 | 14 | -- | 12 | 14 | 14 | 0 |
| | Back | 12 | 12 | 12 | -- | 12 | 12 | 0 |
| | Forefeet | 14 | 14 | 14 | 12 | -- | 14 | 0 |
| | Hindfeet | 14 | 14 | 14 | 12 | 14 | -- | 0 |

distribution of *Bd* among body regions because of the small sample size. Prevalences at the remaining three sites were relatively high: 83% at Kirrama Bridge 11, 88% at Frenchman Creek, and 62% at Birthday Creek (Table 25). Intensity of infection, measured as number of zoospore equivalents totaled across body regions (Table 25), was relatively low at Bridge 1, Bridge 11, and Frenchman Creek (medians of 1, 4.5, and 7 zoospore equivalents, respectively) and substantially higher at Birthday Creek (median of 221.5 zoospore equivalents). However, at all sites some individuals carried very light infections and some carried relatively heavy infections.

Differences in infection intensity among body regions and sampling localities

Our MRBP test rejected the null hypothesis of equal intensity of infection among body regions ($\delta = 2.2125$, $p = 0.0001$), Both the total intensity of infection and the pattern of differences among body regions differed significantly among localities ($\delta = 2.843$, $p = 0.0001$ and $\delta = 2.307$, $p = 0.008$, respectively). Animals at Bridge 11 and Frenchman Creek did not differ significantly in pattern of infection intensity among body regions ($\delta = 2.380$, $p = 0.5363$) or in the total intensity of infection ($\delta = 2.442$, $p = 0.1017$). Animals at Bridge 11 and Birthday Creek differed significantly in pattern of intensity among body regions ($\delta = 2.285$, $p = 0.0055$) and in total intensity of infection ($\delta = 3.389$, $p = 0.0035$). Frogs at Frenchman Creek and Birthday Creek also differed significantly in their patterns of intensity among body regions ($\delta = 2.300$, $p = 0.0071$) and in total intensity of infection ($\delta = 2.504$, $p = 0.0001$).

Figure 96 illustrates the patterns of infection for the five body regions at the three localities with relatively high prevalences of *Bd*. Most individuals at both Bridge 11 and Frenchman Creek had fairly light infections. On average, at these localities infection was most intense on the legs and the abdomen, followed in decreasing order by the hind feet, forefeet and back. At Birthday Creek, where most individuals had more intense infections, intensity tended to be highest on the legs, followed in decreasing order by the hindfeet, the abdomen, the forefeet, and the back.

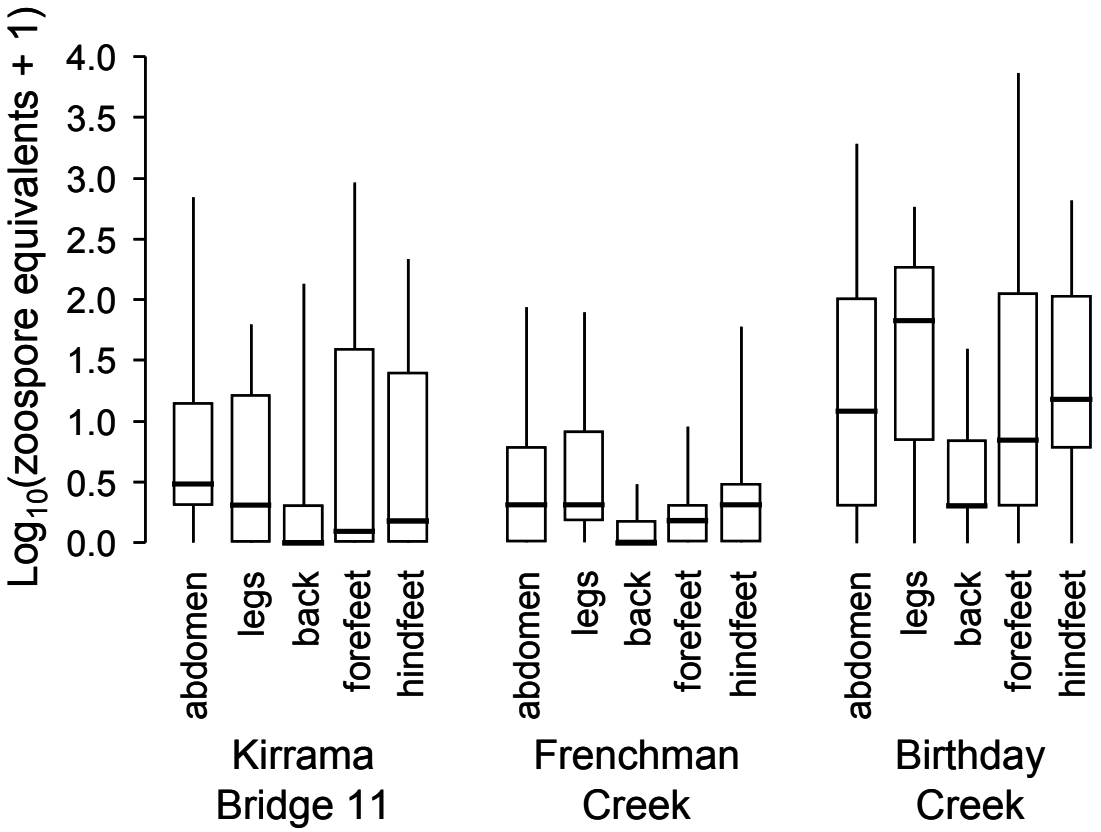


Figure 96. *Litoria genimaculata.* The distributions of infection intensity in number of zoospore equivalents for each body region at the three sites used in analyses, Kirrama Bridge 11 (850m), Frenchman Creek (40m), and Birthday Creek (800m). Heavy horizontal bars represent medians, upper and lower limits of boxes are 75$^{th}$ and 25$^{th}$ percentiles, respectively, and vertical bars represent ranges.

Effects of overall infection intensity on distribution among body regions

Overall, the percentage of total intensity of infection occurring on different body regions differed significantly from that expected if all regions of the body were infected in proportion to their surface area (MRBP, $\delta$= 39.076, P = 0.00005). To determine whether the distribution of *Bd* among regions of the body is related to the overall intensity of infection, for the 56 frogs included in our locality analyses we totaled the estimated zoospore equivalents across all body regions, ranked frogs by this total, and divided them into four groups. Each group contained 14 individuals (Table 26). The *Very Light* rank group included animals that totaled 1-3 zoospore equivalents. The *Light* and *Moderate* rank groups totaled 4-30 and 31-233 zoospore equivalents, respectively. The *Heavy* rank group contained animals that totaled 248-8035 zoospore equivalents. An MRPP test rejected the null hypothesis that the patterns of Bd intensity among body regions did not differ among these four groups ($\delta$ = 2.016, p = 0.0001).

Figure 97 illustrates the distribution of zoospore equivalents on body regions of frogs in each of the four infection rank groups. The abdomen accounted for a median of 58% of the total zoospore equivalents estimated from individuals in the *Very Light* group;

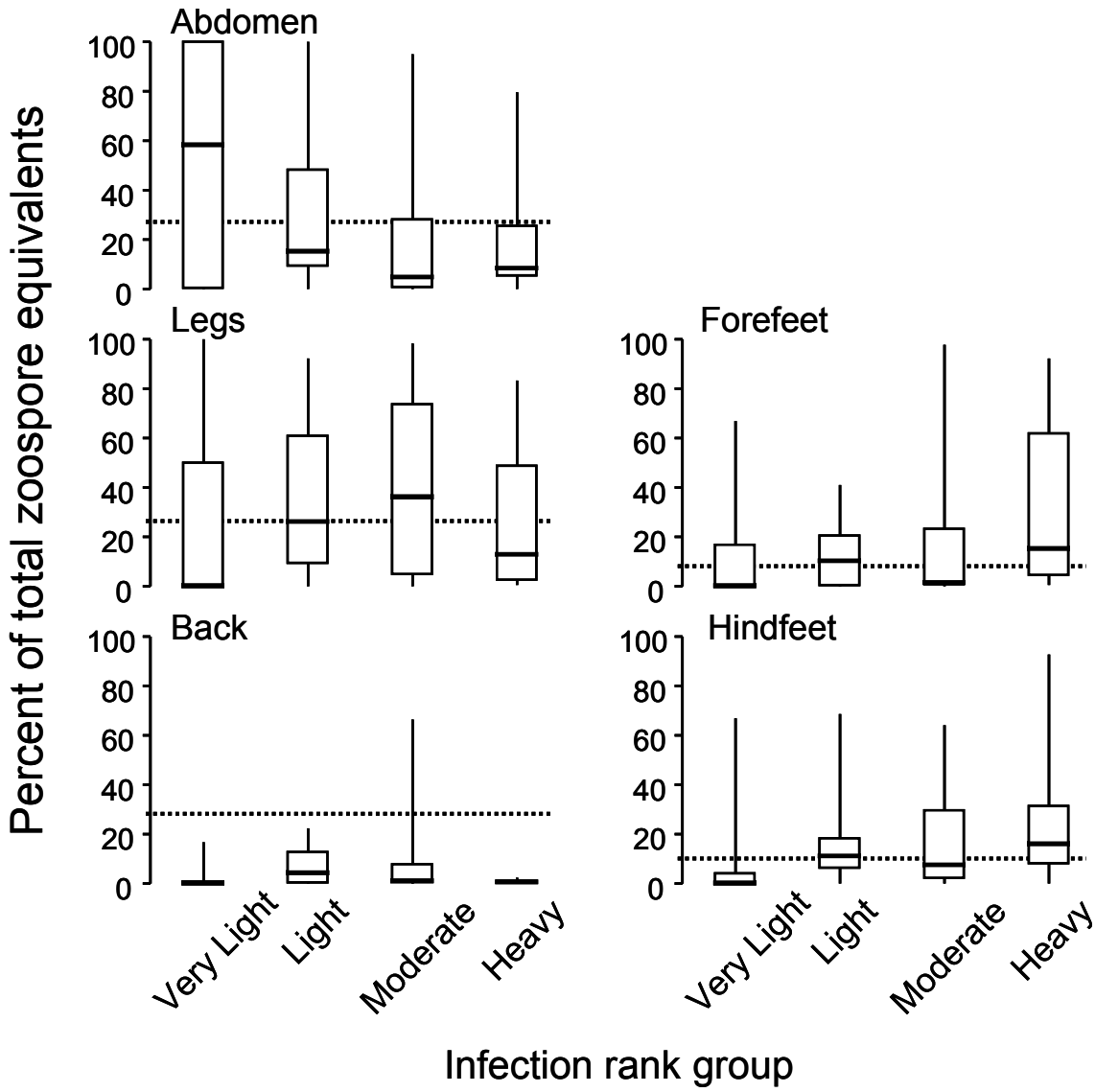


Figure 97. *Litoria genimaculata.* The distributions of infection intensity as percentage of total zoospore equivalents among individuals in four infection rank groups for each body region. Dotted horizontal lines indicate the mean percentage each body region contributes to the total area sampled, and thus delineate the null hypothesis of equal intensity of infection per unit area. Each rank group contained 14 individuals. Plot symbols defined as in Figure 96.

this is over twice the proportion expected based on the relative surface area of the abdomen. The relative intensity of infections in the abdomen decreased as total infection intensity increased, until in the *Heavy* rank group, more than 75% of individuals had a lower proportion of zoospore equivalents on the abdomen than would be expected based on its relative size. The median percentage of zoospore equivalents on the legs in the *Very Light* infection rank group was zero, although more than 25% of individuals had a

greater proportion of the total number of zoospore equivalents on their legs than would be expected based on their relative size. The relative intensity of infection in the legs increased as total intensity increased in the *Light* and *Moderate* groups, then decreased to a median value of roughly half that expected from the relative size of the legs in the *Heavy* group. The back was under-represented in all infection rank groups; only one of the 56 individuals examined had a greater percentage of the total number of zoospore equivalents in the sample from the back than expected based on the relative size of this body area, and most had substantially less than expected. The patterns exhibited by the forefeet and hindfeet were similar across infection rank groups. The medians for both were zero in the *Very Light* group, with slightly more individuals showing percentages of total zoospore equivalents greater than zero in samples from the forefeet. In the *Light* infection ranking group, median percentage of total zoospore equivalents was close to that expected from the relative sizes of the regions for both forefeet and hindfeet, and more than 75% of individuals returned positives for each. Relative intensity of infection in both forefeet and hindfeet decreased slightly in the *Moderate* group, probably reflecting sampling variation. In the *Heavy* infection rank group, the relative intensity of infection in the forefeet and hindfeet increased substantially, leading to median percentages of zoospore equivalents approximately twice as great as expected from the sizes of the body regions.

Examining Figure 97 by looking across body regions within infection rank groups, *Very Light* infections were concentrated on the abdomen, with some individuals also having relatively intense infections on the legs, and tended to be absent from the back, forefeet, and hindfeet. *Light* infections were concentrated on the legs, forefeet, and hindfeet; the median percentages of zoospore equivalents on all three of these body regions are similar to those expected from their relative sizes. Although the abdomens of most individuals with *Light* infections returned positive PCR results, the percentage of total zoospore equivalents was lower on the abdomen than expected from its relative size. The median relative intensity of infection on the back reached a peak in this group, but remained low, with a median percentage of total zoospore equivalents approximately 25% as great as expected from the relative size of the back. The pattern for *Moderate* infections was similar to that for *Light* infections, with even lower relative intensity on the abdomen, and higher relative intensity on the legs. Finally, *Heavy* infections had median relative intensities on the forefeet and hindfeet that were well above the levels expected from the relative sizes of these regions, relative intensities on the legs that were lower than those in *Moderate* infections, and low and very low relative intensities on the abdomen and back, respectively.

Table 26 makes it possible to examine patterns of relationships of positive PCR results among body regions within individuals in each of the infection rank groups. Nine of the 14 individuals in the *Very Light* rank group were positive for only one body region, in each case either the abdomen or the legs. Individuals that were positive for more than one body region tended to be positive on either the forefeet or the hindfeet or both. In the *Light* rank group, eleven of the 14 individuals were positive for both the legs and the abdomen, and most individuals that were positive for either of these regions were also positive for the forefeet, the hindfeet, or both. Only one individual was positive for only a single region, the abdomen. In the *Moderate* and *Heavy* rank groups, almost all individuals were positive on all body regions; even the back was consistently positive,

although as noted in discussing Figure 97, the percentage of the total estimated zoospore number for the back remained low in all infection rank groups. Including the results for the two individuals of the group of 53 that tested negative for both abdomen and legs would make little difference to this general pattern, When we tested the combined hindfeet and forefeet samples for those 53 individuals, only two of them were positive, with infections that would have placed them in the *Very Light* rank group (1 zoospore equivalent) and just into the *Light* rank group (4 zoospore equivalents). These individuals were negative on all other body regions.

## DISCUSSION

### Prevalence, intensity and sampling locality

Populations from three of the four localities we sampled had relatively high prevalences of infection (Table 25), indicating that *Litoria genimaculata* populations are now persisting in equilibrium with the disease and that it does not inevitably result in rapid mortality; this species may thus be serving as a reservoir host. Although prevalences at three of the four localities were high and similar, they differed from one another, and the fourth locality had a very low prevalence. There is no obvious environmental cause for these differences; measured environmental variables were similar at all localities at the times of sampling. It is likely that weather at these localities differs more than is indicated by our short-term measures when it is integrated over the full annual cycle, however the pattern detected was not the expected one, in which higher prevalences occur at upland localities (Woodhams & Alford 2005). The typical upland-lowland differences in prevalence may result from different thermal regimes (Woodhams et al. 2003), and the recent history at Frenchman Creek may simply have not included temperatures elevated sufficiently to reduce the prevalence of *Bd*. Most individuals at three of the four localities had relatively low total intensities of infection, while most at the fourth, Birthday Creek, had relatively high total intensities; this is also likely to reflect the recent detailed history of the locality. The fact that at all localities, some individuals had low total intensities and some had high ones, indicates that although there appear to be effects of the environment and history that operate at the locality level, factors affecting individuals vary on a finer scale.

### Distribution among body regions

Berger et al. (2005a) found no significant differences in the densities of zoosporangia on the ventral surfaces of the head, abdomen, legs, and toes in *Litoria caerulea* suffering from severe chytridiomycosis that were examined post-mortem using histology, but did find lower densities on the dorsal surfaces of the back and calves. Because they examined only frogs suffering from advanced symptoms of the disease, their results provide no insight into patterns of development of infection, or patterns of distribution of infection in asymptomatic frogs. Longcore et al. (2007) examined roadkilled and deliberately collected frogs of seven species collected in Maine, USA. They performed histological diagnoses on sections of skin from the webbing between the toes of the hindfoot, the pelvic area of the ventral abdomen, and (for some specimens) the ventral surface of the tibial region of the hind leg. They were not able to examine all regions for all frogs due to varying degrees of damage to the animals prior to collection. They found that prevalence and intensity of infection varied among species and collection

localities. Overall, they found that prevalences of infection on skin from toe webbings were somewhat higher than on skin from the pelvic region (15.5% vs. 9.8%), and that prevalences were lowest on skin from the tibial region (5.3%). These patterns differed among species. They classified the majority of infections as relatively light, but did not examine how intensity of infection was distributed among body regions within individuals. Puschendorf & Bolaños (2006) found that sections from the fingers, toes, and pelvic patch region of *Eleutherodactylus fitzingeri* returned positive histological diagnoses more often than sections from the gular region and abdomen; however they did not categorize intensity of infection.

Our results from living frogs differ from both histological surveys; as total intensity of infection increased, larger amounts of DNA were found on all ventral surfaces, however, even in heavily infected frogs body regions differed in intensity of infection. In heavy infections, the greatest median percentages of DNA were swabbed from the hindfeet and forefeet, followed by legs and abdomen. In light infections, the percentage of zoospore equivalents was greatest for the abdomen, which accounted for more than twice as many zoospore equivalents as expected from its relative size. The relative intensity of infection on the abdomen decreased as overall intensity increased, while the relative importance legs and feet increased (Figure 97).

The relationships we found between the distribution of *Bd* DNA among body regions and overall intensity of infection could have three causes. They could reflect differences in opportunity for infection; body regions that are more likely to be exposed to infective zoospores from outside the individual should tend to become infected first, and be the first sites to develop heavier infections through local reinfection. They could reflect differences in the vulnerability of body regions to reinfection; if the skin surface of a body region presents a more favorable environment for local zoospore release and reinfection, for example if it tends to retain a film of moisture, that part might become more heavily infected earlier in the process. Finally, they could reflect differences among body regions in their innate resistance to *Bd,* perhaps in the form of localized concentrations of antimicrobial peptides, many of which are effective against *Bd* (Rollins-Smith et al. 2002; Woodhams et al. 2006a, b), or differences in the local structure of the outer layers of the skin, which could also affect vulnerability (Berger et al. 2005a). When frogs are not moving, the ventral surfaces of their feet and legs are almost always in contact with the substrate, whereas their abdomen is only sometimes in such contact. Berger et al. (2005b) found that zoospores growing *in vitro* appeared to be attracted to established colonies and that thalli grow better in clusters. They also found that single zoospores placed on agar usually died. Contact with substrates may maintain a moist microenvironment, allowing high zoospore survival and reinfection rates, and may also increase local reinfection rates by reducing rates of dispersal of zoospores to the external environment.

The intensity of infection on the backs of even heavily infected individuals was always relatively low. Berger et al. (2005a) also found low intensities on the back. This may be due to the high number of serous glands containing anti-fungal peptides located in this region (Rollins-Smith et al. 2002; Berger et al. 2005a), or the relative dryness of the dorsal surface (Berger et al. 2005a), which might inhibit reinfection. *Litoria genimaculata* often spend extended periods on open perches away from water (Rowley & Alford 2007); the relatively low intensities of infection we found on their backs could

result from dryness during these times, or from higher temperatures experienced when the dorsal skin is exposed to direct sunlight; either of these could slow reinfection by *Bd*.

Effects of sampling localities on distribution among body regions

Patterns intensity of infection among body regions were significantly different between Birthday Creek and the other two localities with large enough samples for analysis. These differences among localities did not appear to be a product of differences in elevation, because Kirrama Bridge 11 and Frenchman Creek, which did not differ significantly, are at high and low elevations, respectively, whereas Birthday Creek, which differed from both of those localities, is at an elevation similar to Bridge 11. It is likely that the differences among localities in pattern of distribution on body regions were caused by a combination of the large differences among localities in intensity of infection (Table 25) with the relationship between intensity of infection and distribution of *Bd* among body regions. Most individuals at Bridge 11 and Frenchman Creek fell into our *Very Light* and *Light* infection rank groups, which tended (Figure 97) to be most heavily infected on the abdomen, followed by the legs. Most individuals at Birthday Creek fell into our *Moderate* and *Heavy* infection rank groups, which tended to carry relatively high percentages of zoospore equivalents on their forefeet and hindfeet (Figure 97) and relatively lower (although still high in absolute terms, since these regions are much larger) percentages on the legs and abdomen.

Patterns of development of infections

Our data are consistent with the hypothesis that that the overall intensities of infection we measured are related to the pattern of development of infection in most individuals, rather than to patterns of loss of infection or a combination of patterns of development in some individuals and loss in others. Berger et al (2005a) found that serous glands, which produce antimicrobial peptides (AMPs), are most dense in the dorsal skin of *Litoria caerulea*, and are also relatively dense in the ventral skin of the abdomen and legs, but are less dense in the ventral skin of the forefeet and hind feet. This suggests that resistance to colonization and reinfection by *Bd* should be greatest in the dorsal skin, intermediate in the ventral skin of the legs and abdomen, and lowest in the ventral skin of the feet. Resistance to infection caused by AMPs, and therefore possible reduction in intensity or loss of infection, should occur most rapidly in the dorsal skin, followed by the ventral skin of the legs and abdomen, and finally by the ventral skin of the feet. The lightest infections should thus be concentrated in the feet. This pattern is not the most common one in our data. Some individuals may acquire infections via the forefeet or the hindfeet, or may be losing infections, but the great majority of individuals in the *Very Light* and *Light* infection rank groups were most heavily infected in the abdomen and legs, suggesting that these individuals had infections in early stages of development, and that the abdomen and legs are the most common sites of initial infection. Infections in the two most intense infection rank groups tended to be heaviest in the feet, suggesting that once infections reach these areas, their lower resistance may allow greater densities of *Bd* thalli to develop. It is also very likely that the back is not a common route of infection; all individuals but one had fewer zoospore equivalents on their backs than expected from the relative size of this region, and over two-thirds of individuals in the *Very Light* and *Light* infection rank groups did not return positive results from their back samples. Because we did not test back samples for the 51 individuals who did not test positive on any of the legs, abdomen, forefeet, or hindfeet, it remains possible that we have missed a class of individuals who are infected only on their backs, but the pattern in the remainder of the data makes this seem very unlikely. The abdomen, followed by the legs, may be the most common sites of initial infection because they present relatively large areas for zoospores to establish on, and are often pressed against the substrate, both to allow absorption of water through the pelvic patch , and when frogs adopt a water conserving posture to minimize their exposed surface area (Shoemaker et al. 1992). It is also possible that the nature of the skin in these regions, both of which include parts of the pelvic patch (Shoemaker et al. 1992) in *Litoria genimaculata,* facilitates invasion by *Bd* zoospores.

Implications for swabbing methodology

The increased use of PCR to identify *Bd* infection in amphibians suggests a need for care in the selection of patterns used in swabbing individuals. For results to be comparable between individual researchers and between studies, samples must be collected from a standard set of body regions, swabbed with equal effort. Many publications have described swabbing methods and patterns (e.g., Speare et al. 2005; Hyatt et al. 2007) that could be adopted as standards. However, adoption of a single universal pattern of swabbing several body regions by researchers could inadvertently harm the animals that are sampled. Because populations of *Bd* increase on hosts through

external reinfection, and zoospores may tend to settle very close to the sites from which they are released (Berger et al 2005b), swabbing may artificially increase the rate of dispersal of zoospores across hosts. This might increase the severity of infections on swabbed animals. To prevent this, swabbing could be limited to a single region of the body. If this were done the area most likely to return a positive result should be sampled. However, our results indicate that swabbing any single body region can lead to a high proportion of false negatives in lightly infected animals. A second approach, optimal from the points of view of sensitivity and the minimization of potential harm, is to use separate swabs for each body region. These could then be combined before diagnostic PCR is performed. However, the increased sample volume and time to collect and process samples required by this technique may make it impractical. A third alternative is to swab more than one region, in a pattern that minimizes the probability of transporting zoospores from more heavily infected regions to more lightly infected ones. In *L. genimaculata*, we suggest that for male *L. genimaculata* the ventral surfaces of the legs, the abdomen, and the gripping surfaces of the fore- and hind feet should all be swabbed, with the feet first, followed by the legs and then the abdomen. This would minimize the probability of spreading the infection in lightly infected individuals. Patterns for other species are likely to differ; Longcore et al. (2007) found the feet to be infected at the greatest intensity in lightly infected *Rana* spp. There could also be differences among males, females, and juveniles within species; Rowley and Alford (2007) found differences in behaviour between males and females that could lead to differences in the pattern of development of infections. This suggests that in the absence of species- and class-specific information, our second approach, the use of separate swabs for each body region, should be followed, at least until sufficient data have accumulated to allow an evidence-based pattern to be specified.

It would be useful in future longitudinal studies in which individuals are repeatedly swabbed to swab major regions of the body separately, perhaps combining half of each sample for diagnosis of the presence of *Bd*, and then examining samples from each body region of infected individuals separately. This would clarify initial routes of infection and patterns of development of infections. It would also allow identification of any region of the body producing skin secretions that inhibited the diagnostic PCR (Hyatt et al 2007). This information should aid in understanding how *Bd* is transmitted and why hosts vary in their susceptibility, and could be used to develop species- and class-specific sampling orders for swabbing with single swabs.

*Acknowledgements.* We thank S. Bell, N. Kenyon, V. Hickin, J. Wilkinson, I. Bell, K. Bell, and R. Hansen for assistance with field work. S. Bell provided logistical support and R. Campbell carried out diagnostic PCR assays and instructed SN in the technique. This research was funded by a subcontract to RAA from US National Science Foundation IRCEB grant DEB-0212851 (J.P. Collins, P.I.) and by funds from the Australian National Heritage Trust (Tender 43/04, R. Alford, P.I.). It was carried out under James Cook University Animal Ethics Approval No. A960 (Alford), and Scientific Purposes Permits WITK 03070505 and WISP 03070205 and Forestry Permit INN 05/004, from Queensland.

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***Project 7. Mechanisms of resistance to chytridiomycosis in recovered and recolonised populations.***

Project 7 addresses **Objective 1** by examining the acquisition of resistance by hosts, which may include changes in behaviour as well as or instead of changes in immune function. It addresses **Objective 2** by examining upland populations of species that have survived decline events and of species that have disappeared from areas but recolonised and comparing them to lowland, and now inland, populations of the same species from locations where declines never took place to determine whether the surviving/recolonising populations have greater resistance to chytridiomycosis via skin peptide function. It addresses **Objective 4** by producing data on the extent to which the skin peptides and behaviour of frogs from recovered or recolonised populations reduce their vulnerability to chytridiomycosis. It addresses **Objective 7** by looking for evidence of increased immunity or resistance to chytridiomycosis by comparing resistance to infection and the outcomes of experimental infections between populations that have survived crashes and recovered and populations that have not experienced crashes.

## ***Project 7.1 Have anuran skin peptide defences against an emerging amphibian pathogen responded to natural selection?***

Nicole Kenyon, Ross A. Alford, Sara Bell, and Kirsten Heimann

**Abstract**
The disease chytridiomycosis, caused by the fungus *Batrachochytrium dendrobatidis* (*Bd*), has caused amphibian declines in many regions of the globe. However, its effects vary widely among species; some can persist despite infection while others are driven to local extinction. One factor that may contribute to differences in susceptibility is interspecific differences in innate immune defences, especially in rates of production and effectiveness of antimicrobial peptides (AMPs) against *Bd.* We examined these in two sympatric species of Australian frogs, *Litoria genimaculata* and *L. rheocola.* We collected skin secretions from 216 frogs, from upland populations that suffered declines and disappearances caused by chytridiomycosis and have subsequently recovered or recolonised, and from lowland populations that did not decline, although chytridiomycosis is now endemic. We hypothesised that strong selection pressure exerted by *Bd* could have caused the AMPs of upland populations to diverge from those of lowland populations. We quantified peptide secretion using spectrophotometry and challenged *Bd* cultures with AMPs. Based on the concentrations of AMPs needed to completely inhibit *Bd* growth ($IC_{100}$), both peptides secreted per surface area and AMP effectiveness against *Bd* varied seasonally in both species, suggesting that their innate immune defences may be affected by seasonal changes in physiology, or may be adapted to seasonal fluctuations in environmental microbiota. Overall protection (mL of AMPs at $IC_{100}/cm^2$) of frogs with peptides effective against *Bd* did not differ significantly between high and low elevation populations of either species, however more frogs had effective AMPs at high elevations, suggesting that the stronger selection imposed by *Bd* in high elevation populations has not led to the evolution of different and more effective AMPs, but may have led to a higher proportion of frogs possessing AMPs that are effective against *Bd*.

Keywords: Antimicrobial peptides, chytridiomycosis, anuran, immune defence, innate, decline

### Introduction
Chytridiomycosis is a disease of amphibians, caused by the fungus *Batrachochytrium dendrobatidis* (*Bd*). It is defined as an emerging infectious disease (EID) as it has recently increased its geographical and host species ranges (Daszak, Cunningham & Hyatt, 2001; Dobson & Foufopoulos, 2001; Morse, 1995). The effect of *Bd* infection varies widely among species, ranging from no apparent effect at the population level to local extinction (Lips *et al.*, 2005; McDonald & Alford, 1999; Richards, McDonald & Alford, 1993).

Variation in innate immune defences among species could contribute to the observed variation in susceptibility. A key component of the innate immune system in amphibians is production and secretion of antimicrobial peptides (AMPs), short proteins that hinder initial microbial invasion of the epidermis (Hoffman *et al.*, 1999).

Several studies have shown that *in vitro* AMPs of frogs can kill or inhibit the growth of *Bd* zoospores (Rollins-Smith *et al.*, 2003; Rollins-Smith *et al.*, 2002; Woodhams *et al.*, 2007). There are several aspects of AMPs that can be compared among frog species (Woodhams *et al.*, 2007; Woodhams *et al.*, 2006). First, the quantity of peptides (which includes similar sized peptides and AMPs) a frog secretes per surface area, given a specific stimulus. Second, the effectiveness of AMPs at inhibiting the growth of *Bd* cultures. This is measured as the minimal concentration of AMPs needed to absolutely inhibit the growth of *Bd* cultures ($IC_{100}$). Third, the overall level of protection against *Bd*, which considers both the amount of peptides secreted per surface area and the $IC_{100}$.

Northern Queensland, Australia, is an ideal area to study AMPs as frog populations have been extensively monitored before, during and after chytridiomycosis epidemics, so their responses to these events are well documented. *Litoria rheocola* and *L. genimaculata* have both experienced population bottlenecks. *Litoria rheocola* suffered local extinctions at high elevation sites throughout its range during initial outbreaks of chytridiomycosis in the 1990s, while populations below ca. 400 m were stable (McDonald & Alford, 1999). Populations of this species are slowly recovering at high elevations, most likely by recolonising from lowland populations (Woodhams & Alford, 2005). *Litoria genimaculata* populations at high elevations declined but did not reach local extinction and have subsequently recovered (McDonald & Alford, 1999; McDonald *et al.*, 2005). The lack of declines in both species at low elevations was most likely due to less favourable environmental conditions for chytridiomycosis outbreaks (Berger *et al.*, 2004; Kriger & Hero, 2007; Woodhams & Alford, 2005).

We hypothesised that if AMPs played an important role in the survival of *L. genimaculata* and *L. rheocola* individuals infected with *Bd*, strong selection pressure could have caused the AMPs of upland populations to diverge, increasing their innate protection against *Bd* when compared to lowland populations. We compared AMP production, effectiveness and overall protection against *Bd* between *L. genimaculata* and *L. rheocola* at high and low elevation sites, to test this hypothesis and to provide a better general understanding of intraspecific variation in AMPs.

## Materials and methods

### Sample collection and processing of peptides

Skin peptides were collected from frogs at five sites at three different latitudes throughout the Wet Tropics (Table 27). Each site consisted of a 400 meter transect along a creek. Skin peptides from *L. genimaculata* males were collected from all five sites in winter 2006 and summer 2007. Skin peptides from *L. rheocola* were collected in summer 2007 and winter 2007 at two sites (Windin N Creek and Frenchman Creek; this species was absent from the other sites). Frogs were captured at night using small plastic bags inverted over the investigator's hand and transferred into larger 230mm × 305mm resealable bags (one frog per bag). The resealable bags, containing the frogs, were numbered and the location of each frog on the transect was marked with flagging tape so that individuals could be released at the point of capture after peptide collection. For peptide collection, frogs were temporarily removed from the creek to a nearby vehicle because this process required chemicals that needed to be used away from the creek in case of accidental spillage. A maximum of 10 frogs were sampled per night.

Snout-vent length (SVL) of each frog was measured, the frog was weighed (using a high precision pocket balance PS-50) and skin swabbed using a sterile tubed dry swab (Medical Wire and Equipment MW100, Corsham, Wiltshire UK) that was run across the frog's hands, feet, thighs and ventral surface twice, for diagnostic qPCR assays to quantify infection status *Bd* (Boyle *et al*., 2004). Diagnostic qPCRs were run at James Cook University in the School of Public Health and Tropical Medicine. Each sample was run in triplicate, following the protocol of Hyatt et al. (2007)

**Table 27.** Locations of the five study sites where *Litoria genimaculata* and *L. rheocola* skin peptides were collected.

| ***National Park*** | ***Site*** | ***Elevation*** | ***GPS coordinates*** | ***Frog species*** |
|---|---|---|---|---|
| *Paluma Range* | *Birthday Creek* | *800 m* | *S18º58'54" E146º10'02"* | L. genimaculata |
| *Murray Upper* | *Bridge I* | *100 m* | *S18º12'11" E145º53'00"* | L. genimaculata |
| *Murray Upper* | *Bridge XI* | *850 m* | *S18º12'55" E145º47'48"* | L. genimaculata |
| *Wooroonooran* | *Frenchman Creek* | *40 m* | *S17º18'32" E145º55'16"* | L. genimaculata *and* L. rheocola |
| *Wooroonooran* | *Windin N Creek* | *750 m* | *S17º21'57" E145º42'54"* | L. genimaculata *and* L. rheocola |

Peptide collection followed the protocol described by Rollins-Smith *et al.,* (2006). Following peptide collection, digital images of *L. genimaculata* were taken to allow use of a photographic identification method to eliminate the possibility of taking samples from the same individuals on successive nights. After digital images were taken, frogs were released at their collection point. *Litoria rheocola* does not show a distinct ventral or dorsal pattern that allows recognition of individuals. To avoid re-sampling individuals, frogs of this species were either collected in one night, or at different locations along the creek. For both species it is possible that there may have been some recaptures between seasons, however, mark-recapture studies by other investigators (Richards & Alford, 2005) indicate that long-term recapture rates for these species are very low, making it very unlikely that enough individuals were multiple-sampled to seriously compromise the independence of the data across seasons. If any individuals were double-sampled, the delay between sampling events should have ensured that peptide levels measured in the second sampling should not have been affected by previous sampling. The quantity of norepinephrine injected does not completely deplete the frog of its peptides and recovery time is well within the minimum of five months that elapsed between sampling trips (Rollins-Smith *et al*., 2005).

The samples were eluted from the Sep-Pak filters with 21 mL buffer B (Rollins-Smith *et al*., 2006) using two peristaltic pumps (Gilson Minipuls). Of the total 21 mL sample, 20 mL was placed into a centrifuge tube and stored at - 4ºC. The remaining 1 mL was added to a sterile Eppendorf microtube to determine total skin peptides per sample following the protocol described by Rollins-Smith *et al*., (2006).

The 20 mL peptide samples were concentrated using a rotary evaporator with a water bath, set at approximately 50ºC, to remove the acetonitrile added during elution. Afterwards, samples were stored at - 80ºC for at least 24 hours prior to freeze-drying (Freeze-dryer Model Operon). Peptides are partially hydrophobic and hence we incompletely freeze-dried samples to a volume that would allow reconstitution with sterile HPLC water to a final concentration of 5 mg/mL. Samples were then filter-sterilised using a 0.22 μm Millex filter.

*Bd* growth inhibition assays were performed to a) determine if AMPs were present in the peptide samples and to b) test and quantify their effectiveness against *Bd* cultures. Isolates of *Bd* (Tully-*L. rheocola* 06-LB-1) were cultured following the protocol of Longcore *et al*., (1999) but at 50% nutrient concentration as this increased rates of *Bd* culture growth. Zoospores were harvested by flooding agar plates with 2 mL of TGhL broth medium for 10 minutes (Boyle *et al*., 2003) followed by vacuum filtration through sterile 20 μm spectra/mesh nylon filters (Spectrum 722-05067-000) to remove any zoosporangia that might influence culture growth. The concentration of zoospores was determined by counting live and moving zoospores in three subsamples using a haemocytometer. TGhL was added to produce a final concentration of *Bd* zoospores of $10\times10^{6} \cdot mL^{-1}$. Peptide samples were diluted to 100, 250, 500 and 1000 μg/mL. Ninety-six-well tissue culture treated microtitre plates (Corning Incorporation) were used for growth inhibition assays following the protocol of Rollins-Smith *et al.,* (2006). Each plate contained three peptide samples at each of the four dilutions (five replicates each) with *Bd*, 10 replicates of *Bd* culture at $5\times10^{5} \cdot mL^{-1}$ zoospores (positive control), five replicates of non-viable *Bd* culture that was heat treated for 30 minutes at 60 ºC (negative control), and 19 replicates of the TGhL culture medium (background absorption correction).

Each plate was read daily on the spectrophotometer at an absorbance of 492 nm until one day after maximum growth of the *Bd* culture in the positive controls (day x). Each well of the plate was observed daily under 10 × and 20 × magnification and observations were recorded, including those of bacterial contamination and inhibition of *Bd*. Any well that was noted as contaminated was excluded from analysis as it resulted in higher spectrophotometer readings.

Percentage inhibition was calculated by first subtracting the average absorbance for the negative control from each well at day 0 and day × using the formula:

$$1 - [(\text{day } x - \text{day } 0)/(\text{day } x - \text{day } 0 + \text{positive control})] \times 100$$

An average was taken of all uncontaminated wells per peptide sample per concentration.

Statistical analysis

To determine whether body mass influenced total peptides secreted, we regressed total peptides secreted (μg) on frog body mass (g) separately for each species. We also calculated total peptide secretion per surface area ($cm^2$) similarly to Woodhams *et al*., (2005) by estimating surface area using the equation of McClanahan and Baldwin (1969). The data were $\log_{10}$ transformed before regression analysis. A Mann-Whitney U test (MWU-test) was performed to test for differences in total peptide secretion per surface area between *L. genimaculata* and *L. rheocola*. Because there were no infected

*L. genimaculata* individuals in summer at low elevation, we could not perform a three way ANOVA, including all three factors (infection status, elevation and season).. We therefore performed two two-way ANOVAs (infection status and elevation for the winter samples; infection status and season for samples at high elevation) and a t-test between infected and uninfected *L. genimaculata* at low elevation in winter to test for any effect of infection status and determine whether this variable could be safely excluded from further analyses. None of the analyses indicated that any effects were significant. Infected and uninfected frogs were therefore pooled for the final two-way ANOVA (season and elevation). Only a few infected *L. rheocola* were found during the study and consequently infection status could not be incorporated into the factorial analysis for this species.

Some peptide samples for each species showed no evidence of inhibition of *Bd* at the concentrations tested. This is an important finding in interpreting the extent to which The results for these samples are not commensurate with the results of samples that did show inhibitory activity, but the proportions of samples that fell into each category are a potentially important measure of levels of protection against *Bd.* A series of categorical analyses were carried out. We used Fisher's exact tests for each species to determine whether infection status affected the proportions of samples that fell into each category. If this was not significant, we proceeded to combine the data from infected and uninfected individuals, and used separate Fisher's exact tests to determine whether the proportion of samples showing anti-*Bd* activity differed among sites, elevations, and seasons.

The standard statistic considered in medical and toxicological literature is the $LC_{50}$, the concentration of an agent that is lethal to 50% of the target population (Sanchez-Bayo & Goka, 2007). However, this seems irrelevant in the study of AMPs; a frog surrounded by a concentration of AMPs that reached the $LC_{50}$ for *Bd* zoospores could presumably still become infected and possibly die as a result of chytridiomycosis. We therefore calculated and analysed the $IC_{100}$, the concentration necessary to completely inhibit the growth of *Bd.* This is the same as the MIC (minimal inhibitory concentration) described in Rollins-Smith *et al*., (2003), but potentially less confusing. There were several samples at which no concentration of AMPs tested produced 100% inhibition of *Bd*. In these cases, we used extrapolation to predict peptide concentrations where 100% inhibition would occur ($x = (100/b)/m$), where x is the unknown concentration of peptides, b = y intercept and m= slope of a regression of percent inhibition on concentration.

Because the inhibitory activity of AMPs were measured at few concentrations only and we had to use extrapolation to estimate the $IC_{100}$ for some samples, the data were not truly on an interval scale. For a conservative analysis, we categorised the estimates of $IC_{100}$ into four groups, forming a log series of concentrations: 1 = 50-174 µg/mL, 2= 175-374 µg/mL, 3= 375-749 µg/mL and 4= 750+ µg/mL. Fisher's exact tests were used for each species' data to determine whether there were differences between the $IC_{100}$s of peptides produced by infected and uninfected frogs, between upland and lowland populations and between summer and winter (following the same procedure described in the previous section).

Once the $IC_{100}$ was calculated, the overall protection of the frog against *Bd* was estimated as the total number of mL of solution at the $IC_{100}$ that would be produced per unit surface area:

Overall protection (mL at $IC_{100}$ per $cm^2$) = (total mass of peptides/ SA × $1/IC_{100}$).

The data was $\log_{10}$ transformed before statistical analysis. We repeated the analysis as described in the "total skin peptides secreted" section to determine whether infection status significantly contributed to the variation.

**Results**

A total of 160 *L. genimaculata* and 56 *L. rheocola* were captured to determine prevalence and intensity of infection with *Bd* and to collect skin peptides. Fifty-six *L. genimaculata* and 13 *L. rheocola* tested positive for *Bd*. Except for frogs at Paluma, prevalence of *Bd* was significant lower in summer compared to winter (Fisher's exact test, $P < 0.01$ and $P < 0.01$, respectively; Figure 98). Intensities of *Bd* infection in low elevation *L. genimaculata* populations were generally, although not significant, higher during the winter season (Mann-Whitney U-test, $Z=0.43$, $P=0.66$). Higher prevalence of *Bd* in high elevation *L. rheocola* populations was found during winter at Windin N creek (Mann-Whitney U-test, $Z=-0.64$, $P=0.4$). Overall, *L. genimaculata* had a significantly higher intensity of infection with *Bd* than did *L. rheocola* (Mann-Whitney U-test, $Z=4.17$, $P < 0.001$).

The total amount of peptides secreted by *L genimaculata* and *L. rheocola* were not significantly correlated with body mass ($r^2=0.03$ and 0.001, respectively). Site (ANOVA, $F_{4, 150} = 4.58$, $P < 0.01$ and $F_{1, 52} = 4.60$, $P = 0.04$) and season (ANOVA, $F_{1, 150} = 5.53$, $P = 0.02$ and $F_{1, 52} = 5.68$, $P = 0.02$, respectively) had significant effects on total peptide secretion per surface area in both *L. genimaculata* and *L. rheocola* (Figure 99 and 100, respectively). In addition to the significant main effects, there were significant interactions between the effects of site and season on *L. genimaculata* and *L. rheocola* total peptide secretion per surface area (ANOVA, $F_{4, 150} = 6.89$, $P < 0.01$ and $F_{1, 52} = 4.6$, $P = 0.04$, respectively); this was not sufficient to remove the main effect of site or season. Overall, *L. genimaculata* secreted a significantly lower quantity of peptides per surface area than *L. rheocola* (Mann-Whitney U-test, $P < 0.01$; Figure 101).

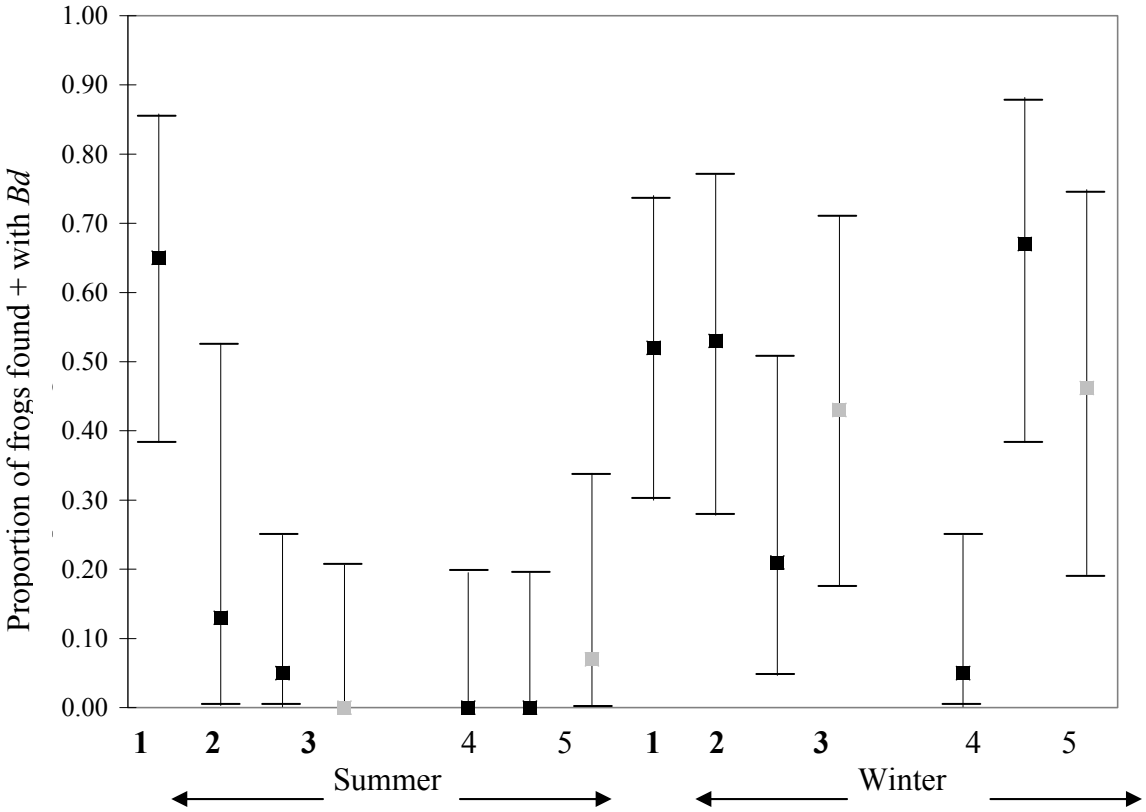


**Figure 98.** Prevalence of *Batrachochytrium dendrobatidis* (means and 95% confidence limits) in *Litoria genimaculata* (■) and *L. rheocola* (▒) individuals from which skin peptides were collected during two seasons at three high elevation sites (**1**= Birthday Creek, **2**= Murray Upper Bridge XI, **3**= Windin N Creek) and two low elevation sites (4= Murray Upper Bridge I, 5= Frenchman Creek).

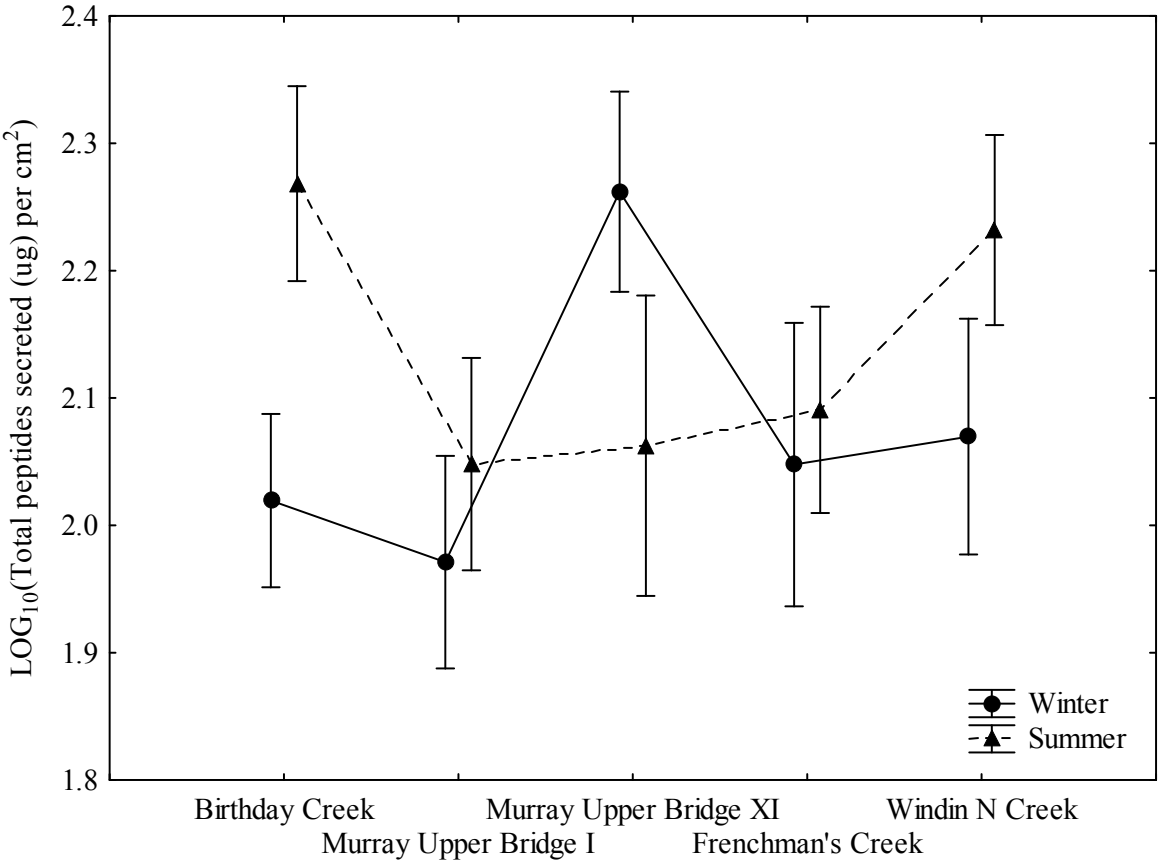


**Figure 99.** Total peptides secreted (μg) per surface area ($cm^2$) by *Litoria genimaculata* (means and 95% confidence limits) at three lowland sites (Murray Upper Bridge I and Frenchman Creek) and two upland sites (Birthday Creek, Murray Upper Bridge XI and Windin N Creek). The lines between data points are to aid in visualisation of the results only.

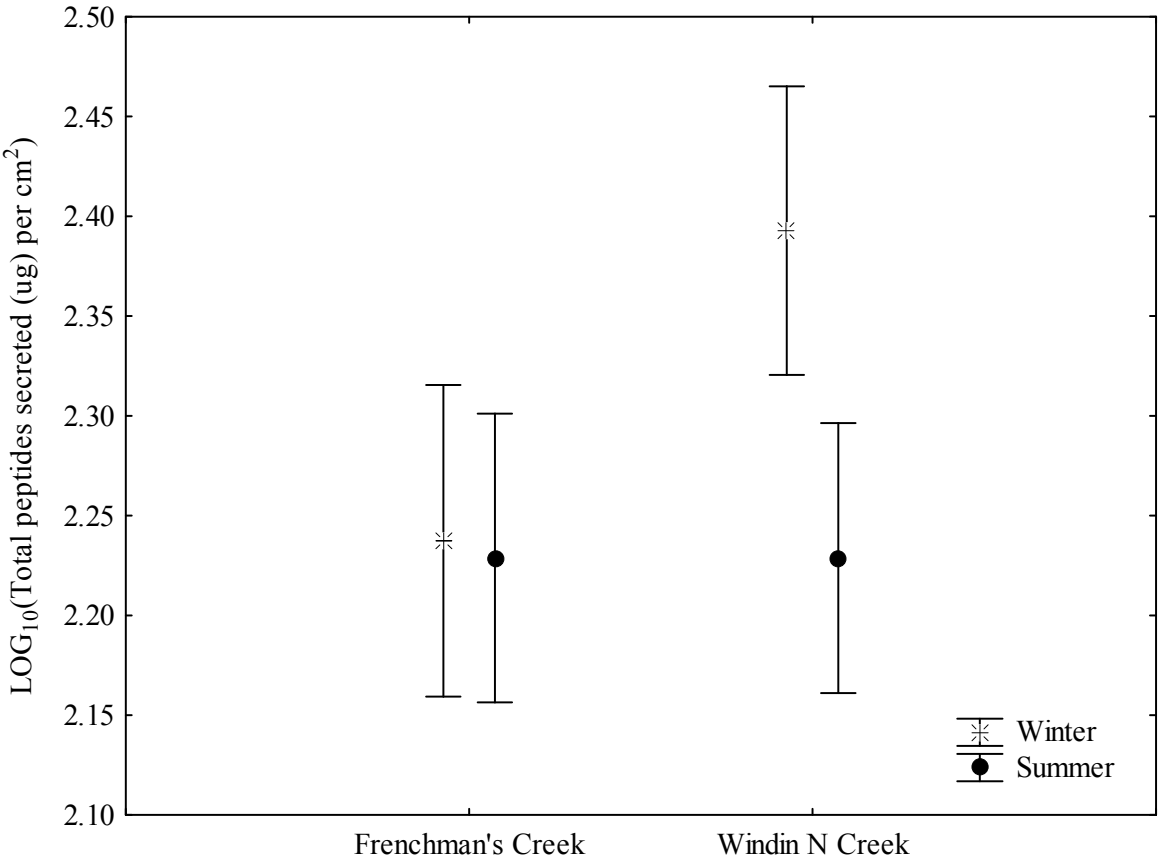


**Figure 100.** Total peptides secreted (µg) per surface area ($cm^2$) by *Litoria rheocola* (means and 95% confidence limits) during two different seasons at one lowland site (Frenchman Creek) and one upland site (Windin N Creek).

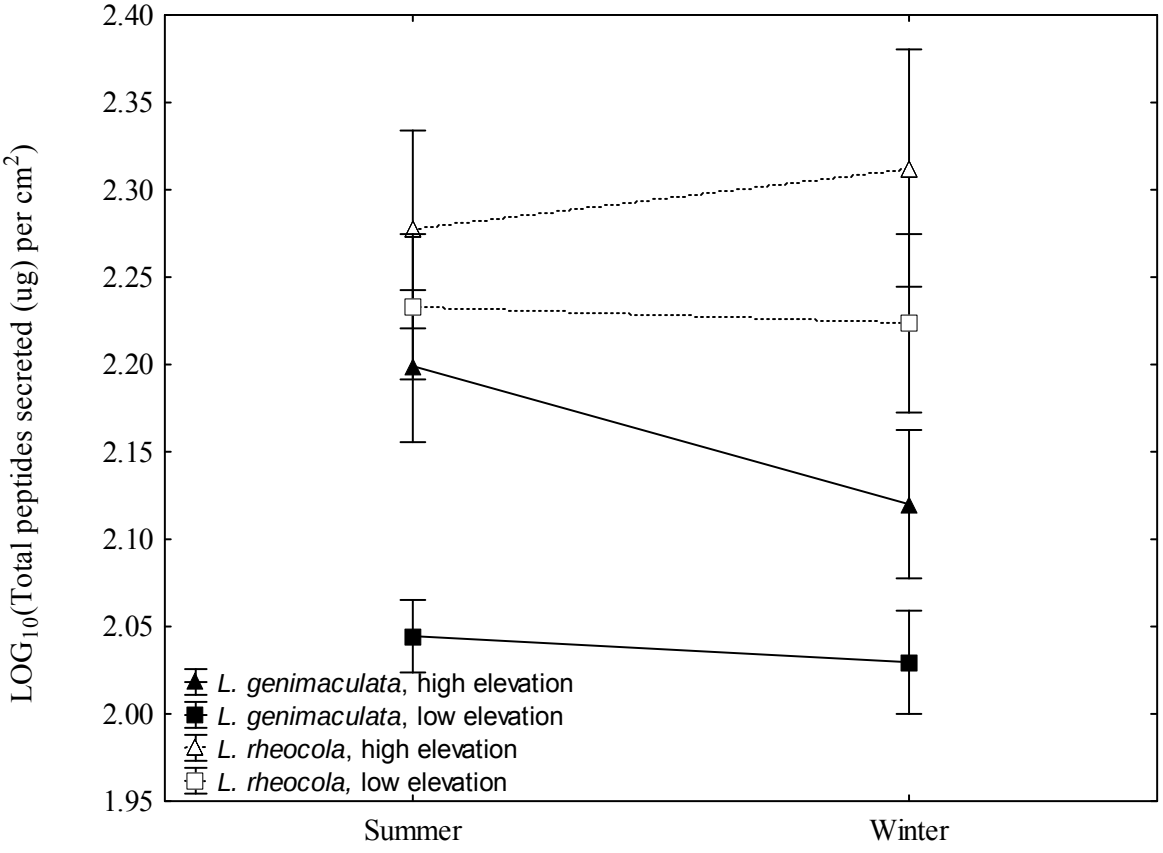


**Figure 101.** Total amount of peptides secreted (µg) per surface area ($cm^2$) by *Litoria genimaculata* and *L. rheocola* (means and 95% confidence limits) in winter and summer at low and high elevations. The lines between data points are to aid in visualisation of the results only.

One hundred and twenty four of the 206 skin peptide samples showed at least some inhibition of *Bd*. The proportions of peptide samples that did and did not inhibit *Bd* differed significantly between *L. genimaculata* and *L. rheocola* (Fisher's exact test, $P < 0.01$). The proportion of *L. genimaculata* samples inhibiting *Bd* was affected significantly by season (Fisher's exact test, $P = 0.01$; Figure 102) and elevation (Fisher's exact test, $P < 0.01$). However, proportions of peptide samples that did and did not inhibit *Bd* did not differ significantly between infected and uninfected *L. genimaculata* (Fisher's exact test, $P = 0.06$). During winter, a higher proportion of *L. genimaculata* peptide samples showed at least some inhibition of *Bd*. Infected and uninfected *L. rheocola* did not differ significantly in proportion of peptide samples that inhibited *Bd* (Fisher's exact test, $P = 0.7$). In *L. rheocola*, there were also no significant differences between winter and summer (Fisher's exact test, $P = 0.5$) and between high and low elevation sites (Fisher's exact test, $P = 0.3$).

There was a significant overall difference in $IC_{100}$ between *L. genimaculata* and *L. rheocola* (Fisher's exact tests, $P < 0.01$) where more peptide samples of *L. genimaculata* required higher concentration to achieve total inhibition of *Bd*. This difference persisted when the data for each season were examined separately. In summer and winter more peptide samples of *L. genimaculata* required higher concentrations to achieve total inhibition of *Bd* than *L. rheocola* (Fisher's exact test, $P = 0.04$; $P = 0.04$, respectively; Figure 103). Within species, there was no significant difference in AMP effectiveness between infected and uninfected *L. genimaculata* and *L. rheocola* (Fisher's exact tests, $P = 0.6$ and $P = 0.4$, respectively). There was also no effect of site on AMP effectiveness for either species (Fisher's exact tests, $P = 0.2$ and $P = 0.6$, respectively). There was a significant seasonal effect on $IC_{100}$ in *L. genimaculata* (Fisher's exact test, $P = 0.03$); more peptide samples in summer required higher concentrations to achieve total inhibition of *Bd* (Figure 103). There was no seasonal effect within *L. rheocola* samples (Fisher's exact test, $P = 0.2$).

*Litoria rheocola* had significantly higher levels of overall protection against *Bd* (mL of AMPs at $IC_{100}/cm^2$) than *L. genimaculata* (Mann-Whitney U-test, $P < 0.01$; Figure 104). *Litoria genimaculata* overall protection did not significantly differ among seasons or elevations (ANOVA, $F_{1, 76} = 2.98$, $P = 0.09$, $F_{1, 76} = 45.0$, $P = 0.50$, respectively) and was not affected by the infection status of frogs. There were no significant effects of season or elevation on overall AMP protection of *L. rheocola* (ANOVA, $F_{1, 40} = 3.04$, $P = 0.09$, $F_{1, 40} = 0.01$, $P = 0.94$, respectively), although the apparent interaction (Figure 105) between these factors (season and elevation) approached significance (ANOVA, $F_{1, 40} = 3.88$, $P = 0.06$).

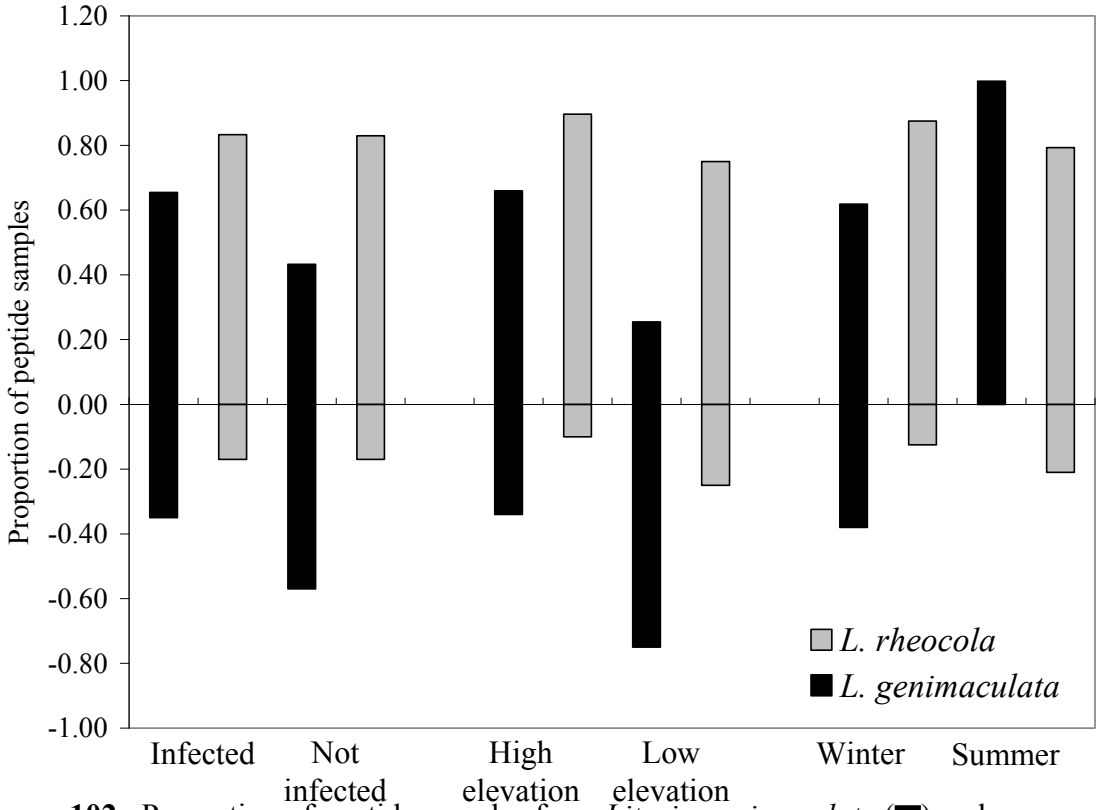


**Figure 102.** Proportion of peptide samples from *Litoria genimaculata* (■) and *L. rheocola* (▒) that did (positive value) and did not (negative value) inhibit *Batrachochytrium dendrobatidis* during challenge assays.

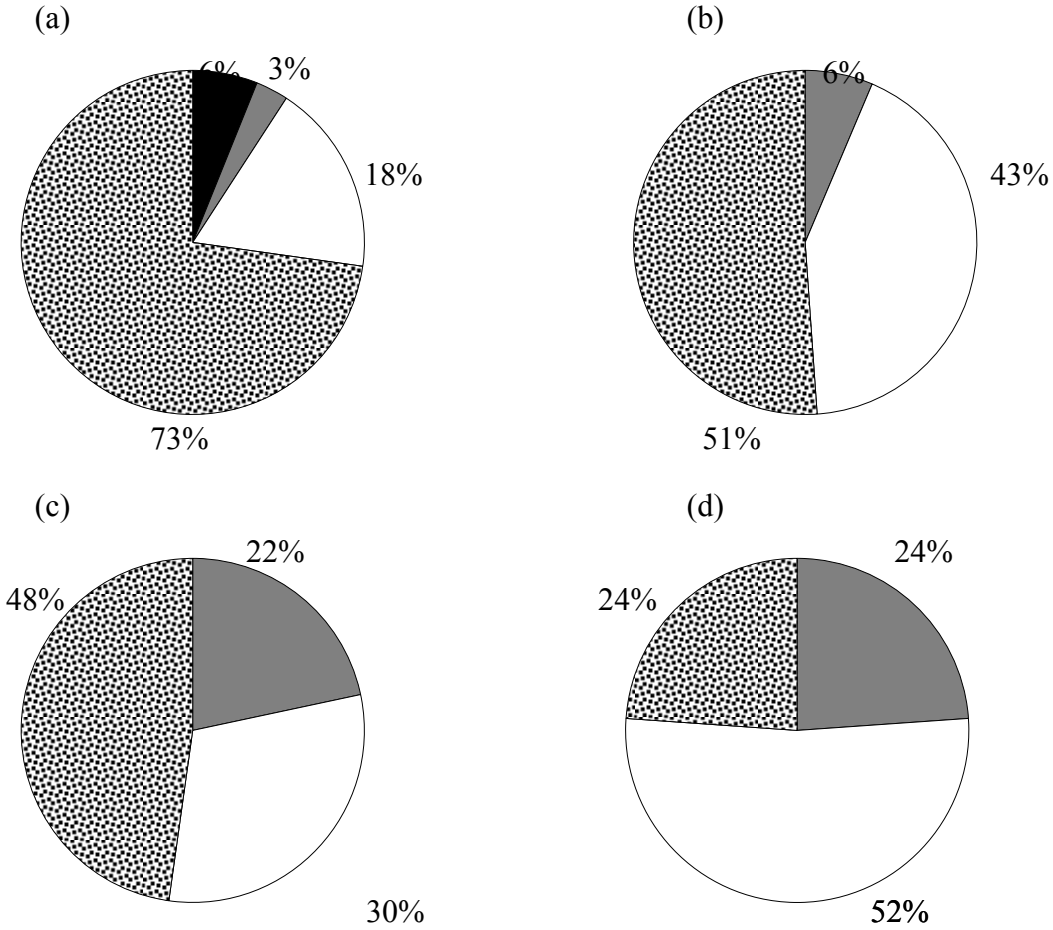


**Figure 103.** Proportions of peptide samples from *Litoria genimaculata* in summer (a) and winter (b) and from *L. rheocola* in summer (c) and winter (d) at the four different inhibitory concentrations, 1 (■) = 0-174 µg/ml, 2 (▒) = 175-374, 3 (□) = 375-749 µg/ml and 4 (▩) = 750+ µg/ml, that completely inhibited *Bd* ($IC_{100}$).

.

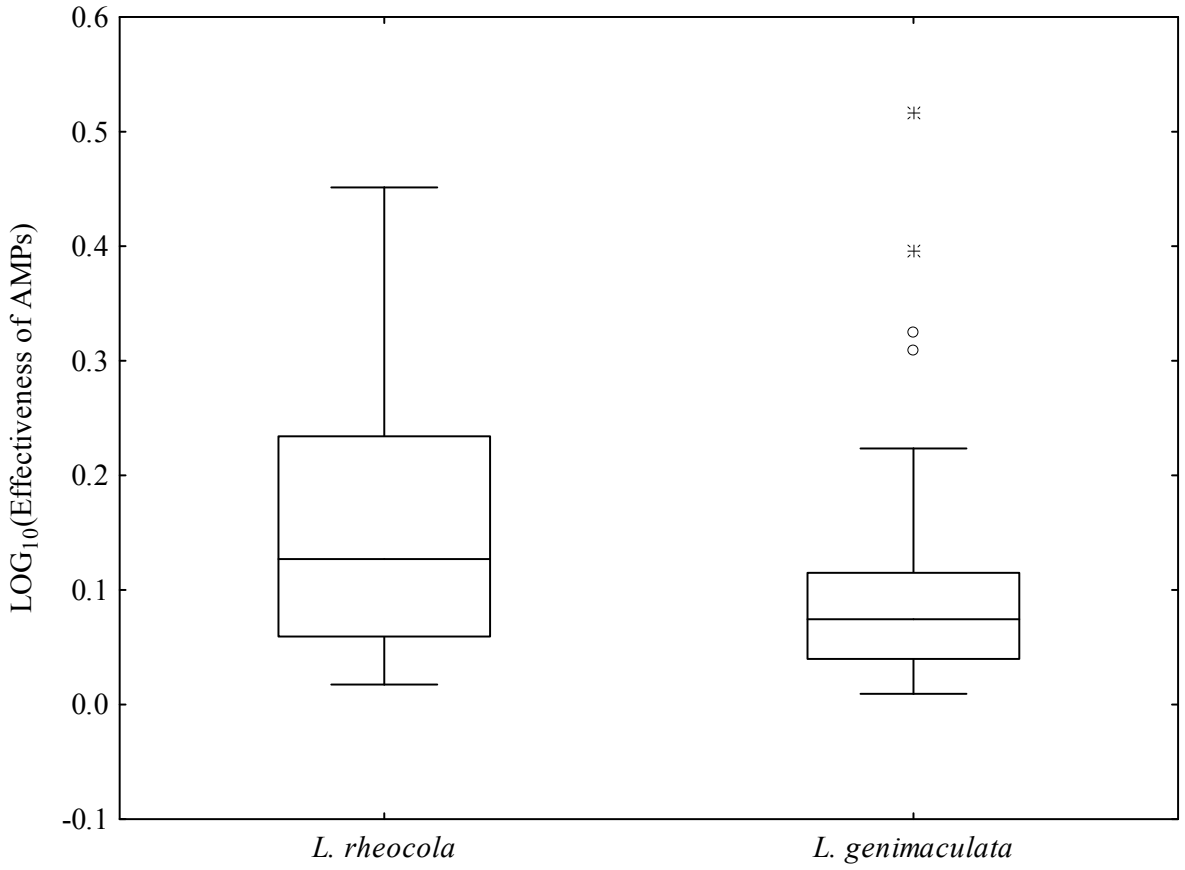


**Figure 104.** Overall protection afforded by AMPs (mass of peptides/ SA × 1mL/$IC_{100}$) in *Litoria genimaculata* and *L. rheocola*. In this Tukey (1977) boxplot, vertical bars indicate ranges, horizontal bar indicates median, boxes indicate upper and lower quartile boundaries.

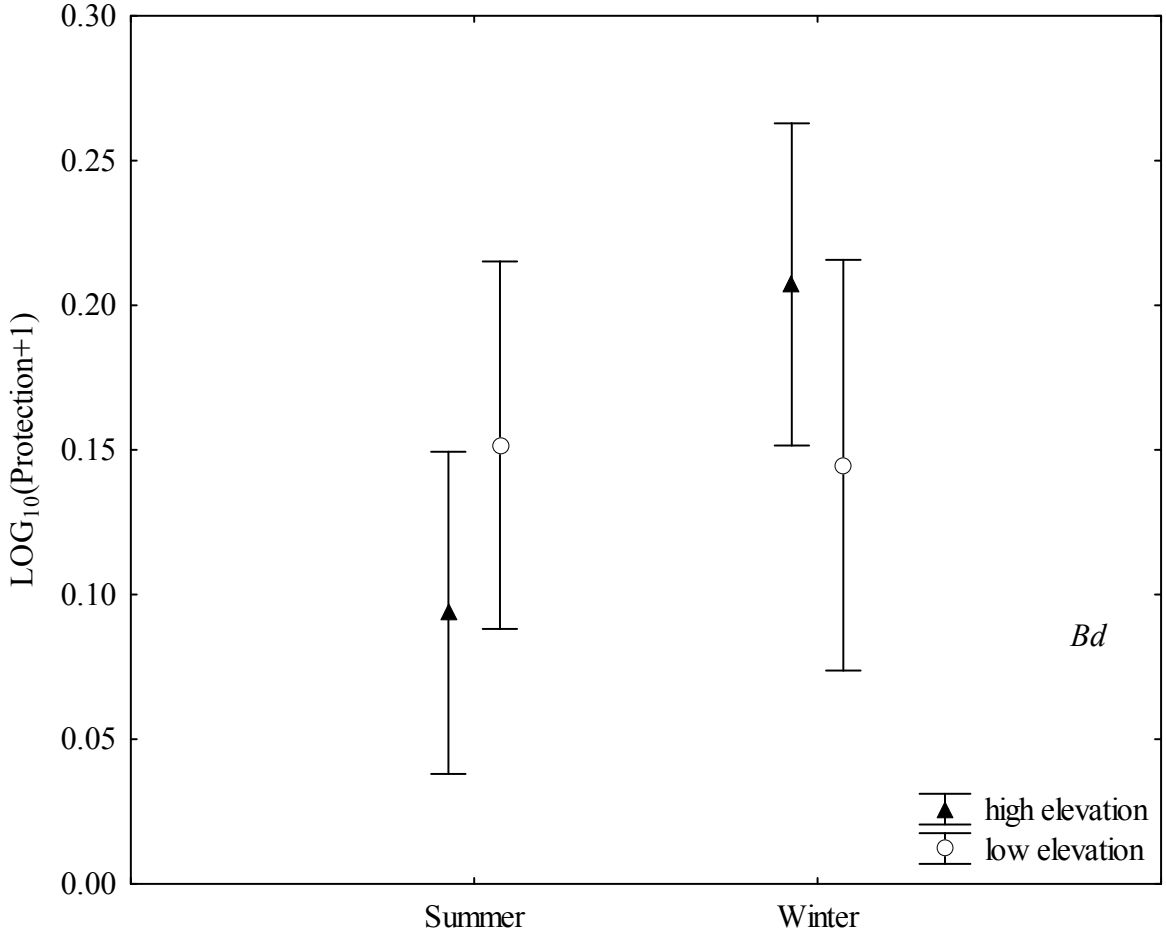


**Figure 105.** Overall protection afforded by AMPs (total peptides secreted × ($1/IC_{100}$) / SA) in *Litoria rheocola* (means and 95% confidence limits) from samples collected during summer and winter at high and low elevation sites.

**Discussion**

Effects of season and elevation on the prevalence of *Bd,* which is usually greater in winter and at higher elevations, have been observed in multiple anuran species in Queensland and New South Wales, Australia (Berger *et al.*, 2004; Kriger & Hero, 2007; McDonald *et al.*, 2005; Retallick, McCallum & Spear, 2004; Woodhams & Alford, 2005). These patterns are most likely driven by temperature, since lower temperatures are more suitable for *Bd* survival and reproduction (Berger *et al.*, 2004; Longcore, Pessier & Nichols, 1999). Lower prevalence of *Bd* infection was detected in summer at most sites, except at Birthday Creek, Paluma National Park, where a high prevalence occurred in *L. genimaculata* in summer. The reasons why frogs at Paluma demonstrated such high prevalence of *Bd* infection in summer remain unclear. Woodhams (2003), who used the same transects, calculated the prevalence to be 10 % or less, which is much lower than recorded during the 2005-2007 period of my study. It is possible that previous studies may have underestimated prevalence (McDonald *et al.*, 2005; Woodhams & Alford, 2005). This is because these studies used histology on removed toe clips to detect *Bd* infection rather than the more efficient qPCR assays (Annis *et al.*, 2004; Hyatt *et al.*, 2007), as the latter method had not been developed for the period data were collected. Low intensity of infection, as was observed in *L. rheocola*, may result in even lower prevalence estimates when histology is used as a diagnostic technique (Hyatt *et al*., 2007). Alternatively, environmental conditions during my study period may have been more favourable for *Bd* compared to the time period of McDonald *et al*., (2005) and Woodhams and Alford (2005) studies.

*Litoria genimaculata* and *L. rheocola* peptide secretion per surface area differed among sites and seasons. Several hypotheses could explain the high seasonal variation. It may be an anuran physiological response to decreased temperatures in winter, resulting in smaller amount peptide per surface area being produced or released during that season. Seasonal variation could also represent an adaptive response to changes in the abundance or composition of the microbial assemblage that the frogs are exposed to. Seasonal variation has been documented in microbial assemblages in the rainforests of northern Queensland; for example Paulus *et al*., (2006) found that microfungal assemblages on decaying leaves of four rainforest tree species at Wooroonooran National Park, northern Queensland, differed significantly between the winter and summer seasons. Studies in other habitats have demonstrated seasonal variation in methanotrophs (a form of bacteria) and epiphytic yeasts (Glushakova & Chernov, 2007; Singh & Kashyap, 2007). The combination of seasonal fluctuations in both quantity secreted and effectiveness suggests that the composition of the AMPs secreted changed seasonally, which would be expected if the seasonal changes were adaptive to fluctuations in the microbiota.

Directional selection, resulting in novel antimicrobial peptide gene sequences, has been observed in *Rana pipiens* (Tennessen & Blouin, 2007). This study concluded that selection may result in affected species producing more effective AMPs against novel microbial organisms. The situation in *L. genimaculata* is more complex. When individuals possessed AMPs that were effective against *Bd,* AMPs from upland populations were not significantly more effective than AMPs from lowland populations, suggesting that selection has not altered the nature of the AMPs possessed by upland frogs. However, significantly greater proportions of individuals in upland populations possessed AMPs that were effective against *Bd*; and at both upland and lowland sites, the proportion of individuals with anti-*Bd* AMPs was

significantly higher in winter. This suggests that selection may have acted to increase the production of existing anti-*Bd* AMPs during winter and in upland populations of *L. genimaculata*. Further studies are needed, investigating the composition of different suites of AMPs in *L. genimaculata* and *L. rheocola*, at high and low elevation sites, in order to determine whether their AMPs have been influenced by natural selection after exposure to *Bd*.

While *L. rheocola* demonstrated a slower population recovery than *L. genimaculata* after the appearance of chytridiomycosis, the species' innate immune defences appear likely to be more successful at inhibiting *Bd* infection than that of *L. genimaculata*. *Litoria rheocola* secreted more peptides per surface area, more individuals secreted peptides that inhibited *Bd,* and overall protection was significantly higher than in *L. genimaculata*. This is in contrast to the findings presented by Woodhams (2003) who observed the opposite trend. However, in that study norepinephrine was not subcutaneously injected but a norepinephrine immersion technique was used instead, which is significantly less effective (Woodhams, 2003). Additionally, sample size was limited which did not allow accounting for other influencing factors, such as season or elevation, which may have affected his results.

This is the first large study on AMPs of two sympatric frog species and results suggests that natural selection caused by the effects of chytridiomycosis on different populations may have acted on the amounts of peptides secreted by *L. genimaculata*. The fact that neither the effectiveness of peptides nor the proportions of individuals secreting effective peptides differed between upland and lowland populations of *L. rheocola* may support the idea that this species recolonised from lowland populations Woodhams & Alford, 2005). The selection pressure experienced by this species while possible recolonising high elevation habitat, is unclear. The results suggest that perhaps they experienced a stronger natural selection pressure than *L. genimaculata*, resulting in more successful innate immune defences inhibiting *Bd* infection. On the other hand, it is possible that other factors, such as microenvironment selection, frequency of frog to frog interactions, or disease avoidance behaviour, may also influence the susceptibility of these frog species to *Bd*.

**Acknowledgements**
The authors are thankful for the qPCR analyses conducted by Stephen Garland at School of Veterinary and Biomedical Sciences, James Cook University and valuable input by Drs. D. C. Woodhams and L. A. Rollins-Smith. This research was conducted under Environmental Protection Agency permit numbers WITK03070505 and WISP 03070205 and Forestry permit INN 05/004 and James Cook University Animal Ethics Committee (A960). This research was supported by a contract (43-04) from the Australian Department of Environment and Heritage.

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***Project 7.2 Behaviour and microenvironment selection in recovered and recolonising populations***

In addition to work on antimicrobial peptides and how they differ among populations with different histories of selection pressure caused by chytridiomycosis, Project 7 aims to compare the behaviour of frogs from populations with different histories, to determine if increased resistance may have resulted from changes in behaviour or microenvironment use. Robert Puschendorf carried out his Ph.D. research investigating how behaviour and microenvironment use differ between recovered, high-elevation populations, and newly-discovered populations on the western fringes of the rainforest that have never experienced catastrophic declines. He is using techniques comparable to those that were used in Project 2, so that his behavioural and microenvironment data can be compared to the extensive library of information available as a result of that project.

In preliminary survey and site selection work carried out during 2006, Robert discovered that an interesting pattern occurs at the western, inland boundary of the Wet Tropics rainforest. At elevations above 800 m asl, healthy populations of *L. nannotis* exist in the dry sclerophyll (Williams 2006). Conspecific populations living at similar elevations within the rainforest have disappeared, although some are presently recovering. At Spurgeon Falls (Figure 12), the transition from one forest type to the other occurs within a short distance, mostly in a very defined way, without a transition. A stream that restricts the spread of fire can define a discrete division between a patch of dry sclerophyll and a wet sclerophyll. As sharply defined as these transitions, so can be the patterns of abundance of frogs between them. In a recent survey, 59 *L. nannotis* were captured in four days of fieldwork at Spurgeon Falls. Fifty-five of these occurred in the sclerophyll, and only four were found in the wet sclerophyll. This held through when sufficient habitat was present and after applying nearly identical collecting effort in both habitats. The environmental gradient in such a small area is quite dramatic, with humidity and canopy cover changing abruptly between forest types. Our observations suggest that a combination of environmental factors such as lower humidity and higher temperature variability due to lesser canopy cover could protect frog populations in the dry sclerophyll from disease outbreaks. Previous experimental research has already shown that frogs exposed to saturated conditions die quicker than those exposed to streams or pool environments (Alford et al. 2002). These saturated conditions mimic those experienced by frogs at high elevation rainforest sites. A comparison between rainforest and sclerophyll forest populations thus could provide a proper setting to investigate how important these factors are in preventing disease spread and outbreaks.

Drawing comparisons with the rainforest is feasible, since the studied species occur on the three forest types, and their interactions with the disease have and continue to be studied in the rainforest. We have already surveyed for the disease in frogs at Spurgeon Falls (sclerophyll, wet sclerophyll, rainforest) and Blencoe Falls (dry sclerophyll; Figure 12). *B. dendrobatidis* has been detected at both sites on *Litoria genimaculata, Litoria nannotis* and *Litoria lesueuri*, all of which are currently being monitored in the rainforest.

Surveys for *B. dendrobatidis* at these sites have shown that disease dynamics diverge across forest types. Microenvironmental profiles differ substantially when compared

between sclerophyll and rainforest populations of the same species. A detailed report on this work appears on pages 69-82 of the report for Tender 42/2004.

Habitats adjacent to the rainforest, such as the dry sclerophyll, could be a refuge for amphibians from chytridiomycosis outbreaks. Understanding what environmental factors are necessary to create these refuges and how frog populations are coming back at higher elevation sites in the rainforest might help us design *in situ* management strategies to help amphibian populations persist at higher elevations sites.

## ***Project 7.2.1 Rediscovery of an Australian stream frog (Litoria lorica) and environmental refuges from chytridiomycosis***

Robert Puschendorf, Conrad J. Hoskin, Ross A. Alford, Keith Mcdonald, Lee F. Skerratt, Scott D. Cashins, Jeremy Vanderwal

In June 2008, 17 years after it was last observed (Cunningham 2002), we discovered a population of one of the high-elevation "rainforest" species that was thought to be extinct, the Armoured Mist Frog *Litoria lorica*, in xeric habitat outside its former known distribution. The new site is on the Carbine Tableland, downstream from a site that has been known for some time to support a small population of *Litoria nannotis*. *Litoria lorica* was commonly thought to be extinct because its known historical range was retricted to rainforest sites at elevations from 640 to 1000 m, within the elevation zone where susceptible species declined to local extinction, and resurveys of known histrical sites had failed to locate any individuals since 1991.

The newly discovered population was confirmed to be *L. lorica* both by morphological comparison to extant preserved specimens and the original species description, and by molecular analysis of small tissue samples, which confirmed that it was related to but distinct from th closely related *L. nannotis* and *L. rheocola*, with which it is sympatric at the newly-discovered site.

### ***Litoria nannotis* and *Litoria lorica* abundance surveys**

Abundance surveys were conducted three times in July 2008, once in September 2008, and once in March 2009 and involved counting and identifying all *L. nannotis* and *L. lorica* individuals located along the transect at night. Because the species were morphologically distinct individuals could be identified without handling.

### **Detection of *Batrachochytrium dendrobatidis* in frogs and tadpoles**

Frogs and tadpoles were sampled for infection status at the end of July 2008 in surveys separate from the abundance surveys, at the peak of winter when temperature is the coolest and prevalence and intensity of infection were likely to be high (Berger et al. 2004; Bradley et al. 2002; Kriger and Hero 2006; McDonald et al. 2005; Retallick et al. 2004; Woodhams and Alford 2005.)

We attempted to catch all visible animals at each localised waterfall or cascade area for swabbing. The exact capture locality of each frog was recorded using a Garmin 60CSX GPS. After swabbing we recorded each individual's sex and age class status (male, female, subadult), snout-vent length (measured using stainless steel callipers) and mass (using a 100 gram digital pocket balance). After measurement and swabbing, the frog was immediately released at the point of capture.

We captured tadpoles by dip netting, and quickly transferred them into individual press seal bags, avoiding any direct handling. Tadpoles were identified according to Richards (1992). Tadpoles of *L. nannotis* and another torrent frog, *L. rheocola*, were captured and swabbed but no *L. lorica* tadpoles were found. Mouthparts were swabbed as in Project 4.5.

Swabs were analysed for the presence of *B. dendrobatidis* using a real-time quantitative Taqman PCR assay (Boyle et al. 2004) at James Cook University, Townsville, Australia. Each sample was run in triplicate. Samples were considered positive if three replicate wells were found to have *B. dendrobatidis* DNA. We chose this stringent criterion to minimise the false positive rate, so our prevalence estimates represent minimum prevalences.

## Results

### Identification of *Litoria lorica* and habitat

The newly discovered population fits all the morphological traits for *Litoria lorica*, including the diagnostic traits that separate this species from the most similar species, *Litoria nannotis* (Davies and McDonald 1979; Cunningham 2002; Hoskin and Hero 2008). Adult size distinguishes the two species, apparent from field measurements taken at the site: *L. lorica* SVL 30-40 mm (mean = 35.2 mm, SD = 2.7, N = 45) versus *L. nannotis* SVL 53-62 mm (mean = 56.4 mm, SD = 3.3, N = 30).

Genetic analysis supported morphological identification of the population as *L. lorica* with samples representing a highly distinct genetic lineage that clearly falls within the Australian 'torrent frog' species group.

The new population of *L. lorica* was located around cascades and large waterfalls on an open, rocky river with perennial flow. The surrounding vegetation in the area is dry sclerophyll woodland, with the nearest well-developed rainforest 6 km upstream. All previously known localities for *L. lorica* were in rainforest. This site has much higher precipitation seasonality and is much drier than the rainforest sites at which this species was previously found.

### Frog abundance estimates

On the 400 m transect, both *L. nannotis* and *L. lorica* were found only in three discrete waterfall/cascade sections, totalling approximately 150 $m^2$. Frogs in these areas were highly clustered, with *L. lorica* and *L. nannotis* mixed together on rocks next to waterfalls and cascades. Within the small areas in which they were found, both species occurred at high densities, with *L. nannotis* (mean = 0.251 individuals/ $m^2$, SD = 0.059) at higher densities than *L. lorica* (mean = 0.159 individuals / $m^2$, SD= 0.064). A maximum of 35 *L. lorica* and 50 *L. nannotis* were observed in March, 2009. In total, there were combined sightings of 307 *L. nannotis* and *L. lorica* during abundance surveys. None of the frogs sighted showed any of the clinical signs of chytridiomycosis such as lethargy, skin sloughing or loss of righting reflex (Berger et al. 1999).

### Prevalence and intensity of infection of *Batrachochytrium dendrobatidis* in frogs and tadpoles

Analysis of skin swabs from July 2008 revealed that 80.6 % of the terrestrial (adult and juvenile) *L. nannotis* sampled were infected with *B. dendrobatidis* (N = 88, 95 % CI = 70.9-88.3 %), while prevalence in terrestrial *L. lorica* was 81.8 % (N = 33; 95 %

CI = 64.5-93.0 %). There was no significant effect of species, class (male, female, sub-adult) or their interaction on infection status (species: Wald chi-square = 0.017, P = 0.896, df = 1; class: Wald chi-square = 0.619, P = 0.734, df = 2; species X class: Wald chi-square = 0.342, P = 0.843, df = 2). All terrestrial frogs were therefore pooled for comparison with tadpoles. All of the 57 tadpoles swabbed (N = 24 *L. nannotis* and N = 33 *L. rheocola*) were infected with *B. dendrobatidis*. The lower 95 % confidence interval for infection prevalence in *L. nannotis* tadpoles was 85.7 % and in *L. rheocola* tadpoles was 89.4 %. No *L. lorica* tadpoles were captured or sampled. Infection prevalence was significantly higher in *L. nannotis* tadpoles than in frogs (Chi square = 12.44, P < 0.001, df = 1). Swabs taken in March 2009 showed that prevalence of infection remained high.

Intensity of infection did not differ significantly between species ($F_{(1,98)} = 0.095$, p = 0.758), class ($F_{(2, 98)} = 0.326$, p = 0.722 ) or the species X class interaction ($F_{(2,98)} = 0.334$, p = 0.716; Figure 2). Infected tadpoles (mean = 2888 zoospore equivalents, SD = 1255; Figure 2) had significantly higher intensities of infection than infected frogs (mean = 418 zoospore equivalents, SD=1255; t = 12.520, P < 0.001, df = 1). As in the abundance surveys, none of the sampled individuals showed any of the clinical signs of chytridiomycosis (Berger et al. 1999).

## Discussion

Little was known about the distribution, ecology, or behaviour of *L. lorica* before it disappeared from rainforest sites. It was therefore listed as critically endangered (Covacevich and McDonald 1993; McDonald and Alford 1999, Cunningham 2002). However, *L. lorica* was generally thought to be extinct because although repeated surveys had been conducted at the locations of previously known populations, the species had not been observed for 17 years (Williams, 2006, McDonald, unpublished). Morphological and genetic analyses clearly show that the population we discovered is *L. lorica.*

The most parsimonious explanation for the current presence of *L. lorica* at this site is that historical surveys had not documented the full extent of its geographic and environmental range; it was probably present at the site throughout the period in which rainforest populations declined. Our rediscovery of this species supports the precautionary approach taken in listing missing amphibian species as critically endangered when there is uncertainty about the species' distribution and ecology.

### Infection of frogs and tadpoles by *Batrachochytrium dendrobatidis*

The prevalences we report in this study are higher than any that have been measured and reported in the literature using molecular techniques to diagnose infection where *B. dendrobatidis* is enzootic (e.g. Kriger and Hero 2006a, b; Kriger and Hero 2007). It is only comparable to infection levels observed during a well-documented epizootic outbreak (Brem and Lips 2008). Contrary to the well-documented population declines in neighbouring rainforest areas (e.g. Schloegel et al. 2006), no historical information is available on pre decline population sizes for these drier areas, but the high abundance of these two riparian species at this site is unmatched in any high elevation areas in the Wet Tropics region. At least in the case of *L. nannotis* it seems to be common to find a higher abundance of frogs on the drier western slopes of Wet

Tropics mountains (Williams, 2006, McDonald unpublished) than anywhere else post decline. This study shows that the average prevalence across *L. nannotis* and *L. lorica* was extremely high (of 85 %, N=121, 95% CI = 78- 92%) and stable, while numbers of individuals also remained stable, suggesting these populations are now coexisting with this highly virulent pathogen. This coexistence may be tenuous; the mechanisms responsible for it and the extent to which the pathogen may be affecting survival and recruitment are unknown. A mark recapture study coupled with disease sampling would shed light into the concealed impacts of *B. dendrobatidis* on this population.

**Potential mechanisms of resistance to chytridiomycosis of the *Litoria lorica* population**

A few Australian species that have suffered large declines from the original outbreaks of chytridiomycosis now coexist with the pathogen where it has become endemic (Australian Government Department of the Environment and Heritage 2006). While this could indicate that a change in the host-pathogen relationship favouring host survival has occurred (as might be expected if host immunity has improved or pathogen virulence has decreased) several species persist only in restricted ranges and/or at lower abundances. In these cases, ongoing disease-associated mortality may inhibit full population recovery long after epidemics have subsided (e.g. Murray et al, in press), and species survival may be locally facilitated by host range overlap with environmental refugia that limit the pathogen's growth (Puschendorf et al. 2009) or virulence (Woodhams et al 2008) or augment the host species' immune response (Andre et al. 2008; Richmond et al. 2009).

In the present case, high prevalence and infection intensities suggest that environmental conditions are suitable for *B. dendrobatidis.* While population declines in the past have resulted in restricted distributions for both *L. nannotis* and *L. lorica*, these study populations do not appear to be declining and no sick or dying frogs have been found, although diseased frogs are still found at present during winter months in the adjacent rainforest (Puschendorf et al. unpublished). Hence, results from this study suggest that frogs are coexisting with *B. dendrobatidis*, despite very high pathogen loads. This could arise in several ways 1) increased tolerance or decreased virulence (or coevolution of host-parasite relationship towards reduced virulence); 2) environmental refugia (not hostile to chytrid since it is so abundant, but augmenting immune response/increase tolerance; 3) mortality is not obvious and population level effects are unknown (Murray et al in press), but it is not enough to cause complete extirpation as it has occurred in high elevation rainforest sites.

The newly discovered population of *L. lorica* occurs in dry sclerophyll woodland, an environment very different from that in the rainforest sites from which the species disappeared. This area has higher surface temperatures, lower annual precipitation and higher seasonality than nearby rainforest sites. It has been experimentally demonstrated that high ambient temperatures can cure frogs of infection by *B. dendrobatidis* (Woodhams et al. 2003; Retallic & Miera, 2007). In rainforest areas *L. nannotis* seems to choose moist and buffered microhabitats ideal for chytridiomycosis, which has been the hypothesized mechanism of why this species has suffered such strong declines compared to other sympatric species (Rowley and Alford 2007a, b). Further research will show if dry forest populations of these two species select different microenvironments that could help them tolerate the pathogen.

The situation of *L. lorica* is different from an environmental refuge that has been described in Costa Rica, where the last known population of the 'rainforest' species *Craugastor ranoides* occurs in the dry forest of the Santa Elena Peninsula, Guanacaste (Sasa and Solórzano 1995; Puschendorf et al. 2005; Zumbado-Ulate et al. 2007). Puschendorf et al. (2009) proposed that this area serves as an environmental refuge from chytridiomycosis-driven amphibian declines by having a unfavourable environment for the pathogen, making infection unlikely.

We hypothesize that the system in which the newly discovered *L. lorica* occur is another environmental refuge from disease-driven amphibian declines but through a different mechanism than the Costa Rica one; in this case *B. dendrobatidis* is present in winter at high prevalence and intensity, but the frogs are apparently coping. The heavy infections found in July may be due, in part, to the relatively low ambient air and water temperatures at that time (because this site is of reasonably high elevation) and its geographical position (downstream from rainforest sites where *B. dendrobatidis* is common (Puschendorf et al. unpublished).

Despite very high prevalence and intensities of infection in July, none of the animals observed in abundance surveys and infection sampling presented any clinical signs of disease. However, even in July, diurnal substrate temperatures at our site are substantially higher than in adjacent closed-canopy rainforest; this may be critical in allowing higher tolerance to the pathogen. Being able to elevate their body temperature, could not only slow down pathogen development, but also increase immune response. In temperate areas, frog's immune system is suppressed over winter and is quickly recovered if temperature is elevated (Cooper et al. 1992, Maniero and Carey 1997) . This does not indicate that infected frogs never develop symptomatic (and possibly lethal) chytridiomycosis, but, taken with the fact that abundance surveys in March 2009 indicated no population crashed occurred over the previously sample winter, does indicate that the population is persisting with relatively low levels of disease, despite very high prevalence of infection by *B. dendrobatidis*.

**Implications for amphibian conservation**

*Litoria lorica* remains critically endangered, since the rediscovered population is relatively common locally but is restricted to a very small area in a single catchment. In regards to management, the first thing to determine is whether this is the only population. Considerable survey effort has been conducted since the original disappearance of the species in 1991 but all of this has been at rainforest sites. Since the species' rediscovery, surveys of stream systems in similar dry sclerophyll habitat elsewhere in the same catchment and in a neighbouring catchment have failed to find additional populations (Puschendorf & Hoskin, unpublished data). More surveys of dry forest stream habitats downstream from rainforest are required in the Carbine Tableland, Thornton Uplands and Windsor Tableland regions before it can be concluded that the rediscovered population is the only remaining population of *L. lorica*.

Assuming for now that this is the only population and that this population has persisted with *B. dendrobatidis* for some time, we outline the following threats and management suggestions.

At present the site is open to public use and subject to a variety of impacts. It should be closed to the public and protected, and impacts should be minimised. The *L. lorica* population should be closely monitored using low-impact techniques, so that any changes in status are detected before they become irreversible. Because the population appears to be coexisting with *B. dendrobatidis*, we do not believe that individuals should be immediately removed to captive species-assurance populations. In conjunction with monitoring, additional data should be collected on the basic biology of the species, which is largely unknown at present; this will facilitate more detailed conservation planning, which might eventually include translocations, and the development of captive maintenance techniques. Because captive techniques for torrent frogs do not exist, their reproductive biology is poorly known and could be problematic because of their unique biology associated to riffles and waterfalls. Development of captive maintenance techniques should therefore commence immediately using the closely related and much more common *L. nannotis* as a surrogate. This approach would make any emergency response more likely to be successful, if monitoring determines that declines are in process (Mendelson et al. 2006).

Our results highlight the potential importance of environmental refuges from disease to amphibian conservation. In both tropical Australia and Costa Rica, species that were thought to be extinct survive in the dry forest areas adjacent to rainforest sites. At least in the case of the Australian refuge, where frogs seem to tolerate chytrid infections better than in the rainforest, it may allow frogs to evolve other mechanisms of resistance since the lesser effects of the disease in these environments will allow time for selection to act. Protecting refuge areas adjoining rainforest is important for the survival of *L. lorica* and is likely to be important for many other frog species around the world.

Our results also highlight the fact that known localities may fail to encompass either or both of the full geographical and environmental ranges of species. Survey efforts focused exclusively on habitats with environmental characteristics matching existing records may therefore fail to detect populations that can be important for species' conservation. Survey efforts should therefore focus on documenting the full extent of the geographical and ecological ranges of species by including areas at or outside the margins of their known ecological ranges. In particular, dry forest habitats bordering rainforest should be seen as targets for survey effort for apparently extinct and declined rainforest amphibian populations globally.

In summary, our rediscovery of *L. lorica* highlights the importance of accurately determining the distribution of threatened or presumed extinct species. It illustrates the need to take a precautionary approach when listing the status of species when there is uncertainty regarding its distribution and ecology. It also highlights the need to look for and conserve populations across the range of environmental conditions that may be occupied by species, because this will reduce the chance of a single threatening process causing species' global extinctions. The rediscovery of *L. lorica* gives hope that small populations of other species that appear to have been driven

extinct by chytridiomycosis may persist in environmental refuges outside their former known distribution.

**Acknowledgments**
RP was supported by an International Postgraduate Research Scholarship from the Australian Department of Education, Science and Training, by the School of Marine and Tropical Biology, James Cook University and by a research contract (43-2004) from the Australian Department of the Environment and Water Resources. Funding for this project was provided by the Australian Department of the Environment and Water Resources, the Skyrail Foundation and a Research and Exploration grant from the National Geographic Society (#8608-09). CJH is funded by the Australian Research Council. Fieldwork would not have been possible without the help of Steve Prutzman, Danielle Saye, Alexander Young, Leighlan Phillips, Shannon Hinrichs and Jamie Voyles. Adrian Arias helped with the exploration of other catchments in the area. Stephen Garland, Ana Carnaval, Kris Murray and Caroline Palmer made helpful comments on earlier versions of the manuscript. Stephen Garland processed the diagnostic samples for the presence of the pathogen. This study was carried out under Scientific Purposes Permit issued by the Queensland Parks and Wildlife Service (WISP 05056608) as approved by the James Cook University Animal Care and Ethics Committee (A1283).

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***Project 8. Epidemiology of chytridiomycosis***

Project 8 addresses **Objective 1** by examining the epidemiology of chytridiomycosis and the large-scale environmental factors that determine the likelihood and outcomes of infection. It correlates climatic factors and water quality in the field with prevalence and intensity of *B. dendrobatidis* infections in frogs, as well as host survival. It addresses **Objective 2** by developing epidemiological models incorporating differences between affected and unaffected populations, and addresses **Objective 4** by using monitoring and modelling to develop an epidemiological framework for understanding and predicting the susceptibility of recovered populations to chytridiomycosis and determining their prospects for long-term persistence. Project 8 is reported on in full in the final report for tender 42/2004.

***Project 9. Studies on Mixophyes balbus and L. aurea surviving in areas contaminated with heavy metals***

Project 9 addresses **Objective 1** by examining populations of the vulnerable species *Mixophyes balbus* and *Litoria aurea* that have survived in sites contaminated with heavy metals or which have high salinity, and investigating whether these factors may decrease the vulnerability of frogs to B.d. It addresses **Objective 2** by examining populations of *M. balbus* and *L. aurea* that have survived in sites contaminated with heavy metals or which have high salinity which may be reducing growth of Bd. This has direct and practical implications for management, as it may be possible to manipulate natural habitats on a local scale or provide artificial habitat to reproduce these conditions.

This project is administratively under the auspices of Tender 42/2004, where it was budgeted and described under objectives 2.2, 2.3, and 2.4, and reporting on it should be present in the final report for that tender.