FINAL REPORT TO

DEPARTMENT OF THE ENVIRONMENT AND HERITAGE

ON

RFT 42/2004

"Experimental research to obtain a better understanding of the epidemiology, transmission and dispersal of amphibian chytrid fungus in Australian ecosystems"

Adult of the rediscovered armoured mist frog *Litoria lorica* (left) and *Litoria nannotis* (right)

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EXECUTIVE SUMMARY

This is the final report for this tender due 31 March 2009. The original tender was three years duration but this was extended to four years early on in the tender once it became clear that it would take some time to get all projects within the tender up and running. This tender is linked to tender 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." Both tenders were undertaken concurrently by the Amphibian Disease Ecology Group at James Cook University. The objectives of the tender are listed below with the major findings. Detailed results follow this executive summary.

Objective 1. **Does *B. dendrobatidis* exist as a free-living organism in suitable habitats, particularly natural water bodies and moist substrates? If so, can we identify and describe these free-living organisms and their biology/ecology?**

Batrachochytrium dendrobatidis exists as a free living organism given that it releases aquatic zoopores into the environment. Our results suggest that motility and possibly survival of these zoospores are decreased in higher nutrient environments due to competition with more robust microflora. In addition, encystment of zoopores and subsequent growth and reproduction of zoosporangia also appears to be decreased in higher nutrient environments and can be explained by an inability to compete with other microflora. We therefore propose that *B. dendrobatidis* is more likely to survive and grow as a saprobe in low nutrient environments. We did detect *B. dendrobatidis* DNA in water, on rocks, leaves and in sand within the stream at very low levels suggesting that *B. dendrobatidis* is widespread and mostly likely surviving as zoospores and possibly small colonies of zoosporangia within biofilms in low nutrient environments. We showed that *B. dendrobatidis* is able to readily disperse within water bodies. We also showed that *B. dendrobatidis* has the ability to adapt to low nutrient environments to maximize its fitness.

The management implications of this are that whilst *B. dendrobatidis* exists within the environment and water and water body substrates are potential fomites for spread, the environment is not a major site for growth and reproduction of *B. dendrobatidis*. It is therefore likely that *B. dendrobatidis* can be readily controlled within amphibian populations by controlling infection within amphibian hosts. It is also likely that transmission of *B. dendrobatidis* is affected by the environment and may be less likely within high nutrient water bodies or other environments that affect the survival of zoospores such as saline or polluted water bodies. In disturbed habitats, modification of water bodies to reduce survival of zoospores may be a viable management option. Potentially infectious water or substrates from water bodies should be treated to kill *B. dendrobatidis* prior to their movement to prevent spread.

Objective 2. **What environmental characteristics (eg. pH, pO2, ion content, nitrate, organic content) of natural water bodies (both permanent and ephemeral) and climate (eg. temperature, rainfall) favour the biology of *B. dendrobatidis*?**

> **Objective 2.1: We will determine whether any of the above environmental or climatic variables are correlated with either intensity and prevalence of infection in frogs and tadpoles or environmental levels of *B. dendrobatidis*.**
>
> **Objective 2.2: Perform a case control study to determine risk factors for survival of *L. aurea* in NSW water bodies with a focus on heavy metal pollution and salinity.**
>
> **Objective 2:3: Determine if there are differences in the growth rate of *B. dendrobatidis* in vitro using water from these sites.**
>
> **Objective 2.4: If heavy metals or salinity appear to have a negative effect on *B. dendrobatidis*, perform experimental infections to evaluate the effect on the natural history of chytridiomycosis.**

The effects of organic content of natural water bodies are explored above under objective 1. Further work on effects of water quality on the epidemiology of chytridiomycosis in the green and golden bell frog *Litoria aurea* has been undertaken by scientists at Newcastle University, Michael Mahony, John Clulow and a PhD Candidate Michelle Stockwell. Their results suggest that higher levels of sodium in water may protect this species from Bd and prevent population extirpation. The results of this work are included in Appendix 1 and will be published by Michelle Stockwell (PhD thesis, in preparation). Some similar collaborative work on the epidemiology of chytridiomycosis in the southern bell frog *Litoria raniformis* has been undertaken with scientists Ray and Cherie Draper from Victoria. It is hoped that this work will be published in the scientific literature by the end of 2011.

We explored the effects of climate, confirming the negative affects of relatively high temperatures on chytridiomycosis such as when minimum temperatures are above an ambient air temperature of 20°C (see also Objective 4 for experimental work showing higher temperatures (32 versus 23°C) increased survival of metamorphs with chytridiomycosis). We also found potential negative effects of high rainfall and high humidity and low humidity. Climate interacts with habitat and life history of species so that the effects of climate can vary. The data collected for this Objective have not been fully analysed but the potential limiting affects of extreme climatic conditions on chytridiomycosis even if they only occur seasonally have been well demonstrated in lowland rainforest populations of frogs. This understanding led to the rediscovery of the armoured mist frog, *Litoria lorica*, during this project in dry sclerophyll forest that is outside the former known distribution of the species in high altitude rainforest. The high altitude dry sclerophyll forest on the western edge of the Wet Tropics is regarded as suboptimal for chytridiomycosis due to its generally unfavourable climate (hotter and drier) for at least part of the year.

The management implications of this work are that species highly susceptible to chytridiomycosis may persist in areas that are climatically unsuitable for chytridiomycosis for at least part of the year. Similarly, unsuitable water bodies for chytridiomycosis may be a refuge for species highly susceptible to chytridiomycosis. Protection of these areas are a priority for management as some may occur outside of current reserves and is currently being addressed for *L. lorica*.

Objective 3.   What is the minimum density of chytrid zoospores in natural water bodies required to infect species of native amphibians?

B. dendrobatidis occurs at low density wihin rainforest streams (as shown in Objective 1) and yet transmission occurs readily (as shown in Objective 7). Transmission is likely to occur at densities of zoospores below our current ability to readily detect them. Therefore, until methods to filter large volumes of water and concentrate zoospores to enable detection are developed we will be unable to answer this Objective.

Objective 4.   Does the density of chytrid zoospores in natural water bodies correlate with intensity of infection of amphibian populations living in those water bodies, and with the level of clinical chytridiomycosis? Can the density of these zoospores in natural water bodies be used to predict periods of high risk of infection for amphibian populations?

The low density of *B. dendrobatidis* wihin rainforest streams as shown in Objective 1 was not correlated with intensity of infection nor with clinical chytridiomycosis. The density of these zoospores could not be used to predict periods of high risk of infection for amphibian populations (see Objectives 1 and 7). Rather density of infected tadpoles and duration of exposure for tadpoles or climatic conditions for frogs were better predictors of risk of infection for tadpoles and frogs, respectively (see Objectives 2.1 and 7). The method of transmission of zoospores such as tadpoles feeding on substrate rather than through direct exposure in the water column can increase the risk of transmission. Therefore the likely method of transmission should be considered when deciding what substrate to sample to detect *B. dendrobatidis* within water bodies.

Objective 5.   How does *B. dendrobatidis* spread between water bodies? Are there non-amphibian vectors of *B. dendrobatidis*?Objective 5.1: River sand as a possible transport vehicleObjective 5.2: Birds as possible vectors

Through *in vitro* studies we show that potential means of translocation may be moist soil and bird feathers. *B. dendrobatidis* survived for up to 3 months in sterile, moist river sand with no additional nutrients added. *B. dendrobatidis* attached to and grew on sterile feathers and were able to be transported by feathers to establish new cultures in media, surviving between 1 and 3 h of drying

between transfers. If these *in vitro* results are valid in the natural environment, the findings raise the possibilities that *B. dendrobatidis* may be translocated by movement of moist river sand and that birds may carry the amphibian chytrid fungus between frog habitats. However, ducks could not experimentally transport viable *B. dendrobatidis* after being exposed to an infected water body. This result may mean that the hypothesis is not plausible, or it may have been due to flaws in the experimental design. We also demonstrated that crustaceans and water dragons are unlikely to be important hosts of *B. dendrobatidis*. The detection of *B. dendrobatidis* DNA within stream sand in Objective 1 is consistent with the possibility of this substrate being a vector. Therefore, potentially infectious substrates from water bodies should be treated to kill *B. dendrobatidis* prior to movement to prevent spread.

Objective 6. **Can *B. dendrobatidis* be eradicated from small natural and artificial water bodies (eg farm dams or backyard ponds)? If eradication is considered possible/practicable, can we identify potential approaches to eradicating chytrid from such water bodies?**

B. dendrobatidis could possibly be eradicated from small water bodies using chemicals which have a disinfectant effect against the organism. We have recently investigated the ability of different products to kill *B dendrobatidis in vitro*. Each of the products tested was intended for purposes other than eradicating *B dendrobatidis* from water bodies. For example, they were tested at concentrations and exposure times applicable to washing tools or treating amphibian larvae etc. However, very effective products could be considered useful for disinfection of whole water bodies. Three of these products, Trigene, F10sc and Betadine, have shown positive results, killing *B. dendrobatidis* at low concentrations. In disturbed habitats, treatment of water bodies to reduce survival of zoospores may be a viable management option.

Objective 7. **How does *B. dendrobatidis* exist within infected frog populations? For example, are all frogs infected, are all tadpoles infected, is survivorship of infected frogs reduced compared to uninfected frogs in the population?**

We have extensively investigated this objective in both frogs and tadpoles. Survivorship of infected frogs is reduced although the analysis on the data presented here has not yet been completed. Analyses have been conducted on previously and contemporaneously collected data to show this (Murray et al 2009, Lee Skerratt unpublished observations). 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 adult and larval stage is likely. The patterns of prevalence and intensity of infection support this. Season affects transmission and development of disease through variations in climate and life history (seasonal recruitment and metamorphosis can affect prevalence in tadpoles). When climatic conditions are optimal then prevalence and intensity of infection of *B. dendrobatidis* can be very high and chytridiomycosis can cause significant morbidity and mortality. This means that wildlife managers should be prepared for seasonal epidemics of chytridiomycosis when conditions are favourable. It also means that *B. dendrobatidis* continues to severly impact amphibian populations although the effects are not obvious unless intensive populations studies such as this one are conducted. However, there is evidence for selection for innate immunity/tolerance within species. The mechanisms for increasing innate immunity/tolerance within species may relate to selection for particular skin and mouthpart characteristics. This is also explored in tender RFT 43/2004. Amphibian populations will recover more quickly and evade the threat of extinction if we can augment this selection process and understand the mechanisms of innate and adaptive immunity/tolerance.

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How Tender 42/2004 Integrates with Tender 43/2004

Both tenders are integrally linked. Several projects span both tenders such as the effect of climatic
conditions and its interaction with the natural history of frogs on outcome of infection (Objectives 2
and 7 in 42/2004 and Objectives 1 and 5 in 43/2004). Even those projects that are confined to one
tender often investigate aspects of chytridiomycosis that are relevant to both tenders. For example,
investigating the reasons for resistance to Bd within frogs in 43/2004 is an essential aspect of
understanding the epidemiology of chytridiomycosis in 42/2004. There has also been the opportunity
to value add to projects within one tender by investigating objectives from the other tender that are
easily addressed. For example it was relatively easy to swab retreat sites, crustaceans and serially
swab individual frogs for objectives in 42/2004 during field work to address objectives in 43/2004. In
addition both tenders benefit from using the same diagnostic expertise, which is the most expensive
and resource intensive part of the projects. Collaboration in this aspect ensures high quality
diagnosis, which is essential for providing high quality data. Both tenders also benefited from a
common pool of skills, knowledge and experience of the scientists and the latest research findings
within each tender.

Scope Items

The objectives of this tender are listed below:

Objectives of tender

Objective 1.   Does *B. dendrobatidis* exist as a free-living organism in suitable habitats, particularly natural water bodies and moist substrates? If so, can we identify and describe these free-living organisms and their biology/ecology?

Objective 2.   What environmental characteristics (eg. pH, pO2, ion content, nitrate, organic content) of natural water bodies (both permanent and ephemeral) and climate (eg. temperature, rainfall) favour the biology of *B. dendrobatidis*? Objective 2.1: We will determine whether any of the above environmental or climatic variables are correlated with either intensity and prevalence of infection in frogs and tadpoles or environmental levels of *B. dendrobatidis*.

Objective 2.2: Perform a case control study to determine risk factors for survival of *L. aurea* in NSW water bodies with a focus on heavy metal pollution and salinity.Objective 2:3: Determine if there are differences in the growth rate of *B. dendrobatidis* in vitro using water from these sites.

Objective 2.4: If heavy metals or salinity appear to have a negative effect on *B. dendrobatidis*, perform experimental infections to evaluate the effect on the natural history of chytridiomycosis.

Objective 3.   What is the minimum density of chytrid zoospores in natural water bodies required to infect species of native amphibians?

Objective 4.   Does the density of chytrid zoospores in natural water bodies correlate with intensity of infection of amphibian populations living in those water bodies, and with the level of clinical chytridiomycosis? Can the density of these zoospores in natural water bodies be used to predict periods of high risk of infection for amphibian populations?

Objective 5.   How does *B. dendrobatidis* spread between water bodies? Are there non-amphibian vectors of *B. dendrobatidis*?Objective 5.1: River sand as a possible transport vehicleObjective 5.2: Birds as possible vectors

Objective 6.   Can *B. dendrobatidis* be eradicated from small natural and artificial water bodies (eg farm dams or backyard ponds)? If eradication is considered possible/practicable, can we identify potential approaches to eradicating chytrid from such water bodies.

Objective 7.   How does *B. dendrobatidis* exist within infected frog populations? For example, are all frogs infected, are all tadpoles infected, is survivorship of infected frogs reduced compared to uninfected frogs in the population?

Outcomes

Specific outcomes of the tender are listed below in detail. We have focused on results not reported in the three previous progress reports of the tender and which have not been published in the scientific literature. Some results require additional analyses which will be completed in 2010 and published in 2010-11 in the scientific literature.

Objective 1.   Does *B. dendrobatidis* exist as a free-living organism in suitable habitats, particularly natural water bodies and moist substrates? If so, can we identify and describe these free-living organisms and their biology/ecology?
SAPROBIC SURVIVAL, GROWTH, REPRODUCTION AND BEHAVIOUR

Laboratory Studies
Growth of *B. dendrobatidis* (Bd) on substrates.
Initial laboratory trials were aimed at developing a method to compare growth of *B. dendrobatidis* (Bd) on different environmental substrates. Initially we attempted growth on unsterilized substrates (sand, gravel, and insect carcasses) in unsterilised pond water as we believed this would be more realistic. However problems with overgrowth of bacterial and fungal contaminants, which suppressed growth of Bd led to the use of sterilised materials. Substrates were placed in 24 well plates with zoospores. As growth could not be observed using an inverted microscope, even on translucent sand, the PCR test was used to quantitate growth. Results from swabbing indicated there was most growth in TGhL media, then sand, then plastic, then gravel. However problems with standardising the amount of surface area of each substrate, where Bd would attach, led to the experiments using scanning electron microscopy (SEM) with the aim of viewing the concentration of sporangia. We explored if *B. dendrobatidis* would grow on substrates other than amphibians by inoculating medium containing various biotic and abiotic substrates with *B. dendrobatidis* and imaging them with SEM (see Appendix 1). The results from this work confirm initial studies that Bd is readily out competed by other microbes. This work suggests Bd probably exists in the environment at low intensity due to microbial competition and may occur at higher intensities under optimal conditions for its survival and growth. This led to the following studies to examine persistence, growth and competitive behaviour of *B. dendrobatidis* in low nutrient environments such as found in rainforest streams.

Persistence of *B. dendrobatidis* (Bd) in low-nutrient stream water microcosms.
In the laboratory, we demonstrated that *B. dendrobatidis* cultures could be maintained for long periods of time in sterile stream water environments with nutrient sources common in stream environments of North Queensland rainforests. Cultures of *B. dendrobatidis* were maintained in 90ml of rainforest stream water for 20 weeks on a nutrient base of 0.05g of either snake skin (*Pseudechis porphyricus*) or sarcophagid flies. No additional nutrients were added throughout the culture period. Active *B. dendrobatidis* zoospores were still observable in both replicates of each food source condition at the time that the experiment was terminated. Furthermore, *B. dendrobatidis* cultures growing on snake skin or flies in stream water microcosms were successfully passaged throughout the 20 week experiment. Our results demonstrate clearly that *B. dendrobatidis* can persist indefinitely as a saprobe feeding on native organic materials in sterile stream water conditions.

We were also successful in demonstrating the persistence of *B. dendrobatidis* in non-sterile stream water microcosms. Zoospores and newly-formed sporangia of *B. dendrobatidis* could be unambiguously identified in non-sterile 90ml flasks containing stream water and snake skin at least 11 days after inoculation. Likely zoospores were seen amongst mixed cultures of other microorganisms up to 25 days post-inoculation. Our efforts to demonstrate longer persistence times for *B. dendrobatidis* grown in non-sterile stream water environments were limited by the lack of an adequate diagnostic test for live *B. dendrobatidis* in mixed-species cultures. We have yet to analyze data from two attempts to demonstrate *B. dendrobatidis* viability in non-sterile cultures several weeks post-inoculation. In the first experiment, we added late stage cane toad (*Bufo marinus*) tadpoles to stream water / snake skin cultures of *B. dendrobatidis* and allowed the tadpoles to feed on the infected snake skin for 5 days prior to euthanasia and preparation for PCR analysis of tadpole mouthparts. In the second experiment, we added a new strip of marked snake skin to similar cultures several weeks after inoculation with *B. dendrobatidis*. After three weeks, the marked snake skin was thin-sectioned, stained with haematoxylin and eosin, and mounted on slides that have yet to be fully analyzed.

Together, these results strongly suggest that *B. dendrobatidis* can survive for days, weeks, or possibly longer periods of time in North Queensland streams using bits of organic matter as nutrient sources. However, additional observations of the stream water cultures of *B. dendrobatidis* suggest that *B. dendrobatidis* zoospores are likely to be very patchily distributed and at very low densities outside of

amphibian hosts in rainforest streams. For example, we observed that, several weeks post-inoculation, sterile snake skin cultures of *B. dendrobatidis* had zoospore densities of less than 0.2% the level observed in TGHL media. In mixed-species environments containing organisms that may either compete with or consume *B.* dendrobatidis, zoospore densities are likely to be lower still. Furthermore, in cultures of *B. dendrobatidis* grown with only snake skin as a nutrient source, both in flasks with stream water and in Petri dishes with snake skin flakes suspended in pure agar, *B. dendrobatidis* sporangia were observed only directly on or in very close proximity to bits of snake skin. Thus, even if *B. dendrobatidis* is widely distributed as a saprobe in North Queensland rainforest streams, it is likely to be at such low densities that haphazard sampling of the stream environment through swabbing or filtering is likely to yield few positive PCR tests.

We further suggest the hypothesis that the persistence of saprobic *B. dendrobatidis* in non-sterile environments, either in the laboratory or in the field, is negatively correlated with dissolved nutrient concentrations in the water. In non-sterile TGHL cultures, *B. dendrobatidis* is rapidly overgrown by bacteria and other microorganisms. However, the first or second order streams in which we have conducted our field studies are very low nutrient environments, with concentrations of dissolved nitrates and phosphates that are likely several orders of magnitude lower than in TGHL solutions. We suggest that the low nutrient conditions found in montane streams in North Queensland favour the persistence of *B. dendrobatidis* through reducing the likelihood of competitive exclusion by other microorganisms.

Finally, in culturing *B. dendrobatidis* in stream water environments and sparse nutrient sources, we anecdotally observed potential differences in the life history of *B. dendrobatidis* when compared to TGHL cultures. Most notable, was the presence of some very large zoospores in the stream water cultures. One such zoospore was measured with a microscope reticle at approximately 9 microns in diameter, much larger than the 3-5 micron range previously reported. Additionally, individual zoospores in stream water cultures appeared to be more motile and possibly longer-living than in TGHL. Sporangia in stream water environments were, in contrast, quite small and few survived to maturity. Sporangia were, as previously noted, highly clustered either on or near snake skin or, when flies were used as a nutrient source, in large clumps (1-2 mm diameter) of sporangia floating within the water column.

Zoospore survival times in leaf detritus collected from stream environments
An experiment was conducted to assess the survival of Bd in non sterile stream sediment.

Methods
Approximately 2 mL of a dense suspension of detritus was collected from a slower flowing pool within a rainforest stream in Tully Gorge National Park. This was brought back to the lab and added to 8 ml of DS (dilute salts; sterile pond water alternative) in a small tissue culture flask. This mixture was then serially diluted 1:10 six times, however only five dilutions were inspected to maintain consistent volumes. Approximately 100,000 Bd zoospores (no sporangia) were added to each flask. Flasks were observed every day for zoospore motility until all activity ceased.

Results

Dilution	Maximum Observed Motility Time
1	1 hour
0.1	1 hour
0.01	1 hour
0.001	1 day
0.0001	4 days
0.00001	7 days

Discussion

Motility is a measure of activity. It is possible that some zoospores may have settled and ceased movement but were still alive. It is also possible that motile zoospores were more difficult to see in the more concentrated dilutions. However, motile zoospores were seen in all dilutions immediately following the addition of zoospores clearly indicating the motility, and possibly survival, of zoospores may be inhibited immediately in dense detritus. Zoospore survival time increased with increasingly dilute samples suggesting survival time may be longer in more dilute environments. No zoosporangia were observed, indicating replication did not take place. These results are consistent with those above indicating that *B. dendrobatidis* may favour low nutrient environments as a saprobe.

Studies on growth at low nutrient conditions (A manuscript has been submitted for publication, also published in an honours thesis (James 2007))
To investigate the hypothesis that Bd grows well and may have a competitive advantage at low nutrients, we examined cultures grown in 1% tryptone down to 0.001% tryptone. Growth was measured using optical density and by counting sporangia. Microscopical observations were used to describe differences in morphology at each concentration. Growth appears healthier on higher nutrient concentrations after 15 days incubation in most cases (Fig1).

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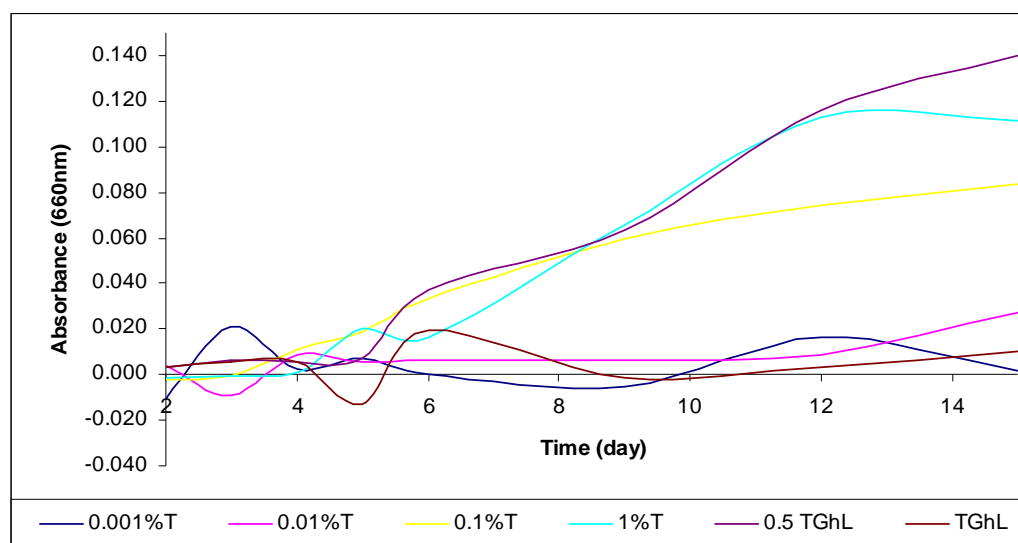
Figure 1: The growth of Bd on various nutrient media concentrations over 15 days. Growth was assessed by absorbance at 660 nm.

In higher nutrients the production and size of the sporangia in addition to the amount of zoospores was greater when compared with the lower nutrient treatments after 9 days incubation. However, the lifecycle was completed more quickly in the lower nutrient conditions, until the lower limit of nutrition for Bd was reached (0.001% tryptone). Zoospore release signifies the end of the Bd life

cycle. The observation of motile zoospores in treatments 0.01% T and 0.1% T occurred on day 4. Motile zoospores were observed in treatment 1% T on day 5 and treatment 0.5 TGhL on day 6. Motile zoospores were not seen until day 15 in treatment of 0.001% T. A competitive strategy of Bd, where a faster growth rate is employed in minimal media in an attempt to establish itself, may be the rationale for the physiological differences.

In addition, there was an increase in the density of rhizoids and the rhizoid covering of the sporangia under lower nutrient concentrations. This may be viewed as a strategy to increase nutrient acquisition at the deficient nutrient concentrations.

This study confirms that although growth rates of Bd are reduced at lower nutrients, it has adaptations that may enable it to be competitive under these conditions. Further studies examining competition under low nutrient conditions are needed (see James 2007 for further information). These findings were replicated by Jamie Voyles, a PhD candidate within the Amphibian Disease Ecology Group at JCU (see Voyles 2009, PhD thesis under examination, for further information).

References
James R.S. 2007. Investigations into the Physiology, Growth and Microbial Community Ecology of *Batrachochytrium dendrobatidis*. Honours Thesis, James Cook University, Townsville, Queensland, Australia.

Competition of Bd with environmental bacteria (Published in honours thesis (James 2007))
As 1) Bd is difficult to detect in the environment, and 2) in laboratory trials, Bd is consistently excluded by other microflora on high nutrient media, we hypothesized that competition in the environment may inhibit its growth. The inhibition of Bd by bacteria collected from soil and water was assessed.

Bacteria were isolated from bank, sediment and water samples from Tully River, before trialling against Bd by streaking on agar plates and observing for a zone of inhibition. Four of 17 bacteria tested inhibited Bd by production of antagonistic compounds. It was interesting that the bank soil samples (where bacteria were more abundant) contained apparently more inhibiting species of bacteria (3/6) than the water samples (1/6) and the sediment samples (0/5). Bacteria from the genera *Pseudomonas*, *Enterobacter/Klebsiella*, *Serratia* and the *Bacillus cereus* group were isolated. The organisms identified from the *Serratia* and the *Bacillus cereus* groups predominantly inhibited Bd. Of particular note is the isolate *Serratia marcescens* which is known to produce chitinase and thus inhibit the growth of fungi in the soil. The results demonstrate that antagonism to Bd is fairly common in environmental microbes. Since it appears Bd can grow under low nutrient conditions, the sediment of the streams and the water itself may be a high priority area to search for saprobic stages of Bd (see James 2007 for further information).

References
James R.S. 2007. Investigations into the Physiology, Growth and Microbial Community Ecology of *Batrachochytrium dendrobatidis*. Honours Thesis, James Cook University, Townsville, Queensland, Australia.

Identifying aspects of *B. dendrobatidis* ecology: dispersal of zoospores in still water.
We examined the movement of Bd in stagnant water. Initially we conducted an experiment in 20 cm upright flasks then transferred the techniques to a vertical column. We completed this experiment successfully demonstrating that Bd moves both up and down within a water column enabling it to disperse and infect tadpoles and frogs throughout a pool of water.

This original vertical column experiment was altered in order to make it easier and more accurate. It is now smaller and examines horizontal movement (Figure 1.1). Zoospores are introduced via a slide holding attached zoosporangia to minimize effects of introduction of zoosporangia and zoospores on their movement. The experiment was left for sufficient time for introduced zoosporangia to release zoospores, and for zoospores to disperse and settle. Zoospore movement was detected by their attachment to slides which were at intervals along the column. The slides were tested for attached Bd by IPX staining and swabbing. In additional laboratory studies, we examined whether Bd moves in response to various stimuli such as light and physical attractants. During our initial experiment, Bd did not move toward light and dark environments nor toward a variety of wavelengths of light. Therefore Bd is likely to disperse throughout a water body irrespective of light conditions. Currently we are modifying techniques to enable us to explore if *B. dendrobatidis* is attracted to particular substances, such as tadpoles (both whole and particular parts), crayfish, stone, agar, fish, soil, insects, and feathers.

Figure 1.1: Container of water with position of slides to detect unassisted horizontal movement of zoospores in a water column.
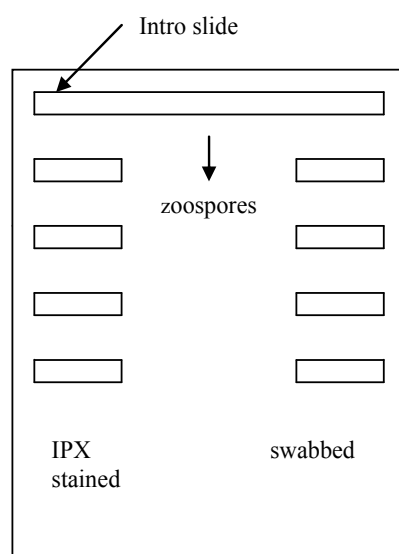
Field Studies
Detecting Bd in the environment
<u>Retreat sites</u> (This has been published in Rowley et al 2007)
During daylight hours, nocturnal frogs (all extant wet tropics species) sit in one position. As these sites are occupied by the frog for at least 12 hours, if Bd is able to persist in the environment, there is opportunity for Bd transmission between the frog and the substrate, particularly in moist retreat sites. In addition, individual retreat sites are often occupied by the same frog for days on end, or may be returned to after nocturnal movement. During the course of tracking species of rainforest stream frogs diurnal retreat sites were recorded and were swabbed for three consecutive days in order to determine whether Bd remained in retreat sites over time, and how long Bd was able to persist in these locations. Retreat sites of two species of Australian rain forest stream frogs (*Litoria lesueuri* and *L. nannotis*) were sampled 0-3 days after occupation during the wet and dry seasons in northern Queensland where chytridiomycosis has been endemic for at least ten years. The intensity and prevalence of infection in frogs during sampling were comparatively low compared with epidemics. Diagnostic quantitative PCR did not detect Bd in any retreat site samples. It thus appears that retreat sites are not a major environmental source of infection when Bd occurs at low prevalence and intensity on frogs. This suggests that control efforts may not need to eliminate the organism from the environment, at least when prevalence and intensity of infection are low in frogs. Simply treating hosts may be effective at controlling the disease in the wild (see Rowley et al 2007 for further information).

<u>References</u>
Rowley J.J.L., Skerratt L.F., Alford R.A. and Campbell R. 2007. Retreat sites of rain forest stream frogs are not a reservoir for *Batrachochytrium dendrobatidis* in northern Queensland, Australia. Diseases of Aquatic Organisms 74: 7-12.

Stream sand and leaves
<u>Introduction</u>
Batrachochytrium dendrobatidis has been detected on rocks adjacent to the stream (Lips et al. 2006) and pond water (Kirshtein et al. 2007, Walker et al. 2007) by quantitative PCR assay, but not from sediment (Kirshtein et al. 2007, Walker et al. 2007).

Additional substrates in the stream environment might support the saprobic existence of Bd as the fungus can be cultured without keratin or keratin derivatives and some chytrid fungi do not depend on a specific substrate for attachment or nutrition. It is possible a long-living or free-living stage of Bd could persist in the biofilm of leaves decomposing in the stream; zoospores of many chytrid fungi can occur in films of water on plants (Carlile and Watkinson 1994) and canopy leaves (Longcore et al. 2005). In addition, some chytrids are host-specific parasites of freshwater phytoplankton (see Ibelings et al. 2004).

The identification of an environmental substrate utilised by Bd for nutrition or attachment would allow temporal and spatial surveys to assess the distribution and abundance of the chytrid, useful knowledge when predicting outbreaks of chytridiomycosis in threatened species, monitoring water bodies prior to reintroduction of extirpated species, and monitoring the spread of Bd through an ecosystem. To improve our knowledge of the environmental reservoirs of Bd, we tested two substrates, stream sand and leaf litter, for Bd. We also compared our success at detecting Bd in sediment with that of existing studies (Kirshtein et al. 2007, Walker et al. 2007)

<u>Procedure</u>
Stream sand and leaves were collected every 20m from the first 100m of a 150-200m transect regularly surveyed for anuran populations in areas where Bd is endemic. Sampling occurred during the austral cool, dry months between May and September in 2006 and 2007 at two locations, Kirrama

within Murray Upper National Park (18º11′S, 145º52′E, elevation 250 m) and Tully Gorge National Park (17º46'S, 145º38'E, elevation 100 m). Sediment and leaf samples were collected by hand and stored in sealed containers below 5°C for 2-4 days until return to the laboratory and storage at -80°C.

We used the MoBio PowerPlant Isolation Kit to extract DNA from 0.05g of superficial tissue scrapped from partially decayed leaves using a scalpel blade and the MoBio PowerSoil Kit to extract DNA from 0.1g of sediment. The suitability of the extraction protocol and DNA quality were assessed by agarose gel electrophoresis and ethidium bromide staining of 10 extracts for each substrate. A PCR assay was used to detect Bd DNA. Real-time PCR analysis, including primers, TaqMan® (Applied Biosystems) probe, and cycling conditions, followed Boyle et al. (2004) with the following modifications. The analysis was performed on the Rotor-Gene™ 6000 (Corbett Research) using Gene-Disc 100 tubes. Fifteen µL reactions were produced by loading 10 µL of PCR master mix, 2 µL of extract and 3 µL of water in triplicate, or 5 µL of positive control standard in quadruplicate. Positive control standards representing 100, 10, 1 and 0.1 zoospore equivalents were prepared and analysed as described by Garland et al. (2009). The PCR reaction mix included the addition of 400 ng/µL of bovine serum albumin (BSA) (Sigma A4161) to reduce inhibition due to environmental contaminants (Garland et al. in press). Ct values (PCR cycle at point of detection) for positive reactions were determined by the Rotor-Gene™ software 1.7 using a threshold of 0.01. In order to test for inhibition of PCR, and the likelihood of false negatives, we performed a separate analysis using the TaqMan® Exogenous internal positive control (IPC) (Applied Biosystems) (0.6x Exo IPC Mix, 0.6x Exo IPC DNA) as an addition to the assay for Bd. Individual IPC analyses were performed on the extracts and the triplicate analysis of water. Extracts producing IPC Ct values 3 greater than the average obtained for water were defined as producing significant inhibition. Those extracts that were negative for Bd and inhibitory were diluted 1 in 4 and reanalysed.

Results
Leaves
Eleven extracts produced at least one positive reaction when the undiluted extracts were analysed. Three produced 3 positive reactions (from 3 analyses) and 1 produced 2 positive reactions. The remainder produced 1 positive reaction. All represented low levels of Bd as represented by Ct values higher than obtained for the 0.1 zoospore equivalent standard (Table 1.1). Eight extracts caused significant inhibition as demonstrated by IPC Ct values greater than 3 above that obtained for water or failed to amplify (data not shown). These 8 extracts were diluted 1 in 4 and reanalysed. The diluted extracts caused no inhibition, 5 were negative for Bd, 3 produced low level positive reactions (Table 1.1). In total, fourteen of the forty-four extracts, or 31.8%, were positive for Bd.

Table 1.1: Results of TaqMan qPCR analysis for the presence of Bd in 44 samples of stream leaf detritus. Ct values are provided for positive reactions. All positive reactions have Ct values lower than obtained for the 0.1 zoospore equivalent (ZSE) standard which recorded an average Ct of 34 (n=4, s.d.=0.39). Ct values above 34 are not quantitative and represent a positive reaction of low concentration. *Extract diluted 1 in 4 due to the undiluted extract causing inhibition to PCR. 0.1 ZSE detected in the PCR would represent 2.5 ZSE in the 0.05g of leaf scrapings for the undiluted extract, and 10 ZSE for the diluted extract.

Sample	Site	Date	# Positive Reactions	Ct (mean)
L6	Tully	13/06/07	0	Negative
L7	Tully	13/06/07	0	Negative
L8	Tully	13/06/07	0	Negative
L9	Tully	13/06/07	0	Negative
L10	Tully	13/06/07	0	Negative
L11	Tully	29/08/07	0	Negative
L12	Tully	29/08/07	3	35.20
L13	Tully	29/08/07	3	37.90

L14	Tully	29/08/07	2	36.70
L15	Tully	29/08/07	0	Negative
L1	Tully	18/10/07	2	36.5*
L2	Tully	18/10/07	1	41.60
L3	Tully	18/10/07	0	Negative*
L4	Tully	18/10/07	0	Negative*
L5	Tully	18/10/07	3	35.9*
L16	Kirrama	18/05/06	0	Negative
L17	Kirrama	18/05/06	1	35.60
L18	Kirrama	18/05/06	1	37.8*
L19	Kirrama	18/05/06	0	Negative
L20	Kirrama	18/05/06	0	Negative
L21	Kirrama	10/07/06	2	37.50
L22	Kirrama	10/07/06	0	Negative
L23	Kirrama	10/07/06	0	Negative
L24	Kirrama	10/07/06	0	Negative*
L25	Kirrama	10/07/06	0	Negative
L26	Kirrama	01/05/07	0	Negative
L27	Kirrama	01/05/07	0	Negative
L28	Kirrama	01/05/07	0	Negative
L29	Kirrama	01/05/07	0	Negative*
L30	Kirrama	01/05/07	0	Negative
L36	Kirrama	12/06/07	0	Negative
L37	Kirrama	12/06/07	0	Negative
L38	Kirrama	12/06/07	0	Negative
L39	Kirrama	12/06/07	0	Negative
L40	Kirrama	12/06/07	0	Negative
L41	Kirrama	24/08/07	1	35.10
L42	Kirrama	24/08/07	1	36.50
L43	Kirrama	24/08/07	3	36.70
L44	Kirrama	24/08/07	0	Negative
L45	Kirrama	24/08/07	1	38.50
L31	Kirrama	03/10/07	1	37.90
L32	Kirrama	03/10/07	0	Negative
L33	Kirrama	03/10/07	0	Negative*
L34	Kirrama	03/10/07	0	Negative

Stream sand

Five extracts, or 5.7%, produced one positive reaction. All represented low levels of Bd as represented by Ct values higher than obtained for the 0.1 zoospore equivalent standard (Table 1.2). No extracts caused significant inhibition as would have been demonstrated by IPC Ct values greater than 3 above that obtained for water. The five positive samples were repeated using 5 μL of extract in the PCR assay. There was no significant inhibition however, 4 of these samples were negative for Bd on re-analysis. Sample S56 recorded three positive reactions with an average Ct of 35.8

Table 1.2: Results of TaqMan qPCR analysis for the presence of Bd in 88 stream sediment samples. Ct values are provided for positive reactions. All positive reactions have Ct values lower than obtained for the 0.1 zoospore equivalent (ZSE) standard which recorded an average Ct of 34.5 (n=4, s.d.=0.74). Ct values above 34 are not quantitative and represent a positive reaction of low concentration. 0.1 ZSE detected in the PCR would represent 5 ZSE in 0.1g of sediment.

Sample	Location	Date	Sample Source	# Positive Reactions	Ct (mean)
S16	Tully	13/06/07	Bank	1	41.5
S17	Tully	13/06/07	Bank	1	37.4
S18	Tully	13/06/07	Bank	0	Negative
S19	Tully	13/06/07	Bank	0	Negative
S20	Tully	13/06/07	Bank	0	Negative
S21	Tully	13/06/07	Bed	0	Negative
S22	Tully	13/06/07	Bed	0	Negative
S23	Tully	13/06/07	Bed	0	Negative
S24	Tully	13/06/07	Bed	0	Negative
S25	Tully	13/06/07	Bed	0	Negative
S26	Tully	29/08/07	Bank	0	Negative
S27	Tully	29/08/07	Bank	0	Negative
S28	Tully	29/08/07	Bank	0	Negative
S29	Tully	29/08/07	Bank	1	42.4
S30	Tully	29/08/07	Bank	0	Negative
S31	Tully	29/08/07	Bed	0	Negative
S32	Tully	29/08/07	Bed	1	36.5
S33	Tully	29/08/07	Bed	0	Negative
S34	Tully	29/08/07	Bed	0	Negative
S35	Tully	29/08/07	Bed	0	Negative
S69	Tully	18/10/07	Bank	0	Negative
S70	Tully	18/10/07	Bank	0	Negative
S71	Tully	18/10/07	Bank	0	Negative
S72	Tully	18/10/07	Bank	0	Negative
S73	Tully	18/10/07	Bank	0	Negative
S74	Tully	18/10/07	Bed	0	Negative
S75	Tully	18/10/07	Bed	0	Negative
S76	Tully	18/10/07	Bed	0	Negative
S77	Tully	18/10/07	Bed	0	Negative
S78	Tully	18/10/07	Bed	0	Negative
S11	Kirrama	18/05/06	Bank	0	Negative
S12	Kirrama	18/05/06	Bank	0	Negative
S13	Kirrama	18/05/06	Bank	0	Negative
S14	Kirrama	18/05/06	Bank	0	Negative
S15	Kirrama	18/05/06	Bank	0	Negative
S1	Kirrama	10/07/06	Bank	0	Negative
S2	Kirrama	10/07/06	Bank	0	Negative
S3	Kirrama	10/07/06	Bank	0	Negative
S4	Kirrama	10/07/06	Bank	0	Negative
S5	Kirrama	10/07/06	Bank	0	Negative
S6	Kirrama	10/07/06	Bed	0	Negative
S7	Kirrama	10/07/06	Bed	0	Negative
S8	Kirrama	10/07/06	Bed	0	Negative
S9	Kirrama	10/07/06	Bed	0	Negative
S10	Kirrama	10/07/06	Bed	0	Negative
S79	Kirrama	29/08/06	Bank	0	Negative
S80	Kirrama	29/08/06	Bank	0	Negative
S81	Kirrama	29/08/06	Bank	0	Negative
S82	Kirrama	29/08/06	Bank	0	Negative

S83	Kirrama	29/08/06	Bank	0	Negative
S84	Kirrama	29/08/06	Bed	0	Negative
S85	Kirrama	29/08/06	Bed	0	Negative
S86	Kirrama	29/08/06	Bed	0	Negative
S87	Kirrama	29/08/06	Bed	0	Negative
S88	Kirrama	29/08/06	Bed	0	Negative
S36	Kirrama	01/05/07	Bank	0	Negative
S37	Kirrama	01/05/07	Bank	0	Negative
S38	Kirrama	01/05/07	Bank	0	Negative
S39	Kirrama	01/05/07	Bank	0	Negative
S40	Kirrama	01/05/07	Bank	0	Negative
S41	Kirrama	01/05/07	Bed	0	Negative
S42	Kirrama	01/05/07	Bed	0	Negative
S43	Kirrama	01/05/07	Bed	0	Negative
S44	Kirrama	01/05/07	Bed	0	Negative
S45	Kirrama	01/05/07	Bed	0	Negative
S56	Kirrama	12/06/07	Bed	1	35.2
S57	Kirrama	12/06/07	Bed	0	Negative
S58	Kirrama	12/06/07	Bed	0	Negative
S59	Kirrama	12/06/07	Bed	0	Negative
S60	Kirrama	12/06/07	Bed	0	Negative
S46	Kirrama	24/08/07	Bank	0	Negative
S47	Kirrama	24/08/07	Bank	0	Negative
S48	Kirrama	24/08/07	Bank	0	Negative
S49	Kirrama	24/08/07	Bank	0	Negative
S50	Kirrama	24/08/07	Bank	0	Negative
S51	Kirrama	24/08/07	Bed	0	Negative
S52	Kirrama	24/08/07	Bed	0	Negative
S53	Kirrama	24/08/07	Bed	0	Negative
S54	Kirrama	24/08/07	Bed	0	Negative
S55	Kirrama	24/08/07	Bed	0	Negative
S61	Kirrama	03/10/07	Bank	0	Negative
S62	Kirrama	03/10/07	Bank	0	Negative
S63	Kirrama	03/10/07	Bank	0	Negative
S64	Kirrama	03/10/07	Bank	0	Negative
S65	Kirrama	03/10/07	Bank	0	Negative
S66	Kirrama	03/10/07	Bed	0	Negative
S67	Kirrama	03/10/07	Bed	0	Negative
S68	Kirrama	03/10/07	Bed	0	Negative

Discussion

This is the first study to detect *Bd* in environmental substrates. Our two and three reaction positive qPCR assays on leaf detritus suggest *Bd* is sporadically distributed at very low densities. Further research is underway to determine if *Bd* presence in leaf detritus shows seasonal variation; however it can be difficult to collect samples during the wet season (austral summer) so attempts will also be made to grow laboratory cultures of Bd on leaves. This process will require development of a live animal test or an RNA assay that will quantify viable Bd as the existing DNA assay may be quantifying non viable Bd in the substrate. Our calculated Bd ZSE from leaf detritus appears too high to be that of non-viable Bd so we are optimistic that this is a relevant finding. Bd may be exiting saprobically in the biofilm on the leaf surface, in which case it may be a source of infection for feeding tadpoles.

Three studies ((Kirshtein et al. 2007, Walker et al. 2007, and the current study) have failed to detect *Bd* in stream sediments other than at very low levels. We therefore assume that Bd is not existing in a saprobic or resting form within this stream substrate to any great extent. However, it is still possible

for this substrate to act as a mechanical vector for spread of Bd given that we detected Bd DNA within it.

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At the time of submission of this report results for water filtration and rock scrapings were unavailable. They were similar to the results above for leaves and sand in the stream. They are included as Appendix 2.

Objective 2. **What environmental characteristics (eg. pH, pO2, ion content, nitrate, organic content) of natural water bodies (both permanent and ephemeral) and climate (eg. temperature, rainfall) favour the biology of *B. dendrobatidis*?**

Objective 2.1: **We will determine whether any of the above environmental or climatic variables are correlated with either intensity and prevalence of infection in frogs and tadpoles or environmental levels of *B. dendrobatidis*.**

EFFECTS OF CHARACTERISTICS OF NATURAL WATER BODIES

The effects of organic content of natural water bodies are explored above under objective 1. Further work on effects of water quality has been being undertaken by PhD candidate Michelle Stockwell and her supervisors Michael Mahony and John Clulow at the University of Newcastle on the epidemiology of chytridiomycosis in *L. aurea*. In addition we are collaborating with Ray and Cherie Draper who are examining this aspect in understanding the epidemiology of chytridiomycosis in *L. raniformis* in Victoria. These two frog species inhabit water bodies with variable environmental characteristics that are ideal for this study. We did not examine futher aspects on the effects of characteristics of water bodies because rainforest stream environments where chytridiomycosis is endemic within our region consistently have high water quality. Therefore studying rainforest streams would be largely uninformative regarding the effects of variation in water quality on *B. dendrobatidis*.

EFFECTS OF CLIMATE

Frogs
Introduction
Temperature is a major climatic factor influencing survival and growth of Bd. Isolates of Bd grow and reproduce between 4-25°C, however maximal growth and pathogenicity occurs between 17-25°C *in vitro* (Longcore et al. 1999, Berger et al. 2004, Piotrowski et al. 2004). Mortalities associated with population declines in Australia often occur during cooler seasons (Berger et al. 1998, 2004, McDonald et al. 2005, Woodhams and Alford 2005). Within infected populations, daily and seasonal fluctuations in ambient air temperature can affect prevalence of Bd and intensity of infection (Kriger and Hero 2007, Woodhams and Alford 2005). Frogs held at elevated temperatures (37°C for <16hr) are able to clear themselves of infection (Woodhams et al. 2003). Environmental temperature may influence the survival of Bd within the adult frog epidermis (Berger et al. 2004, Woodhams et al. 2003) or allow increased host immune function (Andre et al. 2008).

The role of moisture in the physiology and disease dynamics of Bd is less well understood. In culture, the fungus is killed by complete desiccation for longer than 3hr (Johnson et al. 2003). However, the frogs most susceptible to chytridiomycosis are those associated with streams and are therefore unlikely to be exposed to such dry conditions. To determine the effect of climatic variables such as rainfall, humidity and temperature on prevalence of Bd in frogs and intensity of infection, we monitored such conditions at two study sites during a mark-recapture study to determine the dynamics of Bd within infected frog populations (see Objective 7 for full description of study sites and species).

Procedure
We established permanent data loggers (Tinytag *Plus*, Hastings Data Loggers) to record temperature each hour betwen November 2005 and November 2007. Daily rainfall and relative humidity data were sourced from the closest Bureau of Meteorology weather station to each study site. Climatic conditions were then related to the number of frogs, prevalence of Bd and intensity of infection in four species of frog (*Litoria genimaculata*, *L. nannotis*, *L. rheocola* and *Nyctimystes dayi*) at two locations (Murray Upper National Park, 18º11′S, 145º52′E, elevation 250m and Tully Gorge National

Park, 17º46'S, 145º38'E, elevation 100m) as described in Objective 7. From hourly and daily observations of climatic conditions we determined the mean for each variable at intervals (24hr, 7d, 14d, 21d and 28d) before the first night of each survey to determine if they were correlated with the number of adult, male frogs present on the stream transect, the prevalence of Bd, or the intensity of infection (see Objective 7). Animals captured on the first night only of each survey were included in the analyses to ensure environmental factors, and not human disturbance, influenced frog numbers. As inter-specific behavioural differences were likely to greatly influence frog numbers and disease dynamics, non-parametric Spearman's rank correlation coefficients were calculated for each species at each site.

Results

Climatic Conditions at Murray Upper NP

Air temperatures at Murray Upper NP (see Figure 2.1 and 2.2) followed the typical pattern of the austral summer, with mean, minimum and maximum temperatures increasing in September and declining after March. During this time, the maximum daily temperature was often higher than the upper thermal tolerance limit of Bd. In the cooler winter, mean and maximum temperatures were within the thermal range for greatest growth and virulence, however, minimum temperatures may drop below this range. Air temperature never fell below the lower thermal tolerance limit of 4°C for Bd (Piotrowski et al. 2004).

Figure 2.1. Daily mean, minimum and maximum air temperature at Murray Upper NP. Dashed lines represent range of greatest virulence and pathogenicity of *Batrachochytrium dendrobatidis in vitro*.
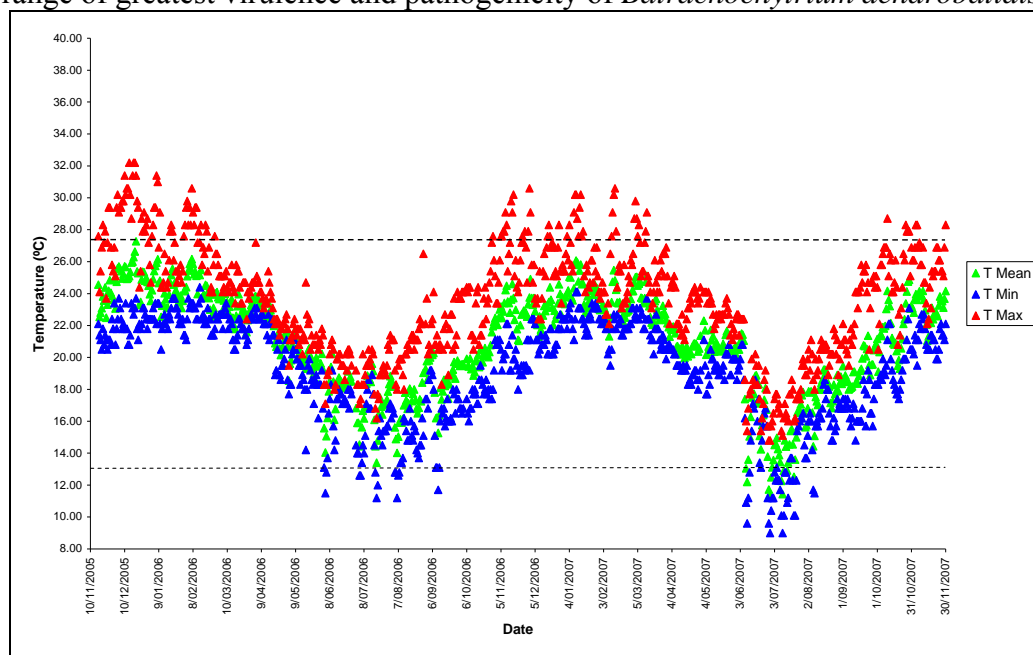
Greatest rainfall also occurred during the austral summer, but was unpredictable and could extend into the winter months (see Figure 2.3-2.4). Maximum relative humidity on any day ranged between 80—100%, with the majority of days at the upper limit due to the dense rainforest canopy above the permanent stream. Minimum daily relative humidity was as its' lowest in the cooler and drier winter months (see Figure 2.5-2.6).

Figure 2.2. Mean, minimum and maximum temperatures at 24h, 7d, 14d, 21d and 28d before each survey for frogs at Murray Upper NP.
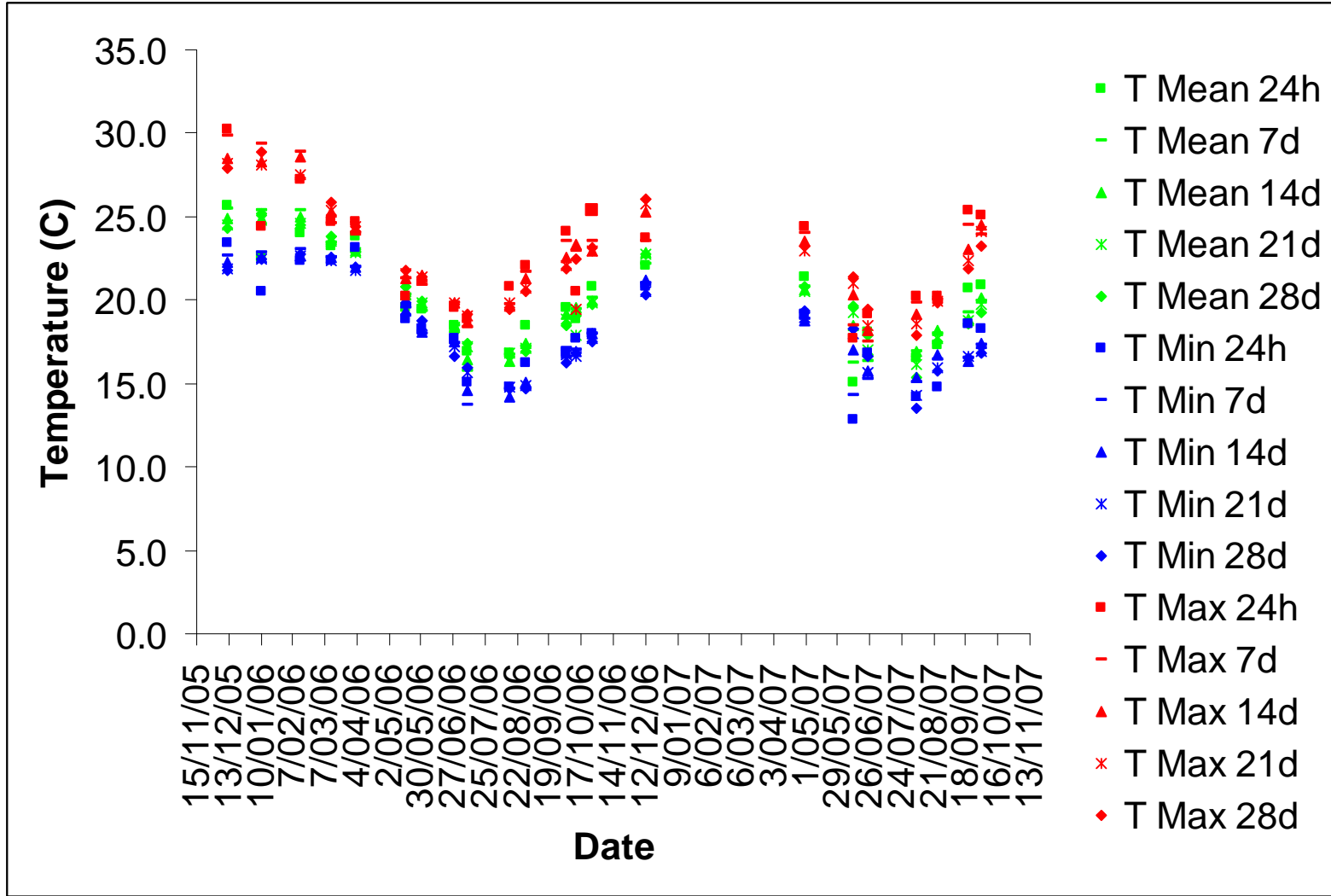
Figure 2.3. Temporal variation in daily rainfall at Murray Upper NP.
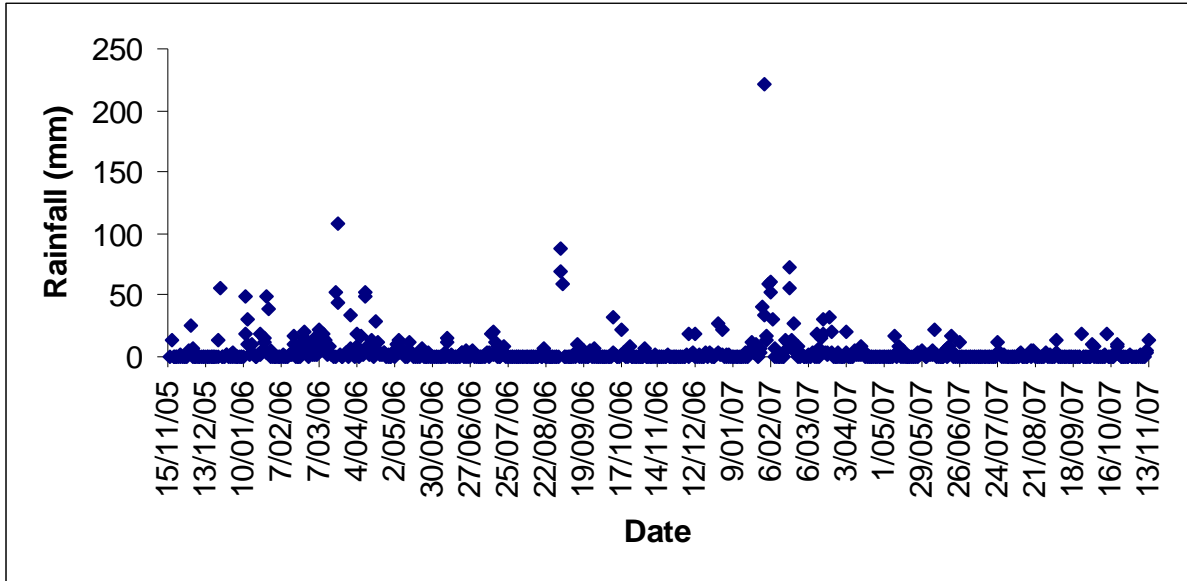
Figure 2.4. Rainfall for 24h, 7d, 14d, 21d and 28d before each survey for frogs at Murray Upper NP.
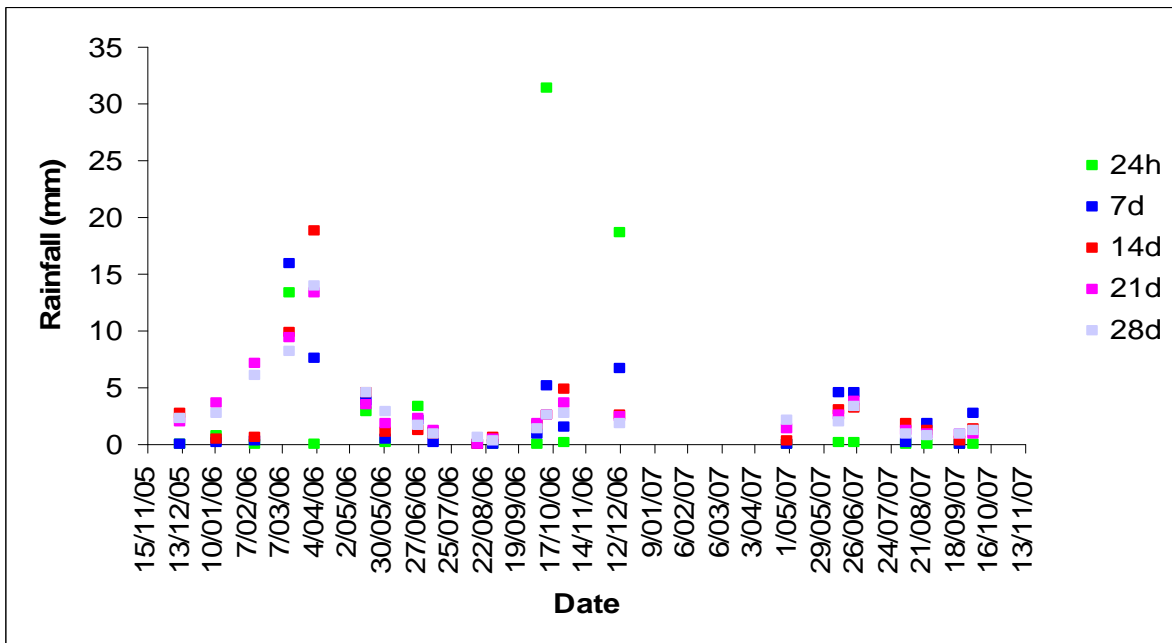
Figure 2.5. Temporal variation in relative humidity (minimum and maximum) at Murray Upper NP.
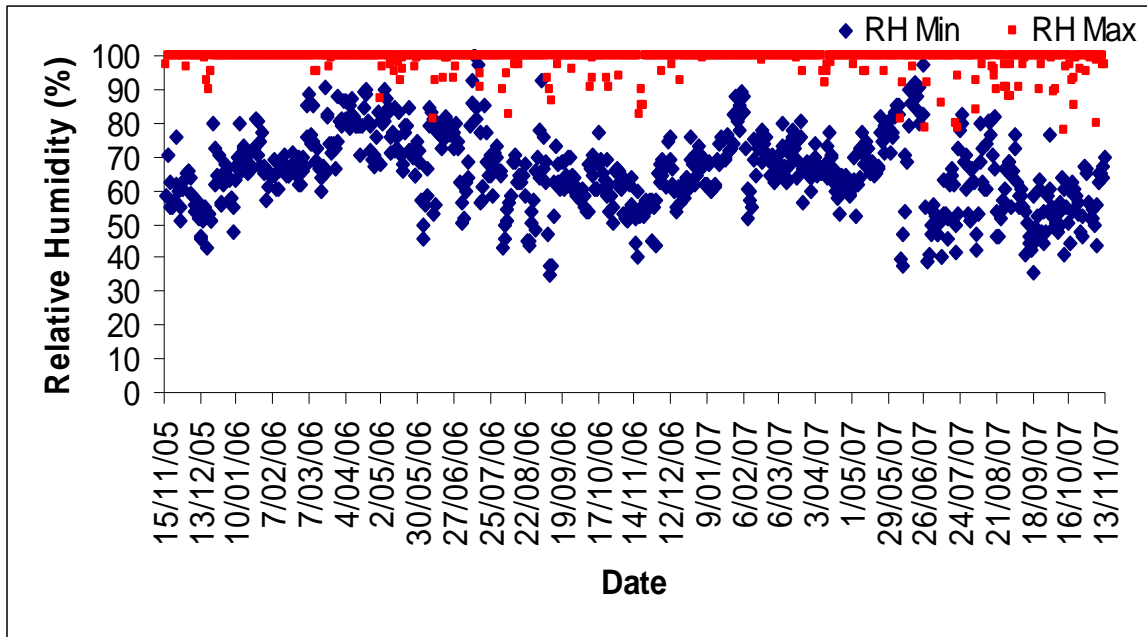
Figure 2.6. Relative humidity (minimum and maximum) for 24h, 7d, 14d, 21d and 28d before each survey for frogs at Murray Upper NP.
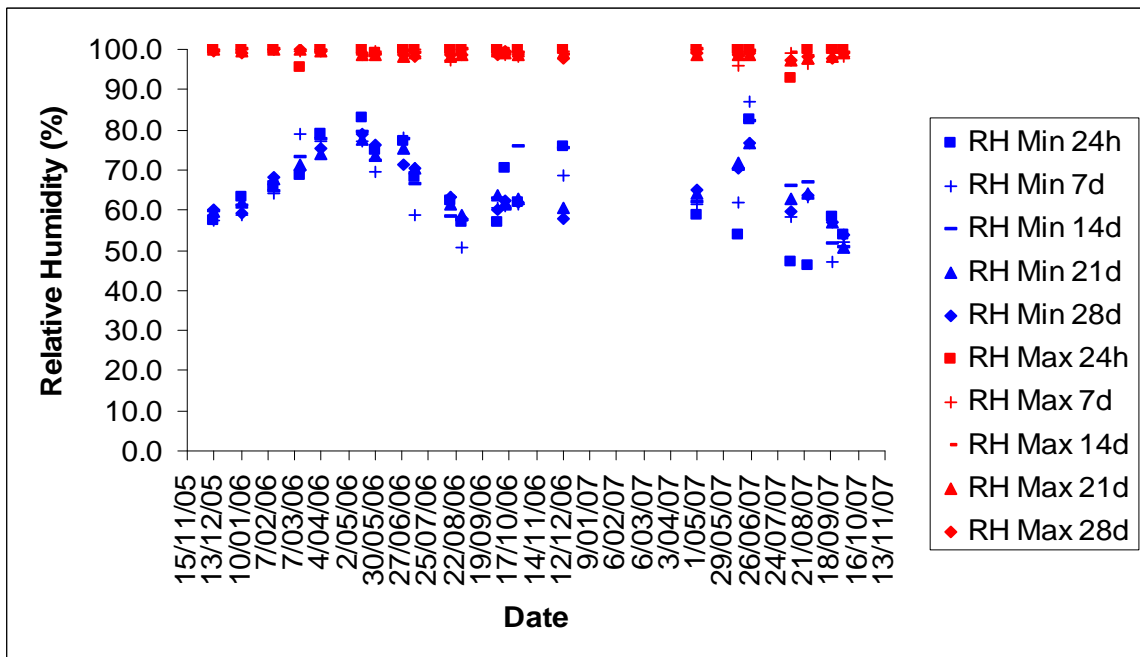
Numbers of *L. genimaculata* (n=96) on the stream at Murray Upper NP were positively correlated with recent maximum air temperatures, but negatively correlated with recent rainfall and longer-term minimum relative humidity. Prevalence of infection with Bd in *L. genimaculata* was negatively correlated with minimum temperatures, rainfall and minimum relative humidity in the weeks before sampling. The intensity of infection was negatively correlated in the 24hr prior to sampling by minimum temperature, minimum rainfall and minimum relative humidity. An increase in maximum relative humidity 3 weeks and minimum temperature 4 weeks prior to sampling was also negatively correlated with intensity of infection.

Table 2.1. Climatic factors correlated with density of *Litoria genimaculata*, prevalence of Bd and intensity of infection (as determined by qPCR) at Murray Upper NP, as determined by Spearman's rank correlation (see also Figures 2.7-2.9).

Climatic factor	Period prior to survey	# Frogs	Prevalence Bd	Intensity of infection (#ZSE)
Mean Temperature	24h	Not Significant	Not Significant	Not Significant
	7d	Not Significant	Not Significant	Not Significant
	14d	Not Significant	Not Significant	Not Significant
	21d	Not Significant	Not Significant	Not Significant
	28d	Not Significant	$r_s=-0.433, P=0.044$	Not Significant
Minimum Temperature	24h	Not Significant	Not Significant	$r_s=-0.606, P=0.037$
	7d	Not Significant	Not Significant	Not Significant
	14d	Not Significant	$r_s=-0.439, P=0.041$	Not Significant
	21d	Not Significant	$r_s=-0.468, P=0.028$	Not Significant
	28d	Not Significant	$r_s=-0.518, P=0.013$	$r_s=-0.581, P=0.047$
Maximum Temperature	24h	$r_s=0.647, P=0.001$	Not Significant	Not Significant
	7d	$r_s=0.452, P=0.035$	Not Significant	Not Significant
	14d	Not Significant	Not Significant	Not Significant
	21d	Not Significant	Not Significant	Not Significant
	28d	Not Significant	Not Significant	Not Significant
Rainfall	24h	$r_s=-0.601, P=0.003$	Not Significant	$r_s=-0.645, P=0.024$
	7d	$r_s=-0.597, P=0.003$	Not Significant	Not Significant
	14d	Not Significant	Not Significant	Not Significant
	21d	Not Significant	$r_s=-0.559, P=0.007$	Not Significant
	28d	Not Significant	$r_s=-0.551, P=0.008$	Not Significant
Minimum Relative Humidity	24h	Not Significant	Not Significant	$r_s=-0.578, P=0.049$
	7d	$r_s=-0.607, P=0.003$	$r_s=-0.495, P=0.019$	Not Significant
	14d	$r_s=-0.578, P=0.005$	Not Significant	Not Significant
	21d	$r_s=-0.682, P=0.000$	$r_s=-0.440, P=0.041$	Not Significant
	28d	$r_s=-0.638, P=0.001$	$r_s=-0.474, P=0.026$	Not Significant
Maximum Relative Humidity	24h	$r_s=0.435, P=0.043$	Not Significant	Not Significant
	7d	Not Significant	Not Significant	Not Significant
	14d	Not Significant	$r_s=-0.474, P=0.026$	Not Significant
	21d	Not Significant	Not Significant	$r_s=-0.746, P=0.005$
	28d	Not Significant	$r_s=-0.534, P=0.010$	Not Significant

Numbers of *L. nannotis* (n=56) were independent of climatic conditions with the exception of a positive correlation with rainfall. Prevalence of Bd and intensity of infection in *L. nannotis* were not greatly affected by the climatic variables examined although the power to detect an effect was lower than for *L. genimaculata*.

Table 2.2. Climatic factors correlated with density of *Litoria nannotis*, prevalence of Bd and intensity of infection (as determined by qPCR) at Murray Upper NP, as determined by Spearman's rank correlation (see also Figures 2.7-2.9).

Climatic factor	Period prior to survey	# Frogs	Prevalence *Bd*	Intensity of infection (#ZSE)
Mean Temperature	24h	Not Significant	Not Significant	Not Significant
	7d	Not Significant	Not Significant	Not Significant
	14d	Not Significant	Not Significant	Not Significant
	21d	Not Significant	Not Significant	Not Significant
	28d	Not Significant	Not Significant	Not Significant
Minimum Temperature	24h	Not Significant	Not Significant	Not Significant
	7d	Not Significant	Not Significant	Not Significant
	14d	Not Significant	Not Significant	Not Significant
	21d	Not Significant	Not Significant	Not Significant
	28d	Not Significant	Not Significant	Not Significant
Maximum Temperature	24h	Not Significant	Not Significant	Not Significant
	7d	Not Significant	Not Significant	Not Significant
	14d	Not Significant	Not Significant	Not Significant
	21d	Not Significant	Not Significant	Not Significant
	28d	Not Significant	Not Significant	Not Significant
Rainfall	24h	$r_s=0.464, P=0.030$	Not Significant	Not Significant
	7d	Not Significant	Not Significant	Not Significant
	14d	Not Significant	Not Significant	$r_s=-0.651, P=0.041$
	21d	$r_s=0.467, P=0.029$	Not Significant	Not Significant
	28d	Not Significant	Not Significant	Not Significant
Minimum Relative Humidity	24h	Not Significant	Not Significant	Not Significant
	7d	Not Significant	Not Significant	Not Significant
	14d	Not Significant	Not Significant	Not Significant
	21d	Not Significant	Not Significant	Not Significant
	28d	Not Significant	Not Significant	$r_s=-0.665, P=0.036$
Maximum Relative Humidity	24h	Not Significant	Not Significant	Not Significant
	7d	Not Significant	Not Significant	Not Significant
	14d	Not Significant	Not Significant	Not Significant
	21d	Not Significant	Not Significant	Not Significant
	28d	Not Significant	Not Significant	Not Significant

Of the three frog species studied at Murray Upper NP, *L. rheocola* (n=93) showed the greatest variation with climatic conditions. Frog numbers were positively correlated with air temperatures, rainfall and relative humidity. The prevalence of Bd was negatively correlated with minimum air temperatures. However, intensity of infection appeared independent of climatic conditions.

Table 2.3. Climatic factors correlated with density of *Litoria rheocola*, prevalence of Bd and intensity of infection (as determined by qPCR) at Murray Upper NP, as determined by Spearman's rank correlation (see also Figures 2.7-2.9).

Climatic factor	Period prior to survey	# Frogs	Prevalence *Bd*	Intensity of infection (#ZSE)
Mean Temperature	24h	r_s=0.515, *P*=0.014	r_s=-0.539, *P*=0.031	Not Significant
	7d	r_s=0.546, *P*=0.009	r_s=-0.582, *P*=0.018	Not Significant
	14d	r_s=0.621, *P*=0.002	r_s=-0.628, *P*=0.009	Not Significant
	21d	r_s=0.613, *P*=0.002	r_s=-0.731, *P*=0.001	Not Significant
	28d	r_s=0.640, *P*=0.001	r_s=-0.727, *P*=0.001	Not Significant
Minimum Temperature	24h	r_s=0.591, *P*=0.004	r_s=-0.670, *P*=0.004	Not Significant
	7d	r_s=0.593, *P*=0.004	r_s=-0.768, *P*=0.001	Not Significant
	14d	r_s=0.654, *P*=0.001	r_s=-0.805, *P*=0.000	Not Significant
	21d	r_s=0.660, *P*=0.001	r_s=-0.829, *P*=0.000	Not Significant
	28d	r_s=0.699, *P*=0.000	r_s=-0.736, *P*=0.001	Not Significant
Maximum Temperature	24h	Not Significant	Not Significant	Not Significant
	7d	Not Significant	Not Significant	Not Significant
	14d	r_s=0.462, *P*=0.030	Not Significant	Not Significant
	21d	Not Significant	r_s=-0.585, *P*=0.017	Not Significant
	28d	r_s=0.497, *P*=0.019	r_s=-0.497, *P*=0.050	Not Significant
Rainfall	24h	r_s=0.458, *P*=0.032	Not Significant	Not Significant
	7d	r_s=0.477, *P*=0.025	Not Significant	Not Significant
	14d	r_s=0.492, *P*=0.020	Not Significant	Not Significant
	21d	r_s=0.612, *P*=0.002	Not Significant	Not Significant
	28d	r_s=0.679, *P*=0.001	Not Significant	Not Significant
Minimum Relative Humidity	24h	r_s=0.515, *P*=0.014	Not Significant	Not Significant
	7d	r_s=0.471, *P*=0.027	Not Significant	Not Significant
	14d	r_s=0.451, *P*=0.035	Not Significant	Not Significant
	21d	Not Significant	Not Significant	Not Significant
	28d	Not Significant	Not Significant	Not Significant
Maximum Relative Humidity	24h	Not Significant	Not Significant	Not Significant
	7d	Not Significant	Not Significant	Not Significant
	14d	Not Significant	Not Significant	Not Significant
	21d	r_s=0.533, *P*=0.011	Not Significant	Not Significant
	28d	r_s=0.566, *P*=0.006	Not Significant	Not Significant

Figure 2.7. Variation in number of frogs at Murray Upper NP with climatic conditionMean Air Temperature
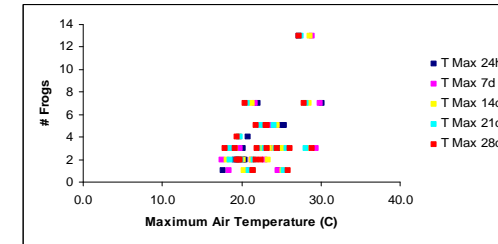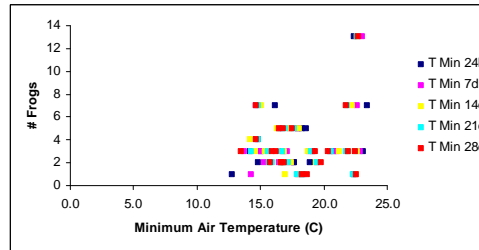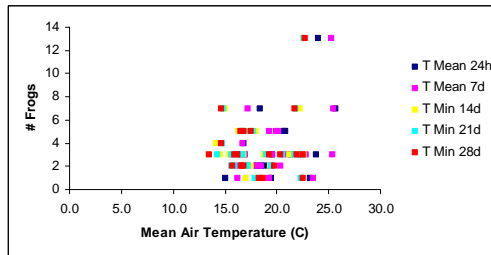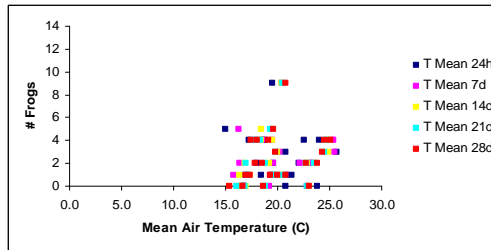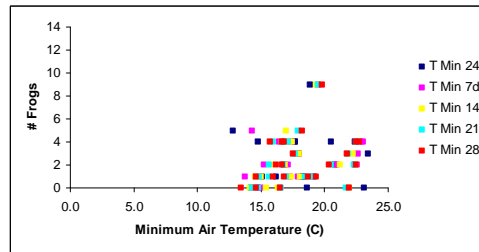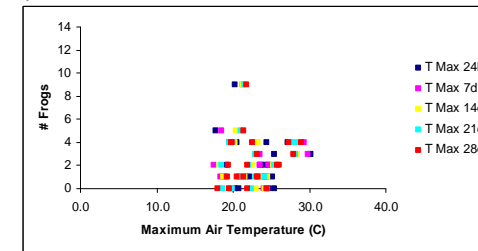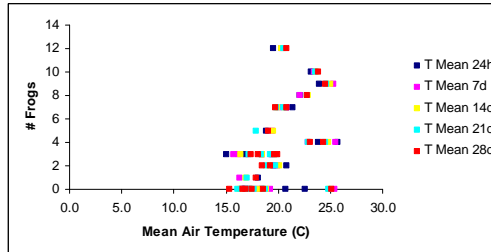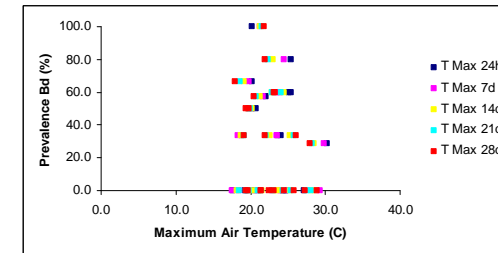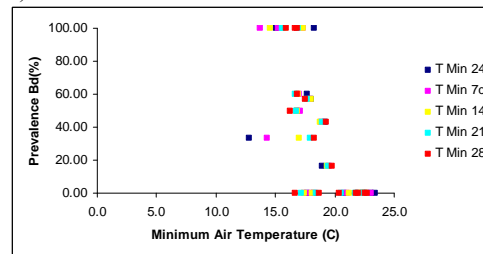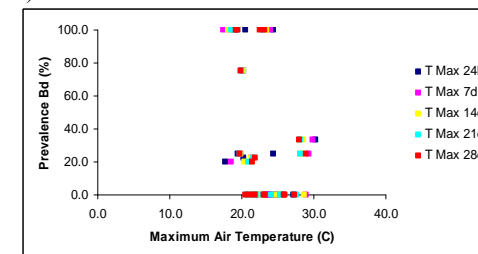
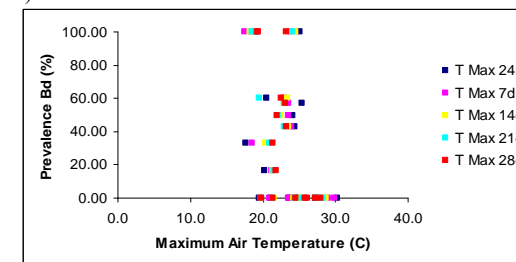a) *L. genimaculata*

d) *L. nannotis*

g) *L. rheocola*

e) *L. nannotis*

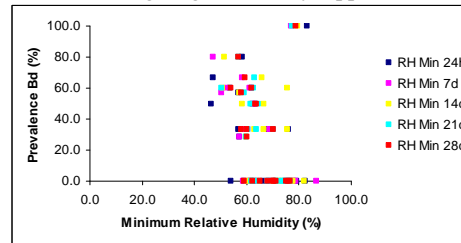h) *L. rheocola*
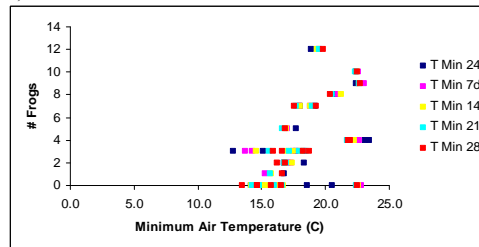
Maximum Temperature

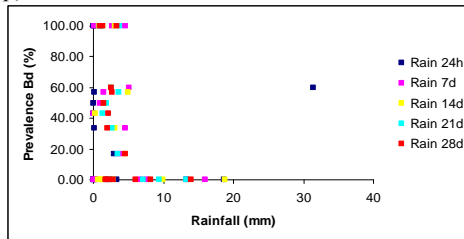c) *L. genimaculata*

f) *L. nannotis*

i) *L. rheocola*
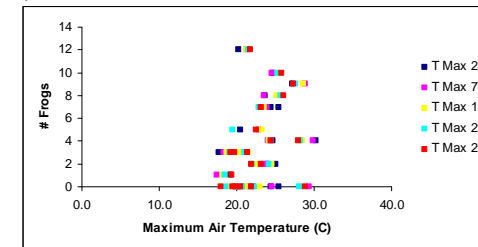
Minimum Air Temperature

b) *L. genimaculata*

Figure 2.7 cont.. Variation in number of frogs at Murray Upper NP with climatic conditions
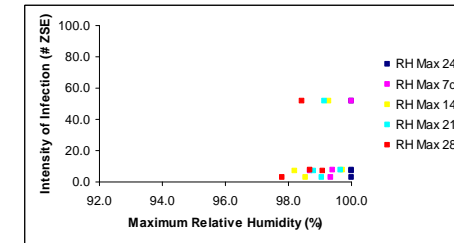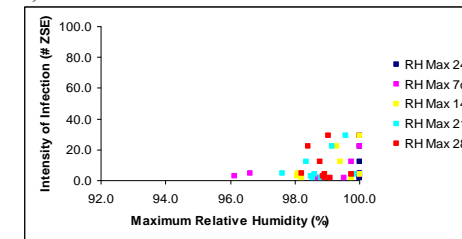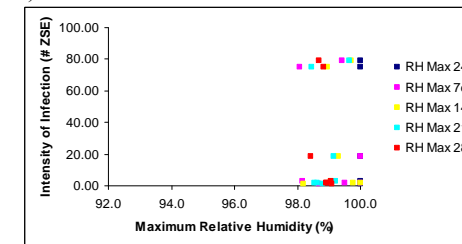Rainfall
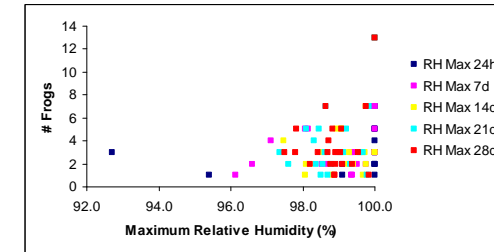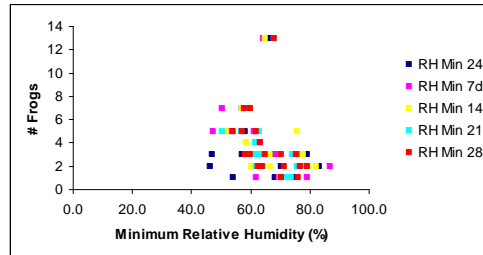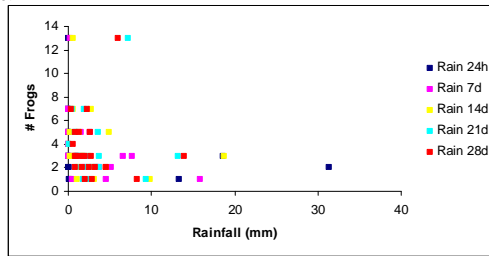
j) *L. genimaculata*
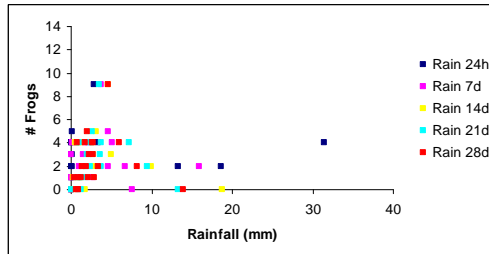
m) *L. nannotis*
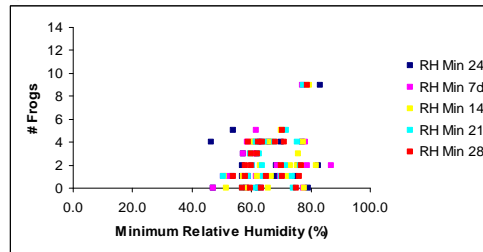
p) *L. rheocola*

n) *L. nannotis*

q) *L. rheocola*

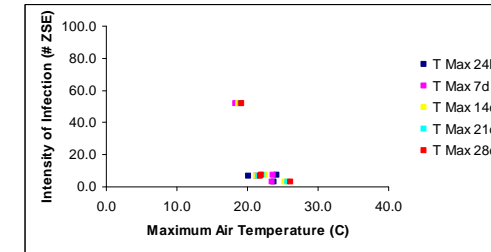o) *L. nannotis*
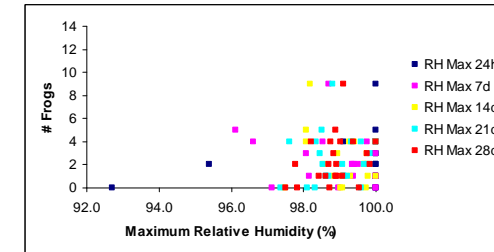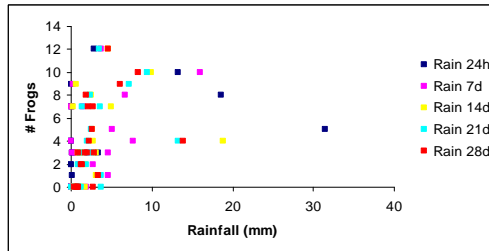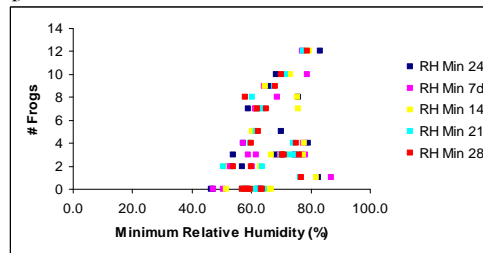
r) *L. rheocola*
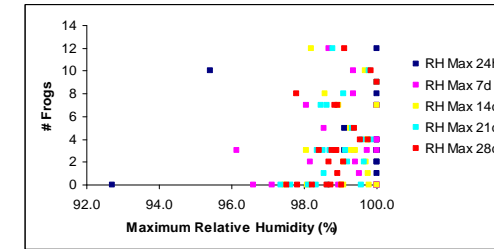
Maximum Relative Humidity

l) *L. genimaculata*

Minimum Relative Humidity

k) *L. genimaculata*

Figure 2.8. Variation in prevalence of *Batrachochytrium dendrobatidis* among frogs at Murray Upper NP with climatic conditions
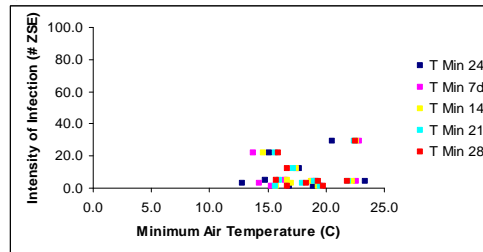
Mean Air Temperature
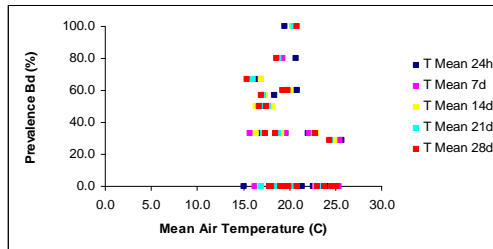
a) *L. genimaculata*
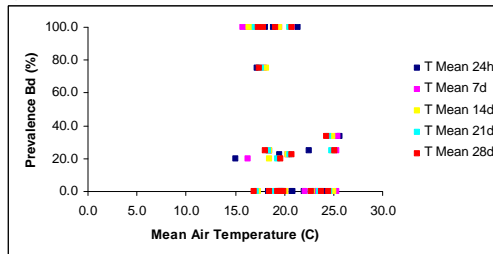
d) *L. nannotis*
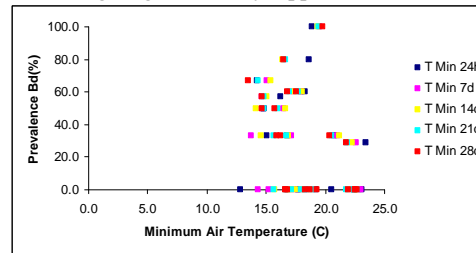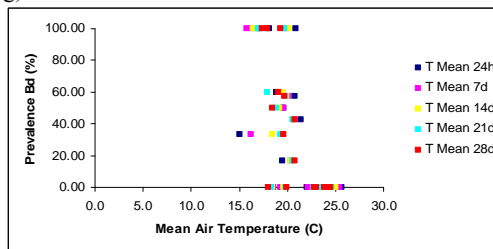
g) *L. rheocola*
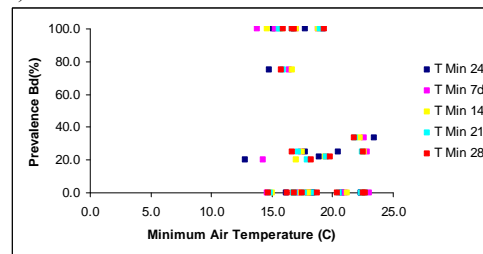
e) *L. nannotis*

h) *L. rheocola*

f) *L. nannotis*

i) *L. rheocola*

Maximum Temperature

c) *L. genimaculata*

Minimum Air Temperature

b) *L. genimaculata*

Figure 2.8 cont. Variation in prevalence of *Batrachochytrium dendrobatidis* among frogs at Murray Upper NP with climatic conditions

Rainfall
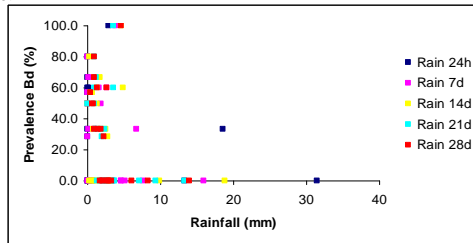
j) *L. genimaculata*
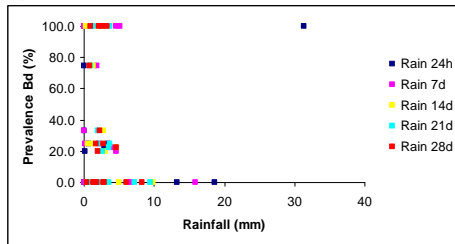
m) *L. nannotis*

p) *L. rheocola*
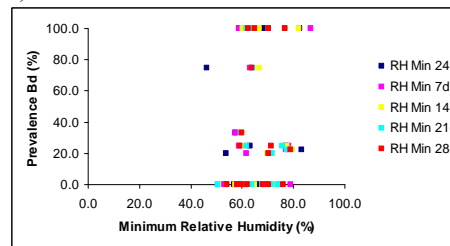
n) *L. nannotis*
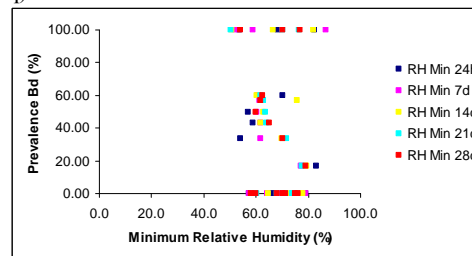
q) *L. rheocola*

o) *L. nannotis*

r) *L. rheocola*

Maximum Relative Humidity

l) *L. genimaculata*

Minimum Relative Humidity

k) *L. genimaculata*

Figure 2.9. Variation in intensity of infection (number of zoospore equivalents) among frogs at Murray Upper NP with climatic conditions
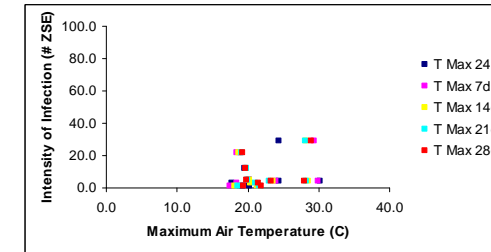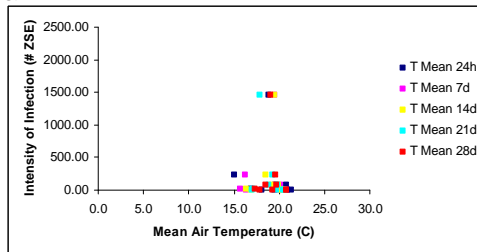
Mean Air Temperature
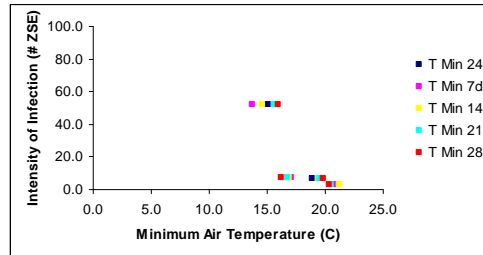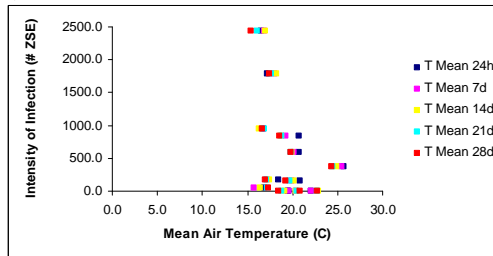
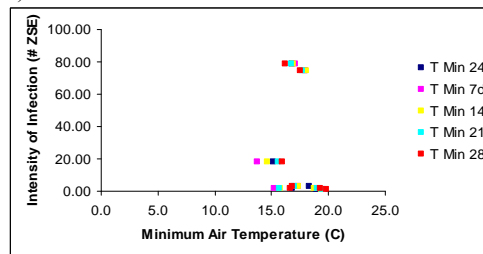a) *L. genimaculata*
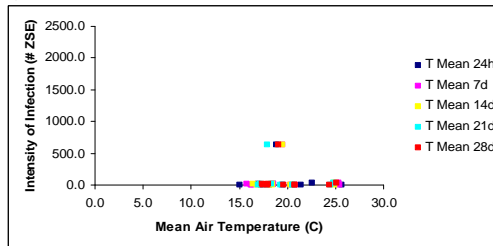
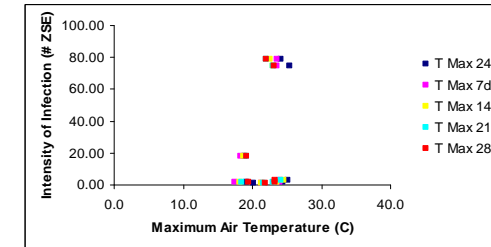d) *L. nannotis*

g) *L. rheocola*

e) *L. nannotis*

h) *L. rheocola*

f) *L. nannotis*

i) *L. rheocola*
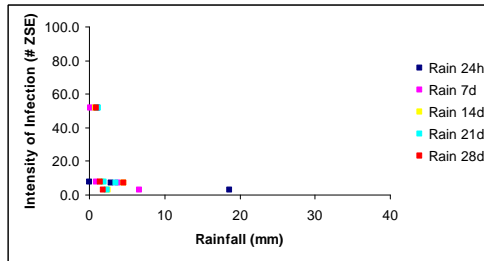
Maximum Temperature

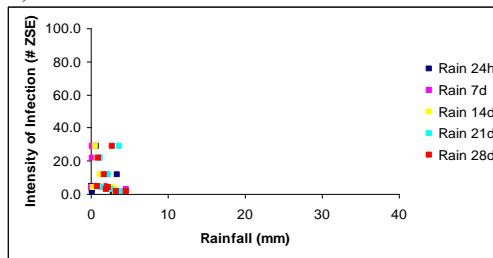c) *L. genimaculata*

Minimum Air Temperature

b) *L. genimaculata*

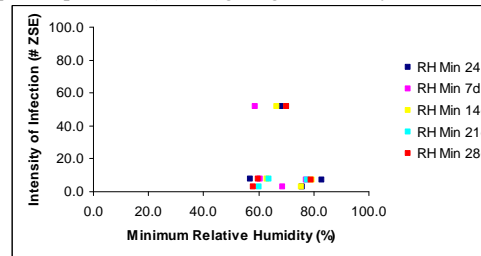Figure 2.9 cont. Variation in intensity of infection (number of zoospore equivalents) among frogs at Murray Upper NP with climatic conditions

Rainfall
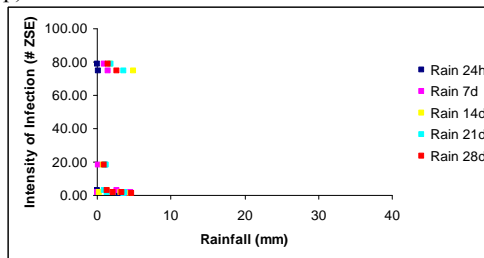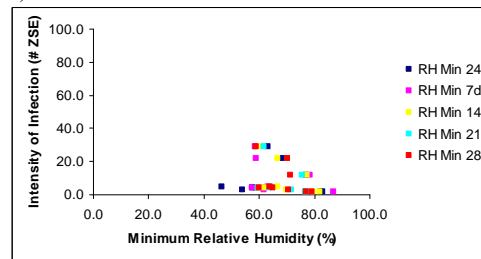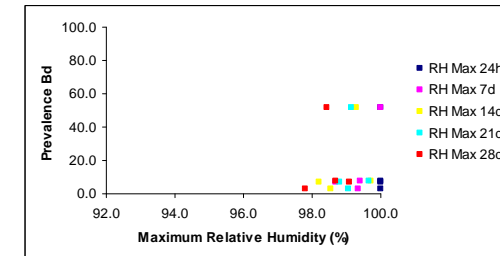
j) *L. genimaculata*

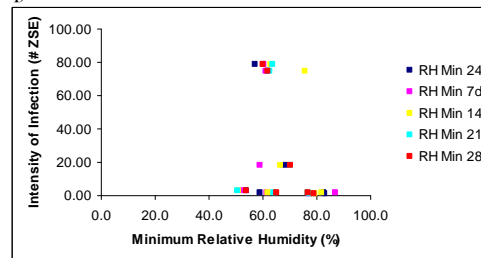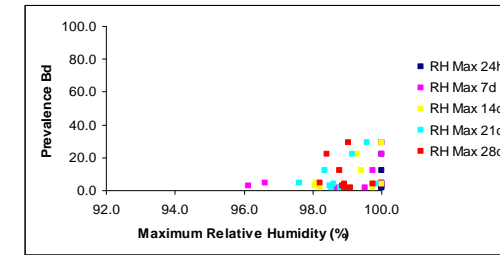m) *L. nannotis*

p) *L. rheocola*

n) *L. nannotis*

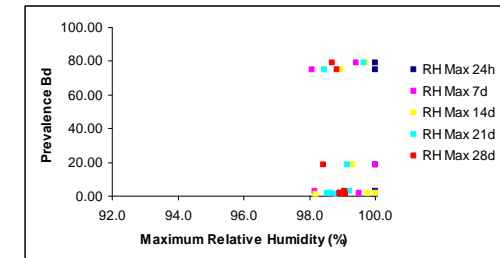q) *L. rheocola*

o) *L. nannotis*

r) *L. rheocola*

Maximum Relative Humidity

l) *L. genimaculata*

Minimum Relative Humidity

k) *L. genimaculata*

Climatic Conditions at Tully Gorge NP

Malfunctioning data loggers precluded the collection of temperature data between July and November 2007. Air temperature at Tully Gorge NP (see Figure 2.10 and 2.11) followed the typical pattern of the austral summer, with mean, minimum and maximum temperatures increasing in September and declining after March. During this time, the maximum daily temperature was often above the upper thermal tolerance limit of *Bd*. In the cooler winter, mean and maximum temperatures were within the thermal range for greatest growth and virulence. However, minimum temperatures occasionally dropped below this range. The lower thermal tolerance limit for Bd (4°C-Piotrowski et al. 2004) was never reached.

Figure 2.10. Daily mean, minimum and maximum air temperature at Murray Upper NP. Dashed lines represent range of greatest virulence and pathogenicity of *Batrachochytrium dendrobatidis*.
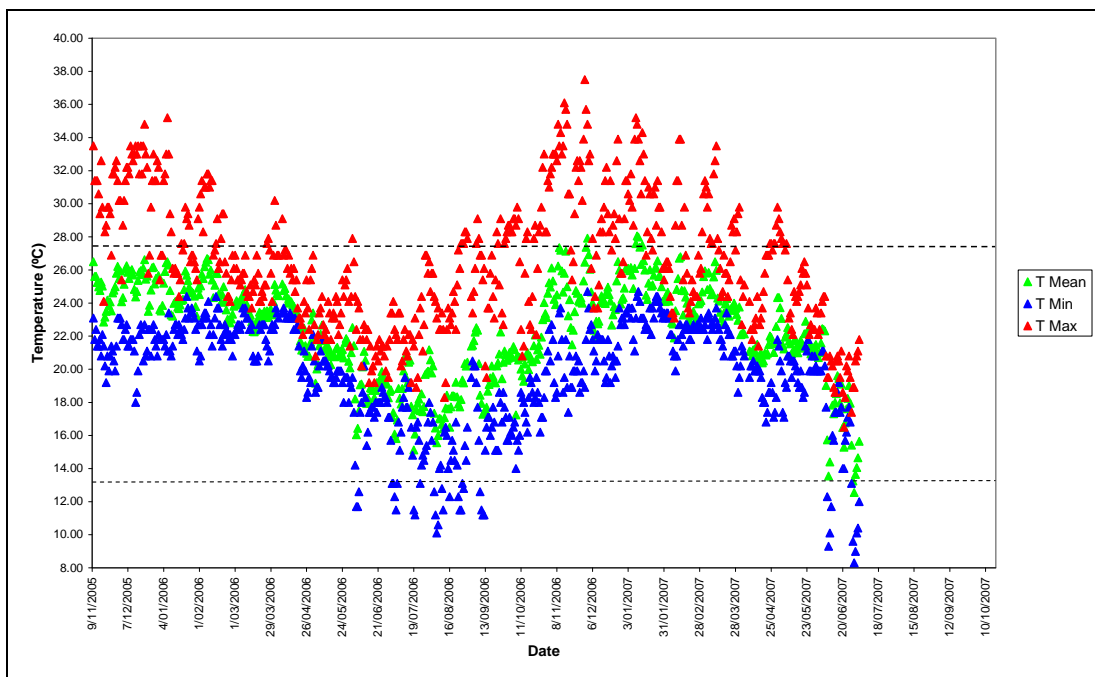
Greatest rainfall occurred during the austral summer, but some winter spring rain was recorded (see Figures 2.12-2.13). Of note is the rain resulting from the close path of Category 5 Cyclone Larry in April 2006. Maximum relative humidity on any day ranged between 80-100%, (see Figures 2.14-2.15) with more fluctuation in the maximum relative humidity than expected for a dense rainforest environment. Minimum relative humidity was at its lowest in the cooler and drier winter months (see Figures 2.14-2.15).

Figure 2.11 Mean, minimum and maximum temperatures at 24h, 7d, 14d, 21d and 28d before each survey for frogs at Tully Gorge NP.

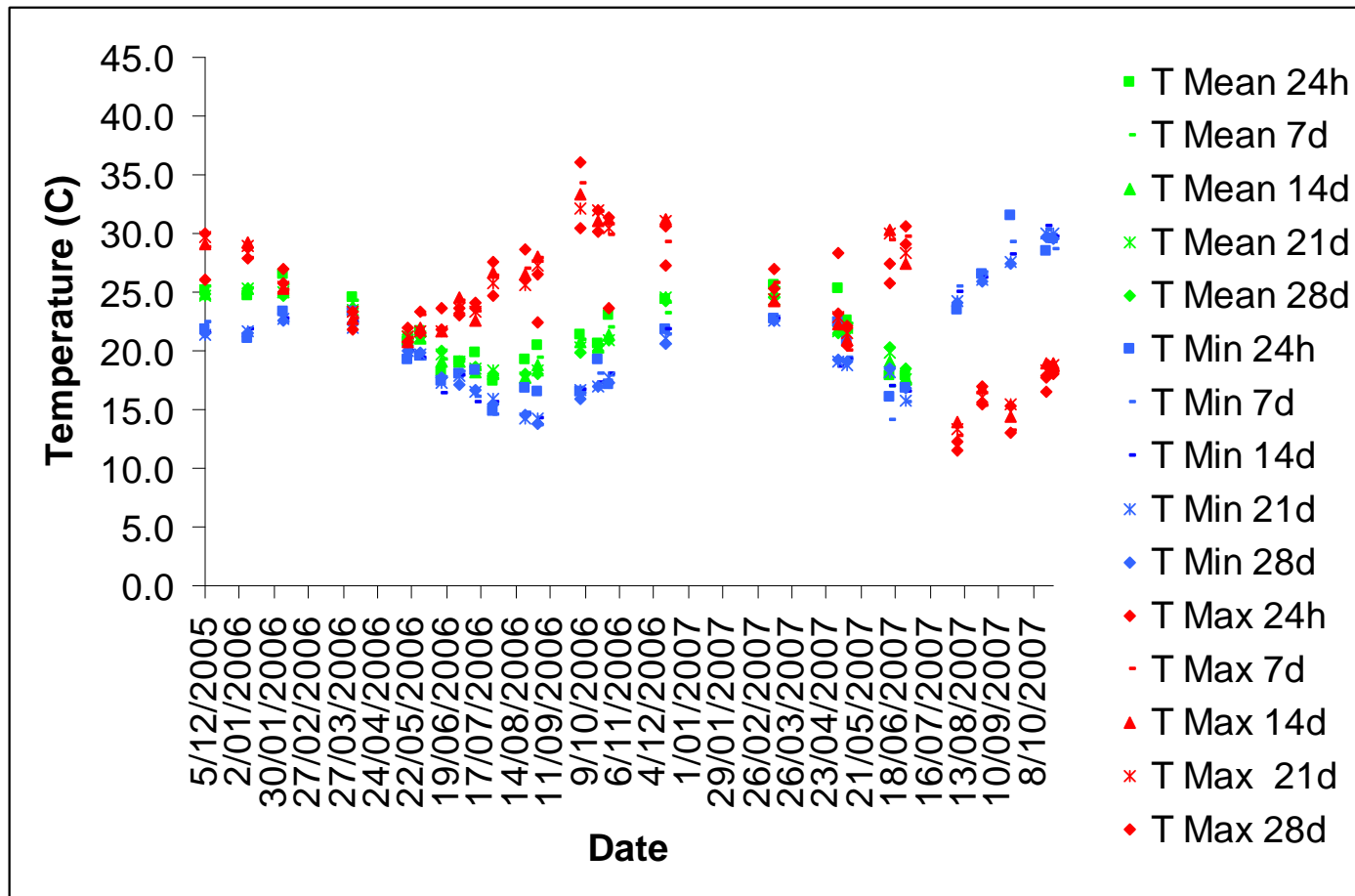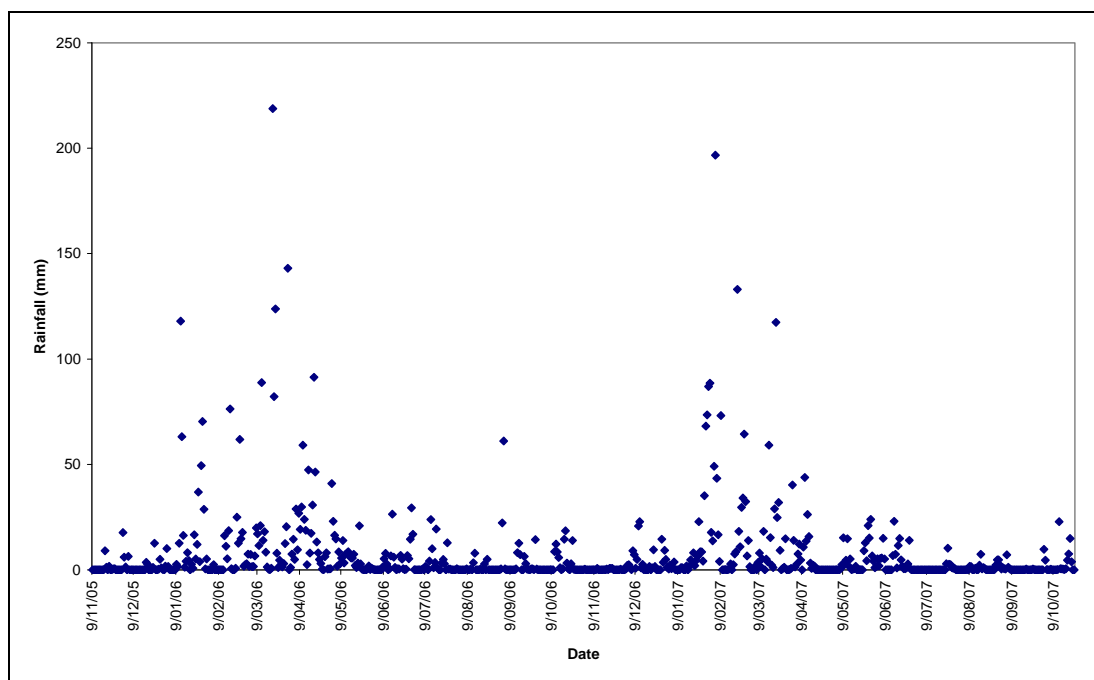Figure 2.12. Temporal variation in daily rainfall at Tully Gorge NP.

Figure 2.13 Rainfall for 24h, 7d, 14d, 21d and 28d before each survey for frogs at Tully Gorge NP.
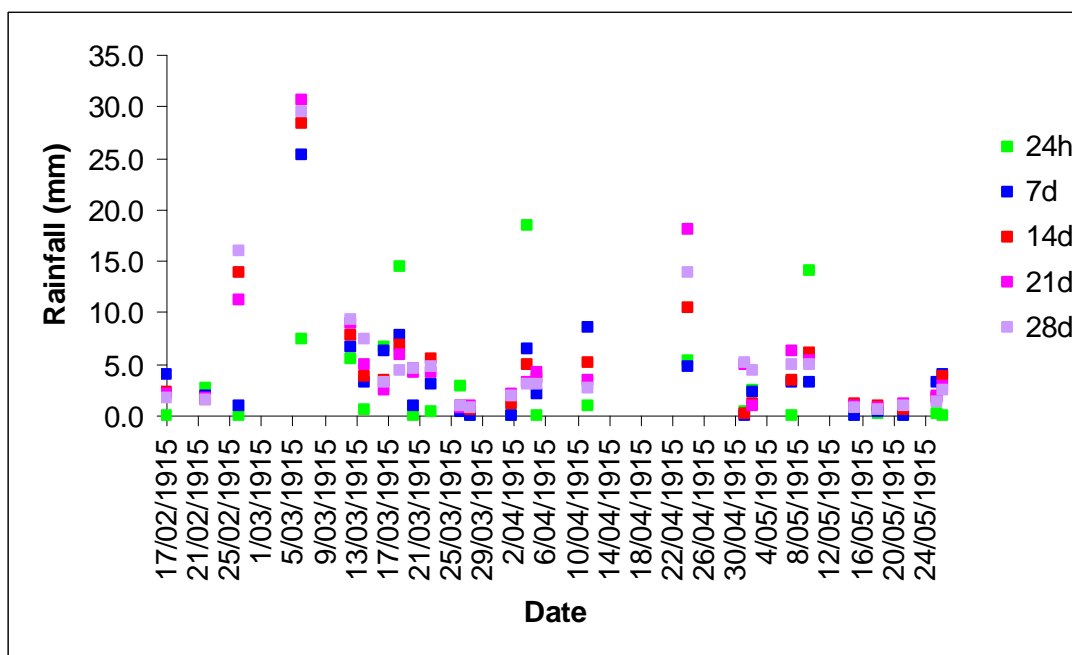
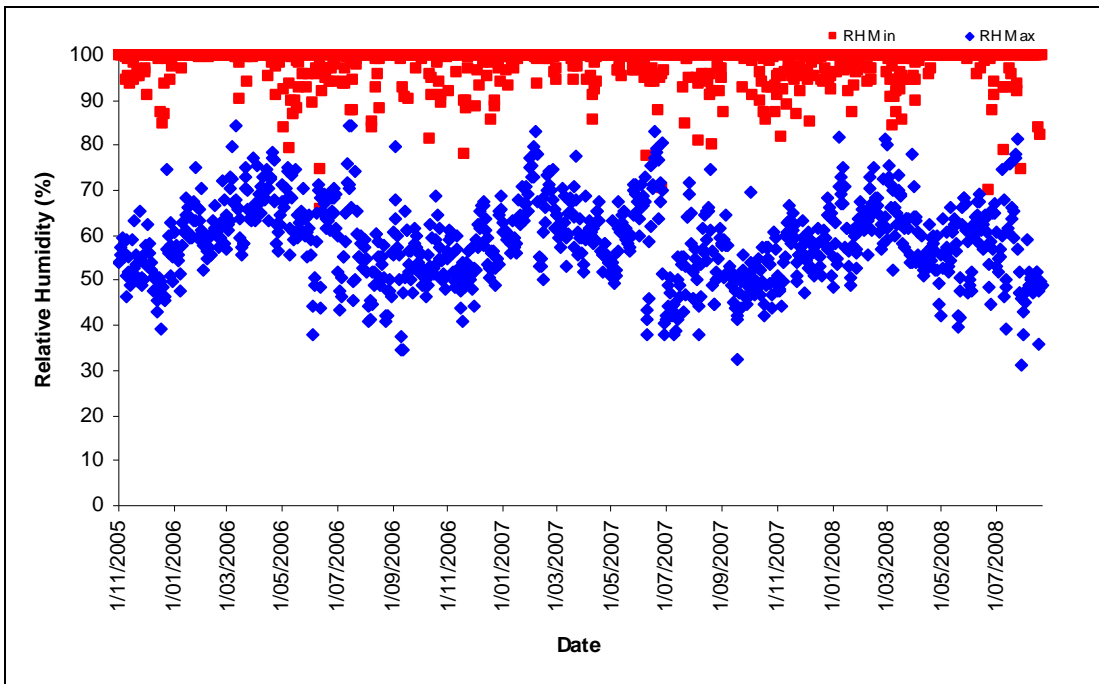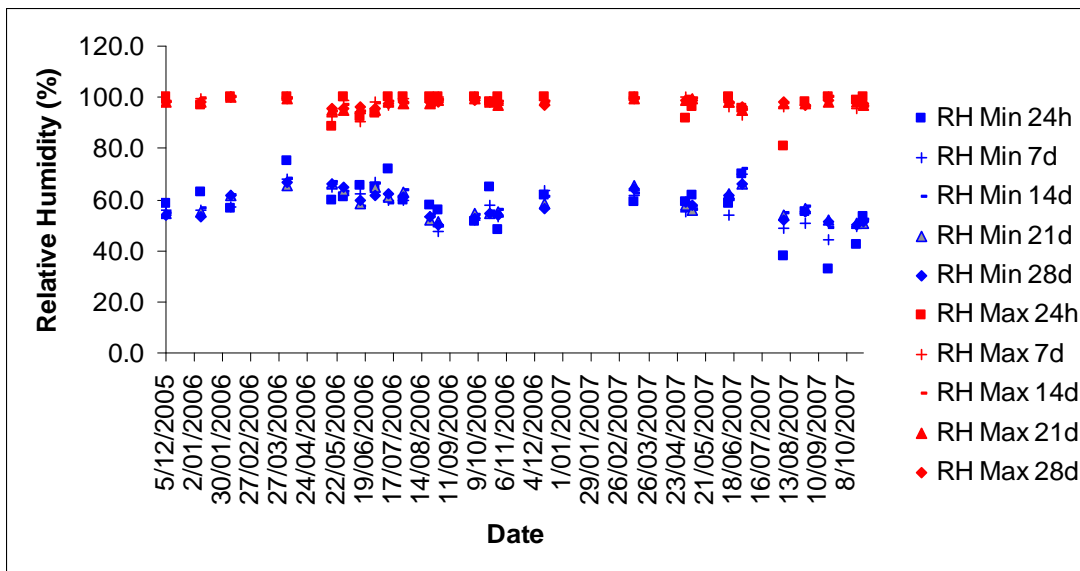Figure 2.14. Temporal variation in relative humidity (minimum and maximum) at Tully Gorge NP.

Figure 2.15. Relative humidity (minimum and maximum) for 24h, 7d, 14d, 21d and 28d before each survey for frogs at Tully Gorge NP.

Due to the low numbers of *L. genimaculata* (n=14) marked and recaptured at Tully Gorge NP, insufficient data exists to determine the climatic conditions favouring Bd infection in this species.

Numbers of *L. nannotis* (n=143) at Tully Gorge NP were positively correlated with mean air temperature between 24hr and 7 days prior. Numbers were also positively correlated with minimum relative humidity in the previous 14-21days. Prevalence of Bd was negatively correlated with mean and minimum air temperatures, recent minimum relative humidity and longer term maximum relative humidity. Intensity of infection was not correlated with any of the climatic conditions under investigation.

Table 2.4. Climatic factors correlated with density of *Litoria nannotis*, prevalence of Bd and intensity of infection (as determined by qPCR) at Tully Gorge NP, as determined by Spearman's rank correlation.

Climatic factor	Period prior to survey	# Frogs	Prevalence *Bd*	Intensity of infection (#ZSE)
Mean Temperature	24h	r_s=0.476, *P*=0.029	r_s=-0.479, *P*=0.028	Not Significant
	7d	r_s=0.518, *P*=0.016	r_s=-0.433, *P*=0.050	Not Significant
	14d	r_s=0.546, *P*=0.010	Not Significant	Not Significant
	21d	r_s=0.495, *P*=0.022	Not Significant	Not Significant
	28d	r_s=0.481, *P*=0.027	Not Significant	Not Significant
Minimum Temperature	24h	Not Significant	Not Significant	Not Significant
	7d	Not Significant	r_s=-0.395, *P*=0.046	Not Significant
	14d	Not Significant	r_s=-0.405, *P*=0.040	Not Significant
	21d	Not Significant	r_s=-0.409, *P*=0.038	Not Significant
	28d	Not Significant	Not Significant	Not Significant
Maximum Temperature	24h	Not Significant	Not Significant	Not Significant
	7d	Not Significant	Not Significant	Not Significant
	14d	Not Significant	Not Significant	Not Significant
	21d	Not Significant	Not Significant	Not Significant
	28d	Not Significant	Not Significant	Not Significant
Rainfall	24h	Not Significant	Not Significant	Not Significant
	7d	Not Significant	Not Significant	Not Significant
	14d	r_s=0.417, *P*=0.034	Not Significant	Not Significant
	21d	Not Significant	Not Significant	Not Significant
	28d	Not Significant	Not Significant	Not Significant
Minimum Relative Humidity	24h	Not Significant	r_s=0.432, *P*=0.028	Not Significant
	7d	Not Significant	Not Significant	Not Significant
	14d	r_s=0.453, *P*=0.020	Not Significant	Not Significant
	21d	r_s=0.411, *P*=0.037	Not Significant	Not Significant
	28d	Not Significant	Not Significant	Not Significant
Maximum Relative Humidity	24h	Not Significant	Not Significant	Not Significant
	7d	Not Significant	r_s=-0.426, *P*=0.030	Not Significant
	14d	Not Significant	r_s=-0.399, *P*=0.043	Not Significant
	21d	Not Significant	Not Significant	Not Significant
	28d	Not Significant	Not Significant	Not Significant

Numbers of *L. rheocola* (n=345) were independent of climatic conditions. Prevalence of Bd was negatively correlated with mean and minimum air temperatures and maximum relatively humidity, but positively correlated with minimum relative humidity. Intensity of infection varied inversely with mean air temperature, rainfall and short-term maximum relative humidity.

Table 2.5. Climatic factors correlated with density of *Litoria rheocola*, prevalence of Bd and intensity of infection (as determined by qPCR) at Tully Gorge NP, as determined by Spearman's rank correlation.

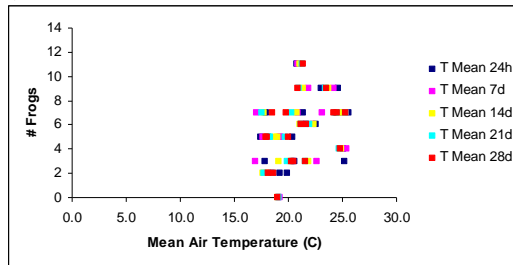| Climatic factor	Period prior to survey	# Frogs	Prevalence *Bd*	Intensity of infection (#ZSE)
Mean Temperature	24h	Not Significant	r_s=-0.573, *P*=0.010	r_s=-0.507, *P*=0.038
	7d	Not Significant	r_s=-0.496, *P*=0.031	r_s=-0.567, *P*=0.018
	14d	Not Significant	r_s=-0.464, *P*=0.045	r_s=-0.500, *P*=0.041
	21d	Not Significant	Not Significant	r_s=-0.539, *P*=0.026
	28d	Not Significant	Not Significant	r_s=-0.515, *P*=0.034
Minimum Temperature	24h	Not Significant	r_s=-0.427, *P*=0.037	Not Significant
	7d	Not Significant	r_s=-0.413, *P*=0.045	Not Significant
	14d	Not Significant	r_s=-0.432, *P*=0.035	Not Significant
	21d	Not Significant	r_s=-0.442, *P*=0.031	Not Significant
	28d	Not Significant	r_s=-0.411, *P*=0.046	Not Significant
Maximum Temperature	24h	Not Significant	Not Significant	Not Significant
	7d	Not Significant	Not Significant	Not Significant
	14d	Not Significant	Not Significant	Not Significant
	21d	Not Significant	Not Significant	Not Significant
	28d	Not Significant	Not Significant	Not Significant
Rainfall	24h	Not Significant	Not Significant	Not Significant
	7d	Not Significant	Not Significant	r_s=-0.626, *P*=0.002
	14d	Not Significant	Not Significant	r_s=-0.475, *P*=0.026
	21d	Not Significant	Not Significant	Not Significant
	28d	Not Significant	Not Significant	Not Significant
Minimum Relative Humidity	24h	Not Significant	r_s=0.559, *P*=0.005	Not Significant
	7d	Not Significant	r_s=0.475, *P*=0.019	Not Significant
	14d	Not Significant	r_s=0.470, *P*=0.021	Not Significant
	21d	Not Significant	Not Significant	Not Significant
	28d	Not Significant	r_s=0.408, *P*=0.048	Not Significant
Maximum Relative Humidity	24h	Not Significant	Not Significant	r_s=-0.472, *P*=0.027
	7d	Not Significant	r_s=-0.444, *P*=0.030	Not Significant
	14d	Not Significant	r_s=-0.426, *P*=0.038	Not Significant
	21d	Not Significant	Not Significant	Not Significant
	28d	Not Significant	r_s=-0.482, *P*=0.017	Not Significant

N. dayi (n=274) numbers positively correlated with mean air temperature, and longer term rainfall and maximum relative humidity. Prevalence of Bd showed a strong negative correlation with mean and minimum air temperature. Maximum relative humidity also negatively affected prevalence and intensity of infection.

Table 2.6. Climatic factors correlated with density of *Nyctimystes dayi*, prevalence of Bd and intensity of infection (as determined by qPCR) at Tully Gorge NP, as determined by Spearman's rank correlation.
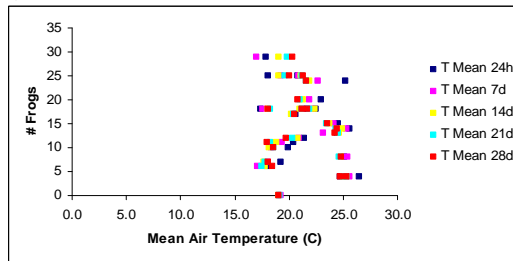
Climatic factor	Period prior to survey	# Frogs	Prevalence *Bd*	Intensity of infection (#ZSE)
Mean Temperature	24h	r_s=0.865, *P*=0.000	r_s=-0.807, *P*=0.000	Not Significant
	7d	r_s=0.817, *P*=0.000	r_s=-0.758, *P*=0.000	Not Significant
	14d	r_s=0.807, *P*=0.000	r_s=-0.785, *P*=0.000	Not Significant
	21d	r_s=0.797, *P*=0.000	r_s=-0.751, *P*=0.000	Not Significant
	28d	r_s=0.798, *P*=0.000	r_s=-0.708, *P*=0.000	Not Significant
Minimum Temperature	24h	Not Significant	r_s=-0.500, *P*=0.011	Not Significant
	7d	Not Significant	r_s=-0.522, *P*=0.005	Not Significant
	14d	Not Significant	r_s=-0.523, *P*=0.007	Not Significant
	21d	Not Significant	r_s=-0.479, *P*=0.015	Not Significant
	28d	Not Significant	r_s=-0.411, *P*=0.046	Not Significant
Maximum Temperature	24h	Not Significant	Not Significant	Not Significant
	7d	Not Significant	Not Significant	Not Significant
	14d	Not Significant	Not Significant	Not Significant
	21d	Not Significant	Not Significant	Not Significant
	28d	r_s=0.407, *P*=0.039	Not Significant	Not Significant
Rainfall	24h	Not Significant	Not Significant	Not Significant
	7d	Not Significant	Not Significant	Not Significant
	14d	Not Significant	Not Significant	Not Significant
	21d	Not Significant	Not Significant	Not Significant
	28d	Not Significant	Not Significant	Not Significant
Minimum Relative Humidity	24h	Not Significant	Not Significant	Not Significant
	7d	Not Significant	Not Significant	Not Significant
	14d	Not Significant	Not Significant	Not Significant
	21d	Not Significant	Not Significant	Not Significant
	28d	Not Significant	Not Significant	Not Significant
Maximum Relative Humidity	24h	Not Significant	Not Significant	Not Significant
	7d	r_s=0.390, *P*=0.049	r_s=-0.409, *P*=0.042	r_s=-0.457, *P*=0.037
	14d	r_s=0.477, *P*=0.014	r_s=-0.553, *P*=0.004	r_s=-0.519, *P*=0.016
	21d	r_s=0.511, *P*=0.008	r_s=-0.478, *P*=0.016	r_s=-0.644, *P*=0.002
	28d	r_s=0.429, *P*=0.029	r_s=-0.511, *P*=0.009	r_s=-0.526, *P*=0.014

Figure 2.16. Variation in number of frogs at Tully Gorge NP with climatic conditions

Mean Air Temperature

a) *L. nannotis*

d) *L. rheocola*

g) *N. dayi*
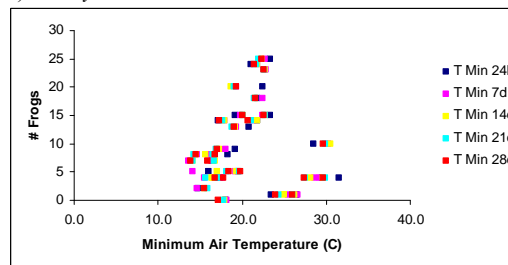
e) *L. rheocola*

h) *N. dayi*

Maximum Temperature

c) *L. nannotis*

f) *L.rheocola*

i) *N. dayi*

Minimum Air Temperature

b) *L.nannotis*

Figure 2.16 cont.. Variation in number of frogs at Tully Gorge NP with climatic conditions
Rainfall
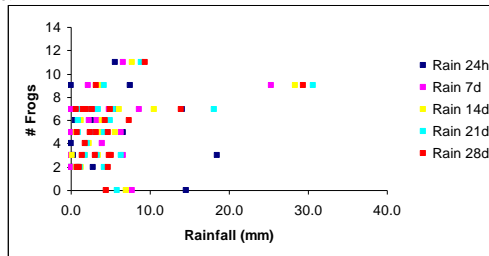
j) *L.nannotis*
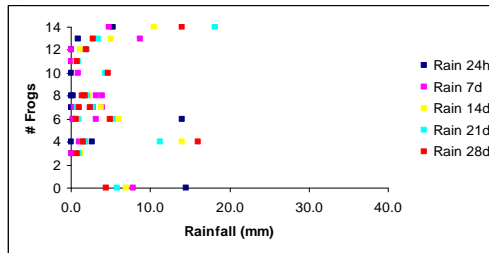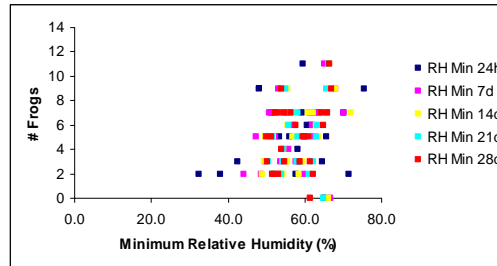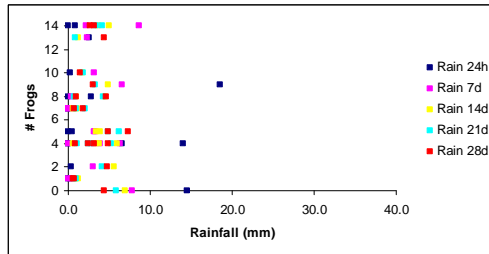
m) *L. rheocola*

p) *N. dayi*

n) *L. rheocola*
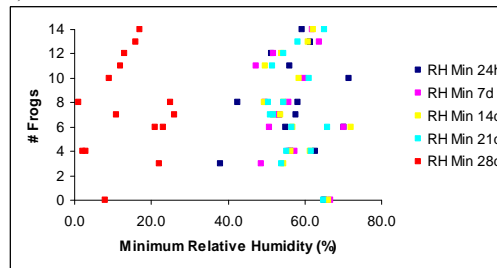
q) *N. dayi*
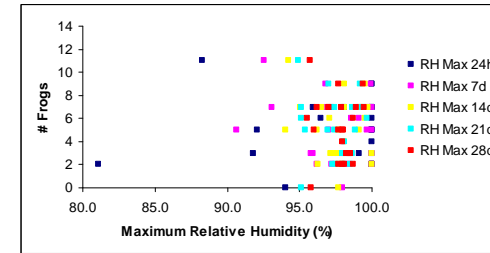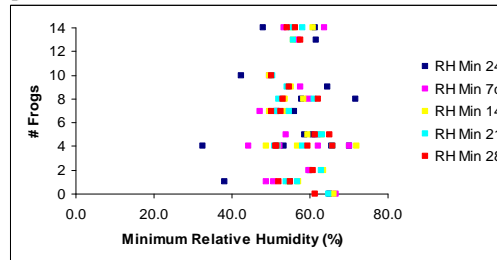
o) *L. rheocola*
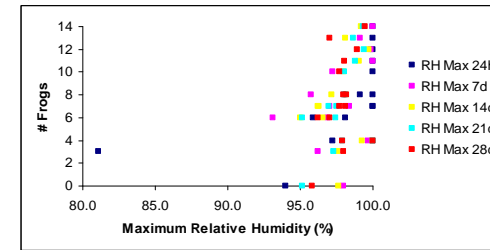
r) *N. dayi*
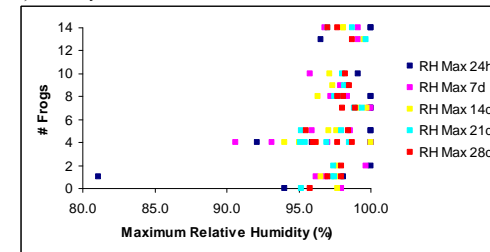
Maximum Relative Humidity

l) *L. nannotis*

Minimum Relative Humidity

k) *L. nannotis*

Figure 2.17. Variation in prevalence of *Batrachochytrium dendrobatidis* among frogs at Tully Gorge NP with climatic conditions

Mean Air Temperature
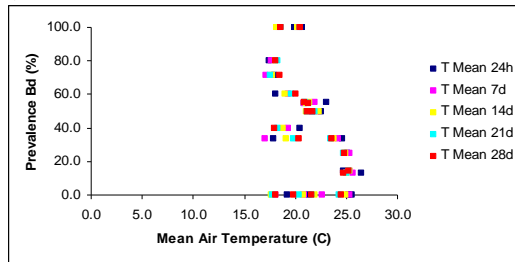
a) *L. nannotis*
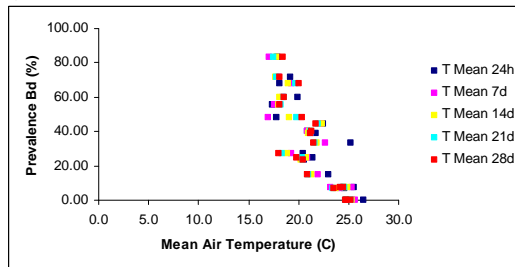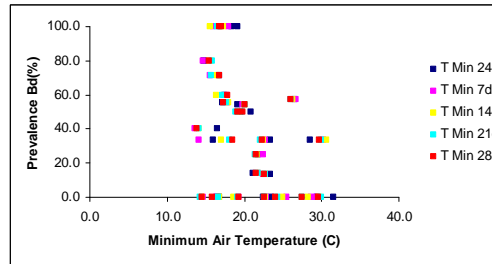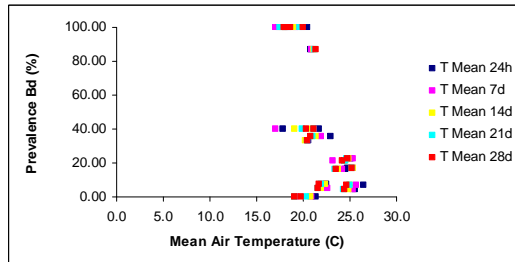
d) *L. rheocola*
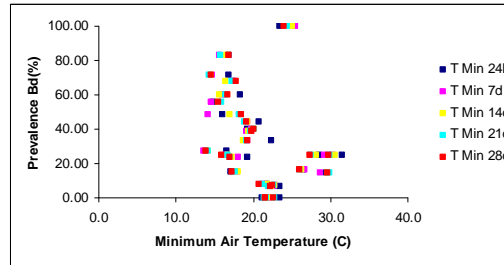
g) *N. dayi*
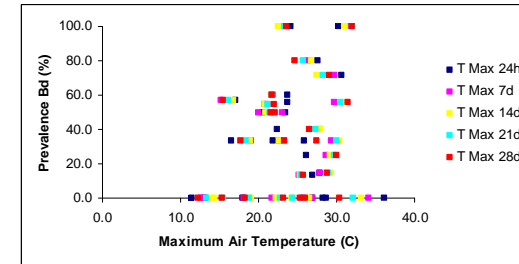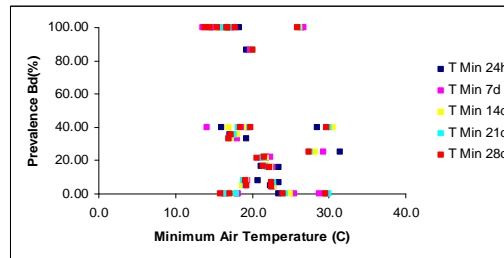
e) *L. rheocola*

h) *N. dayi*

f) *L. rheocola*
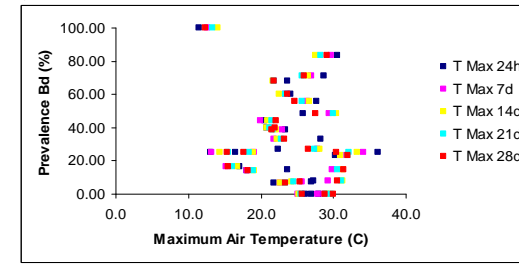
i) *N. dayi*
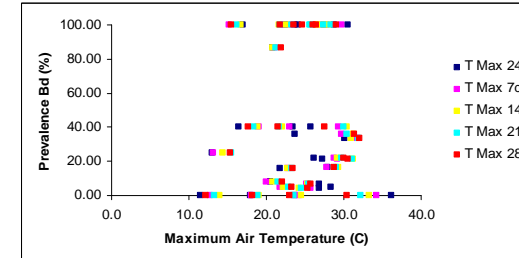
Maximum Temperature

c) *L. nannotis*

Minimum Air Temperature

b) *L nannotis*

Figure 2.17 cont. Variation in prevalence of *Batrachochytrium dendrobatidis* among frogs at Tully Gorge NP with climatic conditions

Rainfall
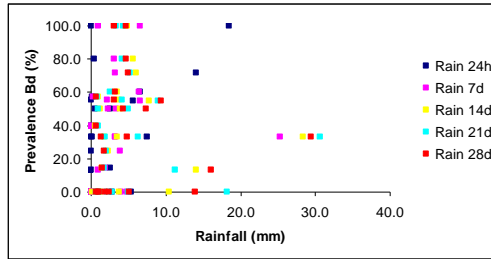
j) *L nannotis*
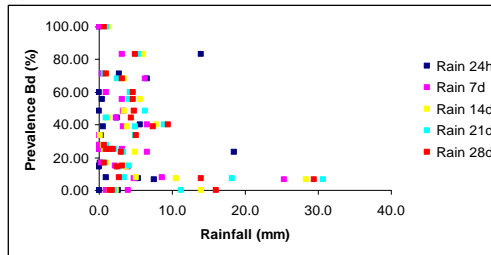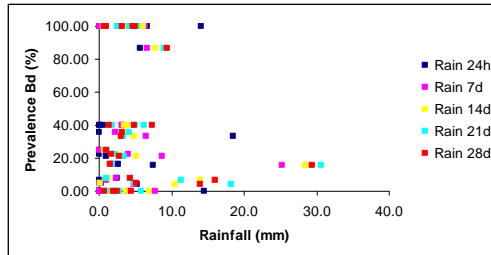
m) *L rheocola*

p) *N. dayi*

k) *L. nannotis*

n) *L. rheocola*
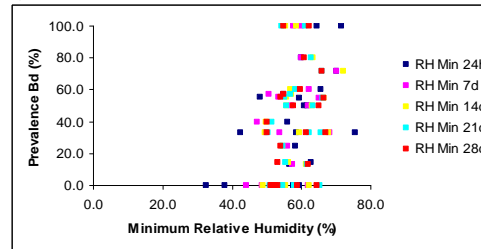
q) *N. dayi*

Maximum Relative Humidity
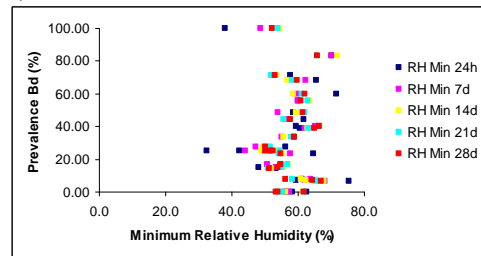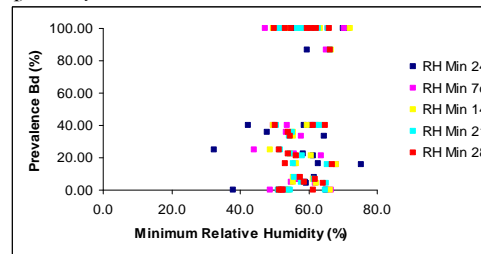
l) *L. nannotis*

o) *L. rheocola*

r) *N. dayi*

Minimum Relative Humidity

Figure 2.18. Variation in intensity of infection (number of zoospore equivalents) among frogs at Tully Gorge NP with climatic conditions
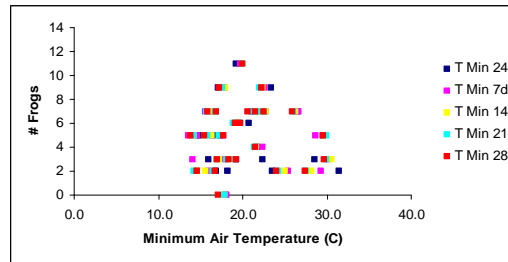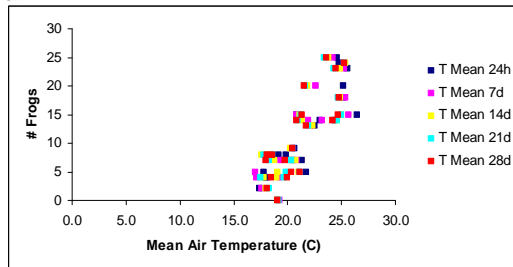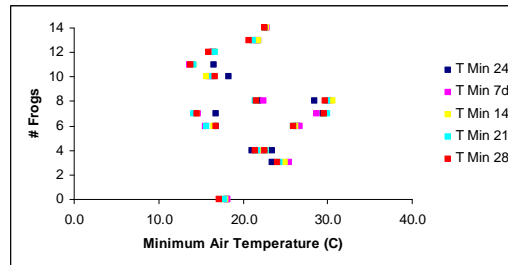
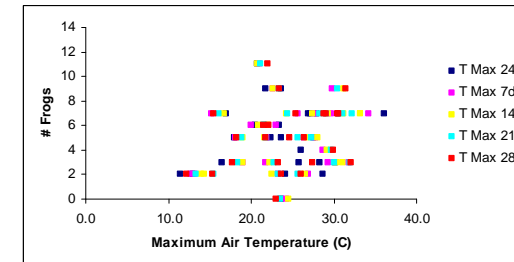Mean Air Temperature
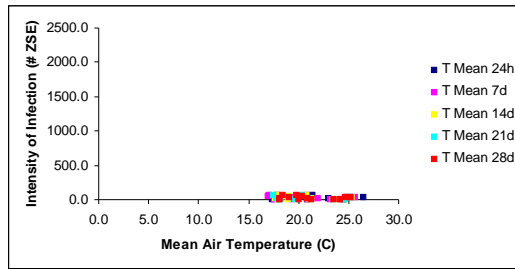
a) *L. nannotis*
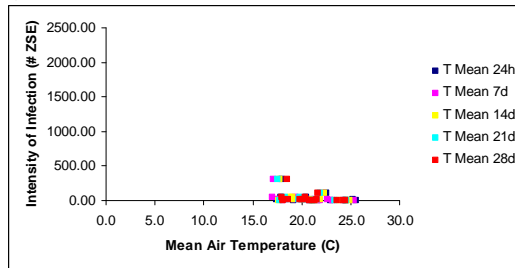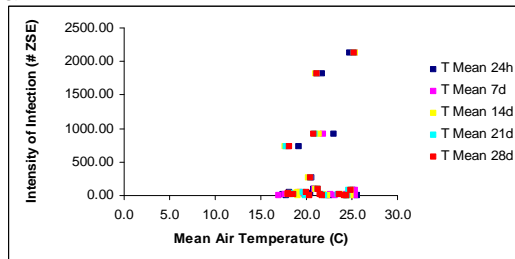
d) *L. rheocola*
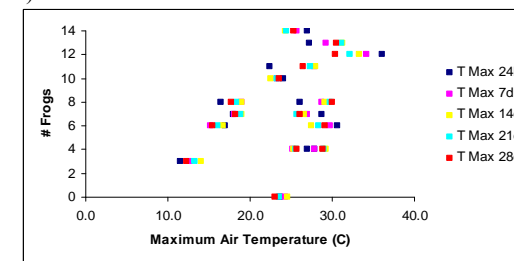

g) *N. dayi*

b) *L. nannotis*

e) *L. rheocola*

h) *N. dayi*
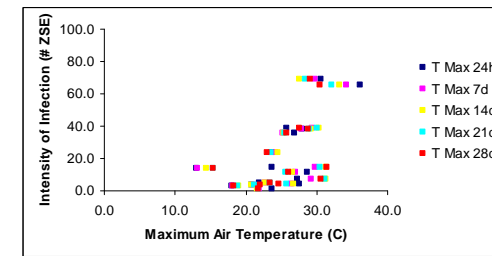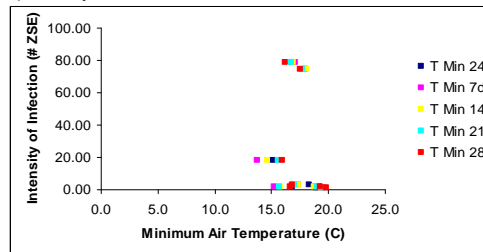
c) *L. nannotis*

f) *L rheocola*

i) *N. dayi*

Maximum Temperature

c) *L. nannotis*

Minimum Air Temperature

Figure 2.18 cont. Variation in intensity of infection (number of zoospore equivalents) among frogs at Tully Gorge NP with climatic conditions

Rainfall
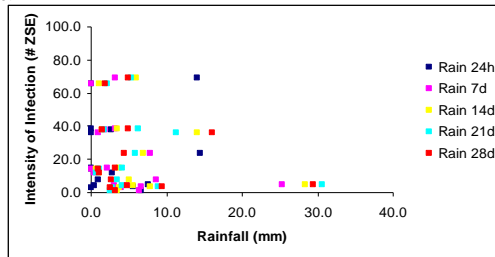
j) *L. nannotis*

k) *L. nannotis*
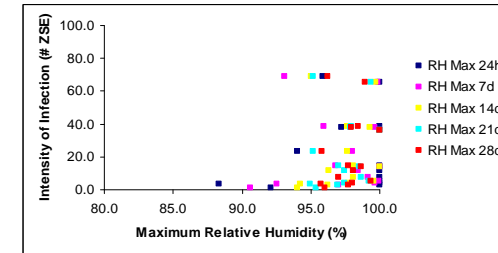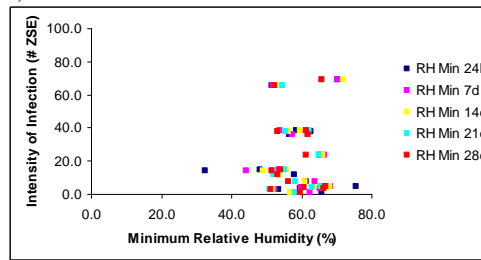
m) *L. rheocola*
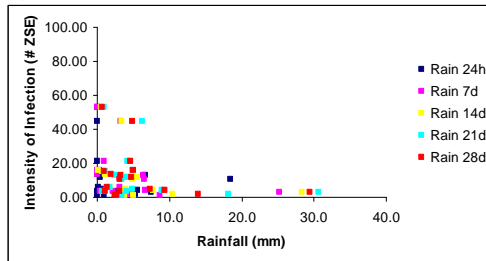
n) *L. rheocola*
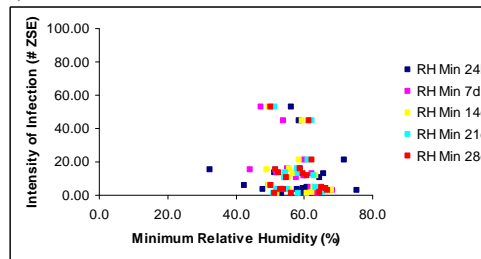
o) *L. rheocola*
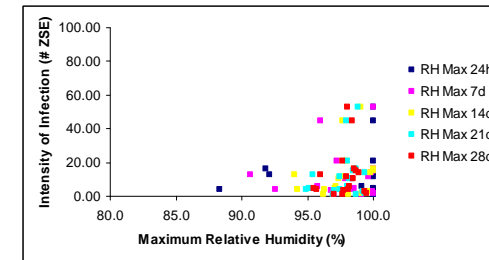
p) *N. dayi*
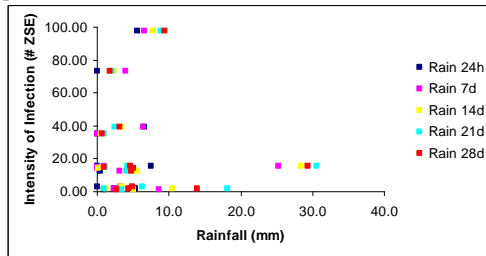
q) *N. dayi*
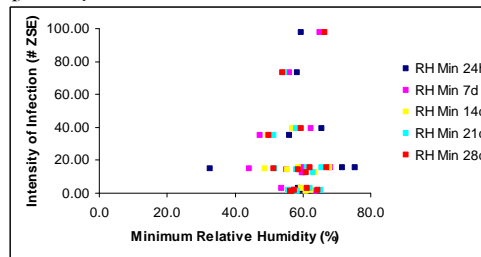
r) *N. dayi*
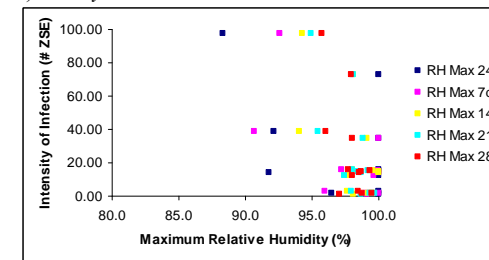
Maximum Relative Humidity

Minimum Relative Humidity

l) *L. nannotis*

Discussion
Our results indicate that the disease dynamics of Bd in the four species of frog studied, *L. genimaculata*, *L. nannotis*, *L. rheocola* and *N. dayi*, at Murray Upper NP and Tully Gorge NP, differ with both species and location. Hence, the disease dynamics in each species at both sites have been discussed separately below. However there were consistencies among species and location. Temperature was generally negatively correlated with prevalence and occasionally intensity of infection. Similarly, rainfall and maximum humidity were negatively correlated and mimimum humidy sometimes positively correlated.

Air temperatures at Tully Gorge NP were above the upper tolerance limits for Bd more frequently than at Murray Upper NP. Higher rainfall was observed at Tully Gorge NP, especially during April 2006 when Category 5 Cyclone Larry passed close to the area. The greater variation in maximum relative humidity at Tully Gorge NP, when compared to Murray Upper NP, is likely due to the significant reduction in the canopy density after cyclonic wind damage and tree-fall. These differences in the climatic conditions between the sites may account for inter-specific variation shown in the relationship between climate and frog numbers, prevalence of Bd and intensity of infection. Numbers of *L. nannotis* and *L. rheocola* were substantially higher at Tully Gorge NP than Murray Upper NP, while the reverse was true for *L. genimaculata*, and this may have influenced our findings at each site. *N. dayi* does not occur at Murray Upper NP.

Climatic Conditions and Chytridiomycosis at Murray Upper NP
Variation among *L. genimaculata*, *L. nannotis* and *L. rheocola* numbers at Murray Upper NP may be explained by frog behaviour and hydration requirements. At elevated temperatures (high maximum temperatures in the 7 days prior), *L. genimaculata* numbers increased at the stream, probably due to the need for hydration. Within the same time period, *L. genimaculata* numbers decreased with rainfall and a corresponding increase in the minimum relative humidity was demonstrated as frogs were able to remain hydrated in the rainforest environment away from the stream. Numbers of *L. nannotis*, a species that remains in close proximity to the stream each day, remained relatively unaffected by climatic conditions, although the number of frogs increased with rainfall and suitable breeding conditions. *L. rheocola* numbers demonstrated a strong association with climatic conditions, increasing as air temperatures rose. Higher rainfall (and by association relative humidity) also resulted in an increase in numbers, likely due to breeding pressure.

It was anticipated that an increase in frog numbers might result in an increased prevalence of Bd, as more frequent contact with infected animals would be likely and zoospores from infected frogs could be shed onto stream-associated substrates. However, results presented in Objective 7 showed prevalence of Bd to be independent of frog density. The prevalence of Bd in *L. nannotis* at Murray Upper NP was also independent of climatic conditions, indicating transmission rates among this species and between frogs and environmental substrates were relatively constant although power to detect effects of climate were lowest for this species at this location. A higher proportion of the *L. genimaculata* and *L. rheocola* populations were positive for Bd infection when minimum temperatures declined. Prevalence of Bd in *L. genimaculata* decreased after recent rainfall, possibly an artefact of frogs moving from the stream into the forest where moisture was then available. This behaviour could result in the negative correlation between prevalence and minimum relative humidity 3 weeks prior to sampling due to lower transmission rates due to less time spent on the stream.

Drew et al. (2006) found temperature to be the most significant predictor of occurrence of *Bd* in Australia. However, rainfall was not found to be a statistically significant factor in predicting the presence of chytridiomycosis (Drew et al. 2006). Our data for *L. genimaculata*, while limited to a small sample size, indicated that rainfall and resulting frog behaviour may influence prevalence of Bd in populations where infection is endemic. This relationship will require further investigation by

means of radio telemetry and determining habitat preferences and infection history of individual *L. genimaculata* to determine if it is significant.

Intensity of infection in *L. nannotis* and *L. rheocola* was largely unaffected by climatic conditions. It is expected that after frogs became infected with Bd, the critical threshold of infection was reached largely by self re-infection as zoospores are released and reattach to the individual host (Carey et al. 2006). However, intensity of infection in *L. genimaculata* may be driven by climatic conditions also influencing prevalence of Bd. The results presented in Table 2.1 are indicative only of a relationship, possibly behavioural, between intensity of infection and temperature, rainfall and/or humidity. The negative correlation between prevalence and minimum temperature suggested the likelihood of infection increased as temperatures fell below that enabling *L. genimaculata* to clear infection by basking and raising body temperature. This may have resulted in the corresponding increase in intensity of infection that occured after low minimum temperatures. In contrast, increased moisture availability after rainfall and with increased relative humidity may have enabled infected frogs to leave the stream and seek more exposed basking areas within the rainforest canopy. Our results require behavioural studies linked with epidemiological studies to determine conclusively if responses to climatic conditions by this species may influence intensity of infection.

Climatic Conditions and Chytridiomycosis at Tully Gorge NP
As insufficient *L. genimaculata* were captured at Tully Gorge NP to allow a comparison between populations at the 2 sites, our understanding of the dynamics of Bd within this species with variations in climatic conditions must rely on the Murray Upper NP population alone. However, differences between *L. nannotis* and *L. rheocola* populations at the 2 sites suggested further studies of *L. genimaculata* at other locations would be beneficial.

Numbers of *L. nannotis* and *N. dayi* increased with mean air temperature. Where as numbers of *L. nannotis* at Murray Upper NP were independent of climatic conditions with the exception of rainfall. The mean air temperature was higher at Tully Gorge NP than Murray Upper NP, perhaps the result of lower altitude or reduced canopy cover and shade, resulting in greater *L. nannotis* activity. Increased minimum relative humidity and rainfall also resulted in increased *L. nannotis* numbers. *L. nannotis* require a moister environment than the sympatric species and do not forage as far from the stream, perhaps due to a greater risk of dehydration (Hodgkison and Hero 2002). Where *L. rheocola* numbers at Murray Upper NP fluctuated greatly with changing air temperature, rainfall and humidity, they were independent of climatic conditions at Tully Gorge NP. *N. dayi* numbers increased significantly with air temperatures, as the species ceased its winter aestivation.

Prevalence of Bd in *L. nannotis*, *L. rheocola* and *N. dayi* decreased significantly as air temperatures increased. Rainfall did not appear to impact prevalence, but relative humidity was a significant predictor of prevalence in *L. nannotis*, *L. rheocola* and *N. dayi*. As minimum relative humidity increased, prevalence in *L. nannotis* and *L. rheocola* increased, possibly as lower moisture availability was inhibitory to Bd survival and growth. However, as maximum relative humidity increased, prevalence of Bd in the three species decreased. A similar response was observed in *L. genimaculata* at Murray Upper NP, and attributed to frogs departing the stream and transmission rates dropping. A corresponding decrease in frog numbers was not observed at Tully Gorge NP, however *L. nannotis*, *L. rheocola* and *N. dayi* are smaller frogs and would dehydrate after a shorter time period. It is possible these species were leaving the stream when temperature and humidity were suitable for short periods only, returning during the evening to hydrate, forage and seek mates, thereby also reducing transmission rates.

Intensity of infection in *N. dayi* was also negatively correlated to increasing relative humidity. This response was not observed in *L. nannotis* or as strongly in *L. rheocola*. This is a preliminary analysis of the data and it is possible that many of these climatic variables are highly correlated or

confounded. We will be checking for correlation and conducting multivariable analysis to account for confounding.

Conclusion
Inter-species variations in frog numbers, prevalence of Bd and intensity of infection may be explained by the effects of climate and its interaction with the habitat occupied by that species. While the role of temperature in chytridiomycosis dynamics has been under investigation in other studies and its negative affects at ambient air temperatures of about ≥27°C (Berger et al 2004) are fairly well understood, none have focused on the role of rainfall and humidity and their possible role in enabling a reduction in prevalence and intensity of infection.

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Rediscovery of an "extinct" Australian stream frog (*Litoria lorica*) in an environmental refuge from chytridiomycosis

(Excerpt from Puschendorf, R. 2010. Environmental effects on a host-pathogen system: frogs and Batrachochytrium dendrobatidis in wet and dry habitats. Ph.D. thesis, James Cook University, Townsville. 145 pp.)

Summary
Chytridriomycosis, caused by the fungus *Batrachochytrium dendrobatidis*, has caused the decline and extinction of amphibians in pristine and apparently undisturbed tropical areas around the world. The Armoured Mist Frog (*Litoria lorica*) is endemic to the Thornton Uplands and Carbine Tableland in northeast Australia. It was not seen between 1991 and 2008 despite considerable search effort and was thought to be extinct. The timing of its disappearance coincided with similar declines of sympatric species in the area, such as *Litoria nannotis* and *Taudactylus acutirostris* due to chytridiomycosis outbreaks. In June 2008, a previously unknown population of *L. lorica* was located. Despite high prevalence and intensity of *B. dendrobatidis* on both sampled frogs and tadpoles, *L. lorica* and *L. nannotis* (a sympatric species) were relatively abundant. This population of *L. lorica* thus appears to be coexisting with *B. dendrobatidis*, which probably caused the extinction of other populations of the species. The environment at the new site is much drier, hotter, and more seasonal than the rainforest sites where extirpation occurred; this suggests environmental conditions at the new site may have allowed the population of *L. lorica* to persist. We suggest that in the face of emerging diseases and other global changes, it is important to more fully understand the ecological ranges of species of concern, and to ensure that conservation efforts are not focused solely on what are thought to be "core" environments.

Introduction
The Wet Tropics in north Queensland, Australia, is one area that has been particularly affected by chytridiomycosis with one species extinct and another seven threatened (Richards et al. 1993; Laurance et al. 1996; McDonald and Alford 1999; Schloegel et al. 2006). The area comprises approximately 1 million hectares of rainforest along with sclerophyll communities dominated by Melaleuca and Eucalyptus (Williams 2006), supporting 27 endemic frog species (Hoskin & Hero 2008).

The Armoured Mist Frog (*Litoria lorica*) is endemic to the Thornton Uplands and Carbine Tableland (Davies and McDonald 1979; Cogger, 2000; Cunningham 2002). It was last seen in 1991 and was thought to be extinct (Covacevich and McDonald 1993; Cunningham 2002). Its disappearance coincided with the declines suffered by sympatric species such as *Litoria nannotis* and *Taudactylus acutirostris* (Richards et al. 1993). In 1990 dead and dying frogs were collected from the same area *L. lorica* was previously known. Necropsies conducted in the late 1990s showed high prevalence and intensity of infection of *B. dendrobatidis* causing chytridiomycosis and death (Berger et al. 1998; 1999; Longcore et al. 1999). It is therefore highly likely that an epidemic of chytridiomycosis was responsible for the disappearance of those species and *L. lorica* from those locations.

Litoria lorica was thought to be a rainforest specialist (Williams and Hero 1998, 2001), but in June 2008, 17 years after it was last seen, we discovered a population of *Litoria lorica* outside its former known distribution in dry sclerophyll habitat. We carried out surveys to determine the spatial extent of the newly discovered population, performed genetic analysis to determine the species identity of the newly discovered population, and took swab samples from tadpoles and adults for non-invasive diagnostic tests of *B. dendrobatidis* infection. Below, we describe the existence of a "believed to be

extinct" species with *B. dendrobatidis,* discussing the conservation implications for such a tenuous coexistence.

Methods

Identification of *Litoria lorica*

Litoria lorica was identified using morphology and genetic data. The morphology of putative *L. lorica* individuals was compared against the species description (Davies and McDonald 1979) and key specific diagnostic traits outlined (Cogger, 2000; Cunningham 2002; Hoskin and Hero 2008). The only species that *L. lorica* could be confused with is *L. nannotis*, a sympatric species that is generally larger in size but otherwise of very similar morphology and ecology. Thus individuals of each species (39 *L. lorica* and 35 *L. nannotis*) were examined in the field and several measurements (including snout-vent length (SVL) and weight) were made.

Toe-pad tissue was taken from three *L. nannotis* and three putative *L. lorica* individuals for genetic analysis. These samples were sequenced for a segment of the mitochondrial cytochrome oxidase I (*CO1*) gene. Extraction and sequencing followed standard techniques, and the primers used were Cox and Coy (Hoskin et al. 2005; Schneider et al. 1998). These sequences were added to extensive sequence data available from the EMBL Nucleotide Sequence database for *L. nannotis* and *L. rheocola* from the phylogeographic studies of Schneider et al. (1998). A neighbour-joining tree was constructed from 510 base pairs (bp) of *CO1*, with *Litoria genimaculata* and *Nyctimystes dayi* used as outgroups. Bootstrap analysis was performed to test the support for relationships. *Litoria lorica* had not formerly been sequenced previously because all samples that were collected pre-decline were formalin-fixed.

Frog abundance surveys

Despite historical occurrences of *Litoria lorica* from rainforests between 640-1000 m a.s.l. in Thorton Uplands and Carbine Tableland (Covacevich and McDonald 1993; Cogger 200; Cunningham 2002; Davies and McDonald 1979; Hoskin & Hero 2008), no abundance information was known. Abundance surveys were conducted along a 400 m transect in the upstream section of the site by walking along the creek at night, counting and identifying all the *L. nannotis* and *L. lorica* located. The species proved to be morphologically distinct (see results), so individuals could be identified without handling. The riparian habitat along the transect was dry sclerophyll woodland on granite hills. Three natural discrete sections of waterfalls and cascades in which the frogs occurred were apparent in the transect. Deeper pools separated these sections. The area of the three distinctively occupied sections was approximated so that abundance per unit area could be estimated.

Detection of *Batrachochytrium dendrobatidis* in frogs and tadpoles

Frogs and tadpoles were sampled at the end of July 2008, at the peak of winter when temperature is the coolest, and prevalence and intensity of infection was 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 captured the animals by inverting an unused 15 x 15 cm plastic bag over our hand and grasping the animal while drawing the bag inside out, thus capturing the frog in the bag without touching it. Captured frogs were then quickly released into larger 21 x 30 cm press seal bag; the small plastic bag used for the initial capture was left inside the larger bag with the frog so that it could later be used to restrain the frog while measuring its weight.

We attempted to catch all visible animals at each localised waterfall or cascade area for swabbing. After capture we placed frogs away from torch light in their individual bags. We used a new pair of

low powder vinyl gloves (Livingstone) to handle each individual as it was swabbed for diagnostic qPCR, measured, and weighed. The exact capture locality of each frog was recorded using a Garmin 60CSX GPS and WGS 84 datum. After swabbing we recorded the individuals' sex and age class status (male, female, subadult), snout-vent length (measured using metric stainless steel callipers) and mass (using a 100 gram digital pocket balance). Frogs were sampled for *B. dendrobatidis* by firmly stroking a cotton swab on their ventral skin three times in each of the following parts: (a) the back of each foot; (b) the pelvic patch on both legs; (c) the ventral surface of the abdomen; (d) each hand, for a total of 27 strokes. Following measurement and swabbing, the frog was immediately released at the point of capture.

We captured tadpoles by dip net, and quickly transferred them into individual press seal bags, avoiding any direct handling. We used well rinsed vinyl gloves and observed tadpoles for any ill effects after handling as suggested by Cashins et al. (2008) to prevent tadpole mortality during handling and changed gloves between each individual to prevent disease transmission. Tadpoles were poured from the zip lock bags into the palm of the hand and secured on the ventral surface between the forefinger and thumb. We then gently stroked a swab 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. Tadpole body length, mass and a score of mouthpart loss was recorded for each individual. Following sampling, tadpoles were returned to the individual bags and held for at least fifteen minutes to confirm their condition before release. Tadpoles were identified according to Richards (1992).

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 apparent prevalences.

When presenting the analysis of *B. dendrobatidis* infection we report prevalence of infection as percentage of individuals of each species infected. We also report the 95% confidence intervals (CI) for true prevalence in the population at the time of sampling. Measures of infection intensity were log transformed prior to analysis (Zar 1999), but are reported in the text as zoospore equivalents prior to transformation (mean ± standard deviation).

Results
Identification of *Litoria lorica*
The newly discovered population of *L. lorica* fit all of the morphological traits that have been described for the species (Cunningham 2002; Davies and McDonald 1979) and fit the major diagnostic traits that separate this species from the very similar *L. nannotis* (*L. lorica* has a smaller adult body size and a more truncate snout shape) (Cogger 2000; Cunningham 2002; Davies and McDonald 1979; Hoskin and Hero 2008). The adult size difference is apparent from field measurements taken at the new site. The SVL of adult *L. lorica* ranged from 30-42 mm (mean 35 mm) and *L. nannotis* from 52-62 mm (mean 57 mm). Sub-adult *L. nannotis* overlap in size with adult *L. lorica*, but *L. lorica* were readily diagnosed by the presence of nuptial pads and accessory spines on the chest and chin (present, although less prominent in females), truncate snout shape, dorsal pattern (more distinctly blotched *versus* more mottled in *L. nannotis*) and ventral colouration (white *versus* cream with grey or brown areas in *L. nannotis*).

Genetic results supported identification of the species as *L. lorica*. The samples from frogs we assigned to this species represent a highly distinct lineage that clearly falls within the Australian 'torrent treefrog' species group, with *L. lorica* being a well-supported sister species to *L. nannotis* (Hoskin, unpublished data). Mean sequence divergence (510 bp *CO1*, Kimura two-parameter) between *L. lorica* and *L. nannotis* is 19.8%, and between *L. lorica* and *L. rheocola* is 24.7%. All

three *L. lorica* individuals sequenced share the same haplotype. The three *L. nannotis* sequenced from the *L. lorica* site sit in the expected position in the tree amongst other Carbine Tableland samples in the 'northern' clade of *L. nannotis* (Cunningham 2002; Schneider et al. 1998).

Figure 2.19. Clustering of *Litoria lorica* and *L. nannotis* beside a waterfall

Frog abundance estimates

Both *L. nannotis* and *L. lorica* were only found around three discrete waterfall/cascade sections of the transect, totalling approximately 150 m^2 of habitat along the 400 m transect. Frogs in these areas were highly clustered, with *L. lorica* and *L. nannotis* mixed together on rocks next to waterfalls and cascades (see supplementary material A). Both species occur at high densities with *L. nannotis* (0.230 ± 0370 individuals / m^2) at higher densities than *L. lorica* (0.140 ± 0.563 individuals / m^2).

Prevalence and intensity of infection of *Batrachochytrium dendrobatidis* in frogs and tadpoles

Analysis of skin swabs revealed that 80.6% of the terrestrial *Litoria nannotis* sampled were infected with *B. dendrobatidis* (N = 88, 95% CI = 0.709-0.883), while infection in terrestrial *Litoria lorica* was 81.8% (N = 33; 95% CI = 0.645-0.930). These prevalences did not differ significantly (χ^2=0.02, DF= 1, P= 0.887). No differences were found among males, females and juveniles for both species combined? (χ^2= 0.393, DF= 2, P= 0.822). The interaction on prevalence between species, life history stages and sex was also …. All of the 57-swabbed tadpoles (Fig. 2, N = 24 *L. nannotis* and 33 *L. rheocola*) were infected with *B. dendrobatidis*. The lower confidence interval of infection prevalence in *L. nannotis* was 0.857 and in *L. rheocola* was 0.894. Infection of *L. lorica* tadpoles could not be determined because none were seen or captured. Disease prevalence was significantly different between frogs and tadpoles (χ^2=12.44, DF= 1, P= <0.001).

There was no significant difference in intensity of infection between species ($F_{1,98}$= 0.095, p=0.758), sex ($F_{2,98}$=0.326, p= 0.722) and no interactions between them ($F_{2,98}$= 0.334, p=0.716; Fig. 2). Infected tadpoles (2888±1255 zoospore equivalents; Fig.2) had significantly higher intensities of infections than infected frogs (418±1255 zoospore equivalents; t=-12.520, DF=153, P= 0.001).

None of the sampled individuals showed clinical signs of lethargy, skin sloughing or loss of righting reflex, all common signs of chytridiomcyosis (Berger et al. 1999).

Figure 2.20. Plots of the environmental space of the Australian Wet Tropics Bioregion (grey); sites where *Litoria nannotis* is found (dark-grey crosses); historical sites for *Litoria lorica* (orange); and points from the site where *Litoria lorica* was rediscovered in this study (red circles).
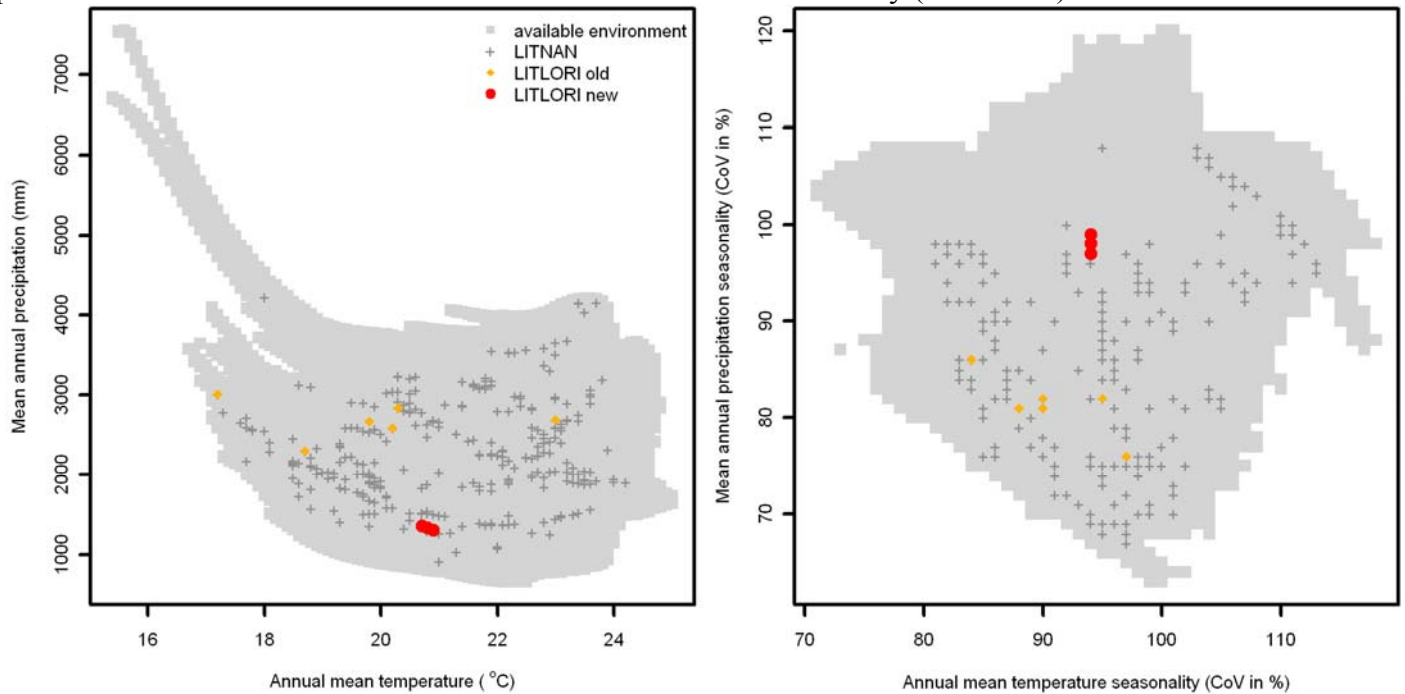
Figure 2.21. Intensity of infection of infected frogs and tadpoles of *Litoria lorica, Litoria nannotis* and *Litoria rheocola*
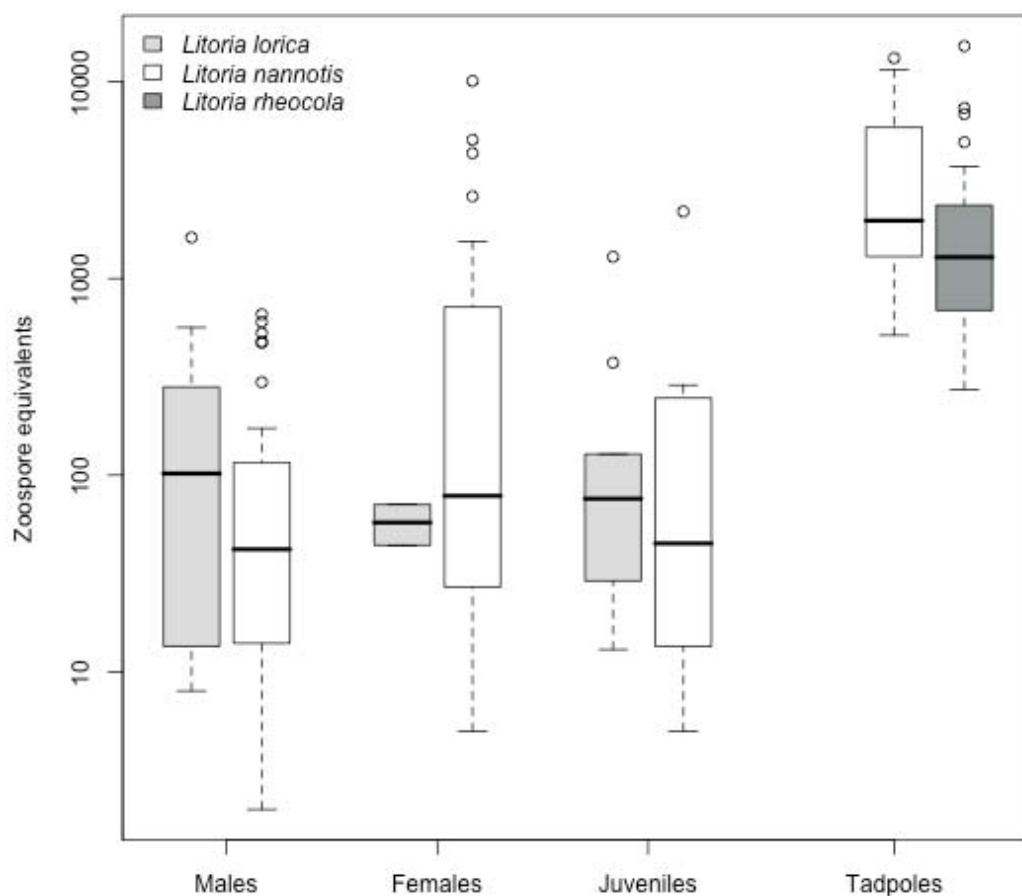
Discussion

Little was known about the distribution, ecology, or behaviour of *L. lorica* prior its disappearance from known sites. Therefore, it was listed as critically endangered (Covacevich and McDonald 1993; McDonald and Alford 1999, Cunningham 2002). However, *L. lorica* was generally though to be extinct because despite repeated surveys at historical sites, the species had not been reported for 17 years (McDonald, unpublished). Morphological and genetic analyses clearly show that the population we discovered is *L. lorica*.

L. lorica was found around cascades and large waterfalls on an open, rocky river with perennial flow. The vegetation in the area is dry sclerophyll woodland; the nearest well-developed rainforest is 6 km upstream. All previously known localities for *L. lorica* were in rainforest. 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 this species as critically endangered, given uncertainty about its distribution and ecology. The species was thought to be restricted to rainforest and it has apparently been extirpated from this habitat. However, we now know from our newly-discovered population that the species can occupy dry sclerophyll close to rainforest and has persisted in at least one site in this habitat.

Infection of *B. dendrobatidis* on frogs and tadpoles
Several frog populations have been found with high *B. dendrobatidis* prevalence without suffering any evident significant effects, although mortality could still be affecting a portion of the population when conditions favour disease development (e.g. Kriger and Hero 2006). A few Australian species that suffered large declines from the original outbreaks of chytridiomycosis now coexist with the disease in restricted distributions and at lower abundance (Australian Government Department of the Environment and Heritage 2006). The prevalences we measured are presently the highest reported in the literature for frogs, with an average across both species of 85.95% (95% CL = 0.78- 0.92). It is not surprising that prevalence and intensity of infection was not significantly different between both species regardless of their sex since they coexist in the same area, splash zones of cascades and waterfalls. Most likely the transmission rates and microenvironments used are similar, which is reflected in the similar infection patterns. The only difference in the infection patterns was between tadpoles and adults. All sampled tadpoles were infected, and they had significantly higher intensities of infection than adults. Although it is difficult to interpret this information since the sampling method and tissues sampled are different between larvae and frogs (only mouthparts are swabbed in tadpoles in contrast with the ventral side of frogs), it is clear that tadpoles are potentially significant contributors to Bd transmission in the stream.

These populations are presently coexisting with the pathogen, but 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. The impact of *B. dendrobatidis* on this population should therefore be investigated using a mark recapture study and molecular techniques.

Potential mechanisms of resistance to chytridiomycosis of surviving *Litoria lorica* population
The present population of *L. lorica* is found in dry sclerophyll woodland, an environment very different from that in the rainforest sites from which the species disappeared (Fig. 1). This area has higher surface temperatures, lower annual precipitation and higher seasonality than nearby rainforest sites. This is similar to what has been reported 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). This area has been proposed to be an environmental refuge from chytridiomycosis-driven amphibian declines (Puschendorf et al. 2008), based on the high abundance of *C. ranoides,* a species that used to be widespread in many lowland areas in the country (Campbell and Savage 2000) and the low likelihood of this pathogen occurring there based on analysis derived from species distribution modelling (Puschendorf et al. 2008).

The presently described system in Australia appears to represent another environmental refuge from disease-driven amphibian declines. The pattern of infection in this Australian refuge is likely in part due to the low ambient air and water temperature (because this site is of reasonably high elevation) and its geographical position (downstream from rainforest sites were *B. dendrobatidis* is common). Despite very high prevalence and intensities of infection, 121 (plus 222 counted frogs during the abundance surveys) individuals of both species presented no clinical signs of disease, even when sampled in winter, the season when the prevalence of *B. dendrobatidis* is greatest and mortality is most often observed in the field although the power to detect clinical disease by intermittent transects is very low (Berger et al. 2004; Kriger and Hero 2006; McDonald et al. 2005, Woodhams and Alford 2005). It is possible *B. dendrobatidis* is affecting frog population dynamics at this site, however, the relatively high abundance of both species compared with adjacent populations of *L. nannotis* in rainforest suggests that the environment at the newly discovered site protects the population from mortality caused by chytridiomycosis to some degree,. It is important to understand the environmental factors and the mechanisms that provide this protection,.

Experimentally, some species of frogs can rid themselves of infection by occupying higher ambient temperatures (Woodhams et al. 2003). Basking behaviour, in which an animal exposes itself to direct sunlight or substrate heated by the sun, has not been observed in *L. lorica* or *L. nannotis* at this site (Puschendorf pers. obs.). The microhabitat used by both of these species seems to be largely similar to that used by *L. nannotis* in the rainforest, where they live in a moist and buffered environment ideal for chytridiomycosis (Rowley and Alford 2007a, b). However, an important difference compared with rainforest populations is that the temperature of the rocks on which the dry forest frogs sit at night is much higher because of the lack of canopy cover (Puschendorf unpublished). Infection usually starts and is highest on the ventral side of the frogs (Puschendorf and Bolaños 2006; North and Alford 2008) so it is possible that a few hours of sitting on warmer rocks might provide some protection, even in winter when night temperatures are coolest. Warmer temperatures might not only slow down the growth of the pathogen, but also help boosting any immune response that might not be possible at lower temperatures.

Implications for amphibian conservation

Litoria lorica remains critically endangered. The rediscovered population is locally common but highly localised 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 more populations (Puschendorf & Hoskin, and Arias unpublished). More surveys of dry forest stream habitat 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. An emergency response is not warranted at this time however an emergency response plan should be drafted in case the population shows signs of decline. This would involve removal of individuals into captivity to prevent their death in the wild and has been a successful strategy for saving several species and populations from extinction including the Panamanian golden frog, *Atelopus zetecki*, the Kihansi spray toad ?, the Wyoming toad and the spotted tree frog, *Litoria spenceri*. Current or potential direct impacts to the site must be minimised. This includes the activities of humans and other agents (e.g., feral pigs and cattle, which are known to occur at the site) that may directly disturb or harm the frogs or tadpoles, or may affect them through impacts to water quality. Also of potential importance are impacts from upstream catchment areas, which may affect water quality at the site. The site should be monitored periodically using low impact techniques to assess population trends and population viability.

At present the population appears stable and is apparently coexisting with *B. dendrobatidis* at a high prevalence and intensity of infection, 15 years after the first disease outbreaks in the area. However, captive assurance colonies should be established to further protect the species against extinction. Captive husbandry and breeding techniques should first be trialed and optimised on more common related species such as *L. nannotis* and *L. rheocola*. We encourage this process to start immediately, which ideally should combine further fieldwork and captive work initially with these two species. Also, the risk of significant impact on the wild population from removing individuals to establish a captive breeding program must first be evaluated and mitigated. In the meantime other important biological information on the species should be collected to guarantee a successful captive breeding program. The identification and biology of the tadpoles, diet of larvae and adults, reproductive biology, are all unknown for this species and fundamental to a successful captive breeding program and for minimizing the loss of individuals. Obviously any significant decline would supersede this prioritisation scheme and warrant an emergency response to save the species (Mendelson et al. 2006).

Another possibility includes translocation of adults or tadpoles to apparently suitable habitat in nearby catchments. Several things would need to be ascertained prior to translocation being attempted. Similar to establishing captive assurance colonies, it would be necessary to determine that the newly discovered population can support removal of individuals. In addition, thorough surveys of possible catchments for introduction would need to be conducted to confidently determine absence of *L. lorica*, and detailed assessment of the factors determining why *L. lorica* is not currently in these catchments would need to be conducted to ensure that threats are not present or are mitigated. It is also possible that individuals could be reintroduced from captive assurance colonies.

Of more general importance is recognition of the role of environmental refuges from disease in amphibian conservation. In both tropical Australia and Costa Rica, it is clear that dry forest areas adjoining rainforest can serve as environmental refuges from chytridiomycosis. Protecting refuge areas adjoining rainforest is extremely important for the survival of *L. lorica* and is likely to be important for many other frog species around the world. Particularly for species that are poorly known, it is important to realize that as well as not encompassing the entire geographical range of species, known localities may not encompass the entire environmental range.

Survey efforts should focus on documenting the full extent of the geographical range by including areas at or outside the margins of the known ecological range of species. In particular, dry forest habitats bordering rainforest should be seen as targets for survey effort for apparently extinct and declined rainforest amphibian populations globally.

Lastly, these species will continue to be threatened unless we can mitigate the impact of chytridiomycosis. This requires research and adaptive management and is an urgent global issue.

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 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.

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Modelling the potential distribution of *Batrachochytrium dendrobatidis* in the Australian Wet Tropics and Cape York Peninsula based on climate

(Excerpt from Puschendorf, R. 2010. Environmental effects on a host-pathogen system: frogs and Batrachochytrium dendrobatidis in wet and dry habitats. Ph.D. thesis, James Cook University, Townsville. 145 pp.)

Knowledge of the ecological and geographic limits of *B. dendrobatidis* is important for amphibian conservation, especially in areas perceived as pristine. An accurate distribution model of *B. dendrobatidis* would enable predictions of potential refugial areas, where environmental variables could limit pathogen impact on amphibian species. A global predictive model of the fundamental niche (*sensu* Hutchinson 1957) of *B. dendrobatidis* already exists (Ron 2005). It provided the best predictions of where this pathogen could be found leading to new local investigations. Although it has been useful in showing the potential niche of the fungus, the model was based on a rather small dataset of occurrences at a coarse resolution (2.5' degrees latitude and longitude) over a large geographic extent; finer scale distributional limits of the fungus were obscured. Thus finer-scale models are needed, particularly in topographically diverse areas such as the Australian Wet Tropics (AWT) and Cape York Peninsula (CYP).

We use Maxent (Phillips et al. 2006) to create climate-based models of the potential niche of *B. dendrobatidis* based on a set of twenty-seven environmental layers available for the AWT and project these models up to CYP. Despite being a relatively novel approach in species modeling, Maxent has been shown to outperform other methods traditionally used in this type of study (Elith et al. 2006).

Uncertainty in model projections are estimated by bootstrapping data (3:1 training:testing ratio) used in 100 model runs and then examining attributes of the 10 best models (as defined by area under the receiver operating characteristic curve (AUC); Hanley and McNeil 1982). Specifically, we examined the average prediction of the 10 best models along with the 95% confidence intervals (Figure 4). This approach allows us to distinguish between areas where the pathogen is predicted to be found with great confidence (i.e., areas of high predictability and low variability among the best runs) from those areas showing high averaged predictability, but also high variability among models. Because conservation planning may require predictions of pathogen presence or absence as opposed to continuous probability values, we also applied a threshold to the ten best models, converting each output into a presence / absence prediction. Although several methods have been suggested to transform continuous prediction into a dichotomous presence-absence outcome (Liu et al. 2005), we chose to select a conservative threshold of the minimum maxent predicted value for the known occurrences.

All 100 models performed better than random with AUC values above 0.5 (0.833±0.035). Models predicted most of the humid areas in the AWT to be suitable for the amphibian chytrid, with the lower confidence interval predicting mostly rainforest areas at mid to high elevations (Figure 4). West of the AWT models predict this pathogen to be mostly absent.

While *B. dendrobatidis* has been found throughout the AWT, but still seems to be absent on frogs sampled for the pathogen in CYP (absent from the 550 individuals sampled) (Fig. 1). Cape York Peninsula seems to have suitable habitat for the amphibian chytrid, and only in the lower confidence interval was it not predicted to occur there. *B. dendrobatidis* has not emerged in that area probably due to CYP's remote location and lack of opportunities for anthropogenic spread. Maybe the intervening drier / hotter region between the AWT and CYP act as a barrier to natural dispersal into more favourable areas of CYP. Similarly the World Heritage Wilderness Area in Western Tasmania is currently free of Bd and is a similarly remote area with few opportunities for anthropogenic spread although natural spread appears more likely compared with CYP.

If the pathogen is ever found in CYP, its effects are hard to predict. While the models developed here predict potentially suitable environment in CYP, suitable environment does not predict major disease outbreaks. As with the AWT, much of the region supports the fungus but it was in a relatively small proportion of the region that the fungus was detrimental to population viability. Modeling pre and post decline frog distributions might provide useful to determine what is the environmental envelope in which disease actually causes population declines. When it is combined with the distribution of the pathogen it might shed more light on which areas with historical declines were related to chytridiomycosis.

Figure 2.22. Orange colour represents areas were *B. dendrobatidis* has been found on amphibians and white represents the area were surveys have not been able to find it in frogs.
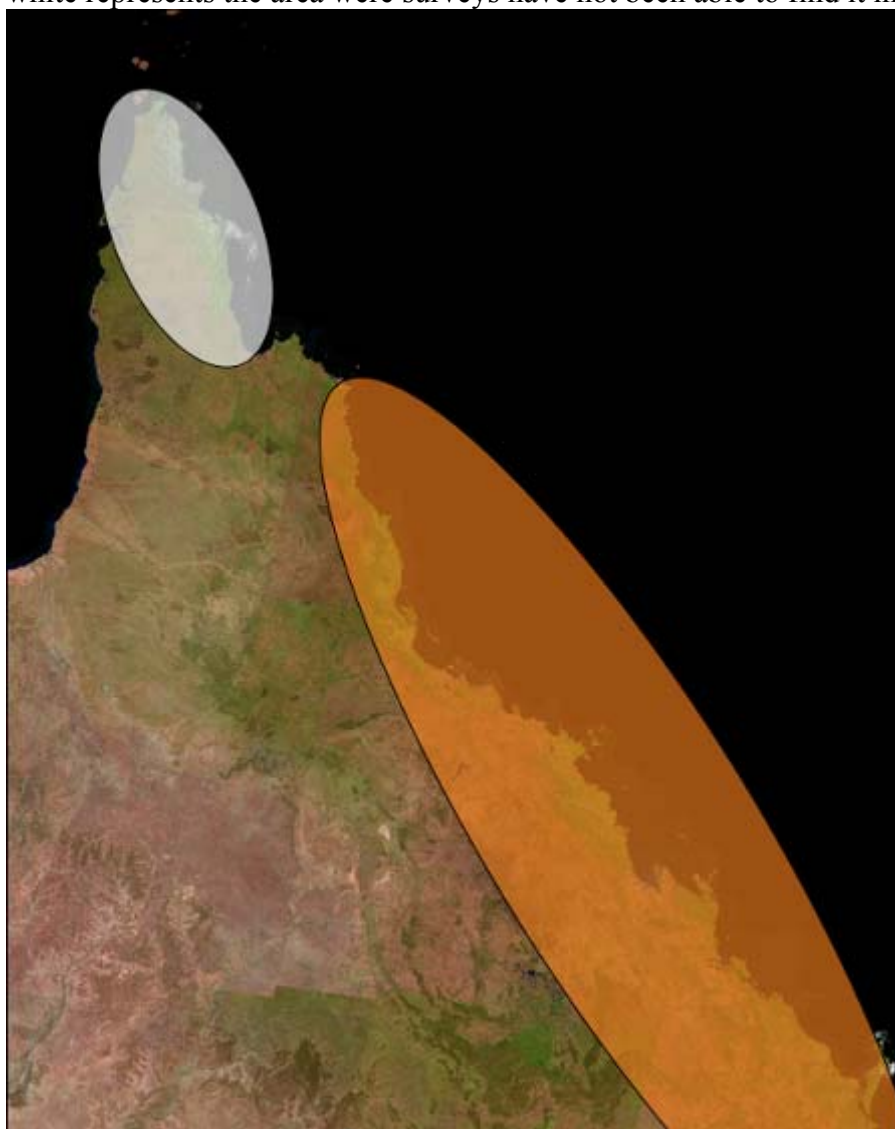
Table 2.7. The two best sampled species, with the sample size and the upper confidence limit of prevalence.

Species	Sample size	Upper confidence limit
Litoria eucnemis	94	0.035
Litoria longirostris	214	0.017
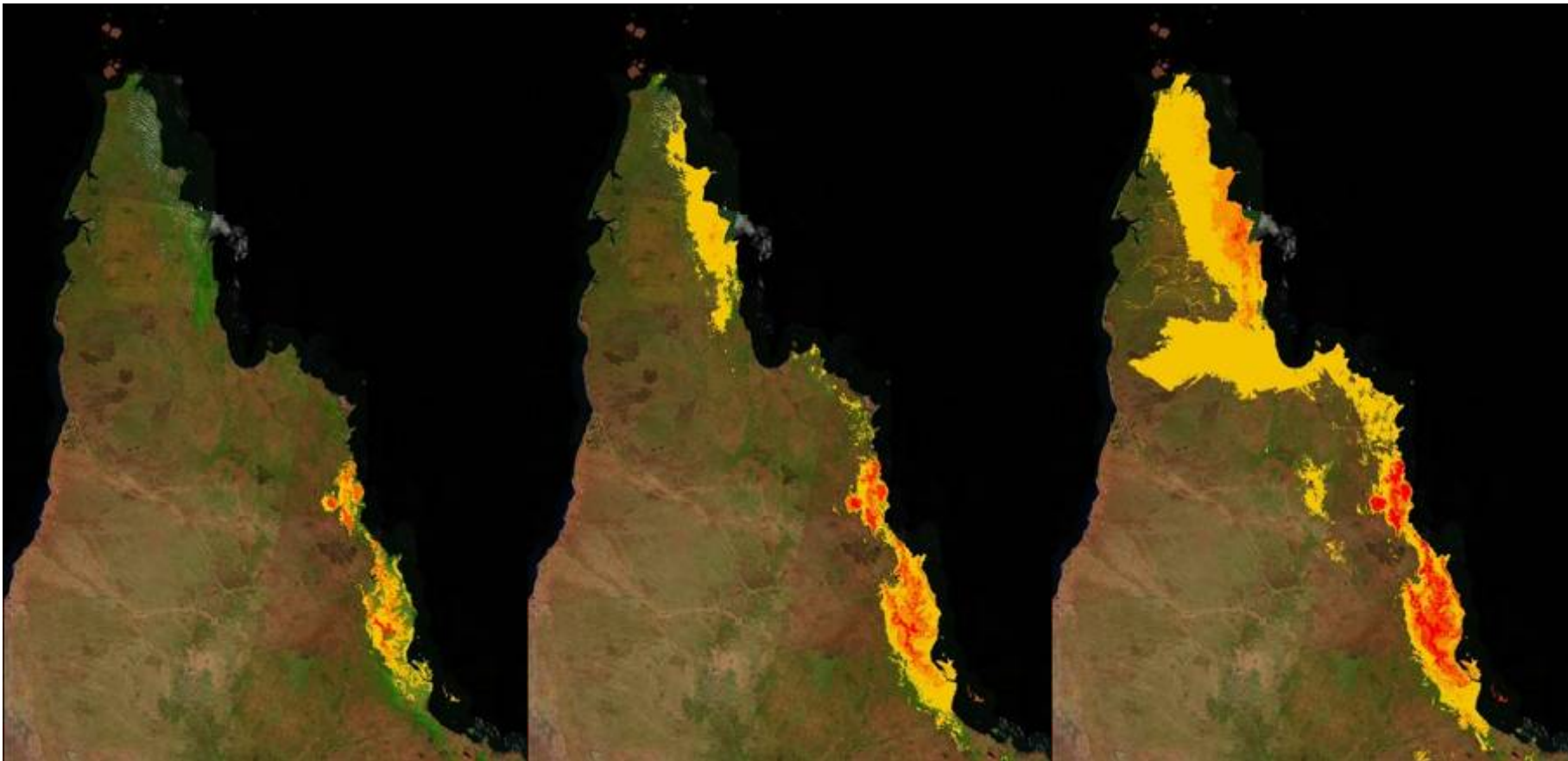
Figure 2.23. Lower confidence interval, average model and upper confidence interval for 100 models run (from left to right). Models were created using data available in the wet tropics and later projected into Cape York Peninsula. Warmer colours represent areas with higher probability of finding the pathogen.

The effect of forest type, behaviour and micro environmental selection on Bd

The effect of forest type: In previous reports we mentioned the pattern found by (Williams et al. 2006) at different highland elevation areas where the amphibian chytrid seems to have the strongest effects on frogs. Throughout their surveys carried out at different localities in the wet tropics the general pattern is that some species have much healthier populations at high elevation dry sclerophyll sites than in the adjacent high elevation rainforests areas. We hypothesized that the effect of the amphibian chytrid could vary between drier and wetter biomes based on these observations, but we still had to document these patterns and understand disease dynamics between these environments. We mostly focused on the waterfall frog (*Litoria nannotis*), since it is an endangered species that suffered strong declines in the rainforest at elevations above 400 m asl, but persisted at lower elevations. To this day, a few populations seem to have recolonised some of these higher sites, but the abundance there is still low.

We chose two pairs of field sites. Each pair studies a population in drier environments (dry sclerophyll), and another adjacent population in a wetter biome (wet sclerophyll or rainforest). We set up data loggers that records temperature and humidity within each transect, to record the environmental differences among them. The Kirrama site is a highland rainforest area (Fig. 5), which we compared to Blencoe Falls (Fig. 6). This last site is mostly surrounded by open dry sclerophyll forest. Waterfall frogs are only found on top of the falls, so that the habitat there is much smaller than the one available at Kirrama.

The other pair of field sites is located on the same catchment close to Mt. Spurgeon. Both forest types, dry (Fig. 7) and wet sclerophyll (Fig. 8) are immediately adjacent in Mt. Spurgeon. We tried sampling each site for one night/ month, but on some occasions it was difficult to get into an area because of bad weather, so that the sampling of one site could have been skipped in some occasions. This was mostly a problem in summer, during the rainy season.

Frog abundance was greater in the drier environments (Figures 9-12). The greater habitat available in Kirrama would suggest that there should be more frogs there than in Blencoe, but this did not occur. The same pattern occurred in the Spurgeon sites, where the abundance in the dry sclerophyll is much higher than in the wet sclerophyll. Also recaptures increased, or at least kept constant in all sites except for Kirrama, where frog abundance seems to have diminished over time after the winter months this year.

Frog mortality was only found in the wetter sites during the July sampling. One dead green eyed tree frog (*Litoria genimaculata*) was found in Kirrama (Fig. 13). In the wet sclerophyll transect in Spurgeon, four green-eyed tree frogs and one waterfall frog were found dead or dying (Fig. 14). The preliminary lab results of these individuals showed strong infections of chytridiomycosis. Sam Young in Cairns is carrying out a full necropsy of these specimens. The difference in disease outcome at sites that are so close by, strongly suggests that the different environmental conditions in these could be responsible for this observed pattern, and potentially the difference in abundance between the dry and wet sites. Under laboratory conditions, *B. dendrobatidis* optimal growing temperature lies between 17-25 °C, and the fungus seems to die above 30 °C (Piotrowski et al. 2004). Moreover, (Woodhams et al. 2003) showed experimentally that frogs could clear the infection when exposed to higher temperatures. There seems to be a correlation of mortality events and colder temperatures during winter in Australia (Berger et al. 2004) and prevalence increases at this time of the year (Woodhams 2003). Humidity could play another important role, since this pathogen does not seem to survive desiccation.

The environmental information collected by the data loggers shows strong differences between the dry and wet environments, even when in short distance with each other. In general both temperature and humidity are much more variable in the drier transects than in the wetter ones in the winter months when the amphibian chytrid becomes a problem and the incidence of frogs dying of

chytridiomycosis increases substantially (Fig. 15-16). The temperature in Blencoe dropped below zero a few times this last winter but the median was in general a few degrees higher than in Kirrama, where temperatures were more buffered and less variable. In the Spurgeon sites these differences are not as dramatic, but still the increased variability in the dry environments vs. the wet persists. Differences in the humidity regimes between the dry and wet environments are much more dramatic, where in the humid environments it does not seem to go below 70%, but in the drier environments it is substantially drier through most winter months.

The idea that the different environmental conditions produce significant outcomes to disease outbreaks is reinforced by the fact that dead or dying frogs were only found in the wet environments.

Behaviour and mircoenvironmental selection: Behaviour studies in lowland populations show that the waterfall frog is more susceptible to become infected and to develop chytridiomycosis than any other sympatric species studied in the region. *Litoria nannotis* mostly lives in waterfalls that are moist and temperature buffered, ideal for chytridiomycosis outbreaks (Rowley and Alford 2007b). This species is frequently found in contact with the stream water, which is one of the mechanisms by which animals can get infected since zoospores infection seems to be correlated with stream water contact. Waterfall frogs also share retreat sites with other individuals, which increase the chance of transmission (Rowley and Alford 2007a). Similar to those studies, we tracked waterfall frogs in our high elevation environments to test if there were any differences in behaviour by which they could have adapted to coexist with the amphibian chytrid in areas were conditions seem to be ideal for disease outbreaks, due to the cooler temperatures. Two sites were radiotracked this year, Kirrama in summer and winter, and Blencoe falls in winter.

The summer tracking at the rainforest site in Kirrama coincided with one of the wettest periods in the region. Frogs moved more than previously recorded, sometimes more than 100 meters, and not only on the creek bed, but also into the rainforest. One gravid female spent several days in the forest, where she would spend the day under the leaf litter. The same individual climbed up to the canopy for one night, but returned the next morning to the creek.

The winter tracking at this same site provided insights on why sometimes we do not find many frogs when surveys are conducted under the wrong conditions for the animals to be detected. At the beginning of this tracking survey, wet and humid conditions allowed us to catch several individuals that were found while they were on the move along the creek bed or at least exposed, close to a waterfall. The following days were extremely dry and cold. The tracked frogs spend most of their time under rocks and boulders, completely out of eyesight, undetectable for a mark recapture survey. The last night before leaving it rained and temperatures climbed up again, with most of the tracked frogs moving overnight, at least a few meters again.

During the October and November mark recapture surveys the forest was mostly dry with almost no rainfall in that period. This was probably a strong influence on the low number of captures found during these trips (Fig. 4). This behaviour suggests that in high elevation rainforest sites such as Kirrama, the right conditions need to occur for the frogs to be out and detectable. Future surveys will show if more humid conditions increase the detectability of these animals. This example illustrates how important it is to understand the behaviour of the studied animals and their interaction with the environment, and how well these studies complement population studies were immigration and mortality rates want to be obtained.

Radio tracking waterfall frogs in Blencoe falls showed how well adapted these animals are to live in drier environment and potentially survive chytridiomycosis outbreaks. A strong storm occurred during the first few days of the tracking period. Although conditions might have ideal for the frogs to move out of the stream environment in the rainforest, none of the tracked individuals did. Some

changed position along the creek, with one moving about 400 meters upstream in one night, but non-wandered around in the adjacent open forest.

Dry conditions occurred during the rest of this survey, in which still some individuals moved at night along the creek, although not as much. Their inability to go out and forage in the open forest that surrounds this area seems to be compensated by a behavioural feeding adaptation. A few gravid females were observed hunting for shrimp in the creek (Fig. 17), which we have not been able to observe in the rainforest and has not been recorded in other dietary studies conducted at lowland rainforest sites (Hodgkison and Hero 2003).

Probably the most surprising discovery is that the tracked individuals spend the day under the water in the stream, which has not been observed in any of the rainforest studies conducted so far. In one instance more than five hours were spend observing one individual, which did not come out of the water at all. This could have profound implications for chytrid dynamics in this system. In experimental work frogs exposed to flowing water had much less of a chance to get sick than the ones exposed to misty environments, typical of the rainforest (Alford et al. 2002). Zoospores are probably washed away under these conditions, which keep the winter infection load lower, and might avoid disease outbreaks.

Further research will show what the environmental threshold is for disease to develop, once this pathogen is endemic in a system. What is clear is that those dry forest environments are important conservation habitats since they seem to be refugia from severe chytridiomycosis leading to mortality. Their value probably increases, if they are adjacent to rainforest habitat. It would provide frog populations with a stock of animals that could potentially reinvade the rainforest habitat where selection for resistance to chytridiomycosis can occur. This selection process has been demonstrated by the development of resistance to avian malaria in Hawaiian birds.

Figure 2.24. The Kirrama field site (High elevation rainforest)

Figure 2.25. Blencoe falls (High elevation open dry sclerophyll forest). The waterfall frog population is mostly concentrated on the area shown in the picture.

Figure 2.26. Spurgeon wet (High elevation wet sclerophyll)

Figure 2.27. High elevation dry sclerophyll

Kirrama (High Elevation Rainforest)

Blencoe (High Elevation Dry Sclerophyll)
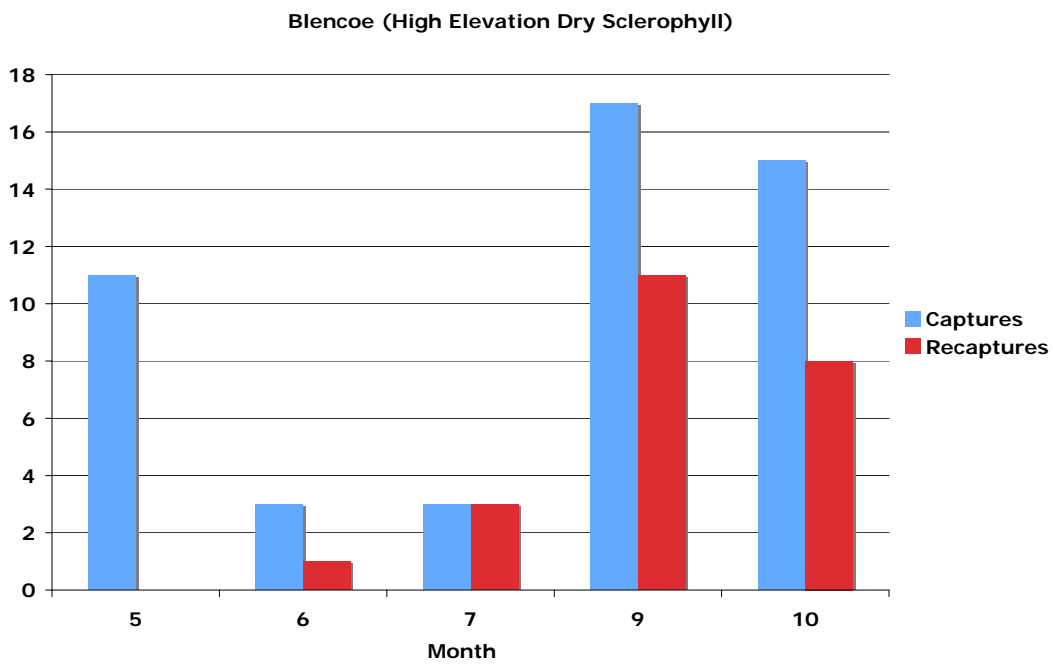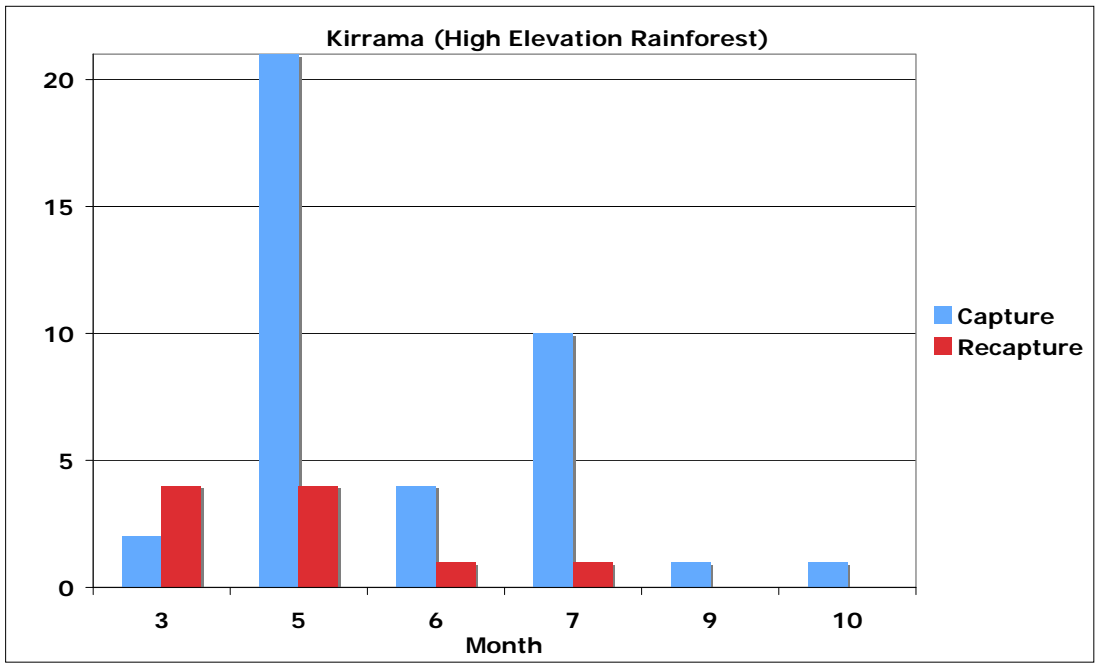
Figures 2.28-2.29. Capture and recapture of frogs during the winter months of 2007 at Kirrama and Blencoe falls
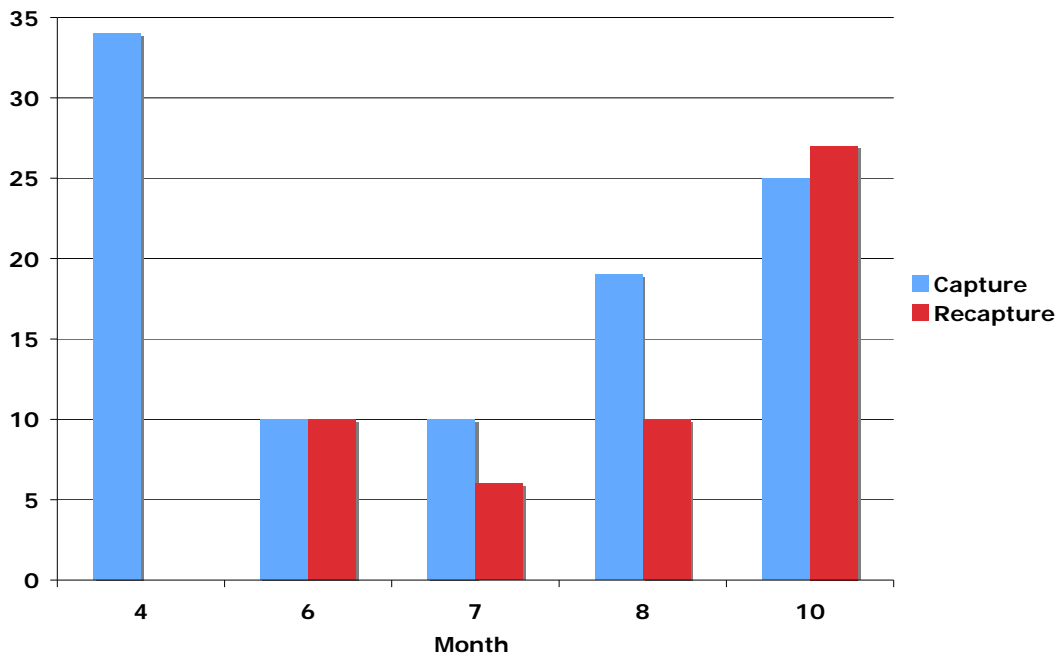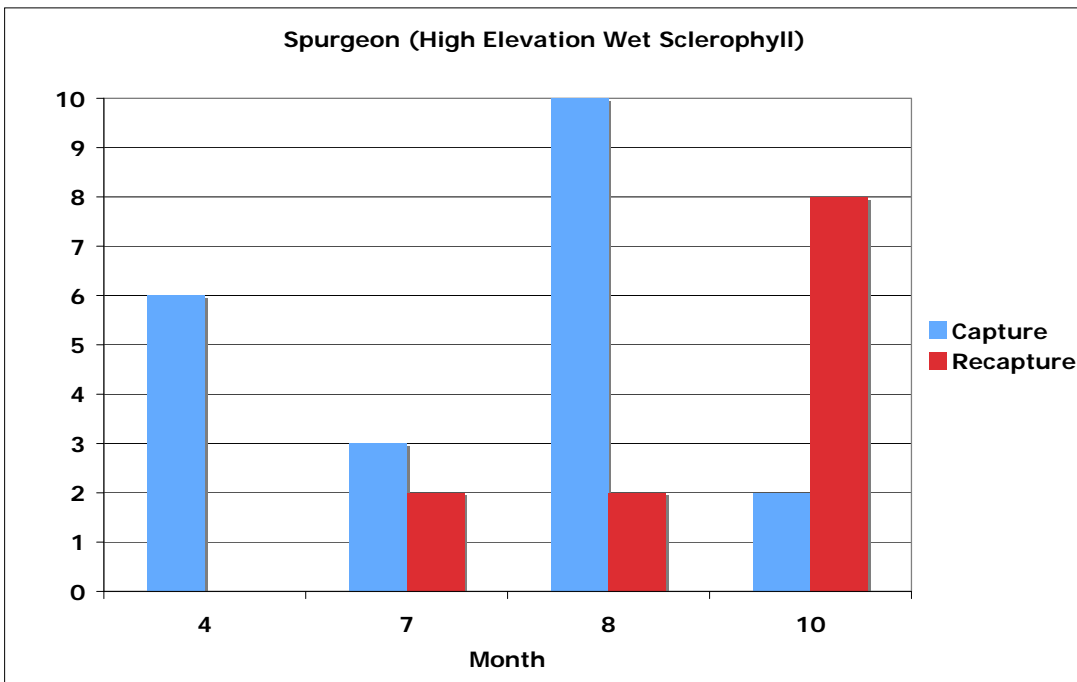
Figures 2.30-2.31. Capture and recapture of frogs during the winter months of 2007 at Spurgeon falls

Figure 2.32. Dead Green eyed tree frog (*Litoria genimaculata*) found floating at Kirrama

Figure 2.33. Dying *Litoria nannotis* found at Spurgeon falls (High elevation wet sclerophyll)

Figure 2.34. Temperature during the winter months of 2007 at the four different sites
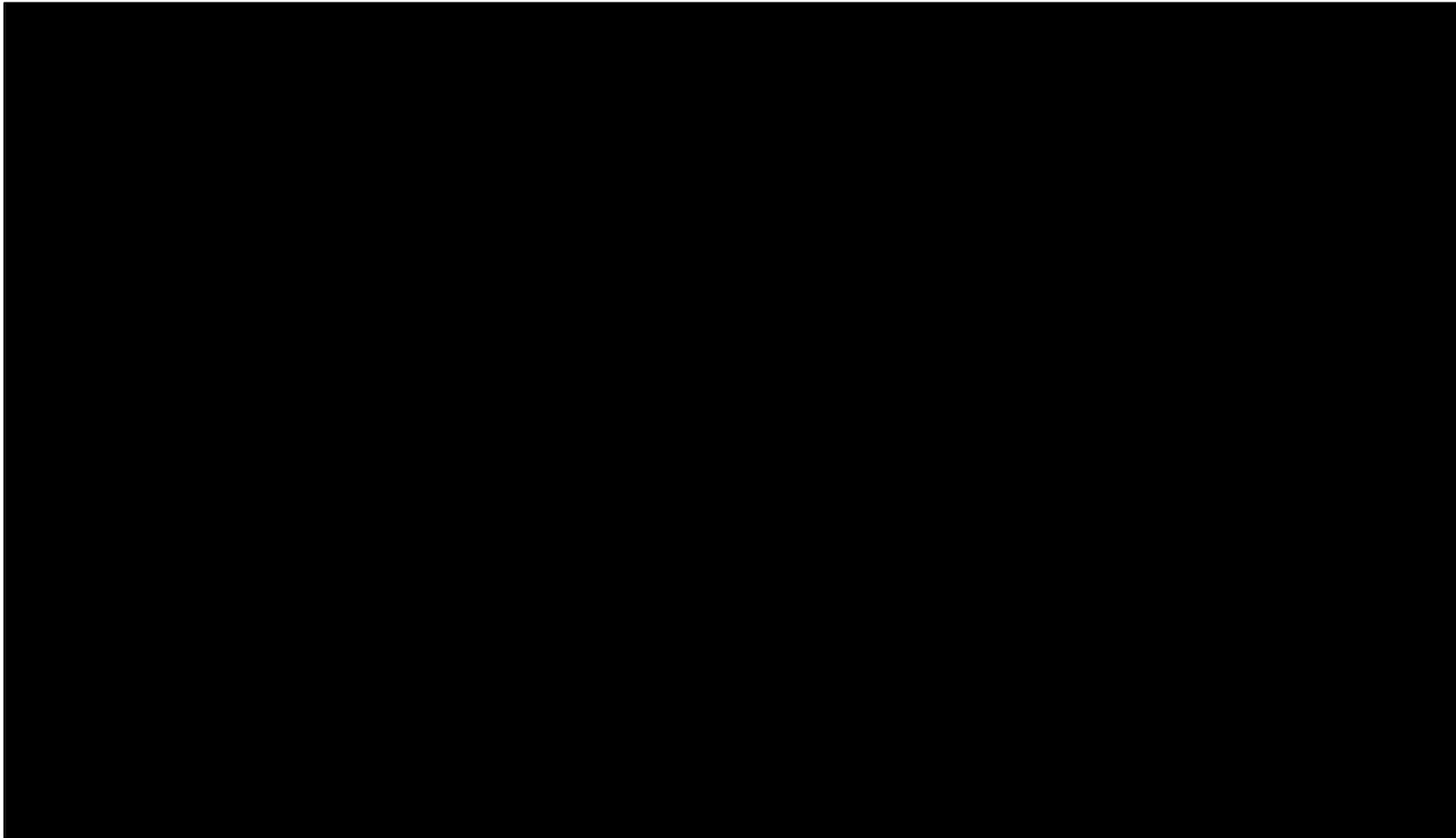
Figure 2.35. Temperature during the winter months of 2007 at the four different sites
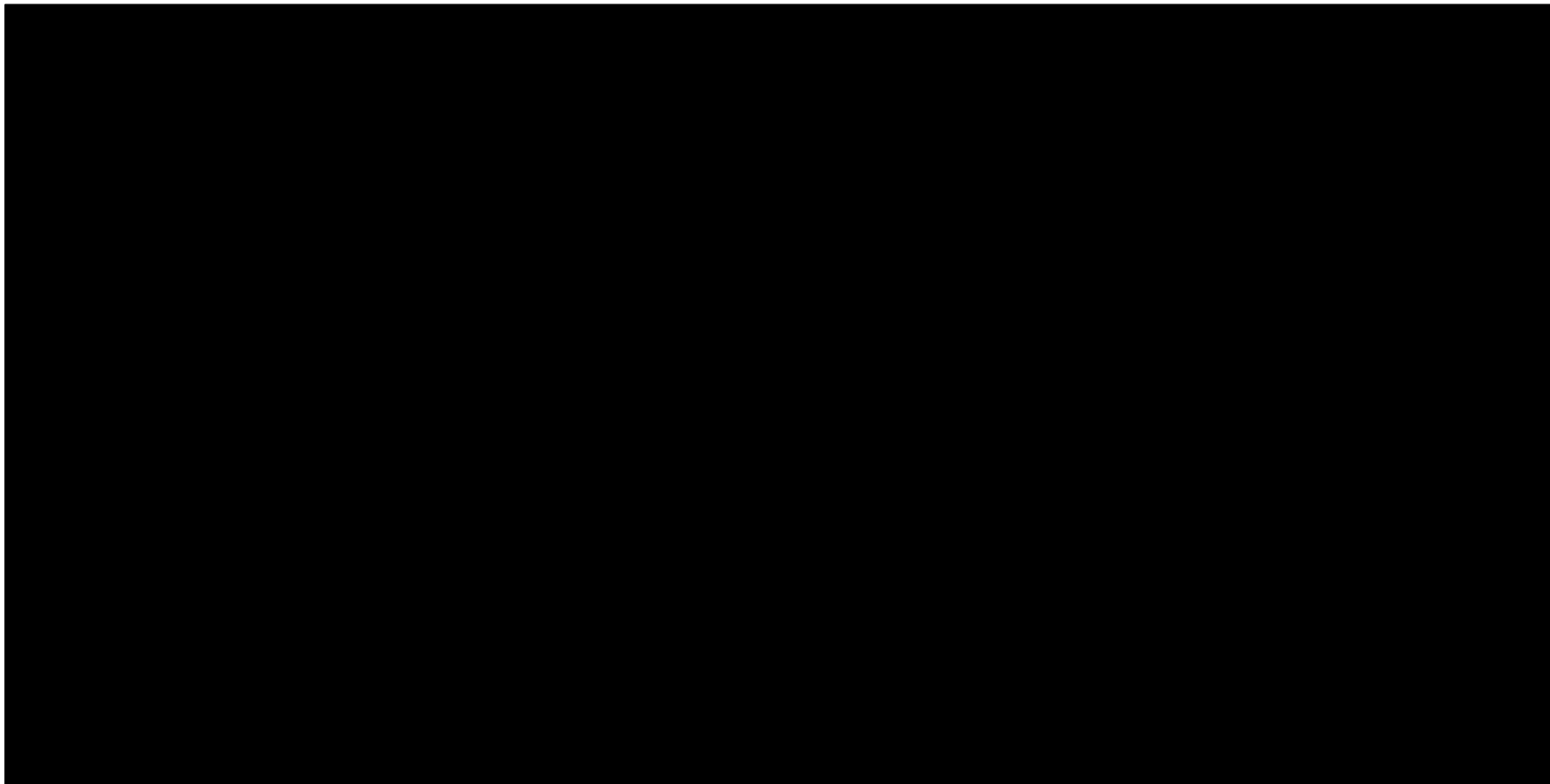
Figure 2.36. Waterfall frog hunting for freshwater shrimp in the dry sclerophyll
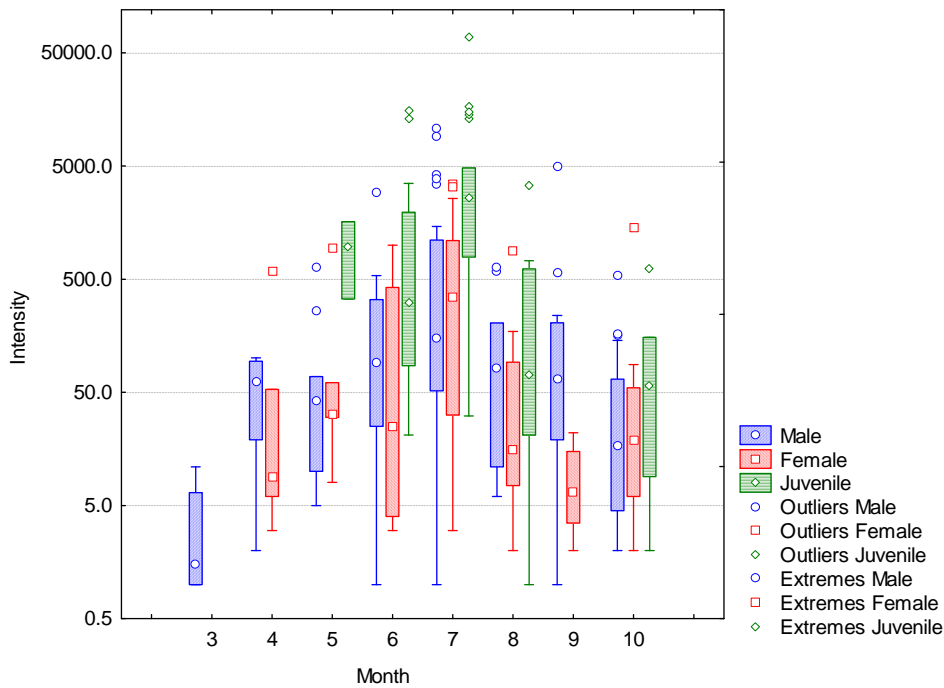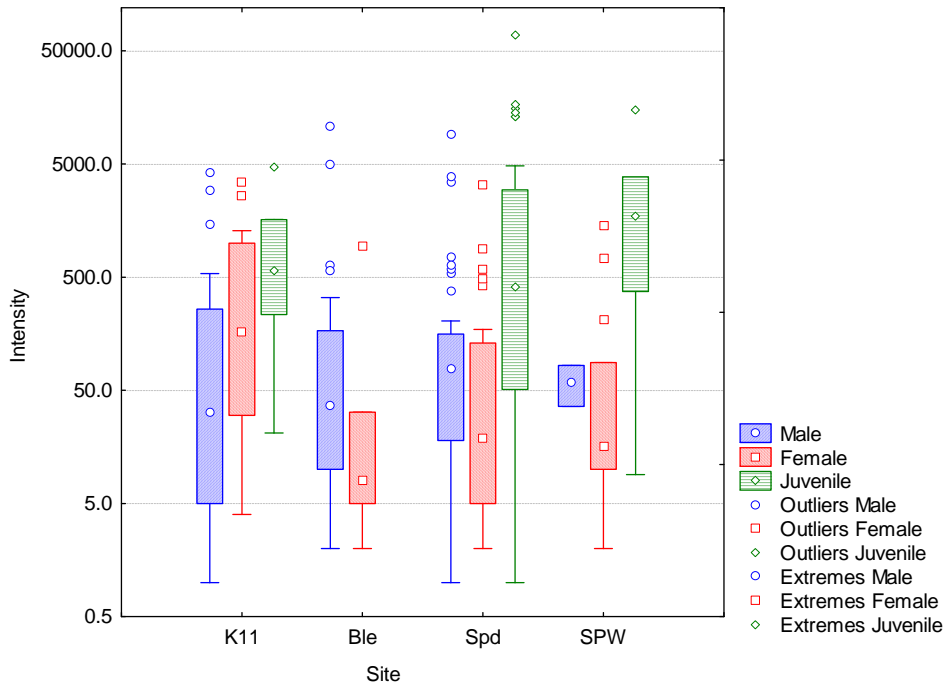
Figures 2.37-2.38. Intensity of infection in relation to site and time of the year stratified by age and sex.

Tadpoles

The effects of climate on the epidemiology of chytridiomycosis within tadpoles in rainforest streams appear to be minimal but are examined in Objective 7. Again temperature has a negative affect when water temperatures are highest around 25°C.

Objective 2.2: **Perform a case control study to determine risk factors for survival of *L. aurea* in NSW water bodies with a focus on heavy metal pollution and salinity.**

Objective 2:3: **Determine if there are differences in the growth rate of *B. dendrobatidis* in vitro using water from these sites.**

Objective 2.4: **If heavy metals or salinity appear to have a negative effect on *B. dendrobatidis*, perform experimental infections to evaluate the effect on the natural history of chytridiomycosis.**

This set of objectives (2.2-2.4) were designed to test the hypothesis that fresh water bodies with some level of salinity or with some heavy metals as pollutants could allow populations of susceptible species of amphibians to remain stable in the presence of Bd. The anecdotal evidence for this came from several sources, one in USA and several in Australia. The USA source was an observation reported at a conference in early 2000 that frogs in Californian ponds that were slightly saline survived while other frog populations in non-saline ponds in the same area declined. However, the evidence was scanty. Better evidence came from observations on the green and gold bell frog (*Litoria aurea*) in NSW which had suffered widespread decline (Mahony 1999). It existed in a number of isolated populations which in the past would have been classified in the past as marginal or unsuitable habitat prior to declines because of salinity or heavy metal pollution from mines or smelters (Ross Wellington, pers comm., 2004). After the discovery of *L. aurea* in a contaminated quarry at the Olympic site at Homebush in Sydney, the site was "cleaned-up" and made into ideal habitat. However, the *L. aurea* disappeared, possibly from chytridiomycosis. Similarly *Mixophyes balbus* in NSW has undergone severe declines, but a recently discovered population in the Southern Blue Mountains lives in water polluted by tailings of a mine. These populations are infected with *B. dendrobatidis*, but mortality is reduced.

The original proposal aimed to test the hypothesis that surviving *L. aurea* populations were in brackish water or in polluted water bodies, and that salinity and heavy metal pollution were having a greater impact on Bd and tipping the balance in favour of the host. The study had significant management importance as in theory it seemed that attempts to upgrade these water bodies, particularly strategies to decrease the level of pollution, may move the host-Bd equilibrium to again favour the pathogen and result in loss of the remnant host population.

Activity on these objectives: By the time the tender was awarded significant changes had occurred in opportunities to research this question. Ross Wellington, our major collaborator, had left the NSW Department of Environment and Conservation, making re-establishing this research connection difficult. Another potential collaborator, Mike Mahoney, at the University of Newcastle had already started a similar project and had received funds from other sources to do this work which was subsequently undertaken by a PhD student, Michele Stockwell. Their findings are included in Appendix 1. They show a negative effect of sodium in water bodies on prevalence, intensity of infection, mortality and population extinction due to Bd. A research group from the University of Woollongong, University of Sydney and Southern Cross University started work on heavy metals (Threlfall et al 2008). A third group, Frog Ecology and Behaviour Group, did intervention research on the effect of increasing salt content on survival success of *L. aurea* (White 2001). Hence, our proposed work under this tender with *L. aurea* in the Hunter-Sydney area became largely redundant owing to groups with closer proximity commencing work on the question.

Discussion

The Newcastle group are finalising their studies and preparing papers for publication. An initial publication (Stockwell et al 2008) described the outcome at a reintroduction site in the Hunter region where 53% of *L. aurea* juveniles tested positive for Bd. Subsequently, chytridiomycosis caused death of this species at this site. Follow up studies suggest that higher levels of sodium in water may protect this species from Bd and prevent population extirpation (Appendix 1). Threfall et al (2008) reported that sites with the largest populations of *L aurea* in the Sydney and Illawarra areas were contaminated by heavy metals, particularly copper and zinc, at levels that exceeded National Sediment Quality Guidelines. *In vitro* tests against Bd showed that copper and zinc inhibited fungal growth, but that this effect decreased with time after 6 days. Although these results supported the hypothesis that heavy metals could inhibit Bd, they were weakened by a failure to monitor frog populations and Bd presence and to include "control" sites were *L. aurea* had disappeared. An intervention study reported by White (2006) described how an artificial pond was filled with NaCl to give a concentration of approximately about 1 g/L and maintained at that level by addition of uniodised salt. Survival of 40 captive bred *L. aurea* released into the pool was high (no Bd related mortality) although growth was slower than expected. In the previous year all *L. aurea* had died with chytridiomycosis being identified as the cause in those examined pathologically. The trial suffered from the limitation of being uncontrolled, but it did provide some support at a low level of evidence for salinity playing a role in protecting *L. aurea* against chytridiomycosis.

References

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Threlfall CG, Jolley DF, Evershed N, Goldingay RL, Buttemer WA. Do green and gold bell frogs Litoria aurea occupy habitats with fungicidal properties? Australian Zoologist 2008;34:350-360.

White AW. A trial using salt to protect green and golden bell frogs from chytrid infection. Herpetofauna 2006;36:93-96.

Objective 3.   What is the minimum density of chytrid zoospores in natural water bodies required to infect species of native amphibians?

B. dendrobatidis occurs at low density wihin rainforest streams as shown in objective 1 and yet transmission occurs readily as shown in objective 7. Transmission is likely to occur at densities of zoospores below our current ability to detect them. Therefore, until methods to filter large volumes of water and concentrate zoospores to enable detection are developed we will be unable to answer this objective.

Developing methods to sample water bodies for Bd (this work has been published as Cashins et al. 2008a and submitted for publication)
Generating an accurate estimate of number of zoospores originally present in the water sample requires an understanding of how many zoospores are likely lost prior to entrapment on the filter membrane as well as how efficiently the DNA from the trapped zoospores can be extracted.   To accomplish this, identical concentrations of Bd zoospores were placed directly into 70% ethanol, directly onto filter membranes, as well as into a mild saline solution for subsequent filtration through filter membranes. By comparing the quantification of zoospores from each of these methods we can estimate the number of zoospores "lost" throughout the process. The ethanol sample was considered the "gold standard" as it was believed very few zoospores could be "lost" as they would be preserved immediately within the ethanol. Surprisingly, the least amount of DNA was extracted from the ethanol samples and the most DNA was extracted from the samples placed directly onto the membranes. An intermediate number was extracted from the samples placed in the saline solution and subsequently filtered. Even more surprising, the number of zoospores calculated to have been on the filter membrane far surpassed the number of zoospores initially placed on the membrane. These results suggest that filtration is a very efficient detection method and the 2 μm Millipore Isopore membranes are a good choice of membrane. They also suggest that the quantitative PCR results may vary depending both on how the sample was taken and how it was processed. This is important when quantitative comparisons between different types of samples and processes are made.

When sampling for the presence of Bd in the field using filtration and qPCR it is vital to prevent cross contamination of samples, as PCR is a very sensitive technique capable of detecting single zoospores. Therefore, it becomes necessary to not only disinfect filtration equipment between samples but also to denature any Bd DNA that may be present. Sodium Hypochlorite (active ingredient in bleach) has been found to effectively kill Bd and is used as a disinfectant of field gear. We tested a series of sodium hypochlorite concentrations and exposure times to determine if sodium hypochlorite can effectively denature Bd DNA. The results show that exposure to 12% sodium hypochlorite for 30 minutes will denature all Bd DNA. This will allow us to sterilise equipment in the field so that we may collect a series of water samples without cross-contamination.

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

Objective 4. **Does the density of chytrid zoospores in natural water bodies correlate with intensity of infection of amphibian populations living in those water bodies, and with the level of clinical chytridiomycosis? Can the density of these zoospores in natural water bodies be used to predict periods of high risk of infection for amphibian populations?**

The low density of *B. dendrobatidis* wihin rainforest streams as shown in objective 1 was not correlated with intensity of infection nor with clinical chytridiomycosis. The density of these zoospores could not be used to predict periods of high risk of infection for amphibian populations (see Objectives 1 and 7). Rather, density of infected tadpoles or climatic conditions for frogs were better predictors of risk (see Objectives 2.1 and 7).

Laboratory transmission experiments

Prior to conducting field-based investigations a laboratory experiment examined transmission of Bd to two native stream-dwelling tadpoles; *Litoria genimaculata* and *Litoria nannotis*. The *L. genimaculata* were reared to the larval stage from collected eggs. *L. nannotis* were captured as tadpoles. This experiment was designed to examine the progression of infection and mouthpart condition of infected tadpoles over time, the effect of infection on time to and size at metamorphosis and the effect of elevated temperatures on metamorph survival. Interestingly, none of the *L. genimaculata* tadpoles became infected following exposure to Bd zoospores in the water (0/61) and all but one of the *L. nannotis* tadpoles were infected prior to exposure (29/30). Therefore, it was not possible to compare the effect of infection on time to and size at metamorphosis. When infected *L. nannotis* tadpoles metamorphosed and first emerged from the water half were kept at 23°C and half were moved to 32°C. Survival in the 23°C treatment (2/10 survived) was significantly lower than in the 32°C treatment (7/9 survived; Fisher exact probability; p=0.0185). Mean days to death, however, did not differ. These results indicate that the timing, behaviour and habitat of infected L. nannotis following emergence may have a large effect on the survival rate of juveniles. Tadpoles that metamorphose when it is warmer and metamorphs that spend a greater percentage of time in warmer places may be more likely to shed infection.

Figure 4.1. Percentage survival and mean days to death in metamorphs of *Litoria nannotis* exposed to different temperatures with chytridiomycosis
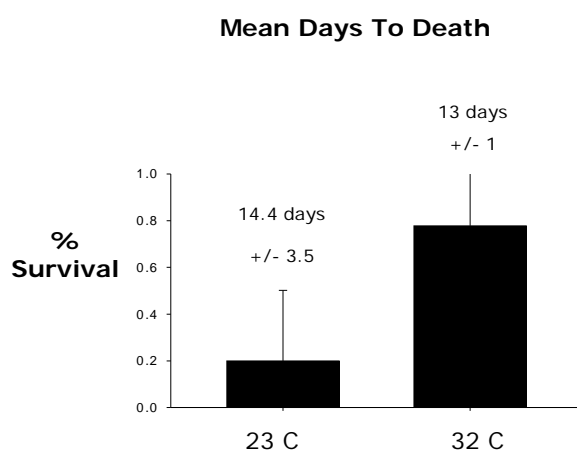
During the above experimental setup it was discovered that latex gloves could be lethal to tadpoles of these species. A series of experiments were conducted to determine which gloves were safe (see Cashins et al 2008 for publication of this work). Because all researchers working on amphibians are strongly encouraged and often required by permit guidelines to wear gloves to minimize the possibility of disease transmission it was important to determine which gloves are safe. Following a series of experiments, we found that latex, nitrile and vinyl gloves were all toxic to some degree.

However, well rinsed vinyl gloves caused no apparent harm. Well rinsed vinyl gloves were therefore used in all subsequent experiments when handling tadpoles was necessary. Gloves have not been found to be toxic to juvenile or adult frogs.

In the laboratory 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). *L. genimaculata* tadpoles were previously found to be resistant to infection following exposure to *Bd* in the water column. One half of *L. caerulea* tadpoles were allowed to graze on feeding plates with Bd actively growing on top of it (Fig. 4.2). 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 4.2. Tadpoles grazing on food plates containing Bd were more likely to be infected (35%, 7/20) than tadpoles that were only exposed to Bd in the water column (0%, 0/20).
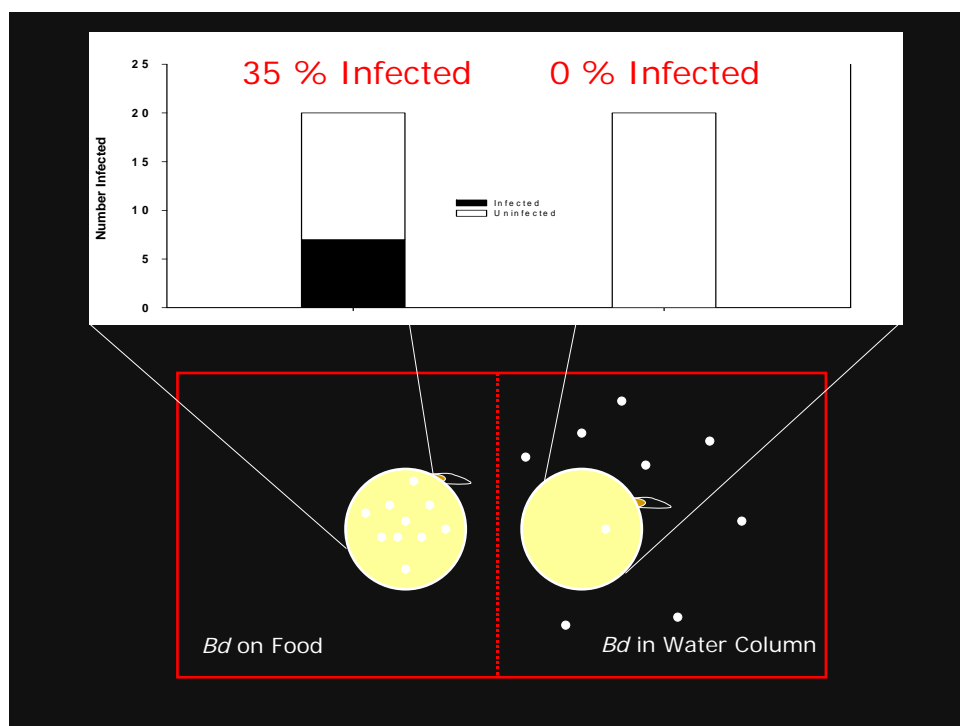
The faeces of some of these PCR positive tadpoles also contained low numbers of Bd zoospores. All infected tadpoles had low infection loads. These results suggest that the method of exposure to Bd may affect the risk of transmission. This is important as it may help explain differential susceptibility to infection in tadpoles of different species. Differential susceptibility as tadpoles could affect survival following metamorphosis. As discussed earlier, under the right conditions, infection as tadpoles can lead to a high rate of metamorph mortality. If tadpoles of a species are infected at a high prevalence the risk of maintaining infection through metamorphosis may increase. These results also indicate that the most efficient method of transmission in the field may be via contact with zoospores on the substrate during feeding as opposed to contact with zoospores in the water column.

In order to address some of these important questions we have developed and are presently trialling 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 (Fig. 4.3). Enclosures will allow us to investigate factors affecting transmission and maintenance of infection through metamorphosis.

Figure 4.3. Tadpole stream enclosure

References

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

Objective 5. **How does *B. dendrobatidis* spread between water bodies? Are there non-amphibian vectors of *B. dendrobatidis*?**

Objective 5.1: **River sand as a possible transport vehicle**

Through in vitro studies we show that potential means of translocation may be moist soil and bird feathers. *B. dendrobatidis* survived for up to 3 mo in sterile, moist river sand with no other nutrients added. *B. dendrobatidis* attached to and grew on sterile feathers and were able to be transported by feathers to establish new cultures in media, surviving between 1 and 3 h of drying between transfers. If these in vitro results are valid in the natural environment, the findings raise the possibilities that *B. dendrobatidis* may be translocated by movement of moist river sand and that birds may carry the amphibian chytrid between frog habitats. However, further studies using sand and feathers containing normal microflora are essential. (This work is published, see Johnson and Speare 2005)

References
Johnson M, Speare R. Possible modes of dissemination of the amphibian chytrid *Batrachochytrium dendrobatidis* in the environment. Diseases of Aquatic Organisms 2005;65:181-186.

Refer to work in Objective 1 on testing stream sand for presence of *B. dendrobatidis* and survival and growth of *B. dendrobatidis* in the presence of other microflora.

Objective 5.2: **Birds as possible vectors**

Summary

Although *B. dendrobatidis* appears to spread as an epidemic wave across newly invaded environments, in its passage along coastal Queensland north from southeast Queensland it traversed areas that appeared unsuitable for ground or water based movement. This suggested that animal carriers, not limited by suitable water bodies, could carry *B. dendrobatidis* across apparently hostile regions. We proposed the hypothesis that birds could be a suitable mechanical carrier of *B. dendrobatidis*. Initial *in vitro* experiments confirmed that Bd could grow on sterile feathers and that Bd could be reisolated from feathers held in air for up to 3 hours (Johnson and Speare 2005). This result confirmed that theoretically an infected bird could transport Bd for a flight time of 3 hours, which depending on species could be 20 km or more. We therefore examined whether *B. dendrobatidis* could grow on a live bird, a much more demanding environment than sterile feathers in a laboratory.

We used three ducklings to investigate whether water birds could spread the fungus into new areas after coming into contact with contaminated water. Ducklings were exposed to water containing *B dendrobatidis* zoospores for 4 hours, and then removed. The feathers and feet were sampled at 6 time intervals, ranging from one minute to 24 hours post zoospore exposure. Swabs were taken from feet and feathers clipped from the ventral surface. For each sample realtime PCR was performed to detect nucleic acid of *B. dendrobatidis* and isolation on agar was attempted. The temperature of the feet and feathers were measured by an infrared device.

B dendrobatidis was not isolated from any of the samples. Contamination due to bacterial and fungal overgrowth was a problem. Positive PCR results were obtained for feathers at 1 min, 30 min and 24 hr and for feet at 2 hr and 3 hr. a number of the feather and feet samples were positive for *B dendrobatidis* DNA. Temperature of the ducks feet and feathers did not exceed 25°C and 27.5°C throughout the experiment.

The experiment demonstrated that DNA of *B dendrobatidis* could be detected on both feathers and feet of ducks immersed in water containing zoospores, but that detection was not consistent over 24 hours. *B dendrobatidis* was not isolated either because it was not viable or because it was outcompeted by bacterial and fungal contaminants.

During the experiment, the temperature of the ducks' feathers and feet remained low enough for *B. dendrobatidis* survival, so it is possible that the *B. dendrobatidis* detected by PCR was in fact alive and viable. An improved technique for isolating *B dendrobatidis* in the presence of other microroganisms is required to answer this question.

Papers published under this objective: Johnson M, Speare R. Possible modes of dissemination of the amphibian chytrid *Batrachochytrium dendrobatidis* in the environment. Diseases of Aquatic Organisms 2005;65:181-186.

Introduction

Once introduced into a new region the fungus *Batrachochytrium dendrobatidis* causes an epidemic that spreads between 15-100 km per year (Laurance *et al* 1996, Lips et al 2008). Although *B. dendrobatidis* appears to spread as an epidemic wave across newly invaded environments, in its passage along coastal Queensland north from southeast Queensland it traversed areas that appeared unsuitable for ground or water based movement (Laurance et al. 1996, 1997, Alford and Richards 1997, Hero and Gillespie 1996). This suggested that animal carriers, not limited by suitable water bodies, could carry *B. dendrobatidis* across apparently hostile regions. We proposed the hypothesis that birds could be a suitable mechanical carrier of *B. dendrobatidis*. We tested this by exposing sterile feathers to 1) zoospore solutions and 2) allowing zoospores to grow on sterile feathers. We discovered that feathers which had been exposed to zoospore solutions for only one minute could harbour viable *B. dendrobatidis* zoospores for up to 1 hour after being removed from the solution and left to dry. Sterile feathers on which zoosporangia had grown could harbour viable *B. dendrobatidis* whilst drying for even longer, up to 3 hours. This work has been published (Johnson and Speare 2005).

There is a significant difference in microenvironment between a sterile feather in a laboratory petri dish and a feather on a live bird. The major differences we could anticipate in theory were 1) competition between bacteria and fungi and possible arthropods on the feathers and *B. dendrobatidis*; 2) presence of antifungal compounds on feathers on the bird's body that would inhibit or kill *B. dendrobatidis*; and 3) a surface temperature on the bird that would be too high for growth and survival of *B. dendrobatidis*.

However, if *B. dendrobatidis* did survive on the feathers or feet of aquatic birds, it could be possible for the birds to act as mechanical carriers of the fungus. For example, a duck could enter a water body in which the fungus is present, and then fly to a new water body where it previously did not occur. The following experiment used live ducklings to test the hypothesis that water birds could introduce the fungus into new areas.

Methods

Ducks:

We used three young muscovy ducks from the same clutch for this experiment. The ducks were kept outdoors prior to the experiment and were fed "turkey starter". At the time of the experiment they were aged about 3 months and each weighed approximately 1300 g. For identification they were marked across the shoulders using a permanent pen.

Exposure solution:

Zoospores were obtained by growing a *B. dendrobatidis* (Tully-Lrheocola-06-LB-1 , P14) culture on TGhL agar plates for 2 days. The plates were flooded with sterile "dilute salts" solution (10^{-3} M KH_2PO_4, 10^{-4} M $MgCl_2$, 2×10^{-5} $CaCl_2$ in water), which was collected after a few minutes wait. A total of 8 mls was added to each plate. An initial 5 mls was added and left for 10 mins before collection. After examination of these plates revealed that there were still zoospores remaining, an extra 3 mls was added, and left for 5 minutes. The concentration of the collected solution was estimated by counting zoospores on a haemocytometer.

The ducks were exposed in tall, cylindrical plastic clothes hampers (Fig 5.1). The containers were thoroughly washed to remove any residues. Ten L of tap water was added to each of the containers the day before the experiment to allow aging. The temperature of the aged tap water was measured and 140 ml of zoospore solution was added, just prior to the ducks being immersed.

Exposure of ducks:
The experiment was performed in a room with air-conditioning set at approximately 20°C. The ducks were cleaned quickly by wiping with a paper towel and then placed in the container holding the diluted zoospore solution. The 10-14 L of water in the container was approximately 13 cm high and resulted in the lowest part of the duck's body (not including the tail) which was approximately 9 cm above the ground, being submerged by about 4 cm. The water level was such that the whole legs and tail, plus the underside of the belly were submerged. The ducks were left in the zoospore solution for four hours.

Sampling of the ducks:
Ducks were sampled before they were exposed to the zoospore solution, and these feathers and feet swabs acted as negative controls. After four hours in the zoospore solution, the ducks were removed. To facilitate handling, the ducks were caught and held using an individual towel for each duck which was reused at each handling. The duck handlers wore gloves. Gloves were changed between ducks and between sampling times to avoid any possible cross-contamination. However, this may have inadvertently removed some of the liquid from the feathers/feet. Ducks were held for one minute, and then the first sample was collected (T=1 min). Ducks were turned upside down and feathers clipped from the underside of the belly which had been submerged in water. Feathers were collected in a sterile petri dish. Each duck had separate scissors, and these were washed between each sampling period with water and sprayed with 70% ethanol. The feet were swabbed, one swab being for PCR analysis, and the other used for isolation. The external temperature of the ducks was measured using an infrared temperature gun. Temperature was measured around the area feathers were sampled, as well as the webbing of the feet and the toes. The ducks were then placed in an upside down washing basket to keep them separated from each other. They were kept in the air-conditioned room throughout the experiment. Ducks were resampled as described at 30 mins, 60 mins, 120 mins, 180 mins and 24 hours post exposure. At 1 week feathers were sampled for PCR only. Up until 180 minutes post exposure, ducks had no access to food or water. After this time they were given a small bowl of water, and a dish of "turkey starter".

Detection of *B. dendrobatidis*
Pilot experiments on techniques showed that *B. dendrobatidis* could be isolated from non-sterile feathers simply by placing the feathers in broth or agar that contained antibiotics. We used three types of agar to maximise the chance of isolating *B. dendrobatidis*. In total there were four methods used to try and isolate *B. dendrobatidis* from feathers. These were:
 1. Placing feather into a well (in a 12 well plate) containing 1ml of tryptone broth
 2. Placing feather on a tryptone agar plate
 3. Placing feather on a TGhL agar plate
 4. Placing feather on an R2A agar plate

Tryptone broth and agar plates consist of 16g tryptone per 1000 ml, with the plates containing 10 g agar per 1000 ml. TGhL agar plates 16 g tryptone, 2 g gelatin hydrolysate, 4 g lactose and 10 g agar per 1000 ml. R2A agar plates were made with 18.2 g R2A (Difco) agar per 1000 ml. The swab from the feet were smeared across the surface of a TGhL agar plate. This was done in close vicinity to the flame of a gas torch to reduce contamination. These feather samples and feet smears were examined at approximately 4 hours, 24 hours and 48 hours after being placed in broth or on agar.

The second feather sample and feet swabs were submitted for real-time Taqman PCR. Feet swabs were processed following the protocol outlined in Boyle et al. (2004) and Hyatt et al. (2007), with some modifications. These are the same methods used to detect chytridiomycosis in amphibians. The feathers underwent a slightly altered process based on pilot trials. During the "extraction" stage, they were treated with double the usual mechanical extraction. They underwent twice the bead beating, and twice the centrifuging that a standard amphibian swab would go through.

Results and discussion
Exposure:
The zoospore solution collected from the agar plates had a final concentration of 115,000 per ml. 140 ml of this solution was added to 10 L of aged tap water, to give a final concentration of 1,587 zoospores per ml (1,587,000 zoospores per L). High concentrations were necessary as preliminary experiments demonstrated that feathers exposed to lower concentrations were less likely to have *B. dendrobatidis* isolated back from them.

When the ducks were first put into the containers, they used their beaks to splash water over the top of their bodies. During the four hours in the exposure solution, all ducks defecated several times and by the end of exposure, the water was quite dirty.
Feathers within the body region that was sampled remained wet up to about 30 minutes post exposure.

Detection of *B. dendrobatidis*:
As is to be expected, no *B. dendrobatidis* zoospores were seen in the negative control feathers or feet smears, taken before the experiment. As for the post exposure samples, none of the feathers or feet smears yielded *B. dendrobatidis*, regardless of the isolation method. The feathers were examined using an inverted microscope at approximately 4 hrs, 24 hrs and 48 hours after they were sampled from the ducks and inoculated onto media. Even the feathers sampled at one minute failed to yield *B. dendrobatidis*. Most feathers did, however, transport mites as well as cell debris to the agar plates. The plates were not contaminated at 4 hours post inoculation, but by 24 hours post inoculation, some of the agar plates were overrun with contaminants (mostly bacterial, and some fungal). The feathers most likely to become contaminated were those sampled whilst still damp. For example, all of the 1 min post exposure feathers, as well as some of the 30 and 60 minute post exposure feathers were contaminated by 24 hours. The feathers and smears were definitely overrun with contaminants by 48 hours. If *B. dendrobatidis* had been present in the samples, it would have been outcompeted by contaminants by 48 hour.

B. dendrobatidis was detected by PCR on feathers and feet, but not consistently (Tables 5.1, 5.2). Two of the 18 samples from feet, and five of the 18 samples from feathers were positive for *B. dendrobatidis* (Table 5.1). Foot swabs were positive only at 2 and 3 hrs. PCR were positive for feathers at 1 min and 30 mins and at 24 hrs (Table 5.2). At 1 min and 30 min, the feathers were still wet from the exposure solution.

The temperature of the duck's feet and feathers remained below 28°C at all times (Table 5.3), with average±std for feathers, webbing and toe of 24.3±2.2, 21.6±1.4 and 22.6±1.7 °C respectively.

Discussion

The attempt to infect feathers on live birds showed that *B. dendrobatidis* could transfer from water to feet and feathers. Persistence over time was also proven with nucleic acid detected at 24 hours on feathers and 3 hours on feet. The detection at 24 hours suggests that multiplication may have occurred, but does not confirm it, and failure to detect *B. dendrobatidis* at one week indicates that *B. dendrobatidis* did not grow on the feathers over the intervening six days. In addition, although there was no possibility of cross-contamination between ducks, it is possible that the towels used for handling individual ducks [wet after the first handling] could have kept some *B. dendrobatidis* alive which in turn could have been re-transferred to the duck on subsequent handlings. However, detection was inconsistent over sampling times and zoospore equivalents were low (Figs 5.1, 5.2). *B. dendrobatidis* was not isolated at any time. Explanations for the failure to isolate *B. dendrobatidis* could be that i) the isolation technique was unsuitable; ii) competition between *B. dendrobatidis* and bacteria inhibited growth of the chytrid fungus on the agar plates; and iii) *B. dendrobatidis* on the feathers and feet was dead. Isolation of *B. dendrobatidis* from frog skin is difficult (Longcore 2000) and rarely achieved since the chytrid fungus seems "reluctant" to grow initially. This may have been a factor in this case. Bacteria will inhibit growth of *B. dendrobatidis* (Longcore 2000, Harris et al 2006) and as overgrowth of bacterial contamination did occur, this may have been an inhibiting factor if *B. dendrobatidis* was alive. Finally, *B. dendrobatidis* may have been dead. Peptides on the skin of amphibians and humans are effective at killing *B. dendrobatidis* (Rollins-Smith et al 2002, Mendez et al 2008). We could find no information in the literature about whether antifungal peptides occurred on bird skin and feathers. An interesting finding was that when these ducks were kept in an ambient temperature of 20°C, the temperature of their feet and feathers was low enough to allow *B. dendrobatidis* to survive and grow.

Conclusion

This experiment failed to demonstrate that ducks could transport viable *B. dendrobatidis* after being exposed to an infected water body. However, it did show that *B. dendrobatidis* could be detected on feathers and feet, but this was inconsistent. The findings do not confirm or negate the hypothesis that birds may function as mechanical carriers of *B. dendrobatidis*. More sophisticated studies may be justified.

Table 5.1. PCR results of feet swabs expressed as zoospore equivalents

	time post exposure						
	pre exp	1 min	30 min	60 mins	120min	180min	24 hr
duck A	0	0	0	0	5	0	0
duck B	0	0	0	0	0	6	0
duck C	0	0	0	0	0	0	0

Table 5.2. PCR results of feathers expressed as zoospore equivalents (Average weight of feather is: 0.0029 g)

	time post exposure							
	pre exp	1 min	30 min	60 mins	120 min	180 min	24 hr	1 wk
duck A	0	21	0	0	0	0	12	0
duck B	0	3	10	0	0	0	0	0
duck C	0	4	0	0	0	0	0	0

Table 5.3. Average temperature during sampling

		time post exposure			
		60 mins	120 mins	180 mins	24 hours
duck A	body	27.5	24.5	25	19.5

	webbing	20	22	21	20
	toe	25	22	23	20
duck B	body	26	24	23.5	21.5
	webbing	21	23	24	21
	toe	21	25	25	22
duck C	body	26.5	25.5	25.5	23.5
	webbing	23	21	23	20
	toe	22	22	23	21

Figure 5.1. Ducks being exposed to zoospore solution and after exposure.

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Additional work not included in Objective 5:

Crustaceans are not hosts of *Batrachochytrium dendrobatidis* (this work is published as Rowley et al 2007)

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Eastern water dragons (*Physignathus lesueurii*) (Reptilia: Agamidae) are not important alternate hosts of the frog chytrid fungus *Batrachochytrium dendrobatidis*

Introduction

B. dendrobatidis DNA has been detected in environmental substrates such as pond water (Kirshtein et al. 2007; Walker et al. 2007) and on the surface of streamside rocks or boulders (Lips et al. 2006), and is regarded as a possible environmental source of infection which stream-associated amphibians

are likely to have contact with. To date, alternative hosts of *B. dendrobatidis* have not been demonstrated (Rowley et al. 2007) however cultures of *B. dendrobatidis* can grow on snake skin (Piotrowski et al. 2004; Windmiller and Robbins unpubl. data), suggesting that susceptible reptiles associated with water bodies may act as both reservoirs of infection and vectors for spread of the disease. The eastern water dragon, *Physignathus lesueurii* (Gray, 1831), is a semi-aquatic, arboreal lizard that perches on emergent rocks and tree branches over hanging creeks and rivers. When disturbed, *P. lesueurii* drops into the water to escape. Its diet includes frogs (Cogger, 1994). The adults are fairly sedentary, moving an average of 76m between captures, however because the recapture rate is low it appears that time spent in habitats where capture is more likely, such as streams, may be infrequent (Thompson 1993). This study surveyed *P. lesueurii* along streams with permanent anuran populations known to be infected with *B. dendrobatidis*, to determine the likelihood of this reptile acting as an important alternate host for the pathogen. We regarded that water dragons would be an important alternate host if infection prevalences in water dragons were similar to those of anurans in the same locations.

Materials and Methods

We captured *P. lesueurii* after chance encounters during surveys of stream-associated anurans in Murray Upper National Park (18º11′S, 145º52′E, elevation 250 m) in the Wet Topics of Australia. Dragons were caught by gloved hand and the ventral surface and inguinal folds swabbed with an MW100 tubed dryswab (Medical Wire and Equipment Co (Bath) Ltd). We wore latex gloves, changed between animals, to prevent transfer of pathogens. Swabs were labelled and stored at <10 °C in the field for up to five days, prior to transport to James Cook University and storage at -80 °C until processing.

A real-time TaqMan® (Applied Biosystems) PCR assay was used to detect *B. dendrobatidis*. The assay followed the protocol described by Boyle et al. (2004) with the following modifications. The analysis was performed on the Rotor-Gene™ 6000 (Corbett Research) using Gene-Disc 100 tubes. A 15 μL reaction volume was produced by loading 10 μL of PCR reaction mix and 5 μL of the diluted sample extract, standard, diluted negative control, or water into Gene-Disc tubes with a CAS-1200™ pipetting robot (Corbett Robotics). The sample extract and negative control were diluted one in 10. Triplicate analyses were performed for each sample, negative control and no-template-control, and quadruplicate analyses undertaken for each standard (100, 10, 1, 0.1 zoospore equivalents). The master mix included the addition of 400 ng/μL of bovine serum albumin (BSA) in order to reduce inhibition of PCR by substances such as humic acids and other inhibitors (Kreader 1996, Garland et al. 2009).

In order to test for false negatives, due to inhibition of the PCR assay, we performed a repeat triplicate analysis incorporating the TaqMan® Exogenous internal positive control (IPC) (0.6x Exo IPC Mix, 0.6x Exo IPC DNA) (Applied Biosystems) into the assay (Hyatt et al. 2007). An extra one in 100 dilution of the extract was performed for subsequent reanalysis when Ct differentials between the sample and the negative control were greater than three. Inhibition would be indicated by Ct values higher than those obtained for the negative control, so a one-sample t-test was performed to compare the sample Ct values. We calculated the exact confidence interval for the proportion of infected water dragons, with the assumption that the proportion infected could be any number in the range of zero to one, with all possibilities being equally likely.

Results

We caught a total of 15 juvenile *P. lesueurii* at Murray Upper National Park in October 2006 (n=8), December 2006 (n=2) and October 2007 (n=5). Captures were limited to opportunistic encounters during routine monitoring of stream-associated anurans.

None of the swabs of these dragons returned positive results for *B. dendrobatidis* by qPCR assay in any of the triplicate wells. These results were not false negatives, as the PCR assay was not inhibited. The Ct value for the swabs from *P. lesueurii* analysed by PCR assay did not vary significantly from the Ct value of the negative control (t=0.616, *P*=0.556, df=12) (see Table 1). The qPCR assay for one swab was repeated in an additional run due to a software analysis failure. The assay for an additional swab was inhibited at the standard concentration and diluted to one in 100. The Ct values for both of these swabs (see Table 1) were lower than that of the negative control and therefore inhibition did not occur at this greater dilution. The exact 95% shortest confidence interval for this 0% estimate of prevalence of *B. dendrobatidis* in water dragons is 0-17%.

Discussion

Juvenile *P. lesueurii* were captured in periods of high prevalence of *B. dendrobatidis* among stream dwelling frogs (Table 2), indicating that the chytrid fungus was present in aquatic habitats. However, all *P. lesueurii* were negative for *B. dendrobatidis* by qPCR assay. Although the upper 95% confidence limit for the 0% prevalence estimate of *B. dendrobatidis* in water dragons was 17% this was much lower than the mean prevalence in frogs of 46% and the lower 95% confidence limit of 36% for frogs at similar times of the year. Therefore, we concluded that water dragons are not important alternate hosts and ceased sampling. However, we cannot rule out low infection prevalences in water dragons. It is unlikely that innate susceptibility to infection varies greatly in different populations of water dragons as this does not occur within species of amphibians. Determining if low prevalence of infection may occur in water dragons would require much larger sample sizes, which are difficult to obtain, or experimental infection trials. The cost versus the benefits of this latter study indicates that it is not currently warranted given the other immediate priorities for research on *B. dendrobatidis*.

Table 5.4. Evidence of lack of inhibition of the PCR assay performed on swabs from *Physignathus lesueurii*. A one-sample t-test (t=0.616, *P*=0.556, df=12) demonstrated that Ct values of the swabs did not vary significantly from the Ct value of the negative control (Mean±StDev= 27.43±0.71).

Dragon #	Replicate Ct Value (calculated from 3 replicates)	Replicate Ct StDev	Notes
1	27.15	0.29	
2	28.21	0.75	
3	27.25	0.76	
4	28.26	0.24	
5	28.18	0.26	
6	27.18	0.07	Ct value of sample statistically compared with Ct value of negative control
8	27.49	0.52	
9	26.81	0.27	
10	26.93	0.61	
11	28.69	0.28	
13	27.08	0.39	
14	27.18	0.10	
15	27.49	0.87	
7	26.87	0.45	Initial software analysis failure, sample repeated in second PCR assay.
12	26.56	0.28	One replicate initially inhibited, PCR assay repeated at 1 in 100 dilution

Table 5.5. Prevalence and intensity of infection of *Batrachochytrium dendrobatidis* in stream-associated anurans in the Wet Tropics.

Month, Year	Location	# Anurans Sampled	Prevalence of *Bd*	Intensity of Infection (Mean # zoospore equivalents, Range)
Oct-Dec, 2006	Murray Upper National Park	113	37%	343, 1-4232
Oct, 2007	Murray Upper National Park	57	54%	213, 1-23,771

As *B. dendrobatidis* is capable of utilising reptilian skin as a nutrient source (Piotrowski et al. 2004, Windmiller and Robbins unpubl. data), our results suggest that the preferred habitat of *P. lesueurii*, such as the forest, limits its exposure to contaminated substrates or, if transmission occurs, does not readily allow progression to infection. This is because *B. dendrobatidis* is sensitive to desiccation (Johnson et al. 2003) and similarly prevalence among forest-dwelling frogs is far less than among stream-associated frogs (Speare et al. 2005; Kriger and Hero 2007). Alternatively, *P. lesueurii* may be an unsuitable host of *B. dendrobatidis*. It appears that stream-associated reptiles such as *P. lesueurii* are not important alternate hosts for *B. dendrobatidis* and are therefore unlikely to be acting as a major reservoir of infection or transporter of the pathogen.

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Figure 5.2. Juvenile Eastern Water Dragon.

Objective 6.   Can *B. dendrobatidis* be eradicated from small natural and artificial water bodies (eg farm dams or backyard ponds)? If eradication is considered possible/practicable, can we identify potential approaches to eradicating chytrid from such water bodies.

Eradicating *B. dendrobatidis* using chemicals. A total of six products tested: Trigene, F10 , Betadine, Phytoclean, Terbinafine and Forexpan fire fighting foam.

B. dendrobatidis could possibly be eradicated from small water bodies using chemicals which have a disinfectant effect against the organism. We have recently investigated the ability of six different products to kill *B dendrobatidis in vitro*. Each of the products tested was intended for purposes other than eradicating *B dendrobatidis* from water bodies. For example, they were tested at concentrations and exposure times applicable to washing tools or treating amphibian larvae etc. However, very effective products could be considered useful for disinfection of whole water bodies. Three of these products have shown positive results, killing *B. dendrobatidis* at low concentrations. The other three were ineffective against *B. dendrobatidis*, or only effective at high concentrations, and should not be considered as possible disinfectants.

CHEMICALS EFFECTIVE AGAINST *B.DENDROBATIDIS*

Trigene, F10sc and Betadine
The findings below are published, see Webb et al (2007).
Identifying efficient and practical disinfectants effective against *B. dendrobatidis* is important to reduce the spread of the disease both in the wild and captivity (Australian Government Department of Environment and Heritage 2006). Our previous studies (Johnson *et al* 2003) investigated the efficacy of 8 disinfectants against *B. dendrobatidis*, and showed acceptable levels of activity for 4, with those containing didecyl dimethyl ammonium chloride (DDAC) being active at the lowest concentration (1 ml L $^{-1}$). Since then, we have evaluated the suitability of three additional disinfectants: two (TriGene Virucidal Disinfectant Cleaner and F10 Super Concentrate Disinfectant) are mixtures of chemicals and one (Betadine Antiseptic Liquid) contains a single active ingredient, povidone iodine. The experiment followed protocol 2 (Johnson et al. 2003), except for minor modifications. Sterile 96 well plates were inoculated with *B. dendrobatidis* zoospores and left to grow for 4 days. The wells were exposed to a range of concentrations of the disinfectants for 1, 5 and 10 minutes. Concentrations recommended by the manufacturer were used as the starting point, and the disinfectants diluted until *B. dendrobatidis* zoosporangia survived. One hundred percent kill of all 8 replicates was required for the treatment to be considered effective. All disinfectants had a 100% efficacy at concentrations recommended by the manufacturers. The lowest concentration capable of 100% kill after exposure for 1 minute was 0.1 ml L^{-1} for TriGene, 0.33 ml L^{-1} for F10 and 100 ml L^{-1} for Betadine. TriGene is the most effective disinfectant found so far, and both TriGene and F10 are more effective than various disinfectants tested in previous studies (Johnson et al 2003). TriGene and F10 are recommended for use in the field over the previously recommended DDAC products, as they are active at much lower concentrations and appear to have no record of environmental toxicity. Any of these three disinfectants is recommended instead of bleach (which seems to be the most commonly used) due to their efficacy at lower concentrations and less hazardous qualities.

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Phytoclean

In 2007 Annie Philips (DPIW-Tasmania) asked that the disinfectant Phytoclean be tested for any disinfectant effect against *B dendrobatidis.*

The plant disease Phytophthora root rot or "die back" is widespread in Tasmania and is putting a number of plant species and communities at risk. The disease is caused by the fungus *Phytophthora cinnamomi* (Rudman 2005). To prevent further spread of the disease within Tasmania, the Department of Primary Industries and Water have developed guidelines which involve washing down machinery, vehicles and equipment when leaving affected areas. The disinfectant "Phytoclean" is recommended as an additive for water used for washdown or foot baths. In late 2007, we were contacted by Annie Philips from the Department of Primary Industries and Water, Tasmania, and asked to examine whether Phytoclean is also effective against the amphibian chytrid fungus, the causative agent of chytridiomycosis. If this is so, then washdown guidelines designed to prevent the spread of root rot might be simultaneously helping to prevent the spread of chytridiomycosis.

Phytoclean is recommended for use at 10% for footbaths and 2 % for washdown of vehicles and washing of hard surfaces and tools (Phytoclean website). The manufacturers recommend that the minimum exposure time of tools, vehicles etc is 30 seconds.

Methods

Phytoclean was tested against *B dendrobatidis* using methods similar to those used in prior disinfectant studies (Johnson *et al* 2003, Webb *et al* 2007). Cultures were grown, using isolate "Tully-Lrheocola-06-LB-1" (Berger *et al* 2005) in 96 well plates for four days, then exposed to Phytoclean various concentrations, for various exposure times. Phytoclean was diluted with sterile water to achieve the required concentration. Each 96 well plate could test 3 concentrations at three different exposure times, with 8 replicates of each. Once the exposure period was up, the plate was inverted and the Phytoclean tipped out. The cultures were rinsed with TGhL broth, then filled with TGhL broth and incubated at 23 degrees. The cultures were checked everyday for activity. One hundred percent kill of all 8 replicates was required for the product to be considered effective. The first tests used concentrations recommended by the manufacturer. When these concentrations were effective, the product was tested at decreasing concentrations until it was no longer killing the *B dendrobatidis*. We tested the minimum recommended exposure time of 30 seconds, as well as 1 minute and 5 minutes.

Results and discussion

Phytoclean is an effective disinfectant against *B dendrobatidis*. A concentration of Phytoclean as low as 0.075% kills *B. dendrobatidis* after an exposure time of only 30 seconds. This concentration is well below the minimum concentration recommended by the manufacturers. This means existing efforts to control Phytophthora root rot using Phytoclean, are also helping to control the spread of *B dendrobatidis*.

As well as testing the effect of Phytoclean on *B dendrobatidis* , we are also looking at its effectiveness against *Mucor amphibiorum,* a fungus which cases a disease known as mucormycosis in some amphibians as well as in platypus. *M. amphibiorum* is a hyphal fungus which cannot be tested in the same way as *B dendrobatidis*. To enable comparison between the two species, we tested *B dendrobatidis* again, using methods similar to those used to test *M. amphibiorum.*

Phytoclean was added to hot TGhL agar, and then plates were poured. The plates contained various concentrations of Phytoclean from 20% down to 0.001%, as well as control plates with no Phytoclean. . A section of healthy culture grown on normal agar plates was excised and placed upside down on a Phytoclean plate. There were three replicates for each Phytoclean concentration and control. Plates were incubated at 23 degrees and checked every day for growth. After 2 weeks, the original agar square from one replicate of each concentration/control was removed from the Phytoclean plate and placed (still upside down) onto a fresh plate with no added Phytoclean. This was intended to show whether the Phytoclean had actually killed the culture, or if it had merely prevented it from further growth. If the culture had not been killed, it would be expected to grow once transferred to a new agar plate. The very low concentration of 0.001% was enough to kill *B dendrobatidis,* and the culture did not on the phytoclean plate, nor did it grow after being transferred to a new agar plate. This suggests that *B dendrobatidis* is quite sensitive to Phytoclean, and continuous exposure to very low concentrations will kill the organism.

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CHEMICALS NOT EFFECTIVE AGAINST *B. DENDROBATIDIS*

Terbinafine

Bonnie Mc meekin (Melbourne zoo) was considering Terbinafine as a treatment for amphibian larvae infected with *B. dendrobatidis.* Terbinafine is available commercially as "lamisil®" (terbinafine hydrochloride tablets) for the treatment of nail fungal infections known as onychomycosis. Bonnie tested the effect of Terbinafine on tadpoles of two species, and found the maximum safe dose for one was 2mg/L and 6 mg/L for the other. The disinfectant effect of Terbinafine on *B. dendrobatidis* in vitro was tested, using a range of exposure times and including a concentration range above and below the values found safe for amphibian larvae. The measure of effectiveness was 100% kill of zoosporangia grown in 96 well plates. There were problems with bacterial contamination, making it difficult to determine whether contamination was killing the *B. dendrobatidis* cultures or if the Terbinafine was. More frequent checking of the cultures revealed that they were often alive and active while still exposed to Terbinafine, suggesting that it is not effective. The highest concentration and exposure combination tested was 10mg/L for 24 hours, and cultures were still alive at the end of the exposure period. As neither species can tolerate exposure to Terbinafine at this concentration, and considering that 24 hours is an excessive amount of exposure, Terbinafine is considered unsuitable for the treatment of amphibian larvae.

Forexpan S fire foam

In 2008 Annie Philips (DPIW-Tasmania) asked that the Fire foam which is widely used in Tasmania, be tested for any disinfectant effect against *B dendrobatidis.*
Forexpan S is a synthetic fire fighting foam concentrate, added to water when controlling forestry or wildland fires. It works by increasing the waters ability to penetrate into burning materials by

reducing its' the surface tension (Angus Fire website, 2009). When fighting bush fires, water may be sucked up from any convenient water body. If this water body is contaminated with *B dendrobatidis*, fire fighters may unintentionally be spreading these diseases into new areas. If the fire foam added to the water has a disinfectant effect on *B dendrobatidis*, this might lower the chances of its spread.

Forexpan S is used at concentrations between 0.1% to 1%. According to operational parks personal, the foam concentrate is only added to the water once it is being sprayed out the hose of the fire truck. Therefore it has a short exposure time before the water enters the environment, which was estimated at less than 30 seconds. If a helicopter is being used to fight the fire, the exposure time was estimated at 5 minutes. Because of *B dendrobatidis* sensitivity to heat, any water which was sprayed directly on, or in the immediate vicinity of the fire would be effectively disinfected. In this investigation we focused on whether the foam might kill any *B dendrobatidis* present in the water before the water left the fire truck or helicopter.

The effect of the fire foam on *B dendrobatidis* was tested using methods similar to those used in prior disinfectant studies (Johnson *et al* 2003, Webb *et al* 2007).

The fire foam was tested at concentrations of 1%, 0.5%, 0.1%, with exposure times of 30secs, 1 min, 5 mins. The highest concentration, 1%, was an effective disinfectant if the exposure time was 1 minute or more. This means that it is possible that water carried in helicopters will be disinfected, if enough fire foam is added to make the water 1% fire foam. Until we get more detailed information about how the product is used, we cannot make any conclusions about its effectiveness against *B dendrobatidis.*

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Antifungal drug Terbinafine as a possible treatment for infected amphibian larvae.

Summary

Terbinafine is a commercially available antifungal drug which may be useful in treating amphibian larvae suffering from chytridiomycosis. Bonnie Mcmeekin (Melbourne zoo) tested the effect of Terbinafine on tadpoles of two species, and found the maximum safe dose for one was 2mg/L and 6 mg/L for the other. The antifungal effect of Terbinafine on Bd in vitro was tested, using a range of exposure times and including a concentration range above and below the values found safe for amphibian larvae. The measure of effectiveness was 100% kill of zoosporangia grown in multiwell plates. There were problems with bacterial contamination, making it difficult to determine whether contamination was killing the Bd cultures or if the Terbinafine was. More frequent checking of the cultures revealed that they were often alive and active while still exposed to Terbinafine, suggesting that it is not effective. Also, controls were often killed as well, suggesting that contamination was responsible. The highest concentration and exposure combination tested was 10mg/L for 24 hours, and wells were still alive at the end of the exposure period. As neither species can tolerate exposure to Terbinafine at this concentration, and considering that 24 hours is an excessive amount of exposure, Terbinafine is considered unsuitable for the treatment of amphibian larvae.

We collaborated with Bonnie Mcmeekin at Melbourne zoo. Bonnie was considering Terbinafine as a treatment for infected amphibian larvae. Terbinafine is available commercially as "lamisil®" (terbinafine hydrochloride tablets) for the treatment of nail fungal infections known as onychomycosis. She tested the effect of Terbinafine on tadpoles of two species, and found the

maximum safe dose for one species was 2mg/L and 6 mg/L for the other. We tested the antifungal effect of Terbinafine on Bd in vitro, using a range of concentrations above and below those which are safe for amphibian larvae.

Methods

Efficiency of Terbinafine tested by following protocol 2 as per Johnson et al 2003, apart from minor modifications. Three Bd strains were used, one isolated from *Litoria caerulea* (Rockhampton-Lcaerulea-99-LB-1), and another two from *L. rheocola* (Mt Misery-Lrheocola-05-LB-1 and Tully-Lrheocola-06-LB-01) (Berger et al. 2005b) depending on which strain was producing the most zoospores at the time. Zoospores were placed in 96-well plates to ensure that all wells contained cultures of approximately the same age. Zoospores were collected from the cultures by filtering 3 day old culture with sterile coffee filters thus removing zoosporangia. Plates were left to grow for 4 days before exposed to Terbinafine. The Terbinafine was obtained by grinding "lamisil ®" tablets in a sterile mortar and pestle and diluted with sterile deionised water. The control wells were treated with sterile water instead of the Terbinafine dilution. The plates were checked periodically after exposure to determine if wells were alive and active, or killed. One hundred percent kill of all 8 replicates was required for the treatment to be considered effective. The first experiments tested the lowest concentrations of Terbinafine, and successive experiments tested higher concentrations (see tables 1-3). In all, the concentrations tested included a range above and below the values found safe for amphibian larvae. One set of experiments tested exposures of 1, 15 and 60 mins, and a further set of experiments tested longer exposures, which were up to 24 hours. In the first set of experiments, the wells were checked after they had been rinsed and filled with broth, while in the second set the wells were checked before they were rinsed (that is, while still exposed to Terbinafine) as well as after. The range of concentrations tested was 0.05mg/L – 10 mg/L.

During the experiments, those wells treated with Terbinafine frequently became contaminated, so the Terbinafine had to be sterilized. Sterilization techniques included U.V light, 95% ethanol and autoclaving at 121 degrees for 15 mins, however none of these eradicated contamination.

Table 6.1.   Ultra violet light exposed Terbinafin, low to medium concentrations
3 Experiments in total:

exp	concentrations	exposure
1	0.5, 1.0, 1.5 mg/L	1,15, 60 mins
2	2.0, 3.0, 4.0 mg/L	1,15, 60 mins
3	4.5, 5.0, 5.5 mg/L	1,15, 60 mins

Table 6.2.   Ethanol exposed Terbinafin, all concentrations.
2 experiments in total:

exp	concentrations	exposure
4	6.0 ,7.0 , 8.0 mg/L	1,15,60 mins
5	1.0, 3.0, 5.0, 7.0, 9.0 mg/L	17 hours

Table 6.3.   Autoclaved Terbinafin, medium-high concentrations
4 experiments in total:

exp	concentrations	exposure
6	5.0, 7.0, 9.0, 10.0 mg/L	18 hours
7	5.0, 7.0, 9.0, 10.0 mg/L	18 hours
8	2.0, 5.0, 7.0, 9.0, 10.0 mg/L (L. rheo tully strain used instead of l. rheo)	24 hrs
9	5.0, 7.0, 9.0, 10.0 mg/L (L caerulea strain used	21 hours

Results/ Discussion

Despite attempted sterilisation, the experimental treatments, and sometimes even the controls appeared contaminated by day 2 or 3 (see table 4). Old and empty zoosporangia were seen to contain tiny jiggling "bacteria". Less frequently, zoosporangia, which had not released zoospores contained these jigglers, and their contents were all bunched up. It appears that this contamination killed the cultures. It is difficult to confirm whether it was the contamination killing the cultures, or if the Terbinafine treatment was a successful antifungal drug. All inactive cultures contained the 'contamination'. In order to decide whether Terbinafine was effective or not, in the experiment with long exposure times wells were checked at the end of exposure while the Terbinafine was still in place, before the wells were rinsed and filled with broth. This was done because usually broth promotes the growth of bacteria. In all these cases the treatment wells were still alive, with motile zoospores present. Although, often the treatment wells would die after the Terbinafine was removed and the well rinsed and filled with broth (see table). This seems to indicate that Terbinafine is not effective, even at the highest concentration and exposure tested (10 mg/L for 24 hours). An ideal antifungal drug for treating amphibians would be effective at low concentrations and exposure times to reduce stress on the host. As neither species can tolerate exposure to Terbinafine at this concentration, and considering that 24 hours is an excessive amount of time for exposure, Terbinafine is considered unsuitable for the treatment of amphibian larvae.

Table 6.4   Results:

Exp	conc and exp	survival comments
1	0.05-1.5 mg/L, 1-60 mins	all treatments still alive after 3 days post exposure, although not healthy. Appears contaminated by next check.
2	2-4 mg/L 1-60 mins	all treatments alive after 1 day post exposure, but inactive by day 4. appears contaminated
3	4.5-5.5 mg/L 1-60 mins	all treatments alive on day 1, but not by day 7
4	6.0 – 8.0 mg/L 1-60 mins	all treatments very active day 1
5	1-9 mg/L 17 hrs	all treatments alive while still exposed to terbinafine (17 hrs), and also alive 5 hours after exposure ended. But inactive by day 3 and appear contaminated.
6	5-10mg/L 18 hrs	all treatments alive after 18 hours (while still exposed), although controls appear more healthy. But controls and treatments inactive by day 3 and appear contaminated.
7	5-10mg/L 18 hrs	all treatments alive after 18 hours (while still exposed) still alive, but unhealthy by day 1 and appear contaminated.
8	2-10 mg/L	all treatments very active at 1 hour exposure. All treatments active after 19 and 24 hours (while still exposed) although controls are more healthy. All treatments and controls inactive by day 1, possibly because of antibiotic broth used, or air conditioner failure.
9	2-10 mg/L	All treatments and controls alive after 21 hours (while still exposed). Treatments appear slightly less active than controls. Controls and treatments inactive by day 1.

Objective 7. **How does *B. dendrobatidis* exist within infected frog populations? For example, are all frogs infected, are all tadpoles infected, is survivorship of infected frogs reduced compared to uninfected frogs in the population?**

Epidemiology of *Batrachochytrium dendrobatidis* in Rainforest Stream Tadpoles

(Excerpt from Cashins, S. 2010. Epidemiology of Chytridiomycosis in Rainforest Stream Tadpoles. Ph.D. thesis, James Cook University, Townsville, Australia. 204 p references to chapters in text apply to chapters in the thesis, not in this report)

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, species closely associate with banks of streams (riparian) 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, species that have tadpoles (non-terrestrial) 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, terrestrial species and species that breed in ponds 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 (see Chapter 5). 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.

This chapter describes 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

See Chapter 5 of Scott Cashin's PhD thesis, Materials and Methods, for description of 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. 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 in Chapter 5. 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 (see Chapter 5 for a discussion of all environmental and ecological variables considered) 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 was 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, Fig. 7.1) with torrent-adapted tadpoles having a higher prevalence of infection than pool-adapted tadpoles (Table 7-1). 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 7-1, Fig. 7.2).
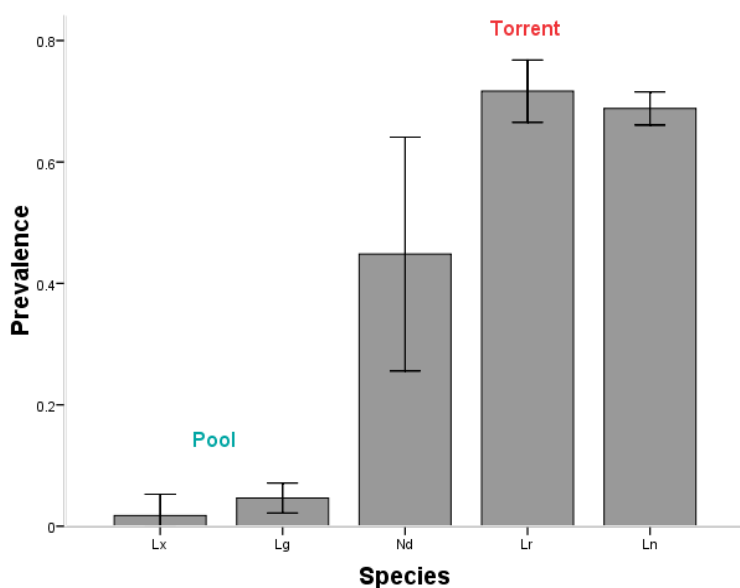

Figure 7.1: Mean prevalence of *B. dendrobatidis* in tadpoles of five species at Tully Gorge NP and Murray Upper NP (2006-2008). Whiskers represent 95% confidence intervals of the mean.

Table 7.1 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

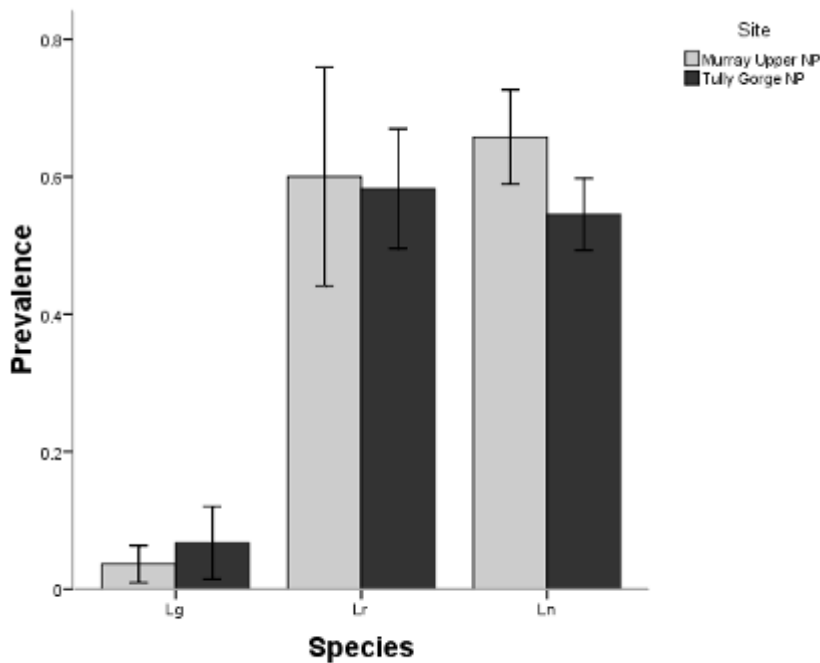* Indicates significant difference in prevalence

Figure 7.2: Mean prevalence of tadpoles between 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 (Fig. 7.3). 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 in *L. genimaculata* is also higher in larger tadpoles (Independent samples T-test: t=-4.580, df=278, p=<.0005, Fig. 7.5), however, prevalence only increased in the largest two size classes (Fig. 7.4). Prevalence in *L. xanthomera* remains very low throughout all size classes.
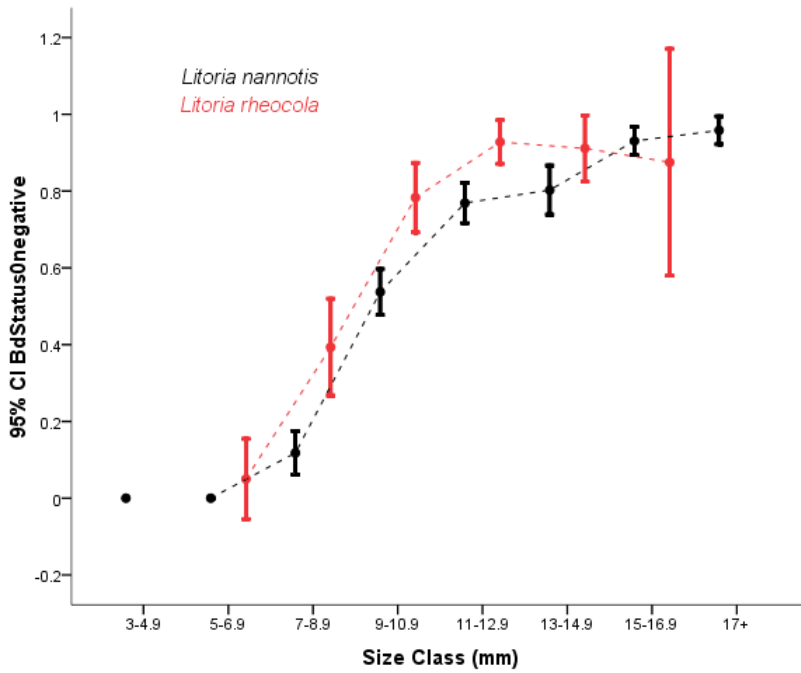
Figure 7.3: Mean prevalence of *B. dendrobatidis* in *L. nannotis* and *L. rheocola* by size class. Tully Gorge NP and Murray Upper NP (2006-2008). Connecting lines are for visualization only.
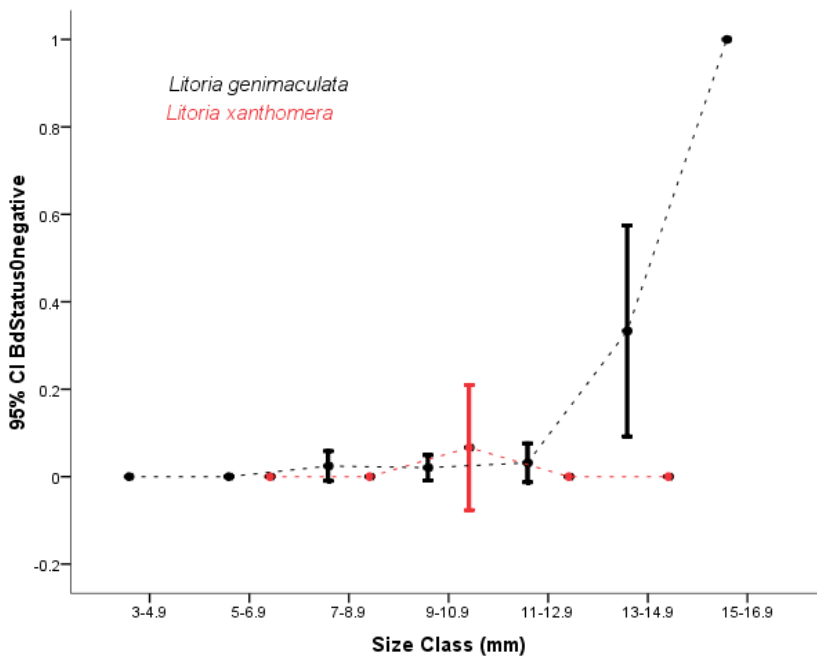
Figure 7.4: Mean prevalence of *B. dendrobatidis* in *L. genimaculata* and *L. xanthomera* by size class. Tully Gorge and Murray Upper NP (2006-2008).. Connecting lines are for visualization only.

Figure 7.5 Mean size of infected and uninfected *L. genimaculata* tadpoles at Tully Gorge NP and Murray Upper NP (2006-2008).

Figure 7.6: 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.
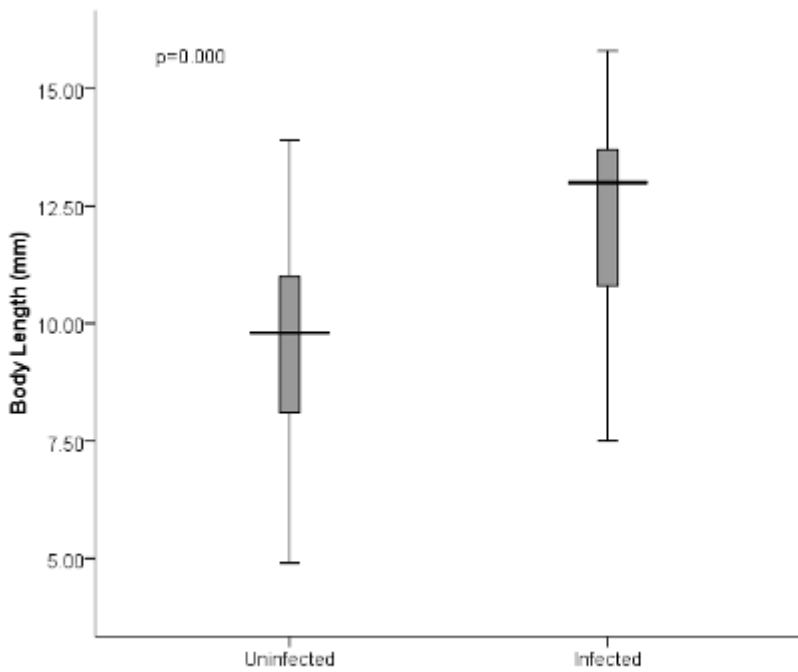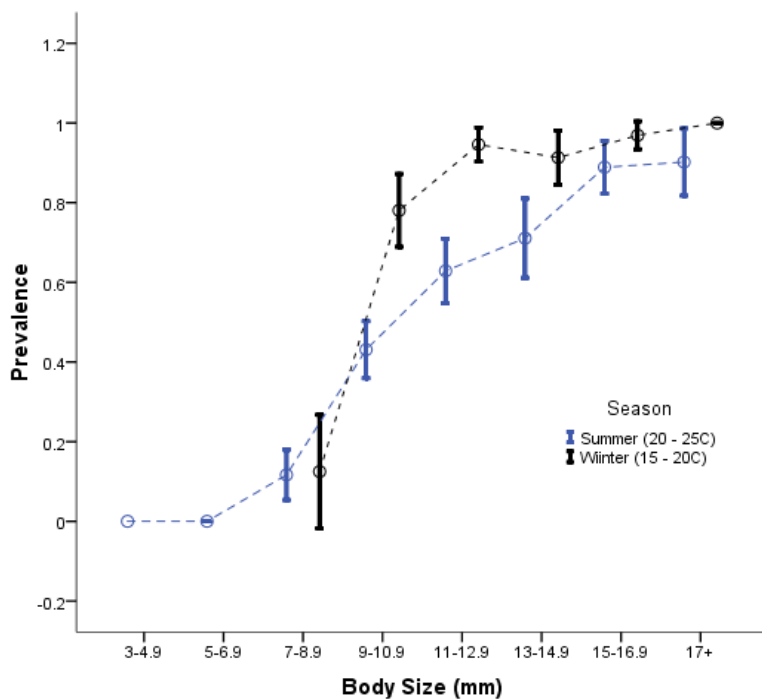
Table 7.2: Chi Square of prevalence by size class and season in *Litoria nannotis* at Tully Gorge NP and Murray Upper NP (2006-2008) *as indicated* **by temperature**

	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

Prevalence by Water Temperature

Yearly water temperature extremes ranged between approximately 15 to 25 C (Figs. 7.9 – 7.10). 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 (Fig. 7.6). 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 a limited duration of exposure to infection risk. 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 (Fig. 7.6, Table 7.2).

The pattern of higher prevalence occurring with cooler temperatures was also observed in the other torrent tadpoles (Fig. 7.7). In contrast, *Litoria genimaculata* were more likely to be infected in warmer water. Tadpoles of the different species were exposed to similar mean water temperatures (Fig. 7.8).

Figure 7.7: 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).
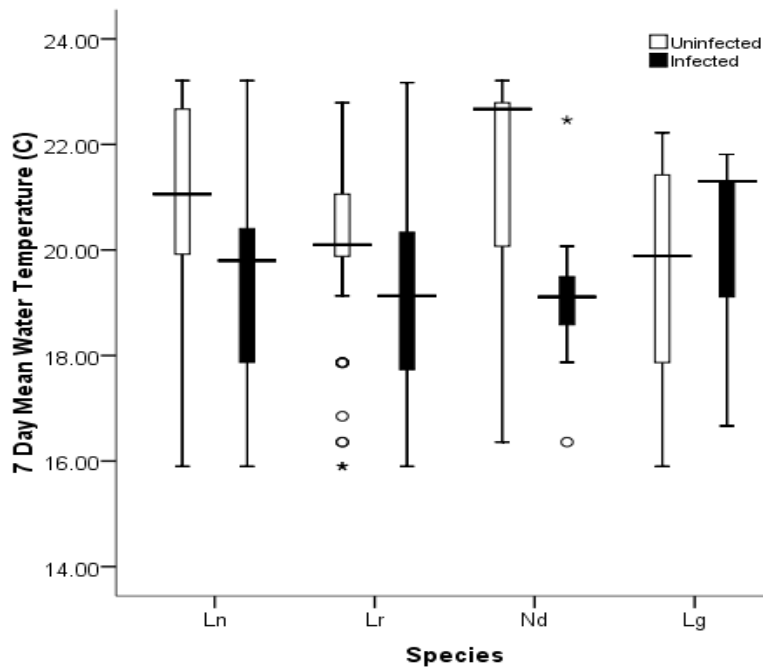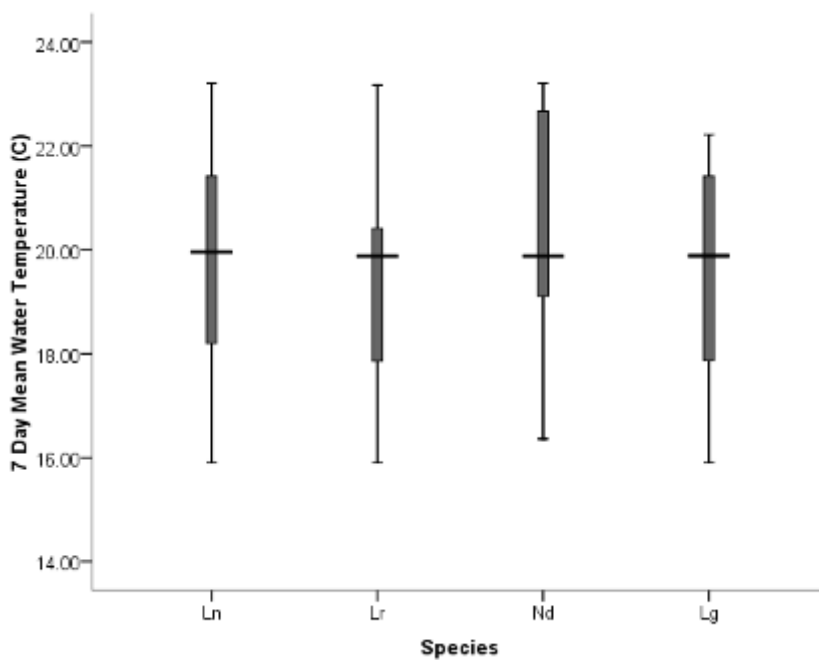

Figure 7.8 Seven-day mean water temperature of stream habitat for combined uninfected and infected *L. nannotis*, *L. rheocola*, *N. dayi*, and *L. genimaculata* prior to capture in Tully Gorge NP and Murray Upper NP (2006-2008).

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 (Figs. 7.9 – 7.10). Periods of increased prevalence in pool tadpoles is explained by the presence of larger size class *L. genimaculata* tadpoles (Fig. 7.5). 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*, and therefore no time of the year when large tadpoles were most abundant (Chapter 5).

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 (Figs. 7.9 – 7.10). This effect of seasonality however, is primarily seen in the medium size-group tadpoles (Fig. 7.11). 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 7.3. 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 (Chapter 5 and Fig. 7.11). This demographic change is seen as an increase of relative abundance in small tadpoles and a decrease of relative abundance in large tadpoles (Fig. 7.11). The seasonal decrease in prevalence within the medium size-group is therefore primarily caused by the largest, infected tadpoles exiting into the large size-group and new, uninfected tadpoles entering from the small size-group (and Fig. 7.11).

Figure 7.9: 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.
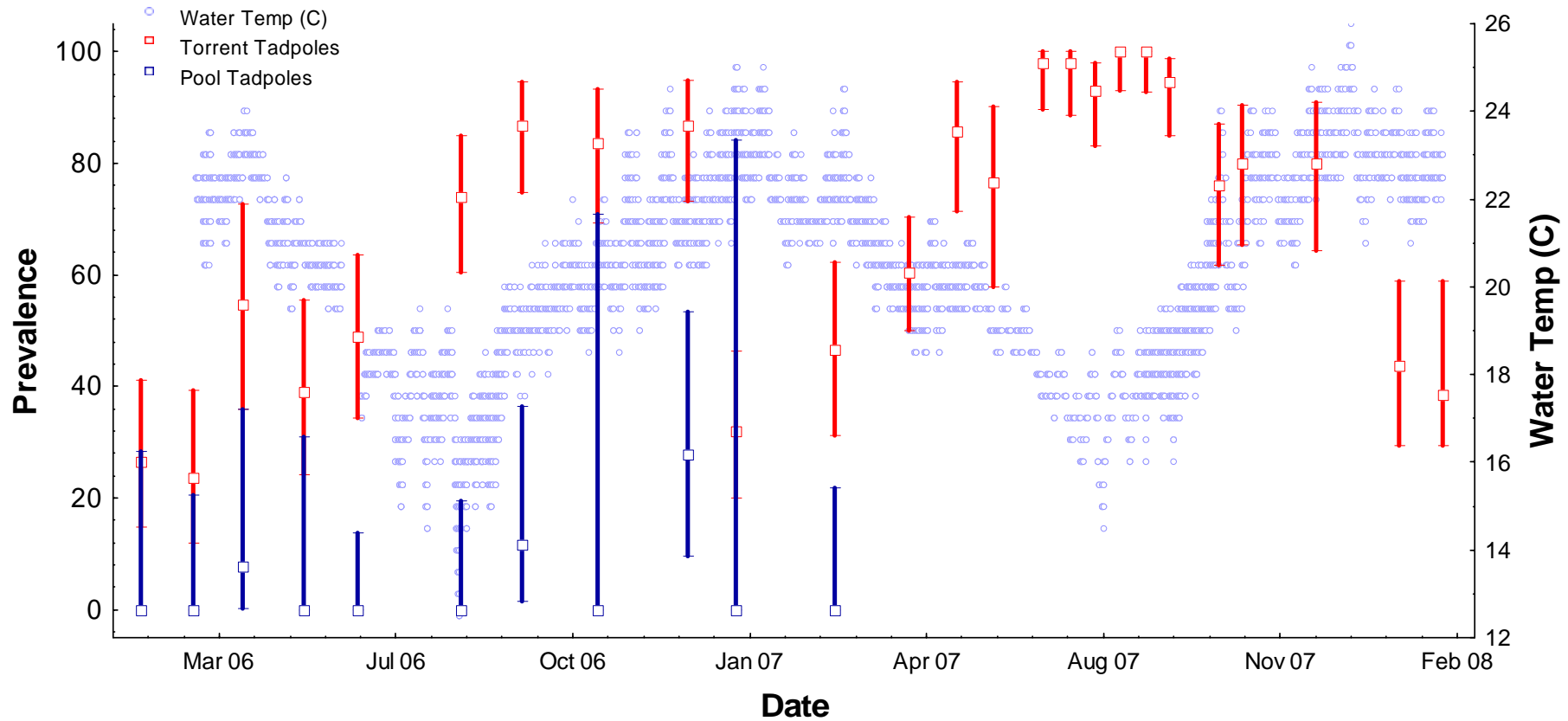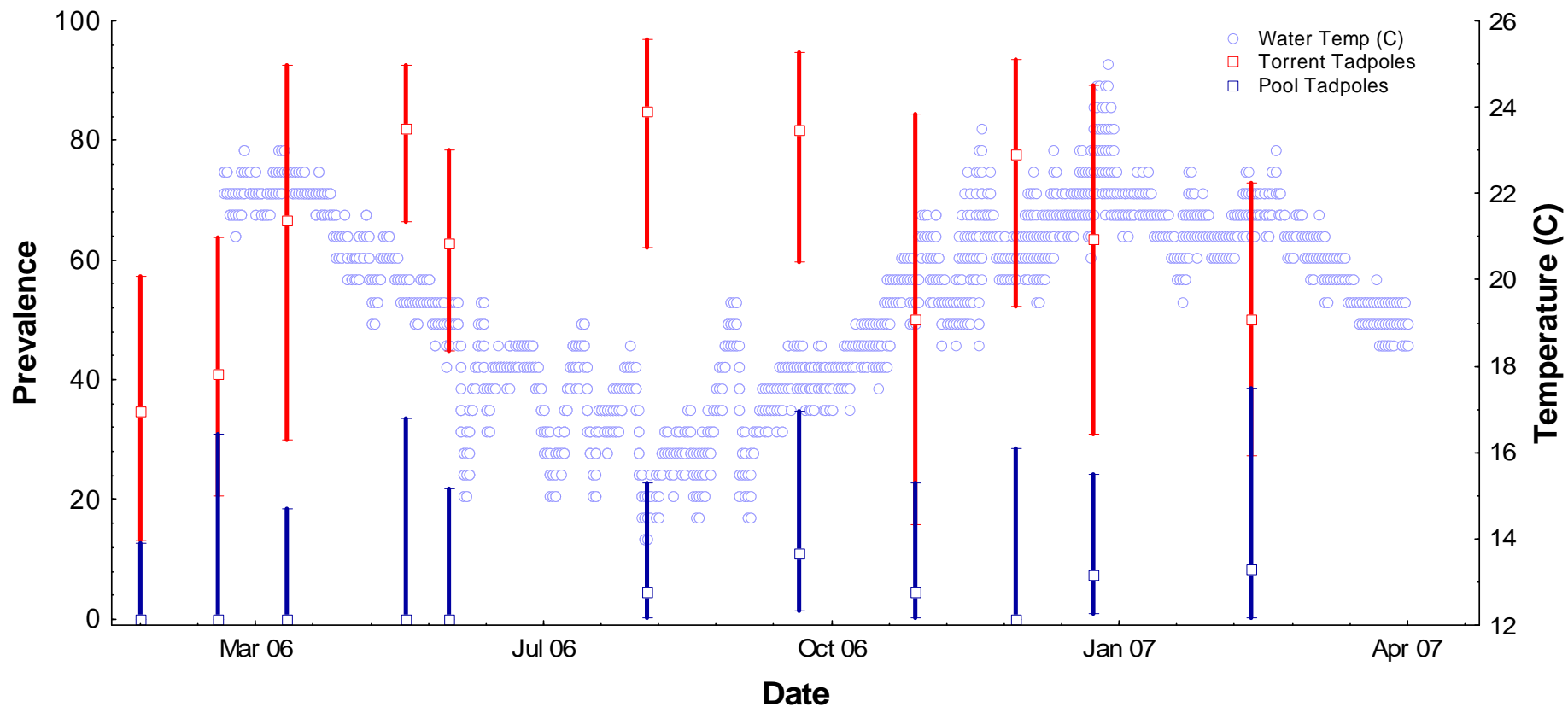
Figure 7.10: Seasonal prevalence of *Batrachochytrium dendrobatidis* in torrent-adapted (*L. nannotis*, *L. rheocola*, *N. dayi*) and pool-adapted (*L. genimaculata*) tadpoles at Murray Upper National Park, 2006 – 2007. Whiskers represent 95% confidence intervals. Blue dots represent the range in water temperature.
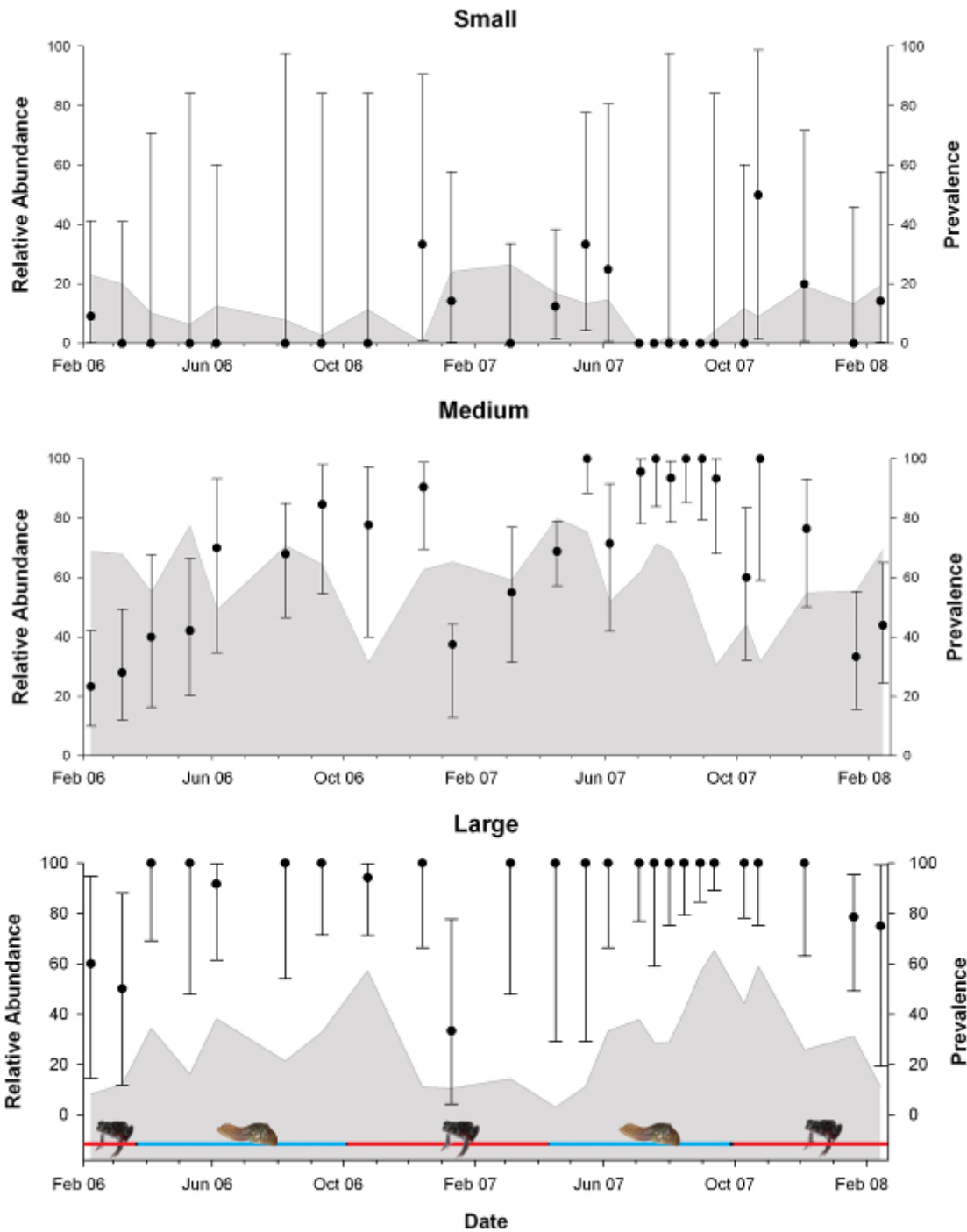
Figure 7.11: Prevalence of *L. nannotis* tadpoles at Tully Gorge National Park (2006-2008). Each panel presents data from small, medium and large size group tadpoles. The shaded gray areas represent the relative abundance of each size group. Whiskers represent the 95% confidence interval. The bar at 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. 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 *L. nannotis* and *L. rheocola* 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).

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			

[a] Model Chi Square for Univariate Model and Whole Model

Intensity of Infection

Infected tadpoles of the different species had significantly different mean intensities of infection (ANOVA, $F_{(4,1050)}$, p=0.0003; Fig. 7.12). *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; Fig. 7.12) 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; Fig.7.17), 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; Fig. 7.16). 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.
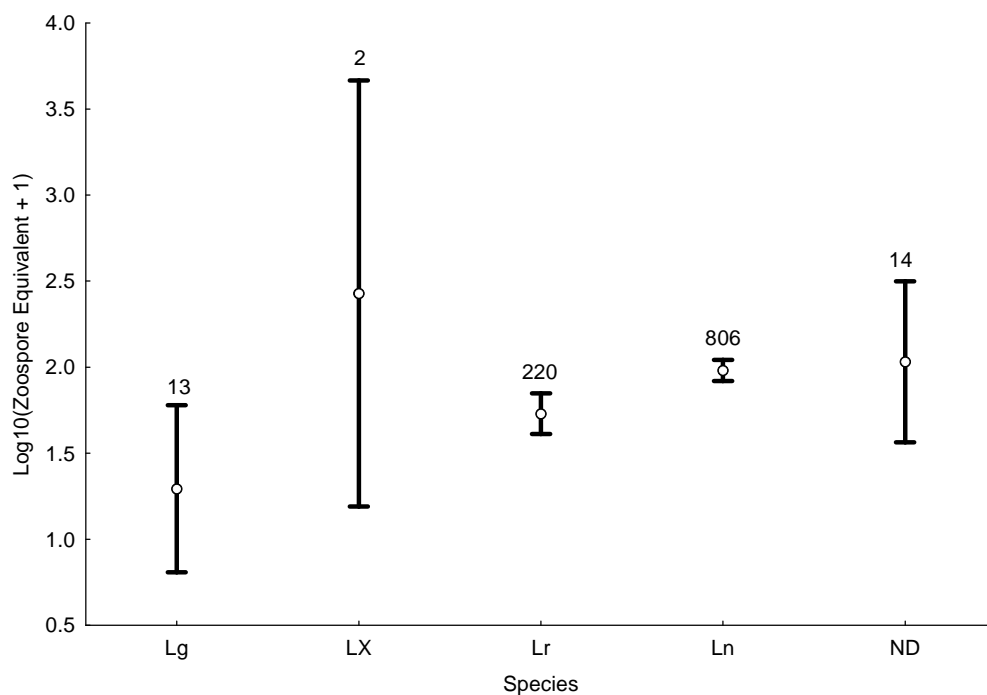
Figure 7.12: Mean intensity of infection in tadpoles of each species. Symbols represent the mean, whiskers represent the 95% confidence interval. Numbers above are the sample sizes.

Figure 7.13: 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 7.14: 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; Fig. 7.13).   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; Fig. 7.14). 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; Fig. 7.15).

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; Fig. 7.17 and *L. rheocola*, p=0.0003; Fig. 7.18). The remaining sampling periods were not 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*.
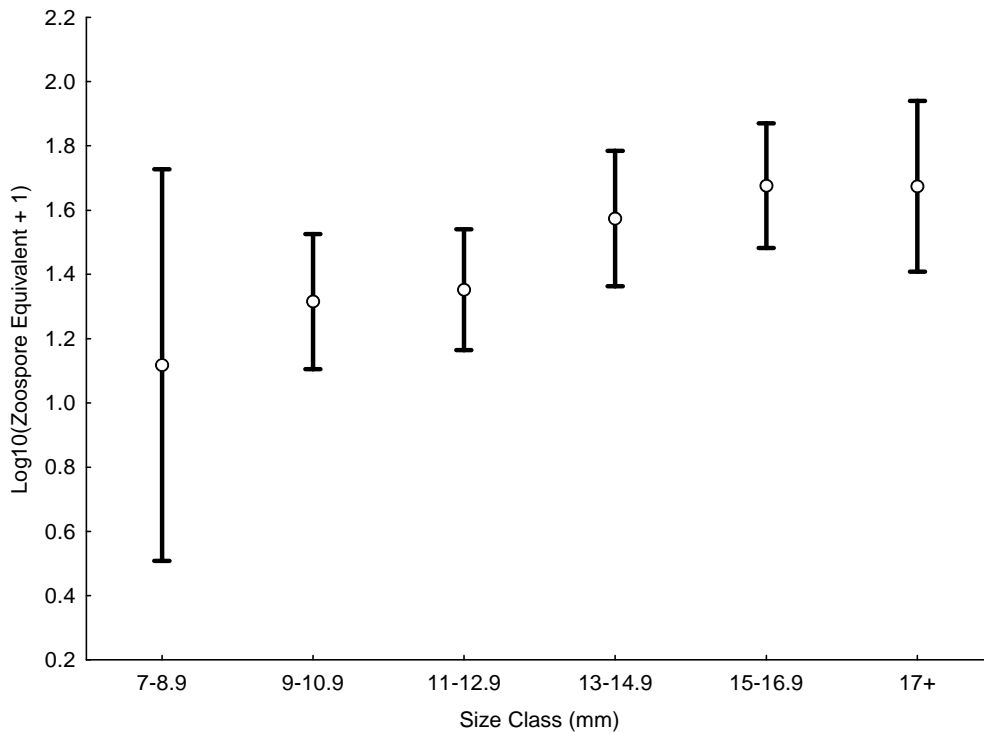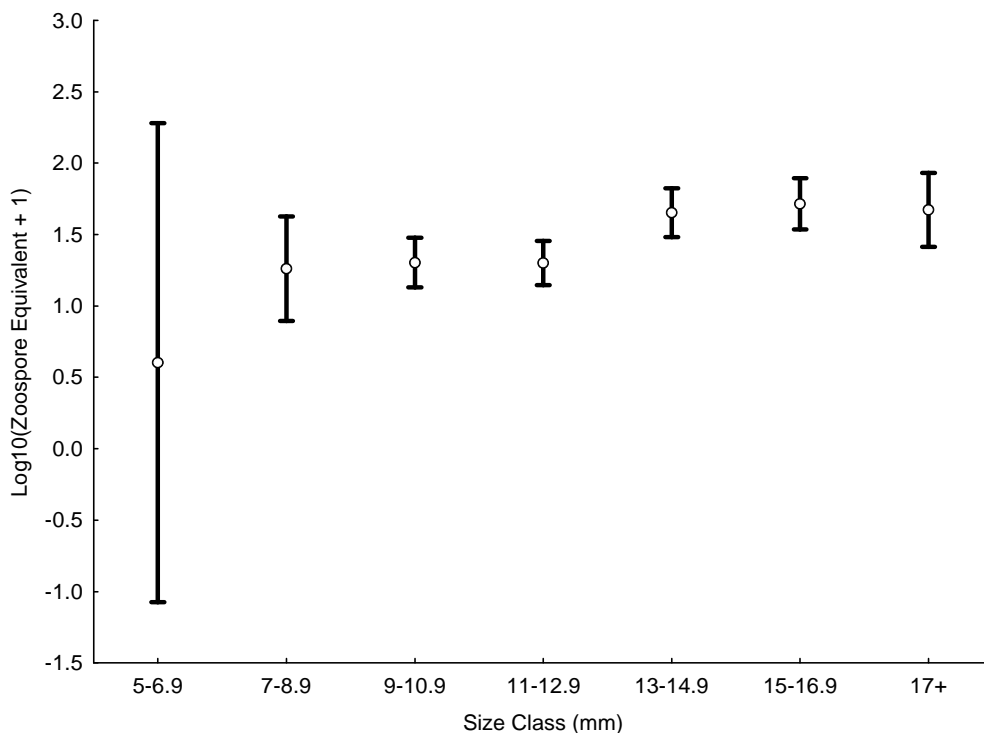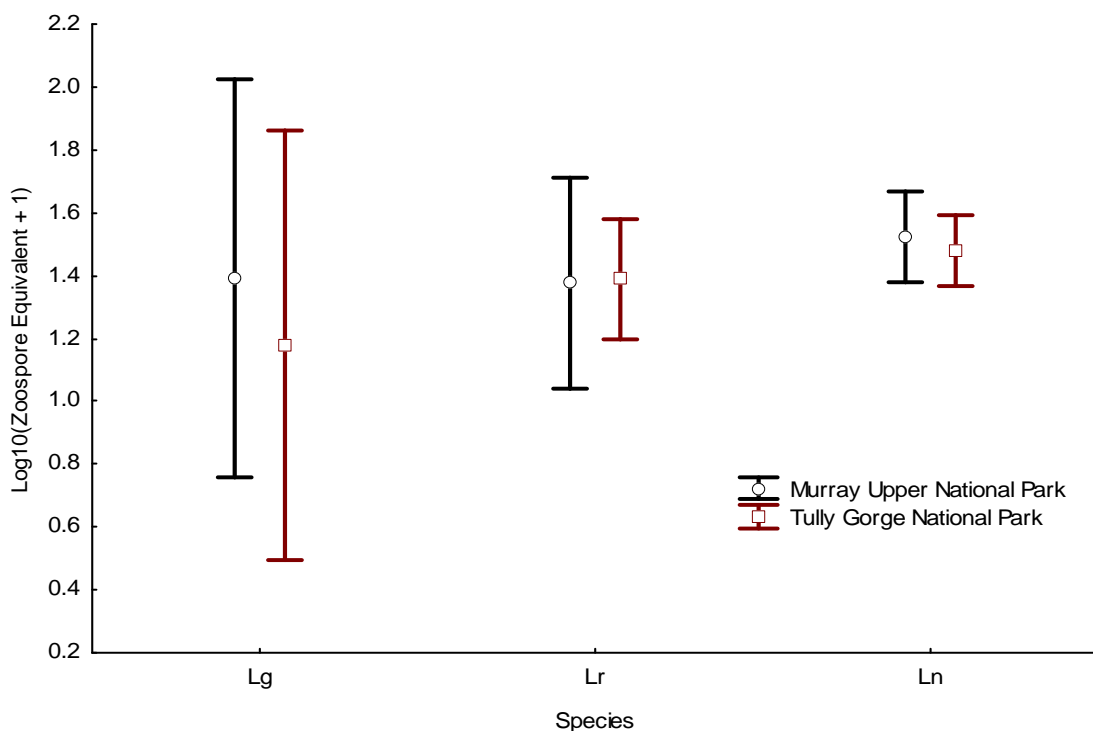

Figure 7.15: Intensity of infection by species and site. Feb. 2006 - 2007. Symbols represent the mean, whiskers represent the 95% confidence interval

Figure 7.1: 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.
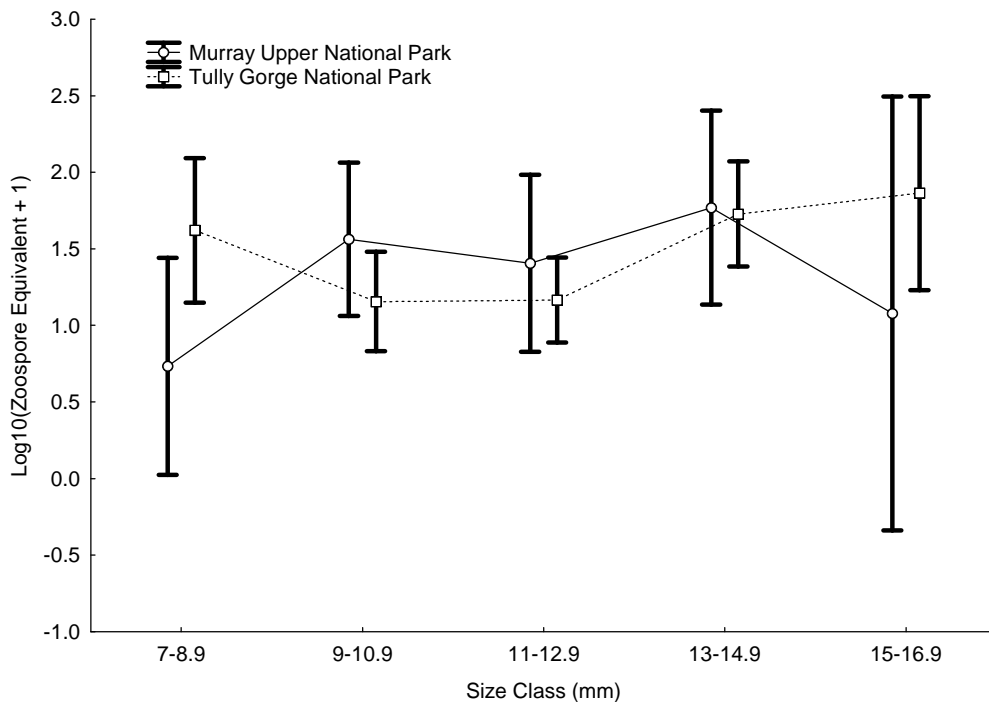
Figure 7.2: Intensity of infection in *Litoria rheocola* by size class and site. Symbols represent the mean, whiskers represent the 95% confidence interval. Connecting lines are a visual aid only.

Figure 7.18: 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.

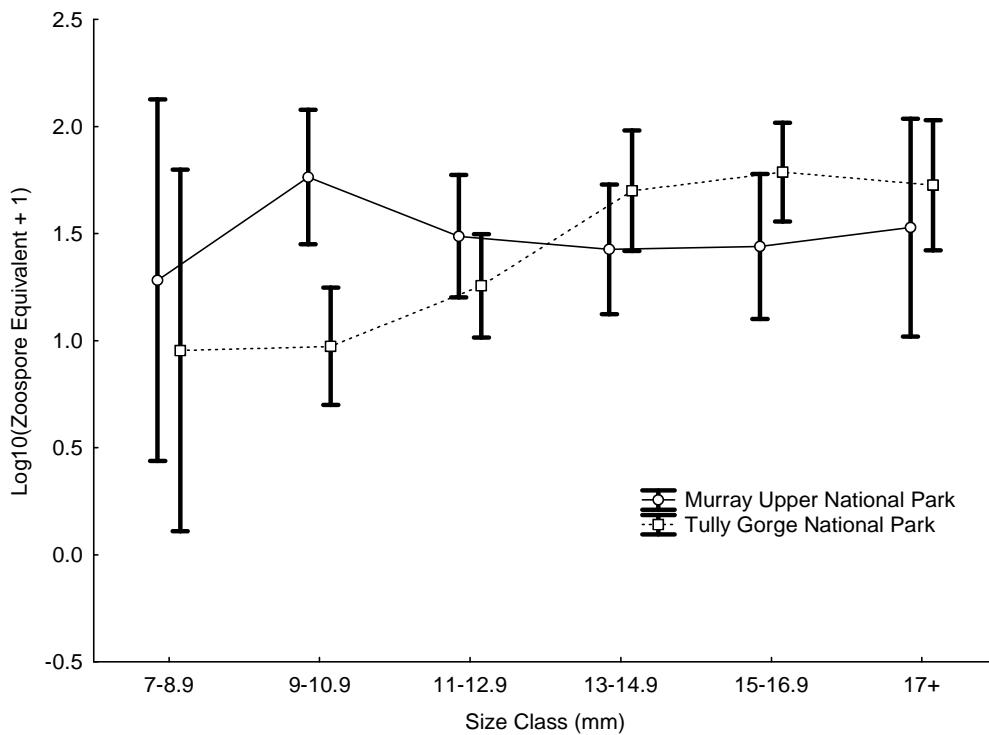Figure 7.19: Intensity of infection of Litoria rheocola by sampling period and site. Symbols represent the mean, whiskers represent the 95% confidence interval. Connecting lines are a visual aid only.

Seasonality

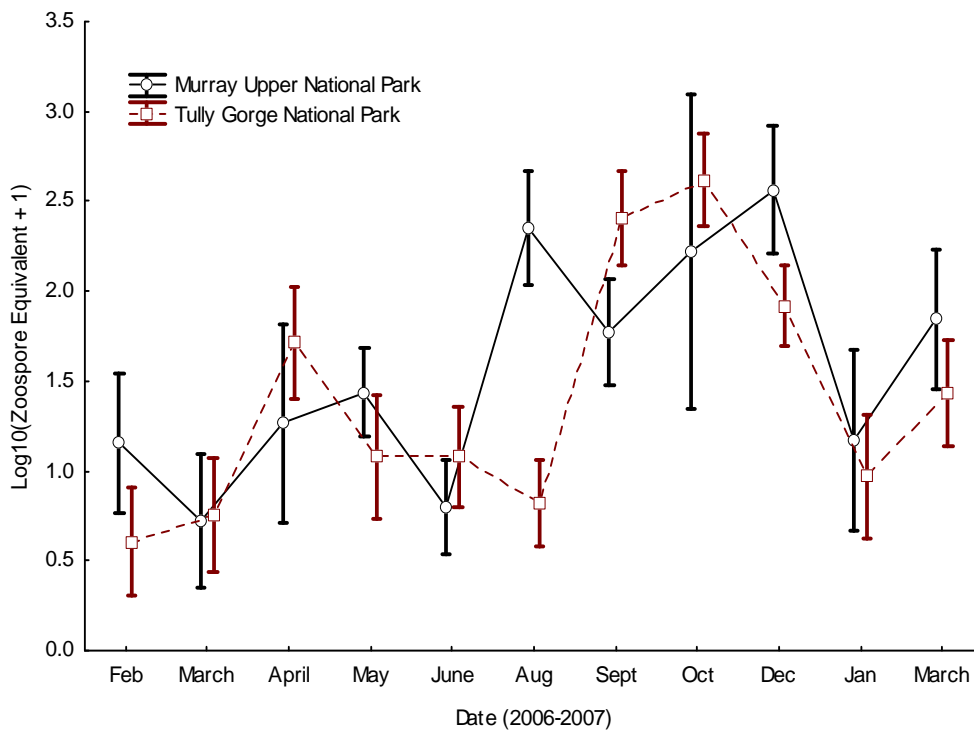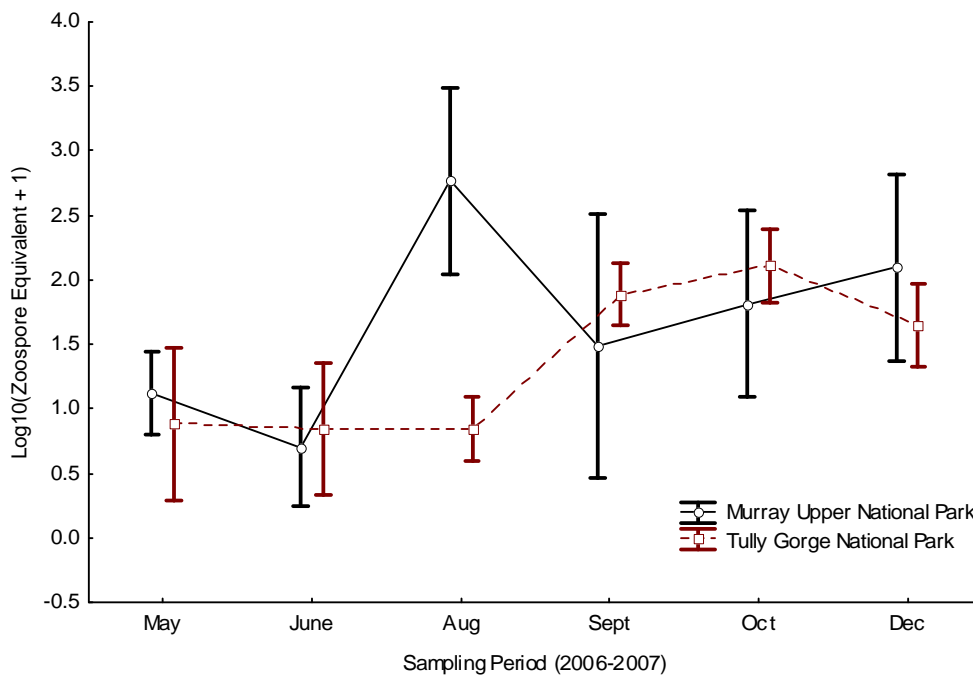Intensity of infection was highly seasonal and differed throughout the year (ANOVA, $F_{(33, 1632)}$, p=0.0000; Fig. 7.20). Peak intensity occurred in spring of 2006 and winter of 2007. The mean intensity of infection in *L. rheocola* was strongly positively correlated with infection intensity of *L. nannotis* by sampling period (Time series cross-correlation r=.865; Fig. 7.21) 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; Fig. 7.21). 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 (Chapter 7). 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 7-3). The whole model explained 20.1% of total variance ($F_{(4, 801)}$, p<0.0005).
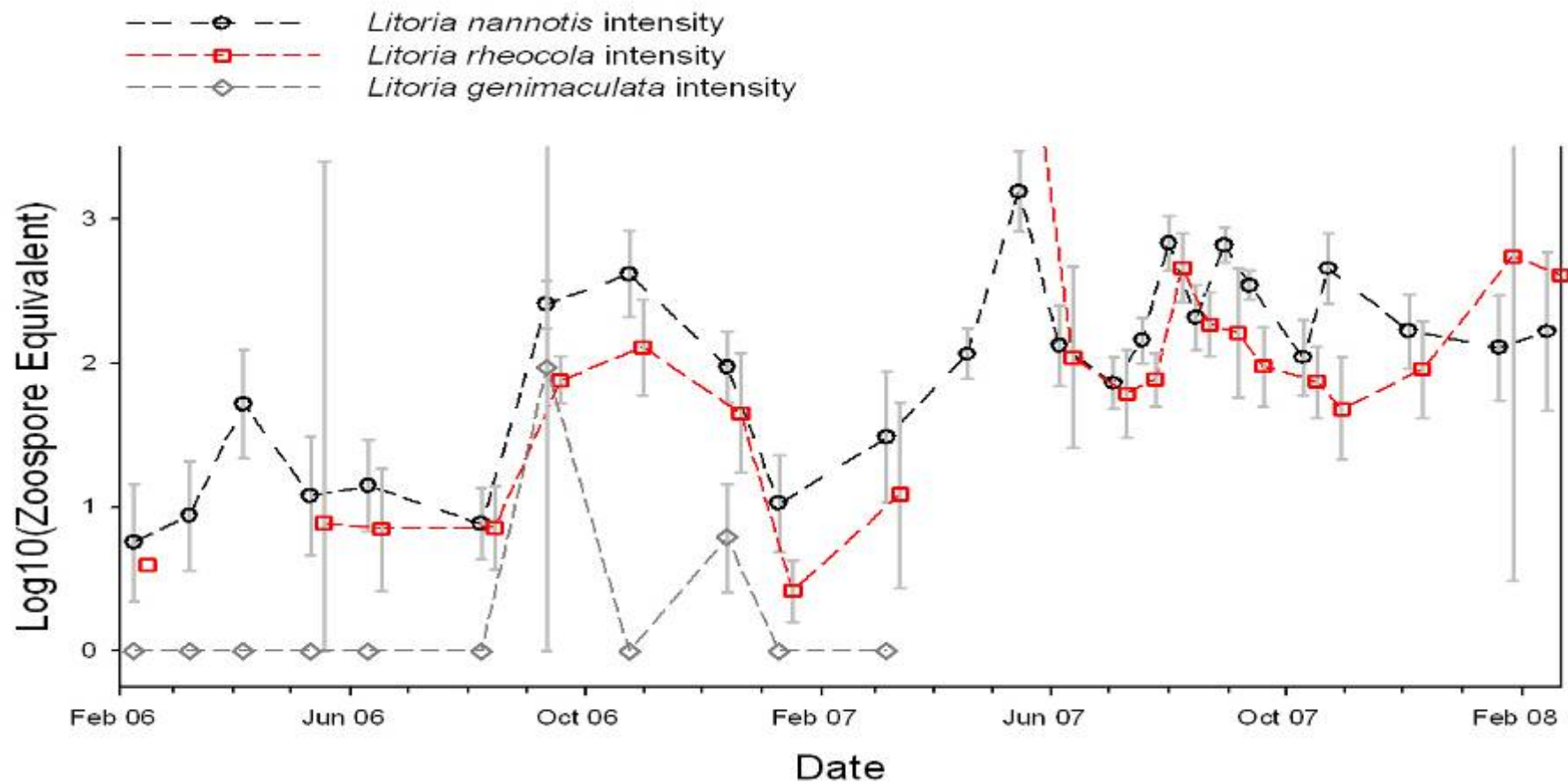
Figure 7.20: Intensity in infected *Litoria nannotis, L. rheocola* and *L. genimaculata* over time at Tully Gorge National Park (2006-2008). Values of 0 were added to indicate tadpoles were captured but were uninfected. Missing values indicate tadpoles were not found.
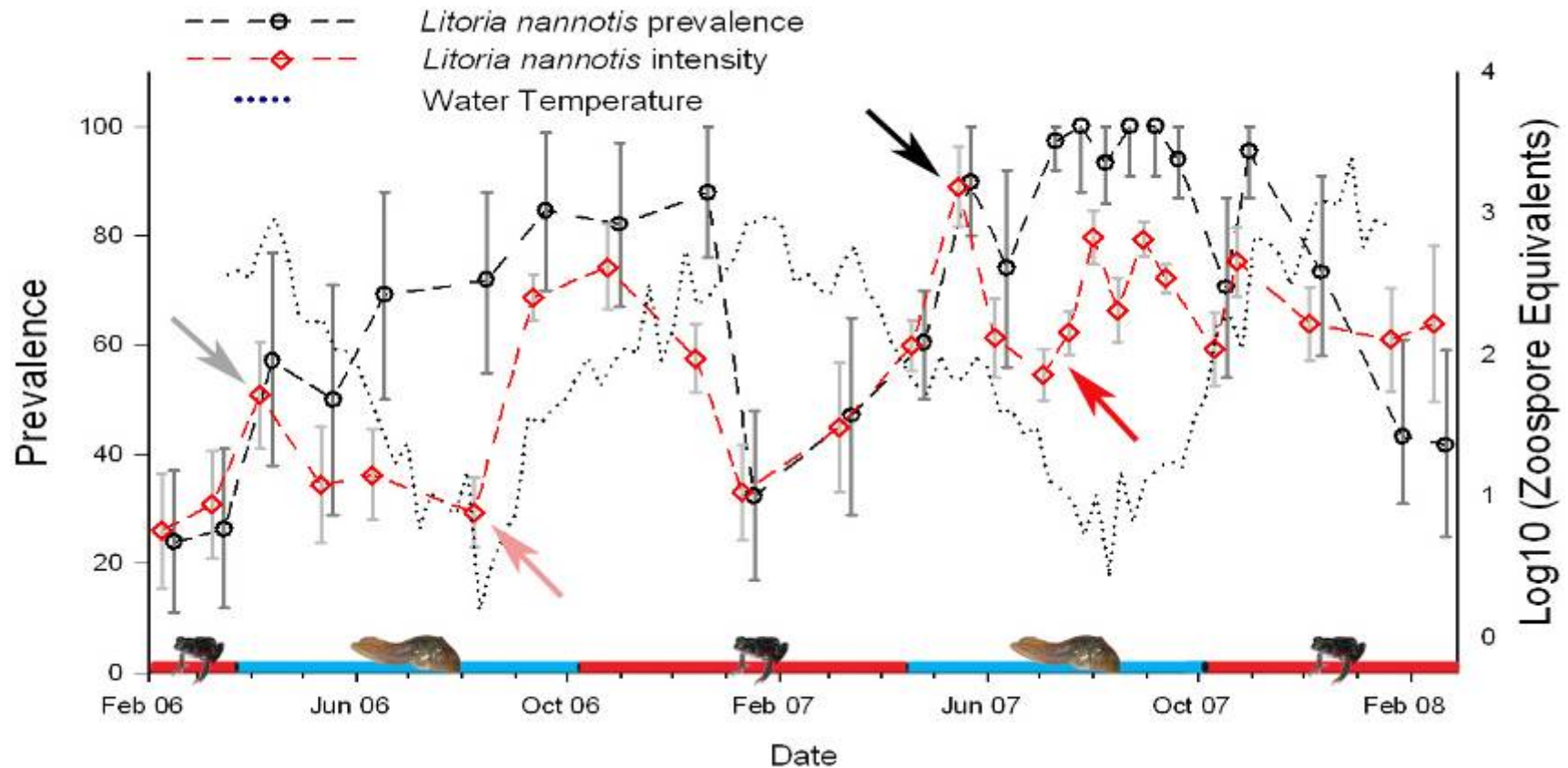
Figure 7.21 Prevalence and intensity of infection over time in *Litoria nannotis* at Tully Gorge National Park (2006-2008). Blue dots represent mean weekly water temperature and are provided as an indication of season. The black arrow indicates the observed start of mouthpart loss. The red arrow indicates 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 7.1: 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 (Chapter 5 Methods). 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 7. 11). 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 number of zoospores released by neighboring tadpoles. The number of zoospores released will be a product of both the density of infected tadpoles and the intensity 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 (Chapter 5). To control for the effect that size has on intensity of infection (Fig. 7.13), 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, Fig. 7.23) revealed that intensity of infection in large size-group *L. nannotis* was moderately positively correlated with density of infected torrent tadpoles.

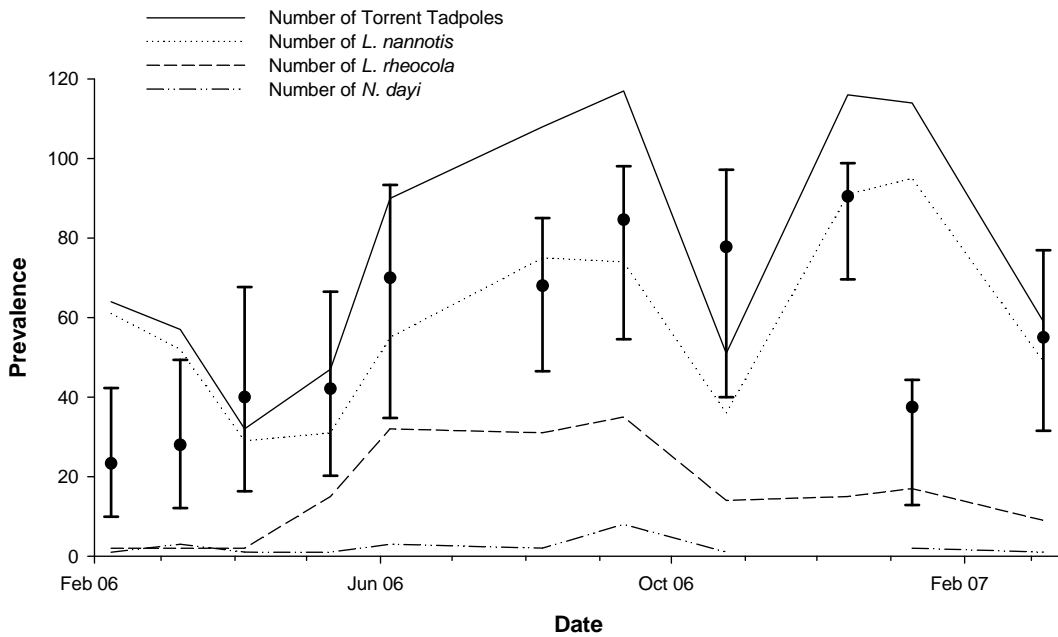Figure 7.22: Prevalence of infection in medium size group *Litoria nannotis* and host density estimates. Tully Gorge National Park, 2006-2007. Squares represent mean prevalence and whiskers are the 95% confidence intervals. Lines indicate host density patterns.
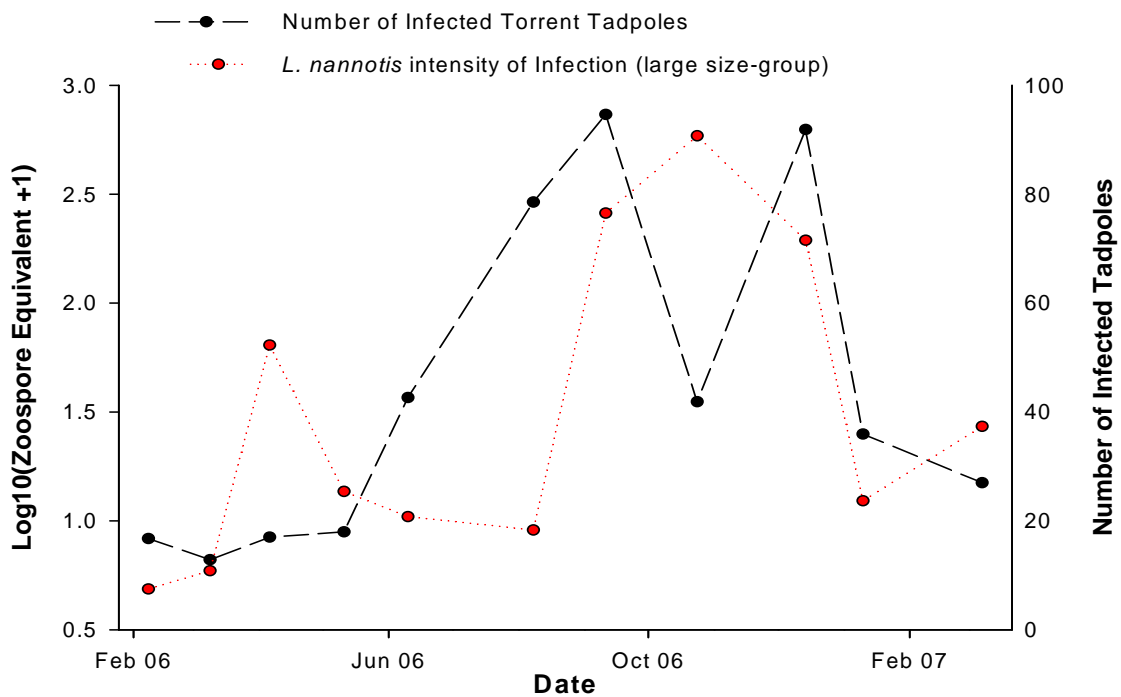

Figure 7.23: Intensity of large size-group *Litoria nannotis* tadpoles and relative density of infected torrent adapted tadpoles, Tully Gorge National Park (2006-2007).

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 7-1). 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, Chapter 5). 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 two *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 of *L. genimaculata* were not significantly different from *L. xanthomera* (Fig. 7.1 and 7.12). *Litoria genimaculata* occupy gently flowing pools that are connected to the main stream (connected pools; Chapter 5). 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 (Fig. 7.5 and 7.17), 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 (Chapter 5). They acquire infection at a small size in the wild (Fig. 7.3) 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 7-2) is inversely related to the velocity of their microhabitat (Chapter 5). *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 7-2 and 7-3).

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 (Chapter 8). 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

<u>Prevalence</u>

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 (Fig. 7.11), 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 (Fig. 7.5, Table 7-2). 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 (Chapter 7) 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.

<u>Descriptive Prevalence Model</u>

A pulse of egg hatching occurs during the summer of each year (Fig. 7.11). Fewer numbers of small, recently hatched tadpoles are found outside of summer, yet infection prevalence in this size group remains low throughout the year indicating they do not hatch infected. Following summer, tadpoles grow over the cooler, drier winter. No metamorphosis takes place over 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 in large tadpoles is at its lowest around February (Fig. 7.23) 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.

1. 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 7-2).

2. 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* (Fig. 7.20). However, intensity did not differ with body size in tadpoles other than *L. nannotis*.

3. 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 is predicted to 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 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 (Fig. 7.21), 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 (Chapter 7). However, prevalence continues to increase (up to 100%) at an apparently slower rate. 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 (Chapter 7) 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 (Chapter 7). 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 (Chapter 7). 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 within size groups (Fig. 7.23). This drop in intensity within size groups 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, in Chapter 7 I show 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 from this chapter 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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Effects of Infection with *Batrachochytrium dendrobatidis* on Tadpoles

(Excerpt from Cashins, S. 2010. Epidemiology of Chytridiomycosis in Rainforest Stream Tadpoles. Ph.D. thesis, James Cook University, Townsville, Australia. 204 p references to chapters in text apply to chapters in the thesis, not in this report)

Introduction

While metamorphosis may appear to represent a clean break from the larval stage, there is a growing body of evidence that demonstrates deleterious conditions experienced by larvae can have latent or carry-over effects on the adult stage and on population dynamics. Latent effects are abundant in the natural world and have been documented in a range of organisms with complex life cycles including gastropods, bivalves, echinoderms, polychaetes, crustaceans, bryozoans, urochordates and vertebrates (Pechenik 2006). Larvae of *Rana pipiens* that were smaller at, or took longer to metamorphose due to higher larval densities were more susceptible as metamorphs to trematode infection (Dare et al. 2006). *Ambystoma opacum* larvae with lower lipid levels experienced significantly decreased survival and fitness as adults (Scott et al. 2007) and short periods of starvation (2 days) in the larval marine snail, *Crepidula onyx*, reduced growth, filtration rates and shell length after metamorphosis (Chiu et al. 2008).

Many organisms, including amphibians, have evolved compensatory responses to negative conditions, allowing recruitment in "off" years at the expense of achieving maximum genetic potential (Metcalfe and Monaghan 2001). However, an introduced pathogen that causes yearly, widespread ontogenetic disruption can generate the equivalent of an "off" year in perpetuity and has the potential to cause cohort effects and alter population dynamics in both the short and long term. Present-day life history traits are shaped, in part, by previous tradeoffs between present and future fitness (Beckerman et al. 2002). For example, water pythons (*Liasis* fuscus) hatched during periods of decreased prey abundance had slower growth rates throughout life, thereby affecting population size structure for decades (Madsen and Shine 2000).

During the first year of sampling (Chapter 6) I observed that some tadpoles, beginning approximately in June experienced significant tooth loss. This loss became progressively more severe in the population and many individuals with high levels of tooth loss appeared to lose body condition. During the second year of study I investigated aspects of infection, tooth loss and body condition in greater detail.

Infection of Batrachochytrium *dendrobatidis* is closely associated with keratinizing cells. In metamorphosed amphibians and some larval salamanders, keratinizing cells occur on the outer

epidermal layer of the skin (Berger et al. 2005b; Davidson et al. 2003). Invasion of these cells by *Bd* zoospores which then develop into zoosporangia causes mortality in susceptible metamorphosed individuals, once a threshold of intensity of infection has been reached (Carey et al. 2006). It is thought mortality occurs by *Bd* infection creating an electrolyte imbalance that leads to cardiac standstill (Voyles et al. 2007). In tadpoles with keratinizing cells, keratinization occurs strictly within the mouthparts. Therefore, infection is restricted to the keratinized teeth, jaw sheath and associated tissues (Marantelli et al. 2004). Tadpole infection is not known to cause death (although see Blaustein et al. 2005). Rather, infection of tadpoles can result in the loss of tooth rows and jaw sheath. Most papers describing *Bd*-associated mouthpart deformities refer to this as "depigmentation" (Felger et al. 2007; Obendorf and Dalton 2006; Padgett-Flohr and Goble 2007; Rachowicz and Vredenburg 2004; Smith and Weldon 2007). As Altig (2007) suggests, this terminology should be updated to reflect current knowledge. Depigmentation may be an appropriate and visually descriptive term for the clinical signs, however, it is an inaccurate pathological description now that histopathology has confirmed the individual teeth and cells within jaw sheath the matrix are physically absent following infection and not simply lacking pigment. I suggest the description "mouthpart loss" be used in place of "depigmentation".

Tadpole mouthparts are comprised of a jaw sheath and a series of transverse tooth rows. Each row is made up of a series of individual teeth, each emanating from a single basal cell. Each visible tooth is stacked upon 2-3 additional teeth which are contained within the sheath (Altig and McDiarmid 1999). When a tooth falls out due to natural processes, the next tooth in line emerges to take its place. This ensures a full complement of teeth at all times. If a *Bd* infected tadpole is missing teeth, this suggests that the basal cells that produce the keratinized teeth have been disrupted in some way by invading zoosporangia and have ceased tooth production (Altig 2007). Presumably, as some cells fail to regenerate teeth, the overall appearance of the tooth row becomes discolored or gray in appearance (S.Cashins pers. obs.). If enough adjacent basal cells are disrupted, a gap appears in the tooth row, and if all cells are disrupted, then no teeth are present. The same would be true for the jaw sheath.

Few studies have examined the effects of infection and the deformity of mouthparts on tadpoles. However those that have, suggest the impacts may be subtle or complex. *Bufo fowleri, Hyla chrysoscelis, Rana blairi* and *R. sphenocephala* experimentally exposed to *Bd* experienced increased time to, and decreased body mass at metamorphosis (Parris 2004; Parris and Baud 2004; Parris and Cornelius 2004). In another experiment, development time in *H. chrysoscelis* was only extended in the presence of *Bd* and a predatory newt (Parris and Beaudoin 2004). Infected *R. pipiens* tadpoles displayed an altered behavioral response to predators that may make them less susceptible to predation (Parris et al. 2006). To date, a direct effect on survival of infected tadpoles has not been found (Parris 2004; Parris and Cornelius 2004). However, mortality due to chytridiomycosis can be extreme soon after metamorphosis when keratinizing cells first develop on the skin of juveniles (Banks and McCracken 2002; Marantelli et al. 2004; Rachowicz et al. 2006).

Based on available evidence, tadpoles are considered to not be substantially impacted by infection (Parris and Cornelius 2004). This has led to the conclusion that the primary impact larval infection may have on a population is as an intraspecific reservoir host; enabling pathogen persistence, or amplification that could drive species extinction even when adult densities are low (McCallum 2005). While this conclusion is appropriate and important, tadpoles of some species may play an expanded role in *Bd* dynamics and population declines. In adults, response to infection is very variable and, like many diseases, is impacted by a range of factors including host environment and behavior (Berger et al. 2004; Richards 2008). Tadpoles, like frogs are an impressively diverse life form. Tadpoles can be classified into 18 ecomorphological guilds based on behavioral and feeding morphologies (Altig and Johnston 1989). In a structurally complex environment, these traits will affect the interaction of the host with the pathogen in both time and space and may lead to very different disease interactions and outcomes within a single environment. However, detailed

information on the effects of infection in the wild are lacking, particularly in tropical streams where declines affecting the greatest number of species occur.

Here, I investigate the relationships between *Bd* prevalence, intensity of infection, host species and mouthpart loss, the pattern of tooth loss over time, and the effect of infection on survival, body condition and host population density. Causes behind species differences in tooth loss and the potential long term implications of larval infection and associated pathology on population dynamics are discussed.

Materials and Methods
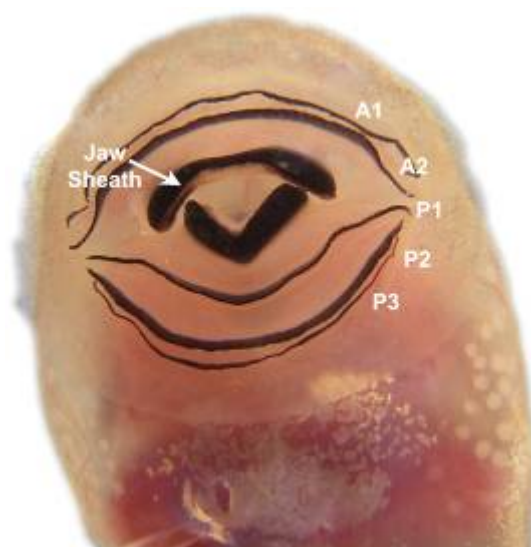
Figure 7A.1: Litoria nannotis mouthparts with labeled tooth rows (A1-P3) and jaw sheath.

Sampling
Tadpole sampling was conducted at Tully Gorge National Park, as described in Chapter 5. Following capture tadpoles were placed into small, individually labeled plastic bags. Torrent-adapted tadpoles naturally adhere to the inside of the bag. For torrent tadpoles, mouthpart loss was described through the bag according to the scores described below. All tadpoles were then transferred to a well-rinsed gloved hand (Chapter 2). Body length was measured (Chapter 5). Pool-adapted tadpoles mouthparts were then examined (with a 10X hand lens if necessary), mouthparts were swabbed and the tadpole was weighed using a portable electronic balance to .05 grams.

For the first year, mouthpart loss was recorded simply as present or absent. Beginning in the second year, mouthpart loss was recorded based on the scores detailed below. Therefore, data for *L. genimaculata* and *L. xanthomera*, which were not extensively surveyed during the second year (see Chapter 6), are only from year 1 and are presented as simply having tooth loss present or absent. Data for *L. nannotis* and *L. rheocola* are presented from year 2 only, when more detailed information was collected.

Tooth loss was noted for each of 5 individual tooth rows. Tadpoles of *L. nannotis* and *L.* rheocola have two tooth anterior (A1 and A2) rows and three posterior (P1 – P3) rows (Fig. 7A.1). Tooth row damage was scored on a scale of 0-6 as follows. 0 = no loss, 1 = discolored (gray) or rough in appearance, but not missing any segments, 2 = more severely discolored (faint) but not missing any segments, 3 = <25% missing, 4 = 25-50% missing, 5 = 50-75% missing, 6 = >75% missing.

Overall tooth loss was ranked, on a scale of 0 to 5, as a measure of the overall condition of all tooth rows, and excludes the jaw sheath. The score was calculated by averaging the percentage of estimated tooth loss on each row. Individual tooth row scores of 1 and 2 were combined into a single "discolored" category. Overall tooth loss scored are as follows. 0 = no loss, 1 = at least one row is discolored (gray, faint or rough) in appearance, but no segments are missing, 2 = <25% missing, 3 = 25-50% missing, 4 = 50-75% missing, 5 = >75% missing.

Jaw sheath loss was ranked on a scale of 0 to 5 as follows. 0 = no loss, 1= thinning, 2 = <10%, 3 = 10-50%, 4 = 50-90%, 5=>90%. The scales for tooth loss and jaw sheath loss were created differently to accommodate and more accurately represent the different patterns of loss in the respective keratinized structures.

Results

During year 1, prevalence of tooth loss and infection was very low in tadpoles of *Litoria genimaculata* and *L. xanthomera* (Table 7A.1 and Table 7A.2). Due to the very small effect of *Bd* on these tadpoles, they were unable to be analyzed further. In contrast, tadpoles of *L. nannotis*, *L. rheocola* and *Nyctimystes dayi* had high prevalence of mouthpart loss and *Bd* infection during year 1 (Table 7A.3, mouthpart data not shown). As a result, mouthpart loss in tadpoles of these species was examined in more depth in year 2. *N. dayi* is not included in analyses due to small sample size.

Prevalence (Tooth Loss)

The proportion of tadpoles with no loss, discoloration, <25%, 25-50%, 50-75% and >75% loss differed significantly between infected and uninfected individuals of both *Litoria nannotis* (x^2 (5, 688) = 174.1, p < 0.0005) and *L. rheocola* (x^2 (4, 154) = 57.43, p < 0.0005, Fig. 7A.2). *Litoria nannotis* tadpoles with no loss ($x^2(1,572)$ = 123.7, p < 0.0005) and with discolored mouthparts ($x^2(1,320)$ = 13.83, p = 0.001) had lower prevalence of *Bd* than tadpoles with any greater level of tooth loss. Tadpoles, with tooth loss greater than discoloration, were combined as prevalence of *Bd* did not differ between groups ($x^2(3,171)$ = 4.4, p = 0.224). *Litoria rheocola* with normal mouthparts had lower prevalence of Bd than tadpoles displaying any level of tooth loss, including discoloration ($x^2(1,121)$ = 41.5, p < 0.0005). In contrast with *L. nannotis*, *L. rheocola* with discolored or any category of missing teeth did not differ in prevalence among these groups ($x^2(1,108)$= 0.03, p = 0.857). The sample size for *L. rheocola* with >75% loss was small (n = 2) and was removed for analyses but included in Fig. 7A.2.

Prevalence (Jaw Sheath Loss)

The proportion of tadpoles with no loss, thinning, <10%, 10-50%, 50-90% and >90% jaw sheath loss was significantly different between infected and uninfected tadpoles of both *L. nannotis* (x^2 (5, 616) = 75.2, p < 0.0005, Fig. 7A.3) and *L. rheocola* (x^2 (4, 151) = 30.9, p < 0.0005, Fig. 7A.3). However, over 60% of tadpoles of both species with no loss were still infected. Prevalence was 100% in tadpoles of both species presenting any level of jaw sheath loss (except for 1 *L. rheocola* with 50-90% jaw loss that was uninfected as determined by PCR)
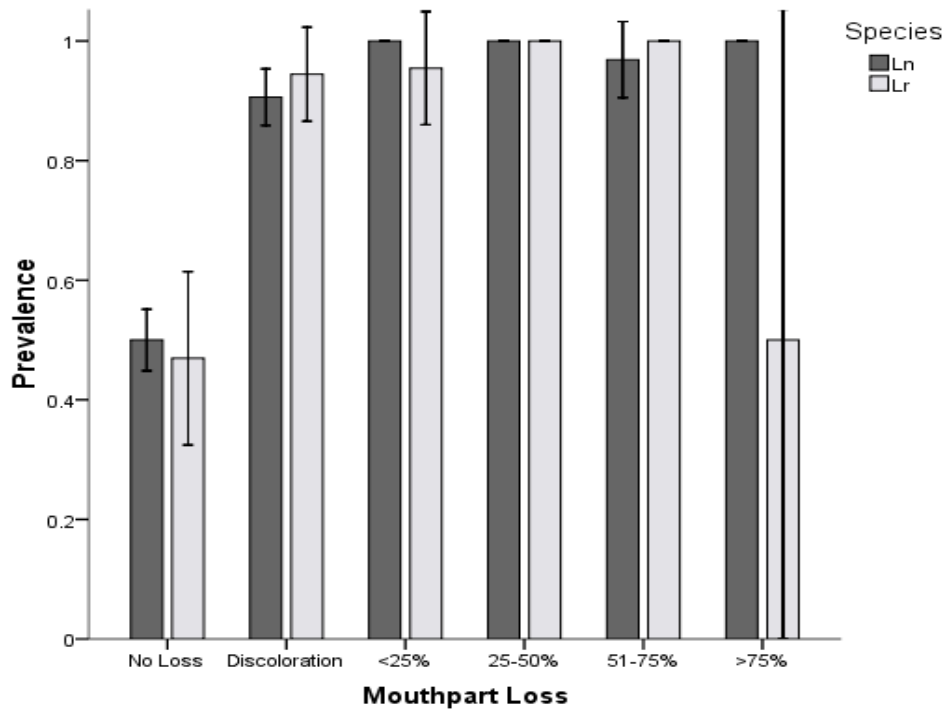
Figure 7A.2: Prevalence of *B. dendrobatidis* infection in *L. nannotis* and *L. rheocola* at Tully Gorge NP by degree of overall mouthpart loss (2007-2008).
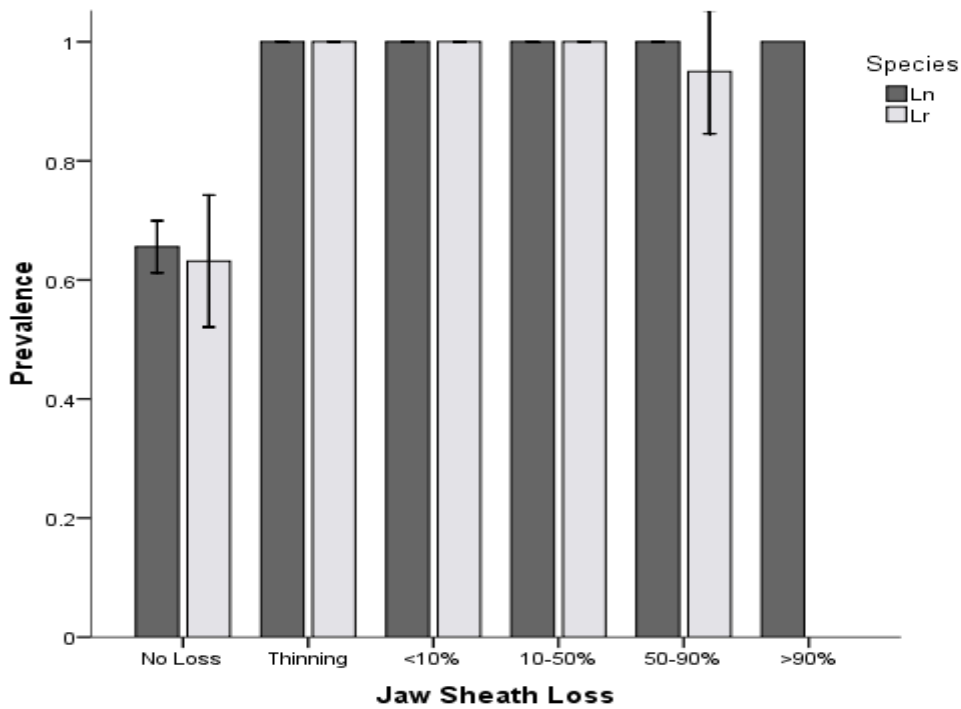

Figure 7A.3: Prevalence of *B. dendrobatidis* infection in *L. nannotis* and *L. rheocola* at Tully Gorge NP by degree of jaw sheath loss (2007-2008).

<u>Binomial Logistic Regression</u>

Binomial logistic regression was employed to assess if mouthpart loss or jaw sheath loss was the better predictor of *Bd* infection and if there were any differences in this predictive ability between *L. nannotis* and *L. rheocola*. Data was from Tully Gorge NP between 2007 and 2008. For the analysis, infection status was the response variable, and species, mouthpart loss score and jaw sheath loss score were the predicting variables. Mouthpart loss was found to be a significant indicator of infection status while species and jaw sheath loss were not (Table 7A.1). This indicates that once mouthpart loss was accounted for, there was no significant relationship with the remaining two variables. The results also show that once mouthpart loss reaches 25%, additional tooth loss does not improve the ability to predict infection status (Table 7A.1).

Table 7A.1: Binomial logistic regression examining the predictive ability of jaw sheath loss and tooth loss on infection with *Batrachochytrium dendrobatidis* in *L. nannotis* and *L. rheocola* at Tully Gorge NP (2007-2008).

Variable	Coefficient	SE	Wald	df	p	Odds ratio	Lower 95% CI	Upper 95% CI
Species			1.444	1	0.230	1.409	0.805	2.465
Jaw Sheath Loss Score			1.703	5	0.888			
Thinning	18.965	0.004	0.000	1	0.996	0.000	0.000	
<10%	18.690	0.005	0.000	1	0.997	0.000	0.000	
10-50%	18.424	0.006	0.000	1	0.998	0.000	0.000	
50-90%	2.142	1.641	1.703	1	0.192	8.517	0.341	212.530
>90%	20.463	0.000	0.000	1	0.100	0.000	0.000	
Tooth Loss Score			48.492	5	0.000			
Discoloration	1.963	0.315	38.844	1	0.000	7.122	3.841	13.205
<25%	3.284	1.026	10.243	1	0.001	26.682	3.571	199.354
25-50%	19.661	0.003	0.000	1	0.995	0.000	0.000	
50-75%	1.433	1.216	1.389	1	0.239	4.191	0.387	45.424
>75%	0.605	1.634	0.137	1	0.711	1.831	0.074	45.071
Intercept	-0.208	0.270	0.591	1	0.442	0.812		
Whole Model			256.784	11	<.0005			

<u>Sensitivity/Specificity</u>

Visual assessment of mouthpart loss in both *L. nannotis* and *L. rheocola* had very high specificity as an indirect technique for the detection of *B. dendrobatidis* infection (Table 7A.3). Therefore, there were very few false positives in tadpoles presenting mouthpart loss (from discoloration to complete loss). However, sensitivity was low. Therefore, there is a high likelihood of obtaining false negatives, which would lead to an underestimation of prevalence if mouthpart loss was the only method of *Bd d*etection employed. When the minimum visual assessment threshold for a *Bd*-positive individual is increased so that only tadpoles with missing gaps in tooth rows are considered infected, specificity increases (*L.* nannotis; 99.5%, *L. rheocola*; 93.3%), however sensitivity decreases (*L.* nannotis; 33.5%, *L. rheocola*; 55.1%, Table 7A-3).
Visual assessment of jaw sheath as an indicator of infection status produced similar results. Specificity was very high, however, sensitivity was low.

Due to the low prevalence of mouthpart loss and infection, mouthpart loss as an indicator of infection for the pool tadpoles (*L. genimaculata* and *L. xanthomera*) had very high specificity and very low sensitivity (Table 7A.2). In the case of *L. xanthomera*, the sensitivity was 0% simply because no mouthpart loss was observed.

Table 7A.2: Evaluation of overall tooth loss as an indirect test of infection with *Batrachochytrium dendrobatidis* in *Litoria genimaculata* and *L. xanthomera* compared with PCR. Prevalence is the rate of PCR infected tadpoles in each visual assessment category. Percent accurate is the percentage of cases the visual assessment correctly identified. Sensitivity is the number of visually positive tadpoles divided by the number of PCR positive tadpoles; high values = low rate of false negatives. Specificity is the number of visually negative tadpoles divided by the number of PCR negative tadpoles; high values = low rate of false positives.

Species	Mouthpart	Visual Assessment	PCR Result		Prevalence	Percent Accurate	Sensitivity	Specificity
			Bd-	Bd+				
L. genimaculata	Tooth Rows	Fine (-)	259	12	4.4%	95.6%		
		Abnormal (+)	6	1	14.3%	14.3%		
		total	265	13		93.5%	7.7%	97.7%
L. xanthomera	Tooth Rows	Fine (-)	63	2	3.1%	96.9%		
		Abnormal (+)	0	0	0.0%	0.0%		
		total	63	2		96.9%	0.0%	100.0%

Table 7A.3: Evaluation of overall tooth loss as an indirect test of infection with *Batrachochytrium dendrobatidis* in *Litoria nannotis* and *L. rheocola* compared with PCR. Prevalence is the rate of PCR infected tadpoles in each visual assessment category. Percent accurate is the percentage of cases the visual assessment correctly identified. Sensitivity is the number of visually positive tadpoles divided by the number of PCR positive tadpoles; high values = low rate of false negatives. Specificity is the number of visually negative tadpoles divided by the number of PCR negative tadpoles; high values = low rate of false positives. The tooth loss category of "discolored" was considered as both visually *Bd* positive and negative for comparison.

Species	Mouthpart	Visual Assessment	PCR Result Bd-	Bd+	Prevalence	Percent Accurate	Sensitivity	Specificity
L. nannotis	Tooth Rows	Fine (-)	198	203	50.6%	49.4%		
		Discolored and Abnormal (+)	15	305	95.3%	95.3%		
		total	213	508		69.8%	60.0%	93.0%
		Fine and Discolored (-)	212	338	61.5%	38.5%		
		Abnormal (+)	1	170	99.4%	99.4%		
		total	213	508		52.9%	33.5%	99.5%
	Jaw Sheath	Fine (-)	156	297	65.6%	34.4%		
		Abnormal (+)	0	163	100%	100%		
		total	156	460		51.8%	35%	100%
L. rheocola	Tooth Rows	Fine (-)	26	23	47.0%	53.0%		
		Discolored and Abnormal (+)	4	104	96.3%	96.3%		
		total	30	127		82.8%	81.9%	86.7%
		Fine and Discolored (-)	28	57	67.1%	32.9%		
		Abnormal (+)	2	70	97.2%	97.2%		
		total	30	127		62.4%	55.1%	93.3%
	Jaw Sheath	Fine (-)	28	48	63.2%	36.8%		
		Abnormal (+)	1	74	98.7%	98.7%		
		total	29	122		65.0%	60.7%	96.6%

<u>Intensity</u>
Because mouthpart loss is a more precise indicator of infection status than jaw loss (see binomial logistic regression results above), the following analyses focus on mouthpart loss data. *Litoria rheocola* with >75% loss were excluded from the analyses due to small sample size (n=2).

Intensity of infection differed significantly among tooth loss categories in both *L. nannotis* (ANOVA, $F_{(5, 502)} = 17.6$, $p<0.0005$, Fig. 7A.4) *and L. rheocola* (ANOVA, $F_{(4, 127)} = 16.5$, $p<0.0005$, Fig. 7A.4). In infected animals of both species, mean intensity was lowest in tadpoles with no apparent loss. Intensity increased in tadpoles displaying discoloration and tooth loss of <25%. As tooth loss severity increased from this point, mean intensity tended to decrease, although it was not statistically significant. Results from Tukeys HSD posthoc test indicate that mean intensity in *L. nannotis* differed between tadpoles with no tooth loss and all other categories except >75% loss. Tadpoles with >75% loss had mean infection intensities equivalent to infected tadpoles without tooth loss. Intensity was not different among any groups displaying discoloration or tooth loss.

Although infection intensity was lower in *L. rheocola* than *L. nannotis* the pattern of intensity in relation to tooth loss was very similar to that of *L. nannotis*. Mean intensity in *L. rheocola* tadpoles with no tooth loss was significantly lower than tadpoles with any level of tooth loss. Mean pathogen load peaked in tadpoles with <25% tooth loss and then tended to decrease, however, intensity was not statistically different among animals displaying any level of tooth loss.
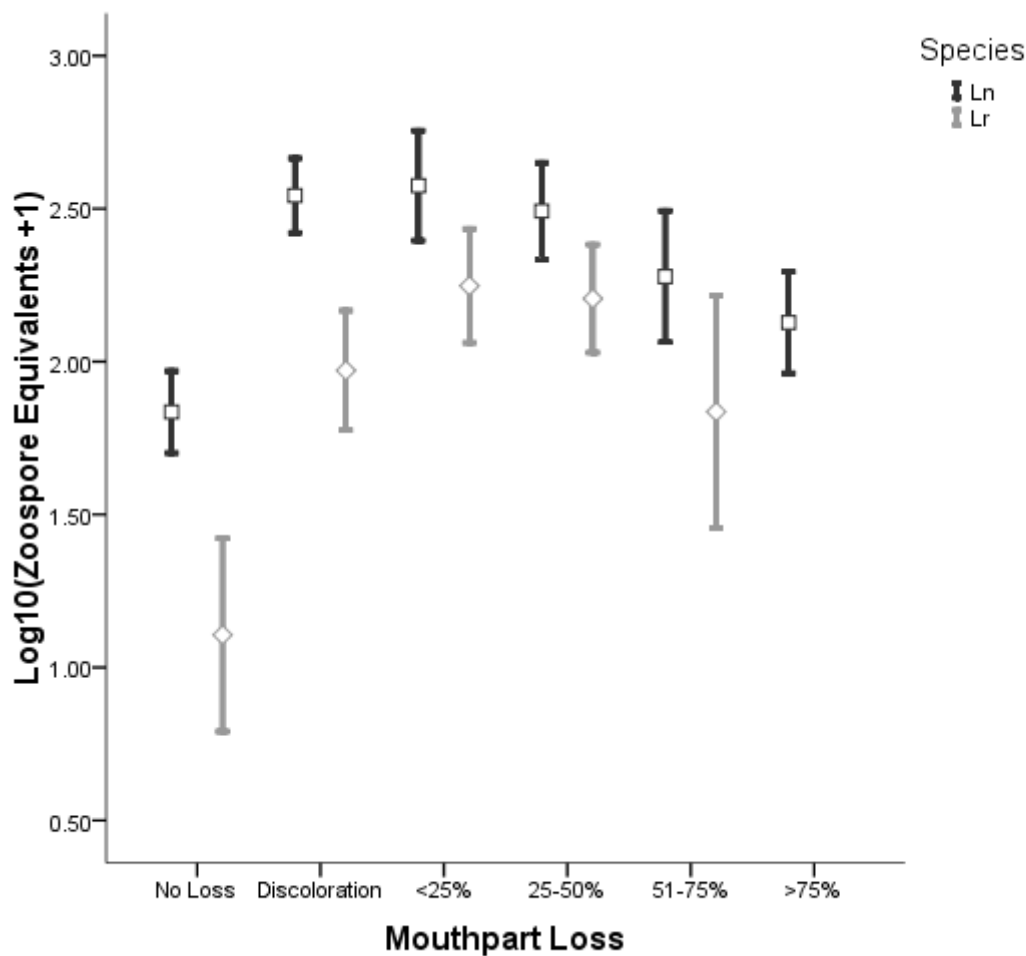
Figure 7A.4. Intensity of *Batrachochytrium dendrobatidis* infection in *L. nannotis* and *L. rheocola* by degree of overall tooth loss.

Size

Because *L. nannotis* and *L. rheocola* are of different sizes (Chapter 5) and sample size for *L. rheocola* is smaller, this analysis focuses on *L. nannotis*. Uninfected *L. nannotis* tadpoles were primarily smaller tadpoles without tooth loss (Fig. 7A.9). Relatively few large size group tadpoles with normal teeth were uninfected, however, the only large tadpoles that were uninfected had normal teeth and, in one case, discoloration (Fig. 7A.9). Although large size group tadpoles had very high prevalence of infection and high intensity (Chapter 6) they did not have high levels of mouthpart loss. The number of large tadpoles, in fact, decreased with increasing mouthpart loss (Fig. 7A.9) until no large tadpoles were captured with >75% tooth loss. Tadpoles suffering advanced stages of tooth loss (>50% loss) were primarily medium size group tadpoles. Nearly all small size group tadpoles had no tooth loss, with only one small size group tadpole captured displaying loss greater than discoloration Fig. 7A.9).

Seasonality

The results show that prevalence was high (90.6 – 100%) in tadpoles with any level of tooth loss (from discoloration to >75% loss), and in tadpoles with larger body sizes (Fig. 7.XXX and 7A.9). Therefore, uninfected tadpoles were primarily present in the population only when small, new tadpoles entered the population (Fig. 7A.10). Recruitment occurred throughout the year but particularly during spring-summer and coincided with periods of metamorphosis (Chapter 5)

The severity of tooth loss within the population was dynamic within and across size classes over time. Beginning in the summer of 2007 (January) there was no observed tooth loss until May 15 (Fig 7A.11). Leading up to this time, the mean intensity and prevalence of infected individuals in the population increased (Fig. 7.15, Fig. 7A.12). From May 15, tooth loss began to intensify while mean infection intensity decreased. When tooth loss severity peaked around July 18, mean intensity was 10x lower and all tadpoles were missing over 25% of their teeth. However, only medium size group tadpoles were missing over 75%. Over the following two months, until September 21 this pattern was reversed. Overall mouthpart condition improved, the number of tadpoles in the large size group increased as tadpoles developed towards metamorphosis and mean infection intensity trended upwards until September 12, around the time the period of metamorphosis began. From here, the number of large size group tadpoles decreased and the number of small to medium size group tadpoles increased, with the majority of small tadpoles uninfected and with no mouthpart loss. Intensity in infected tadpoles trended downward until sampling stopped at the culmination of the year-long cycle on February 14, 2008.

Although tooth loss data was not recorded during the first year of the study in 2006-07, we can compare patterns in the intensity of infection data (Fig. 7.14) with patterns seen in 2007-2008. While no definitive relationships with tooth loss can be drawn, the same general pattern of infection intensity, which may be indicative of tooth loss patterns, was seen in 2006-07 as well as 2007-08: Infection intensity of infected individuals begins low in summer, then increases over autumn before decreasing temporarily in winter (as tooth loss severity increases). Intensities then increase again (as mouthparts recover) before peaking in spring and then declining back into summer as frogs metamorphose and new tadpoles enter the population.

Progression of Infection

Jaw Sheath

As overall tooth loss intensified, so did loss of the keratinized jaw sheath (Fig. 7A.5). Normal jaw sheaths were observed only in tadpoles with none to low levels of mouthpart loss. Jaw sheath loss, ranging from thinning up to 50% loss, was present when overall tooth loss was, on average, 25% or more, and extreme jaw sheath loss (>50%) was primarily present in only those tadpoles with severe overall tooth loss. Therefore, it is apparent that jaw sheath loss follows and does not precede tooth row loss. This conclusion is also supported by Fig 7A.3 that shows prevalence of infection in tadpoles with normal jaw sheaths is higher than in tadpoles with normal tooth rows, indicating that tooth rows display signs first.

Tooth Rows

As overall tooth loss increased in severity, individual tooth rows were not affected equally. The outermost posterior (P3) and anterior (A1) rows (Fig. 7A.1) suffered significantly more loss than the three interior rows at all stages of overall tooth loss, except when overall loss exceeded 75% (Fig. 7A.6). Row P3, compared to A1, had significantly greater mean individual tooth loss except when overall tooth loss was greater than 50%.

Prior to the period of peak overall tooth loss, increasing loss is associated with increasing intensity of infection and prevalence. It is logical to conclude that the tooth rows showing the initial damage are the ones that are more heavily infected and the tooth ones that recover first are the rows that are least infected. When overall tooth loss was first apparent and as overall loss was increasing in severity, the outermost posterior row (P3) was the most severely affected, followed by the outermost anterior row (A1), followed by the remaining three interior rows. During the period of peak mouthpart loss, the interior rows suffered tooth loss equivalent to the exterior rows (Fig. 7A.11). Following peak loss period, as teeth recovered, the reverse pattern was seen. The condition of the interior rows improved

first, while the exterior rows often remained visibly damaged. As the interior rows recovered to near normal, the exterior rows of many individuals remained damaged, with P3 in generally worse condition than A1.

Habitat

Over the period of peak mouthpart loss, when mean overall loss was greater than 25% (July 4 – Aug 1 2007, Fig. 7A.7), tadpoles with increasingly severe mouthpart loss tended to occupy habitat with slower mean flow rates. Differences in mean flow rate by overall tooth loss score was significant for *L. nannotis* (ANOVA, $F_{(3, 94)} = 3.055$, $p = 0.032$) and near significant for *L. rheocola* (ANOVA, $F_{(3, 37)} = 2.613$, $p = 0.066$). Tukey's HSD posthoc test indicated, for *L. nannnotis*, that tadpoles with greater than 75% loss occupied habitat with a slower mean water flow rate than tadpoles with <25% loss. There were no other statistically significant comparisons.
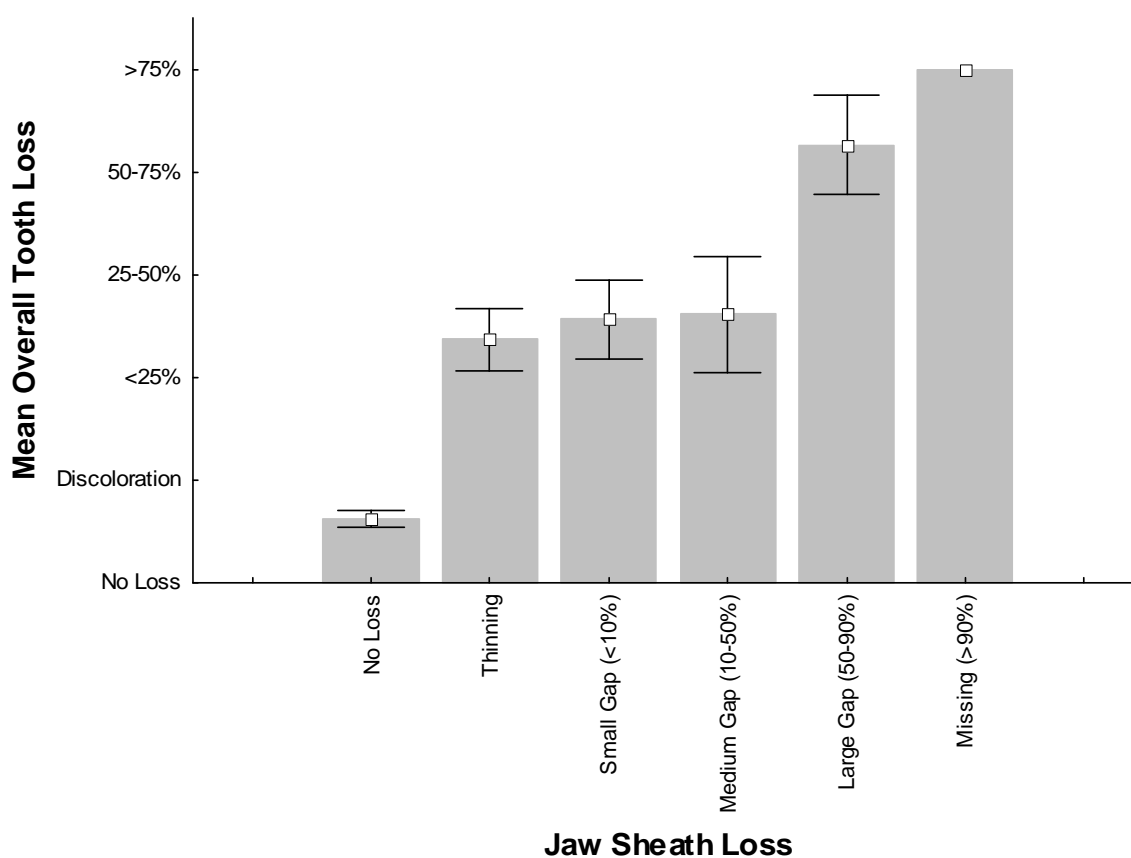
Figure 7A.5: Mean overall tooth loss score of *L. nannotis* and *L. rheocola* tadpoles in Tully Gorge NP with varying degrees of jaw sheath loss (2007- 2008).
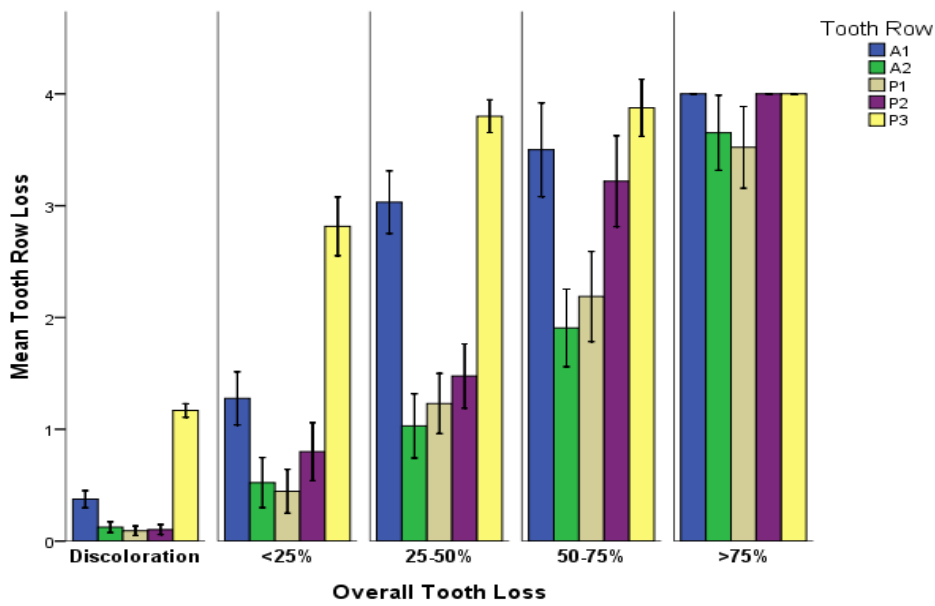
Figure 7A.6. Mean individual tooth row loss by varying degrees of overall tooth loss in *Litoria nannotis* and *L. rheocola* in Tully Gorge NP (2007-2008).
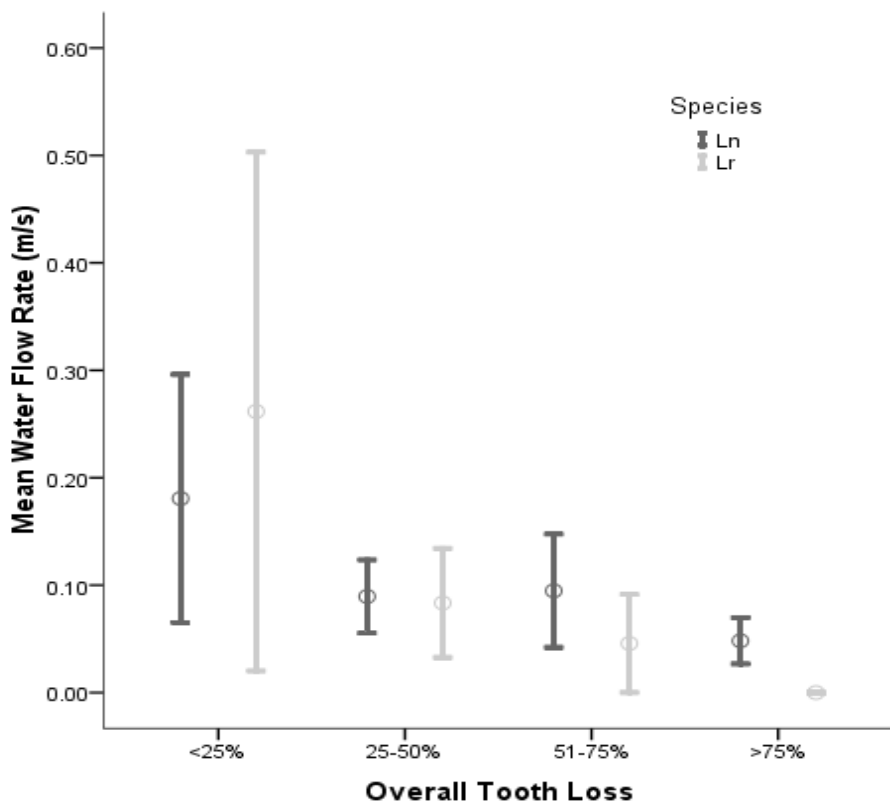
Figure 7A.7. Mean water flow rate where Litoria nannotis tadpoles with varying degrees of overall tooth loss were captured in Tully Gorge NP (2007-2008).
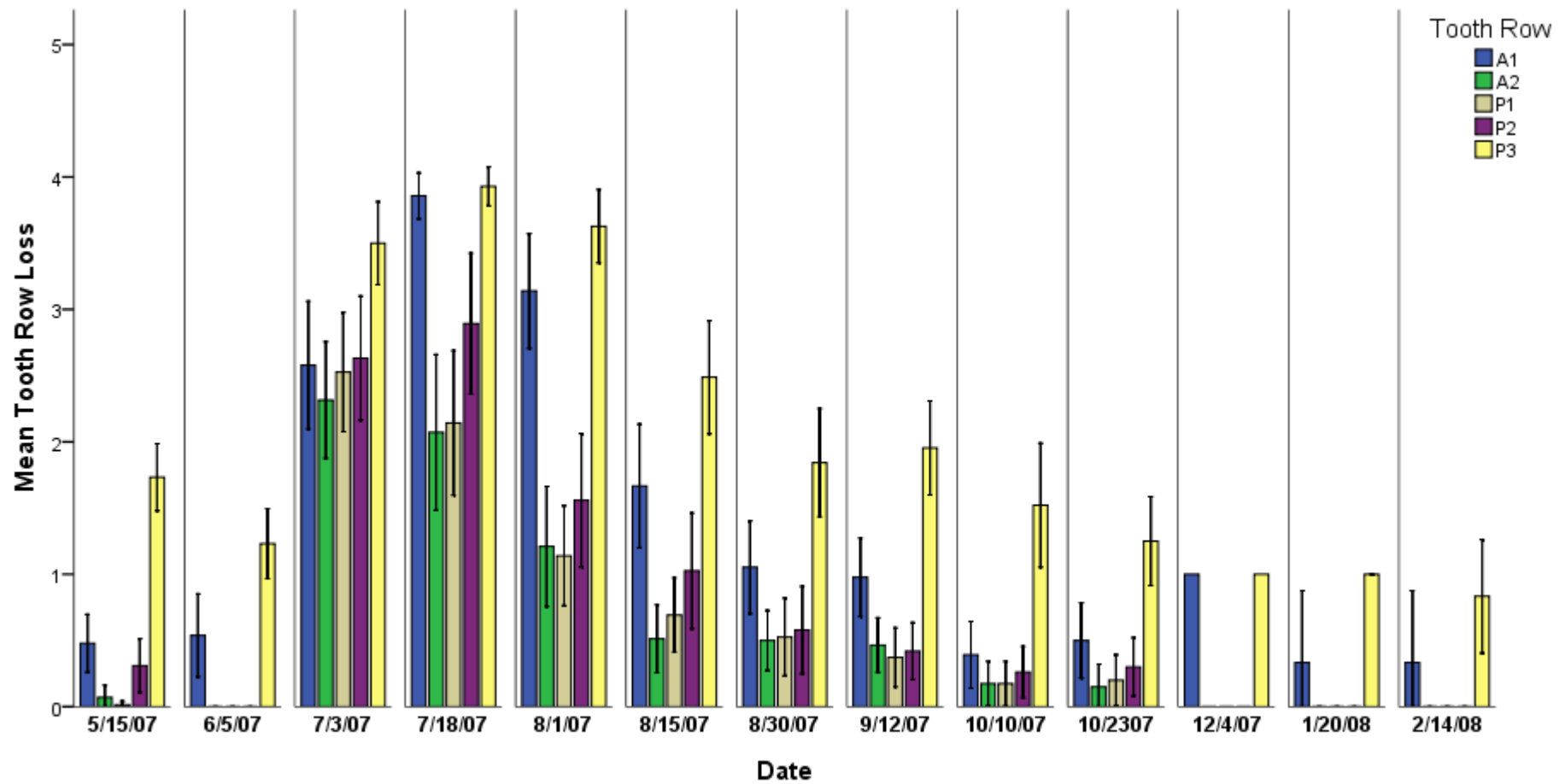
Figure 7A.8. Mean individual tooth loss in *L. nannotis* in Tully Gorge NP over time. Graph begins when tooth loss was first observed
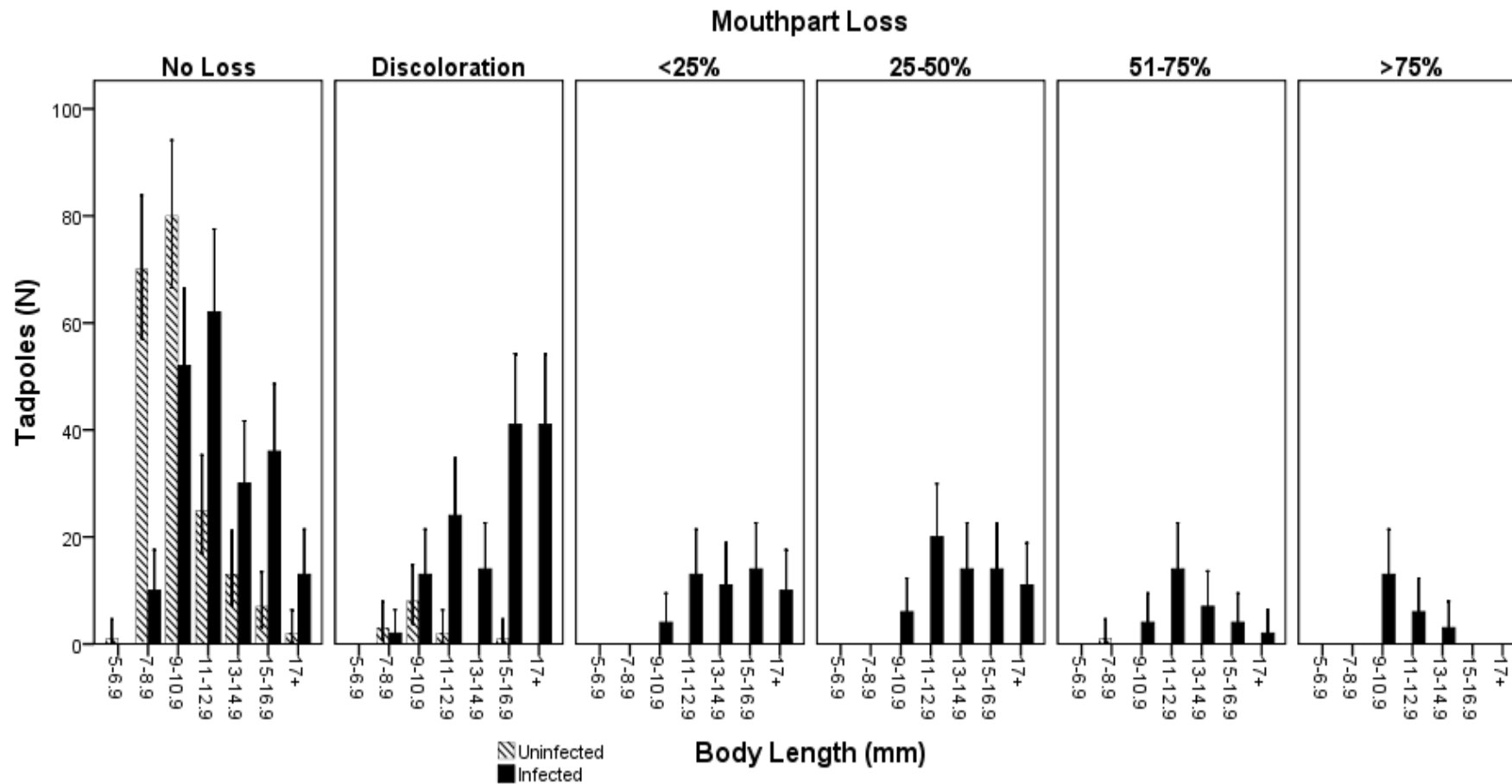
Figure 7A.9. Numbers of infected and uninfected *Litoria nannotis* tadpoles in Tully Gorge NP by overall tooth loss and size class (2007-2008).
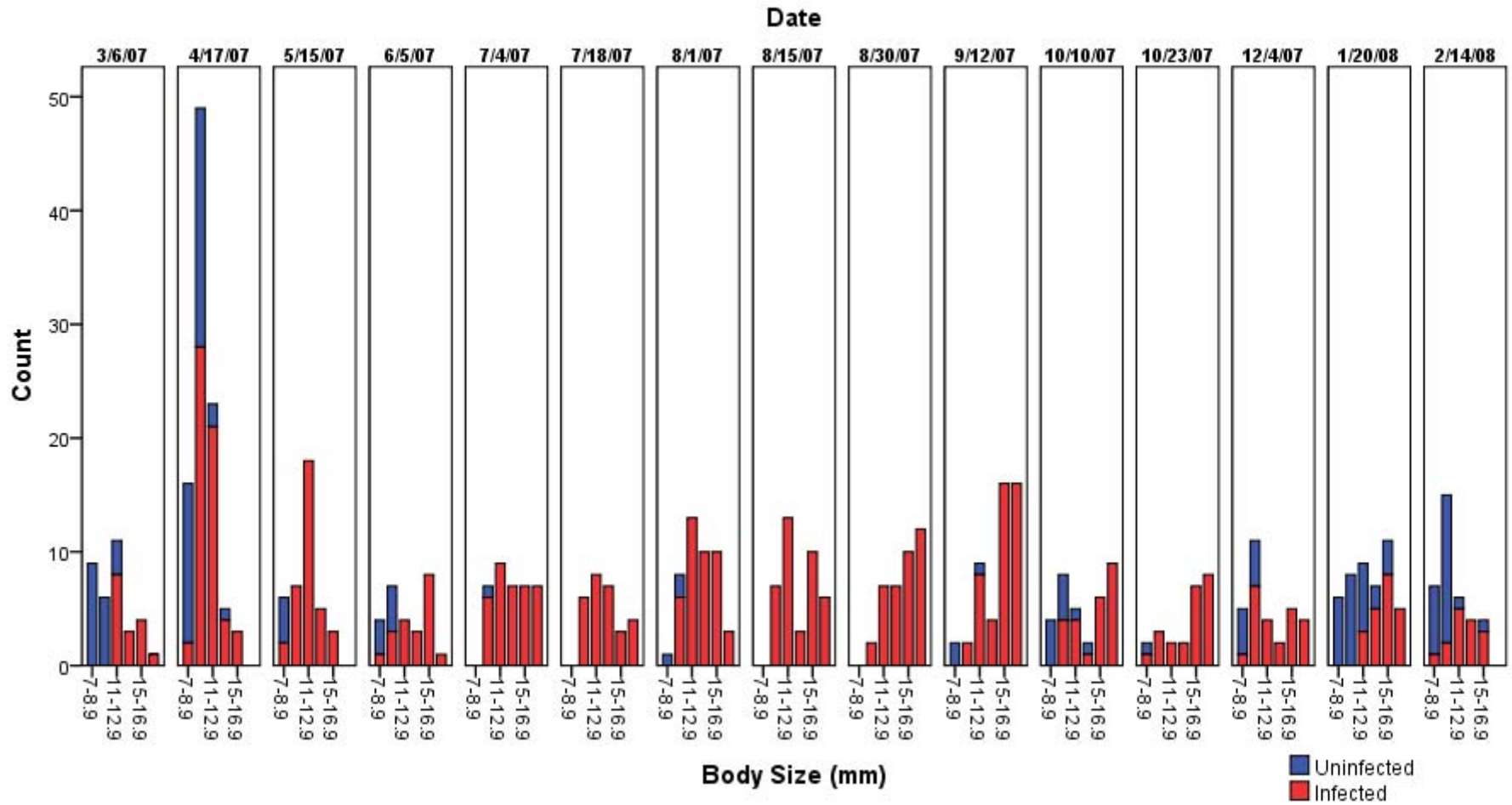
Figure 7A.10. Number of *Litoria nannotis* tadpoles in Tully Gorge NP infected by size class over time (2007 – 2008).
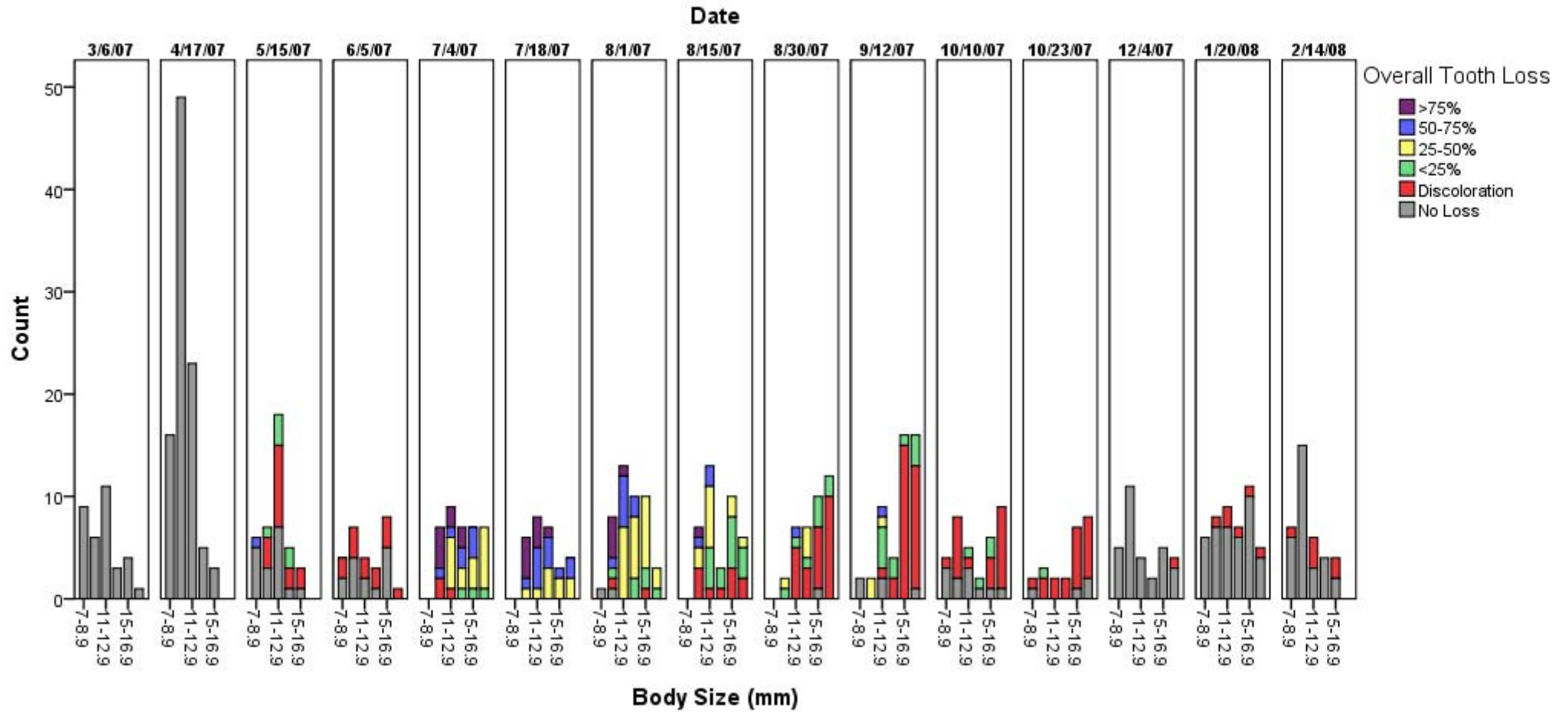
Figure 7A.11. Overall tooth loss in *L. nannotis* in Tully Gorge NP by size class over time (2007- 2008).
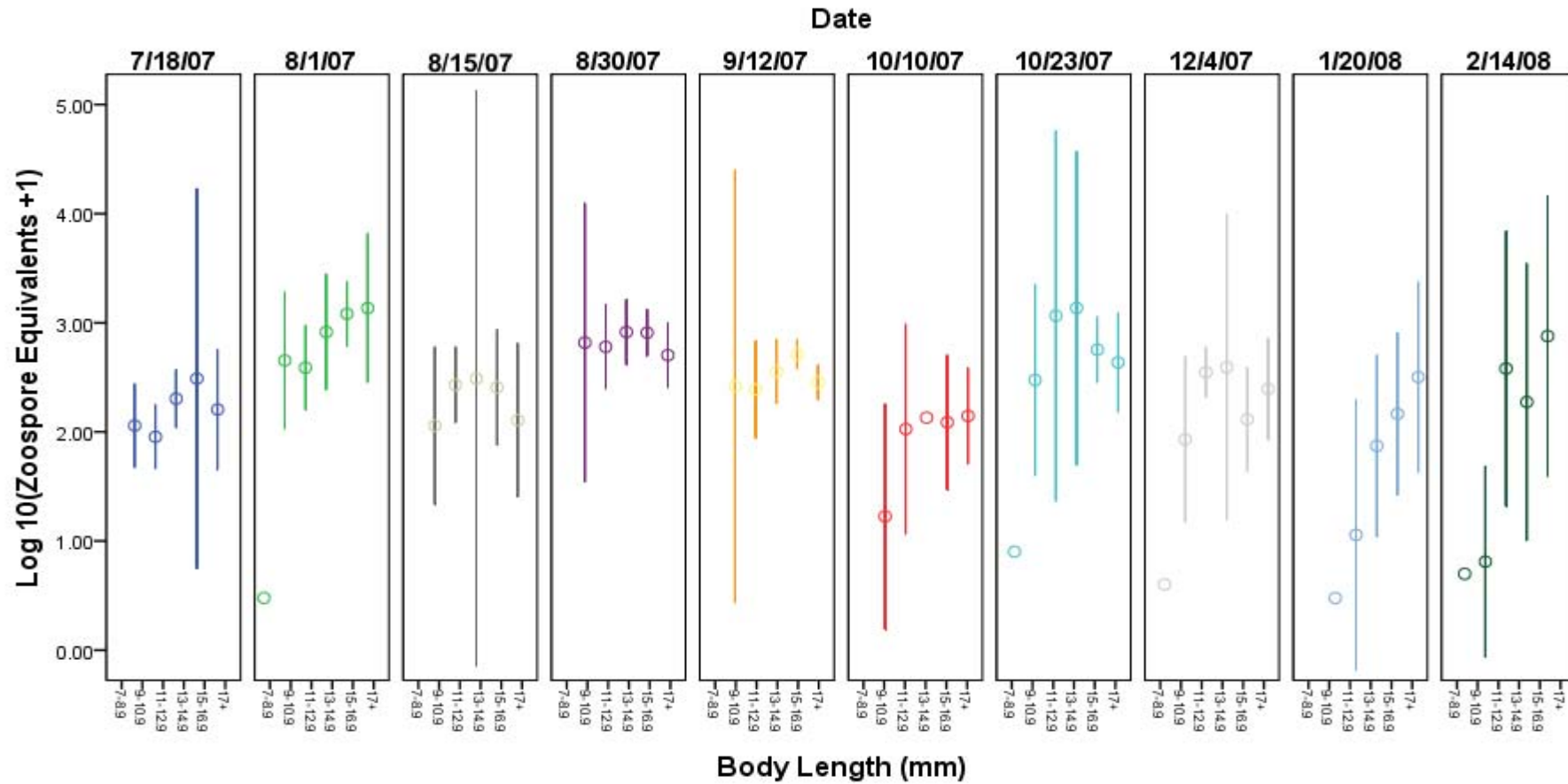
Figure 7A.12. Intensity of infection in *Litoria nannotis* tadpoles in Tully Gorge NP by size class over time (2007- 2008).

Body Condition

Tadpole body condition was estimated by first calculating the best fit exponential regression between body length (mm) and mass (g) using Quasi-Newton estimation methods (*L. nannotis*: R^2 = 93.4%, *L. rheocola* R^2 = 92.88%). From this, the mass residual for each tadpole was determined. These residuals represent the difference in mass from the expected for a given a body size. The mean and 95% confidence intervals of the residuals were then calculated for each sampling period and overlaid onto a stacked bar graph of overall tooth loss over time for both *L. nannotis* and *L. rheocola*. When body condition is good and weight is greater than average for a given size, the residual value will be above zero. The mean value of mass residuals significantly decreased as mouthpart condition decreased over time and increased as mouthpart condition improved (ANOVA, *L. nannotis*, $F_{(13, 579)}$, p = 0.000000; *L. rheocola*, $F_{(12, 121)}$, p = 0.00000, Fig 7A.13). Following recovery, in *L. nannotis*, mean value of mass residuals then significantly decreased during the period of metamorphosis and recruitment (Fig. 7A.13).

Observations

Over the course of the two-week peak overall loss period, many of the tadpoles captured with >50% loss were physically suffering from an apparent severe decrease in body condition (Fig. 7A.14). These tadpoles had very little to no food in the gut, poorly developed tail musculature, were physically weak and were poor swimmers during streamside captive observation. These apparently weakened tadpoles would stroke their tail a couple of times for locomotion, then stop in mid water column and drift until contact with the substrate was made. In contrast, healthy tadpoles swim by forcefully stroking their tails and accelerating through the water column until contact with the substrate is made.
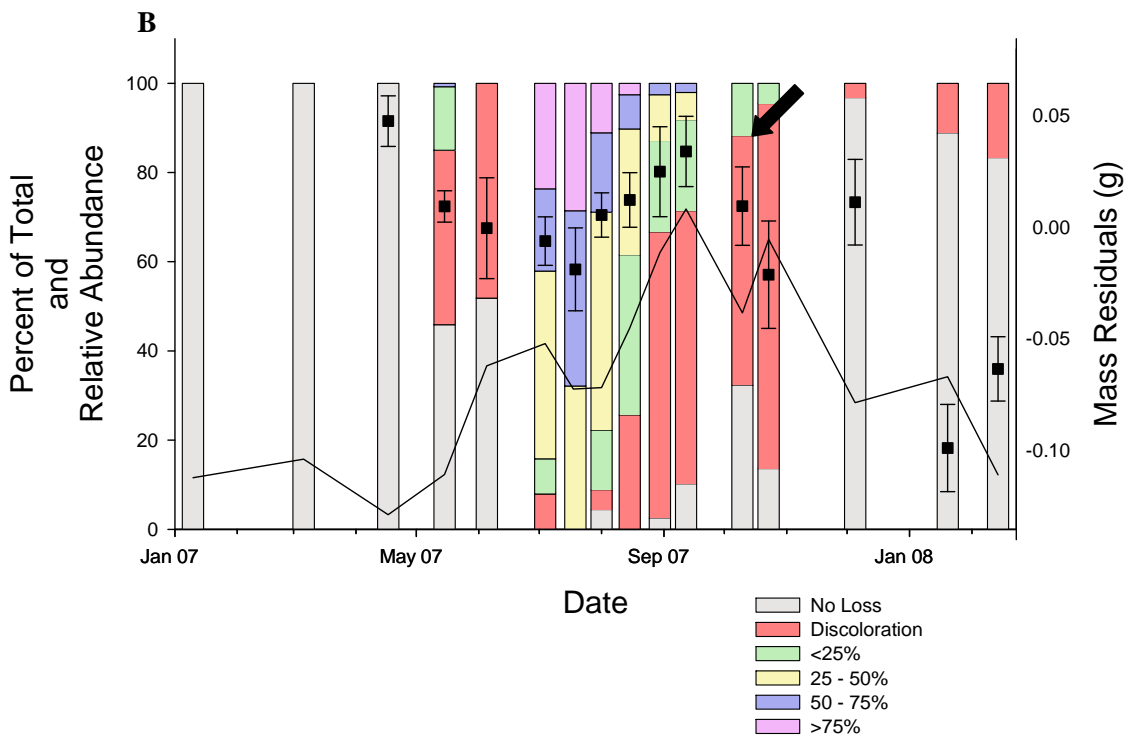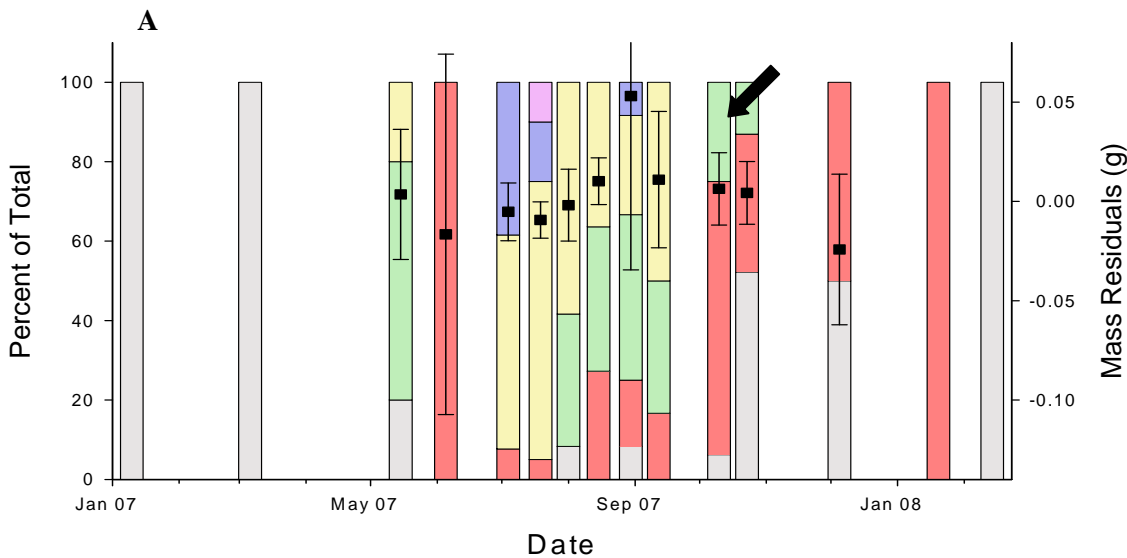
Figure 7A.13. Box Plots represent mean mass residuals with 95% confidence intervals and are overlaid onto stacked bar graphs representing degrees of tooth loss over time. A) *Litoria rheocola*, B) *L. nannotis*. Tully Gorge NP (2007-2008). The solid black line in B is the relative abundance of large size group tadpoles. Black arrow indicates beginning of metamorphosis

On at least two occasions, segments of tooth rows dislodged from a severely weakened *L. nannotis* mouthpart during handling and swabbing. The dislodged segments of teeth were found on both the glove following handling and on the swab tip following swabbing. Five emaciated and weak tadpoles died during handling. Apart from infrequent glove-related mortality (Chapter1), no other tadpoles died following handling during field sampling, indicating body condition was the significant factor leading to death.

Figure 7A.1. Left: A healthy *L. nannotis* with normal tooth rows, full stomach and well-developed tail musculature. Right: An unhealthy *L. nannotis* with >75% overall tooth loss and 50-90% missing jaw sheath, empty stomach, and poorly developed tail musculature.

Effect on Population Size and Development

In order to determine if the tadpole population size significantly changed following the period of peak body condition loss, population estimates on a 5 meter section stream at Tully Gorge were conducted. *Litoria nannotis* tadpoles were exhaustively sampled by two people using the dip net methods described in Chapter 5. Sampling was conducted on May 15, 2007 when tooth loss was first detected and on August 16, 2007 immediately following the period of peak tooth loss but before the period of metamorphosis and recruitment. Sampling occurred in 15 minute blocks, separated by 5 minute breaks. As the stream is an open system it is not feasible to truly exhaust the population. Linear regression of the number of tadpoles captured per sampling block by the total number of tadpoles captured provides an estimate of the total number of tadpoles present. The *L. nannotis* population was estimated at 184 tadpoles on May 14 and at 190 tadpoles on August 16, indicating a significant decrease in the population was unlikely to have occurred (Fig. 7A.16).

In an initial effort to investigate if tadpoles metamorphose at a smaller size as a result of mouthpart loss, a comparison was made with a site on the Carbine Tablelands on the McCleod River where mouthpart loss in torrent tadpoles due to *Bd* infection does not appear to be as severe. However, more study is needed to confirm this. Tadpoles within the largest size class (>17 mm) were significantly larger at the McCleod River (M = 21.2 mm, SD = 2.36) than at Tully NP (M = 18.1 mm, SD = .63; $t(54)$ = -6.74, $p < 0.0005$; Fig. 7A.15). Many factors can affect size at metamorphosis (Chelgren et al. 2006), however, these results provide tentative support to the hypothesis that mouthpart loss may affect size at metamamorphosis. For this analysis, only tadpoles from 8/30/07 and 9/12/07 were selected at Tully Gorge NP in order to include only the largest available tadpoles as this was the period just prior to metamorphosis. McCleod samples were from 7/28/08, the middle of Winter, and the predicted peak period of mouthpart loss.
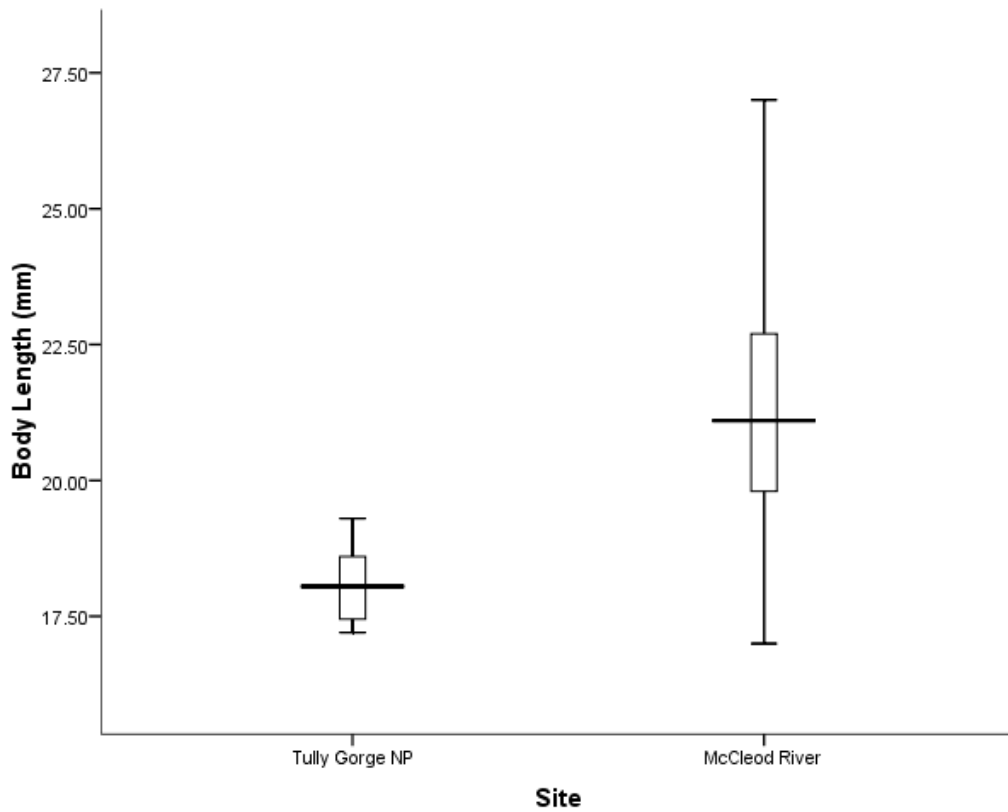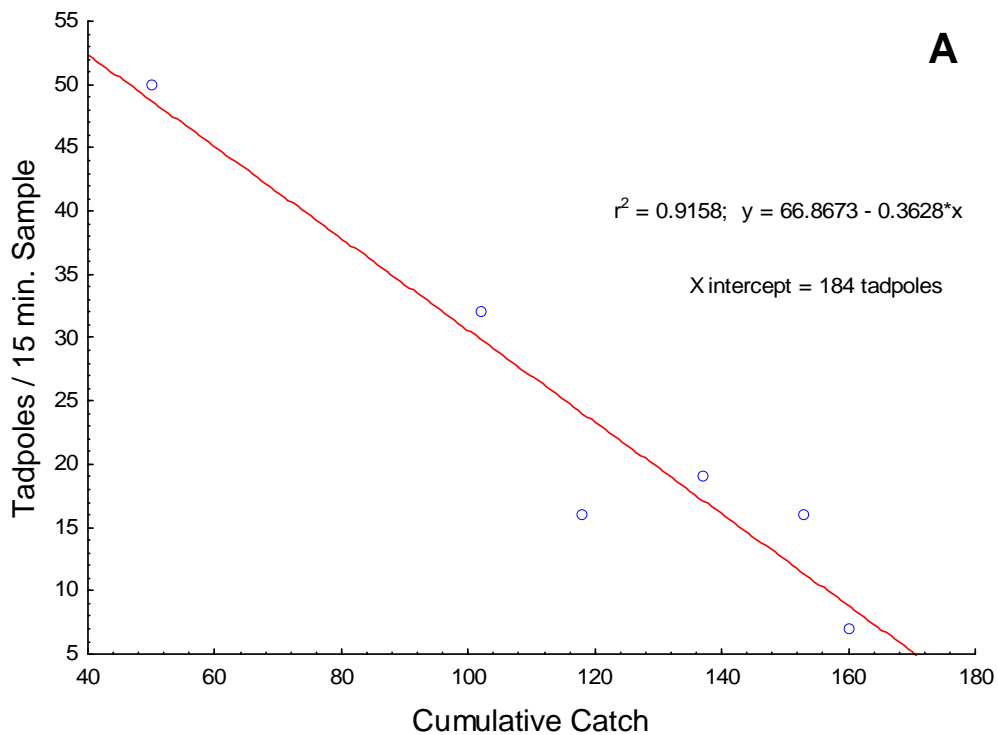
Figure 7A.14. Box Plot of *Litoria nannotis* tadpoles with body length >17 mm at Tully NP and McCleod River. Tully NP data are from the 4 weeks prior to the onset of metamorphosis.
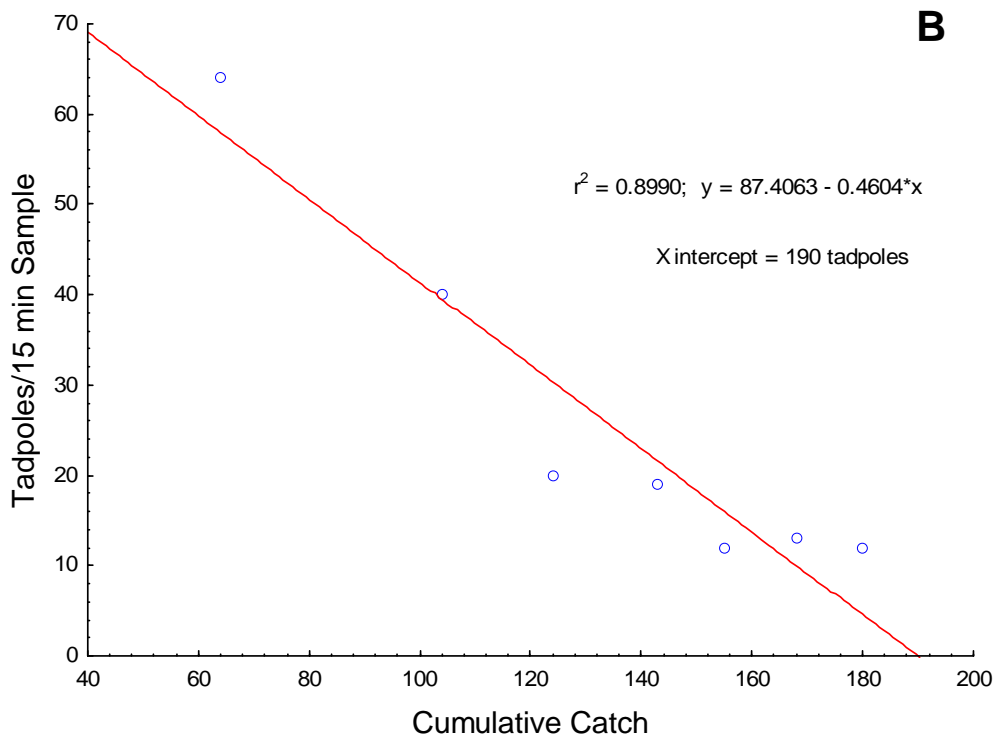

A

$r^2 = 0.9158$; $y = 66.8673 - 0.3628 \cdot x$

X intercept = 184 tadpoles

B

$r^2 = 0.8990; \; y = 87.4063 - 0.4604*x$

X intercept = 190 tadpoles

Figure 7A.15. Population estimate of torrent tadpoles in a 5 meter stretch of stream at Tully Gorge NP before and after peak mouthpart loss. A) May 15, 2007. B) August 16, 2007

Discussion

This study indicates that *Bd* can interact with larvae in a variety of ways, likely as a result of differences in host behaviour and microhabitat. This is the first evidence of a direct negative effect of *Bd* infection on tadpole health and condition, and the first evidence that tadpoles can recover mouthparts following loss. Infection with *Bd* causes the loss of keratinized mouthparts. In torrent adapted tadpoles these mouthparts are essential for acquiring food from rock surfaces. As mouthpart loss increased in severity, tadpoles apparently lost the ability to feed effectively and body condition decreased until many tadpoles were visibly skinny and physically weak. Mouthparts, however, were able to recover, allowing feeding to resume and body condition to improve, preventing widespread mortality. I discuss the possible effects a seasonal cessation of larval growth and development could have on a population and the usefulness of mouthpart loss as an indicator of infection.

Mouthpart Loss and Prevalence of *Bd*

During surveys at Murray Upper NP and Tully Gorge NP, mouthpart deformities in the form of missing teeth and reduction in the keratinized cells of the jaw sheath, were common in tadpoles of *L. nannotis*, *L. rheocola* and *N. dayi* and strongly associated with *Bd* infection. Deformities were very rare in *L. genimaculata* and were not strongly associated with infection. However, during field enclosure experiments (S. Cashins, unpublished), mouthpart loss in *L. genimaculata* was found to be strongly associated with infection, indicating that the absence of mouthpart loss during this field study was due to absence (or low intensity) of infection rather than a resistance to mouthpart loss. Deformities were never observed in *L. xanthomera*.

Oral deformities have been associated with *Bd* infection in larvae of a number of species (Fellers et al. 2001; Lips et al. 2004; Marantelli et al. 2004; Symonds et al. 2007; Vredenburg and Summers 2001). Knapp and Morgan (2006) found the presence of oral deformities to be highly predictive of *Bd* infection at both the individual and population level (86% and 92% correctly predicted respectively). However, deformities can have a number of causes apart from disease, including temperature, contaminants, physical damage and phylogeny (Drake et al. 2007; Padgett-Flohr and

Goble 2007; Rachowicz 2002). It also appears that larva of some species may exhibit no mouthpart deformities when infected with *Bd* (Knapp and Morgan 2006). To date, all detailed data on mouthpart deformities in *Bd* infected populations have come from pond dwelling tadpoles in temperate regions (with the exception of Symonds et al. 2007). This is the first information on infection and deformities in torrent adapted tadpoles and tadpoles in rainforest streams. More data is needed on tadpole mouthpart deformities in rainforest streams as this is where the majority of species affected by *Bd*-associated declines reside. A number of qualities separate rainforest streams from temperate ponds that could affect the prevalence of non-disease deformities. Tropical streams are often permanent water bodies with a small minimum to maximum temperature range. Therefore, the stress of seasonally cold temperature or evaporating habitat leading to overcrowding are unlikely causes of deformities. Many *Bd*-associated declines in tropical rainforest streams have occurred within protected areas, limiting the effects that pesticides or contaminants may have on deformities.

Indirect Indicator of Infection
Both the overall tooth loss score and the jaw sheath loss score returned similar results: loss as an indicator of infection was more specific than it was sensitive. Compared to overall tooth loss, jaw sheath loss in torrent tadpoles had a higher specificity but sensitivity was much lower (Table 7A.3). This indicates that a longer duration of infection is required before loss of jaw sheath cells is noticeable (Fig. 7A.5). This delay leads to an increase in false negatives when using jaw sheath loss as an indicator of infection. The binomial logistic regression indicated that tooth loss was a better predictor of infection status than jaw sheath loss. This is because it is a more sensitive test, having a reduced number of false negatives. However, if the goal of sampling tadpoles is to identify whether *Bd* is present in a population, such as during a broad scale mapping effort (Skerratt et al. 2008); using jaw sheath score to select individuals for subsequent direct testing (PCR or histology) would be preferred as jaw sheath score is the more conservative test with a higher specificity. For researchers inexperienced in distinguishing a "discolored" tooth row from a "normal" tooth row or a "thinning" jaw sheath from a "normal" jaw sheath, a modification of the overall tooth loss score would provide a simpler, potentially less ambiguous, and equally effective method as jaw sheath loss score for identifying infected individuals (Fig 7A.5). Collapsing "no loss" and "discolored" tadpoles into one "uninfected" category and considering any tadpole with missing segments of teeth to be "infected" is as effective as jaw sheath loss and removes the need to distinguish between a "thinning" and a "normal" jaw sheath. Others have concluded that jaw sheath loss is the preferred indicator of infection status because deformities to jaw sheath are less likely to occur due to factors other than *Bd* (Knapp and Morgan 2006; Obendorf 2005). If uninfected torrent tadpoles are desired, overall tooth loss score is the appropriate indirect screening tool due to a lower rate of false negatives (higher sensitivity).

This and previous studies indicate the relationship between *Bd* infection and mouthpart loss will vary considerably with species and habitat (Knapp and Morgan 2006; Padgett-Flohr and Goble 2007; Smith and Weldon 2007). Before mouthpart loss is used as an indicator of infection in a new species, a trial should be run to establish the strength of the relationship.

Mouthpart Loss and Intensity of *Bd*
The intensity of infection in infected tadpoles was lowest in tadpoles with no loss, then increased to a peak intensity at loss less than 25%, before tending to decline as loss increased above 25%. In *L. nannotis* there was no significant difference in intensity between tadpoles with no loss and tadpoles with >75% loss. The increasing intensity of infection with increasing tooth loss is consistent with an infection spreading across the mouthparts that disrupts tooth production leading to more advanced loss. Interestingly, intensity begins to decline as overall tooth loss increases above 25%. The decrease in keratinized mouthpart structures may cause a decrease in suitable substrate for infection, leading to the decline in intensity. Mouthparts were able to recover, and as they recovered *Bd* intensity increased. Intensity of infection over two years was best explained in a multiple regression

model of body length, rainfall, site and solar radiation (Chapter 6). Mouthpart loss was not included in this analysis because it was considered a sign and not a cause of infection intensity.

Figure 7A.12 shows that until mouthparts began to recover on August 15, 2007, intensity of infection increased with size as indicated by the multiple regression model. However, after mouthpart loss peaked and mouthparts began to recover, the pattern changed from a linear relationship to a bell shaped relationship; medium sized tadpoles had a higher intensity and smaller and larger tadpoles had a lower intensity. As large size-group tadpoles left the population and small tadpoles hatched (Fig. 7A.11, December 4, 2007) the original pattern of increasing intensity with body size returned (Fig. 7A.12). This shifting intensity of infection pattern suggests either large tadpoles had developed resistance (as discussed in Chapter 6) and/or the cessation of feeding due to mouthpart loss was impeding development such that only those tadpoles with recovered mouthparts and decreased infection intensity were able to grow and transition through to the larger size classes. In support of this, Figure 7A.13 indicates *L. nannotis* tadpoles stopped entering the large size-group during peak mouthpart loss. Only after mouthparts began to recover did tadpoles resume movement into the largest size group and metamorphose.

Interestingly, it appears that infection may "burn itself out" although infections are rarely cured completely (Chapter 6). Once infection progresses to the point where few keratinized teeth remain (and the tadpole is in poor condition), intensity drops, at which point the mouthparts begin to re grow (and intensity increases again). This drop in intensity due to tooth loss may provide a small reprieve allowing mouthparts the chance to re grow. However, in order to achieve the near full recovery of mouthparts population wide that was observed (Fig. 7A.13), even as infection persists at an elevated level, some form of resistance or tolerance is required (discussed in Chapter 6).

Histological examination of preserved tadpoles is needed to investigate possible mechanisms for the apparent resistance and/or tolerance. This work is ongoing. However, I conducted detailed descriptions of loss by individual tooth row at the macroscopic level as an indirect indicator of infection patterns.

Progression of Infection

The progression of mouthpart loss can be used as a measure of the progression of infection. Understanding where infection begins is important to understand where and how transmission occurs. I monitored the progression of infection via indirect means by recording the condition of each tooth row. Tooth Rows that experience loss first are assumed to have been infected first.

Jaw Sheath Loss

I observed jaw sheath loss only in tadpoles with missing tooth rows, indicating that jaw sheath loss follows tooth loss. This is in contrast to the pattern seen in *Rana* muscosa and *R. aurora* where jaw sheath loss either precedes tooth loss, or jaw sheath loss is present but tooth rows are normal (Nieto et al. 2007; Rachowicz and Briggs 2007). This may indicate a difference in how these tadpoles become infected with *Bd*. The jaws of the ranids may have closer contact with *Bd* in the environment compared to the torrent tadpoles.

Tooth Row Loss

The outermost tooth rows (A1 and P3) were the first to show damage and the last to recover (Fig. 7A.8). This strongly suggests that the outermost rows are the initial point of transmission and are in closest contact with environmental *Bd*. Torrent tadpoles are believed to use the outermost tooth rows to aid in attachment to the substrate in flowing water (Altig and Johnston 1989). The increased substrate contact of the outermost tooth rows could explain the pattern of infection and supports the hypothesis that torrent tadpoles acquire infection through contact with substrate (Chapter 6). The interior tooth rows were next to show significant loss. During the recovery phase, the interior rows

were the first to reappear, suggesting that the outermost rows were more severely damaged and, thus took longer to recover.

It is also possible that repeated re-infection of the outer rows from environmental zoospores contributed to a longer recovery time. Considering tadpoles appear to be the main driver of infection dynamics in tadpoles (Chapter 6), understanding the relative importance of external re-infection vs. self re-infection may have important management implications. The rate of infection will drive intensity of infection, and in turn, tadpole health and the numbers of zoospores shed into the water. If self re-infection is the primary driver of infection, then the progression of clinical signs and intensity of *Bd* within an individual should proceed independent of external factors. Removing tadpoles from the stream would eliminate the zoospores produced by that tadpole but would have no effect on other tadpoles. However, if external re-infection is important, removing tadpoles and altering the density of infected individuals should decrease the rate of transmission and the intensity of *Bd* in tadpoles across the population. This hypothesis should be tested further in the lab as manipulating density of infected tadpoles in the field could be used to reduce transmission rates between tadpoles and frogs in streams.

Habitat

Over the period of peak mouthpart loss (July 4 – Aug 1, 2007), *Litoria nannotis* tadpoles with greater than 75% loss were found to occupy significantly slower water flows than tadpoles with less than 25% loss. Tadpoles with extensive loss were often skinny and frail. This suggests tadpoles with heavy mouthpart loss may select habitat with slower water flows to conserve dwindling energy reserves or may be excluded from high energy flows due to an inability to maintain position. Tadpoles with severe loss were observed to be weak and had difficulty swimming even within a sorting tray. These tadpoles are likely to have had difficulty navigating swift flow rates without being swept downstream.

Body Condition

The severe loss of teeth due to infection very likely reduced or eliminated the ability of torrent tadpoles to extract nutrients from the surfaces of rocks. This restriction in feeding ability apparently caused a significant decrease in body condition prior to metamorphosis. This decrease was severe in some individuals, likely causing incidental mortality. This population-wide period of starvation during development appears to have affected growth and time to metamorphosis. Based on results from other studies, this could have negative carryover effects on adult fitness, traits and population dynamics.

The goal of tadpoles is to maximize size and reduce the time to metamorphosis, as this improves the chances of escaping predation, avoiding desiccation and establishing good fat stores for survival while terrestrial foraging skills develop (Pfennig 1992; Wilbur 1980). The growth and development, and thus the timing of metamorphosis in anurans is plastic in response to a number of factors, including but not limited to tadpole density (Gromko et al. 1973; Scott 1994), predation (Benard 2004; Lardner 2000; Nicieza et al. 2006), temperature (Blouin and Brown 2000; Smith-Gill and Berven 1979), photoperiod (Wright et al. 1990) water level (Denver et al. 1998) and food availability (Alford and Harris 1988; Morey and Reznick 2000). The often complex interaction of these factors leads to an important tradeoff between the risk of remaining in the larval environment and the risk of transitioning to the unknown terrestrial environment.

Werner and Gilliam (1984) proposed that for organisms with a significant life history shift, the optimum size and age for metamorphosis is based on the minimum ratio of size specific mortality (μ) to growth rate (g). The Wilbur-Collins (1973) model of amphibian metamorphosis is similarly based on the idea that developmental rate is influenced by growth rate. Under consistently low nutritional conditions, growth and development is predicted constant, and metamorphosis occurs when a minimum body size is obtained. The minimum size is likely established evolutionarily by decreased

survival in tadpoles below the limit. If nutritional conditions improve and growth rate increases, time to metamorphosis (developmental rate) increases to take advantage of abundant aquatic resources, and tadpoles metamorphose at a larger size. If a tadpole is above the minimum metamorphic size and growth rate decreases as a result of deteriorating conditions, metamorphosis is accelerated to avoid unfavorable aquatic conditions. Therefore, periods of decreased nutrition are predicted to either accelerate or retard metamorphosis depending on developmental stage (Wilbur and Collins 1973). Alford and Harris (1988) experimentally tested this hypothesis and showed that changes in nutrient availability during various stages of ontogeny can have significant effects on time to, and size at metamorphosis. Tadpoles that experienced nutritional deficiencies following a period of high nutrition took longer and metamorphosed at a smaller size than tadpoles that experienced an increase in nutrition following initial low nutrition.

Here, starvation following loss of mouthparts is functionally equivalent to a decrease in available nutrients due to external conditions and is therefore predicted to have a similar impact on the tradeoff between time to and size at metamorphosis. A period of population wide starvation in the middle of growth and development could significantly affect not only larval survival, but also development, time to and size at metamorphosis and fitness during the adult stage.

Calef (1973) found that 94% of *Rana aurora* tadpoles could survive without food for at least 4 weeks. However, tadpoles did not grow and were reported as 'not healthy' at the end of the starvation period. Based on the time-series distribution of overall loss in *L. nannotis* (Fig. 7A.11) the maximum period of starvation, or severely reduced feeding efficiency, as a result of >75% tooth loss, for any individual tadpole was between 4-8 weeks. For many individuals, the time spent at >75% loss is believed to be less than 4-8 weeks based on the recovery rate of tooth loss in the population. Regardless, many *L. nannotis* tadpoles are likely capable of surviving starvation periods of this duration.

Although many captured tadpoles were visibly skinny, in poor condition and occasionally near death, population density estimates obtained by exhaustive sampling before and after peak overall loss do not indicate the presence of widespread mortality (Fig. 7A.16). These weakened tadpoles, however, may have a decreased ability to avoid predators or maintain position against the water current (Fig. 7A.15).

The period of starvation did not appear to cause significant mortality, however, the potential under different circumstances to do so, exists. If tooth recovery is delayed causing an increase in the period of severe overall loss and starvation, mortality of tadpoles is predicted to increase. Lower temperatures, for instance, may decrease the physiological ability of basal cells to produce teeth. Tadpoles at higher elevations or during cold spells, therefore, may be more susceptible to mortality from starvation. Even in the absence of mortality, a period of nutritional deficiency may have significant effects on growth, development and even on the fitness of adults.

No tadpoles from the large size group were observed with >75% overall loss. This suggests that 1) tooth loss did not progress to advanced levels in already large tadpoles and/or 2) tadpoles ceased growth, and only entered the large size class following recovery of teeth. The time series graph of overall loss (Fig. 7A.11) and relative abundance of large tadpoles (Fig. 7A.13) reveals the demographic shift from a population dominated by small and medium size-group tadpoles to one dominated by large size-group tadpoles occurred once mouthparts and body condition began to recover following July 18. The data indicate tadpoles recovered mouthparts, resumed growth, added body mass and quickly entered the large-size group with primarily discolored mouthparts. Metamorphosis was observed after 9/12/07 when metamorphs and tadpoles with emergent legs were first observed and the number of large size-group tadpoles subsequently decreased as individuals metamorphosed and entered the terrestrial environment.

Theoretical Model

As mouthparts first began to disappear, growth rate began to decrease. According to the Wilbur-Collins model, tadpoles of adequate size to metamorphose would do so to escape the deteriorating conditions. Although no tadpoles were observed metamorphosing at this time, there was a reduction in the number of large size-group tadpoles between July 4 and July 18 when mouthpart loss peaked. Any tadpoles too small to metamorphose at this time would be required to remain in the stream and attempt continued growth. Increased mouthpart loss due to *Bd* infection then causes the remaining tadpoles to lose body mass. Once mouthpart loss increases above 25%, the intensity of infection drops, likely as a result of the pathology causing a decrease in substrate (teeth) suitable for infection. The decrease in infection intensity due to tooth loss and the apparent development of resistance or tolerance may allow teeth an opportunity to regenerate. As teeth regenerate, feeding ability increases and body mass begins to recover allowing development towards metamorphosis to resume. However, because of the slow growth rate imposed earlier by mouthpart loss (analogous to crowding in Wilbur-Collins) tadpoles metamorphose near the allowable minimum size.

This proposed model leads to the prediction that tadpoles that do not suffer severe mouthpart loss should metamorphose at a larger size. Experimental evidence has shown infected tadpoles metamorphose at smaller sizes in other species (Parris and Cornelius 2004). Unfortunately, an empirical test of this hypothesis in the field is difficult as few data exist on torrent tadpole body sizes prior to the emergence of *Bd* and *Bd* is present at all currently known torrent tadpole sites. Further, a range of factors are known to affect body size at metamorphosis in tadpoles.

However, a site in dry schlerophyl forest where *Bd* is present provides the best opportunity to test this prediction. Drier sites, may serve as a refugia from *Bd* associated rainforest declines due to the less *Bd* hospitable environmental conditions (Puschendorf et al. 2005). Based on initial sampling it appears that tadpoles at this site may not suffer severe mouthpart loss. A survey was conducted during the peak of winter, when mouthpart loss should be most severe. Mouthpart loss was observed, however at levels below that seen during peak winter at Tully Gorge NP. The size of the tadpoles at this site were, as predicted, significantly larger than at Tully Gorge NP. Increased size at metamorphosis in populations such as this may assist in generating a healthier adult population.

Although many traits of larvae and adults are decoupled by metamorphosis (see Chapter 1 for discussion), some traits are linked and adult fitness can be negatively affected by growth and development in the larval stage (Nicieza et al. 2006). Negative effects on larvae during development have been shown to impact many classes of organism with complex life cycles (Pechenik et al. 1998), amphibians included. Altwegg and Ryer (2003) tracked *Rana lessonae* and *R. esculenta* over 3 years and found individuals that metamorphosed at a smaller size or had longer developmental times were at a significant disadvantage on land. Small juveniles had decreased terrestrial survival, slower growth and smaller size at maturity. Scott (1994) manipulated *Ambystoma opacum* larval densities in field enclosures, then tracked them following metamorphosis for 6 – 7 years. He found that animals in the higher density treatments had lower lipid stores at metamorphosis, were smaller and older at first breeding, had smaller clutch sizes, and possibly lower survival. 21% of low density individuals returned to breed compared with only 6% in the high density treatment. Chelgren et al. (2006) found that *Rana aurora aurora* tadpoles with decreased food availability as larvae had significantly decreased survival following metamorphosis. Gervasi and Foufopoulos (2008) reported tadpoles that accelerated metamorphosis due to manipulated pond drying had decreased immune responses following emergence. Other researchers have found similar effects of larval condition on adult survival and fitness (Berven and Gill 1983; Scott et al. 2007; Smith 1987).

Smaller clutch sizes and longer time to first reproduction are important life history traits that directly affect fitness and population growth (Scott 1994). These more subtle negative effects, along with reduced juvenile survival due to high prevalence of *Bd* at metamorphosis could reduce recruitment and negatively affect population growth, thereby hindering the ability of populations to recover

following an epizootic decline. Near 100% prevalence at metamorphosis and a yearly period of larval starvation due to a loss of teeth could have serious long term effects on population recovery and size.

Species such as *L. genimaculata* whose larvae are largely unimpacted by *Bd* would be predicted to have recruitment unaffected by infection and thus should experience a faster rate of recovery following an epizootic decline of adults. Unhindered recruitment may help explain why *L. genimaculata* was particularly resilient following decline and appeared to quickly return to pre-decline levels compared with species having torrent adapted tadpoles. Species with torrent adapted tadpoles have had difficulty recovering, particularly above 400 meters (McDonald and Alford 1999). Association with permanent water (Lips et al. 2003; McDonald and Alford 1999), air temperature (Berger et al. 2004; Woodhams et al. 2003) and *Bd* strain differences (Berger et al. 2005a; Retallick and Miera 2007) are important factors affecting adult susceptibility to chytridiomycosis and are also expected to play a role in population recovery.

While this study was not able to directly investigate the effect of larval starvation on adult fitness, the abundance of studies demonstrating the negative effects undersized larvae can have warrants that this, heretofore neglected topic receive more attention. It is important to determine if similar patterns and results are found in other tropical stream systems, particularly in the Central and South America.

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How does *B. dendrobatidis* exist within infected frog populations?

Study Sites

Tully Gorge National Park (17º46'S, 145º38'E, elevation 100m) and Murray Upper National Park (18º11′S, 145º52′E, elevation 250m) in the Wet Tropics, Australia, were surveyed bi-weekly during the cool, dry, winter months and monthly during the hot, wet summer. Each site was visited for 2-3 nights on every trip.

Both sites were relatively undisturbed rainforest streams. The rocky stream substrate ranged from small pebbles (<0.5cm diameter) to large boulders (>10m diameter). Stream height varied greatly depending on seasonal rainfall (wet summers and dry winters) and streams contained waterfalls, riffles and pools. A 150m (Tully Gorge NP) to 200m (Murray Upper NP) marked transect was established along each stream.

Study Species

Four sympatric anurans (*Litoria genimaculata*, *L. nannotis*, *L. rheocola* and *Nyctimystes dayi*) were monitored during the study. The former 3 species are found at both Tully Gorge and Murray Upper NPs. *N. dayi* is found at Tully Gorge NP only.

***Litoria genimaculata* (Horst 1883) (Green-eyed Tree Frog)**

L. genimaculata is a scansorial frog restricted to tropical rainforest between Townsville and Cooktown. It suffered short-term population declines (McDonald and Alford 1999) due to chytridiomycosis but has recovered and remains abundant compared with sympatric species that have declined or disappeared (McDonald et al. 2005, Richards and Alford 2005).

Reproduction is distinctively seasonal, occurring during September to March (Richards and Alford 2005). This is a relatively long breeding season, so males spend only short periods actively trying to attract females and feed extensively from the abundant prey (Richards and Alford 2005). Adult males account for most of the frogs on the stream, but recaptures of marked individuals are rare, suggesting an open population with a high turnover of frogs moving between the stream and adjacent forest (Richards and Alford 2005). Individuals may remain away from the stream for several days, spending most time in the canopy and potentially moving along and between streams (Rowley and Alford 2007). Females are rarely captured on the stream (Richards and Alford 2005) and move further than males, perhaps due to greater food requirements, reduced desiccation risk because of larger body size, or lack of site fidelity when compared with males (Rowley and Alford 2007). Adult frog numbers along a stream are greatest between October and February and decline substantially in winter as they disperse away from the stream (Richards and Alford 2005). Tadpoles metamorphose throughout spring and summer (Richards and Alford 2005). Juveniles disperse into the forest and small adult males return in June to September when ready to breed (Richards and Alford 2005). Hibernation does not occur but activity levels are greatly reduced as a response to lower environmental temperatures and/or reduced abundance of invertebrate prey (Richards and Alford 2005).

***Litoria nannotis* (Andersson, 1916) (Waterfall Frog)**

L. nannotis formerly occurred throughout the Wet Tropics from Paluma to Cooktown (EPA/QPWS 2005). Chytridiomycosis-related declines in *L. nannotis* occurred above 400m during the early 1990's, with persistence of populations below this altitude. Some recovery has occurred since, as lowland populations recolonise upper altitudes (McDonald et al. 2005).

L. nannotis is an obligate stream-dweller and stream-breeder with both males and females using the stream as primary habitat throughout the year (Hodgkison and Hero 2001), often grouped together (Hodgkison and Hero 2002). Frogs predominantly remain at the stream during the day, sheltering behind waterfalls or between rocks or basking in splash zones adjacent to waterfalls (Hodgkison and

Hero 2001). Activity increases at night as frogs move into more exposed positions on the stream and a small proportion venture a short distance into the forest but always return before sunrise, at the risk of desiccation (Hodgkison and Hero 2001). Females may venture further than males (Hodgkison and Hero 2001, Rowley and Alford 2007) but neither sex are known to move between streams (Rowley and Alford 2007). *L. nannotis* are predominantly sedentary (Hodgkison and Hero 2001, Rowley and Alford 2007), and demonstrate high site fidelity (Phillott unpublished data). Less activity occurs during winter (Rowley and Alford 2007).

Litoria rheocola (Liem, 1974) (Common Mist Frog)
The pattern of chytridiomycosis-related declines in *L. rheocola* is similar to that of *L. nannotis*. Formerly distributed from Lumholtz National Park to Amos Bay in the Wet Tropics, *L. rheocola* has disappeared above 400m although lowland populations remain stable (EPA/QPWS 2005).

L. rheocola is a stream breeding frog, however it inhabits more generalized creek habitat than *L. nannotis* (Liem 1974), preferring slower moving sections of water (Hodgkison and Hero 2002). Breeding occurs year round (EPA/QPWS 2005) but may peak between March and July (Hodgkison and Hero 2002). Males show greater fidelity to the breeding site (Hodgkison and Hero 2002) than females which forage away from the stream. Juveniles utilise the streamside vegetation more often than the stream itself (Hodgkison and Hero 2002). Activity is reduced in the dry winter (Hodgkison and Hero 2002)

Nyctimystes dayi (Gunther, 1897) (Australian Lace Lid)
The pattern of chytridiomycosis-related declines in *N. dayi* is similar to that of *L. nannotis* and *L. rheocola*. Once distributed throughout the Wet Tropics between Paluma and Big Tablelands, upland populations have contracted while lowland populations <300m remain unaffected (EPA/QPWS 2005).

N. dayi utilises similar stream habitat to *L. rheocola* (Liem 1974), however is far less abundant in the cooler dry season (Hodgkison and Hero 2002) and may be absent from the stream for weeks. At this time it is presumed frogs aestivate in refugia or adjacent to the stream (Hodgkison and Hero 2002). Sexual variation in stream fidelity is similar to that of *L. nannotis* and *L. rheocola*.

Capture-Mark-Recapture Study
We searched the stream transect by spotlight starting approximately half an hour after dusk. Frogs were caught by hand, while wearing single-use latex gloves to minimise the chance of transferring pathogens between individuals. All frogs were measured using 30cm stainless steel Vernier callipers. Size and sexual characteristics were contributing factors when categorising frogs as :
> Juveniles
> Immature (no sexual characteristics)
> Adult males (nuptial pads and/or mating calls)
> Adult females (ovarian eggs).

Toe-tipping was used to mark each individual with a unique number. Excision occured at the most distal inter-phalangeal joint. This marking method is the only reliable means of identifying small individuals or species lacking persistent, individually-identifiable natural markings (see Phillott *et al.* 2007). It did not appear to adversely affect long term recapture rates (Phillott *et al.* unpublished data) or inflammation rates of marked digits (Phillott *et al.* unpublished data). However, there is the possibility that all invasive marking methods may influence frog behaviour and/or survival to some slight degree. Animals were marked at the first capture only, according to the numbering scheme in Figure 7B.1 which allocates frogs a unique number based on the pattern of toes removed. Previously marked frogs have their unique number established and additional toes were not removed. Frogs were released at the point of capture.

Figure 7B.1 Pattern of toes removed during toe-tipping, allowing 699 frogs of each species to be marked. Diagram by Rebecca James.
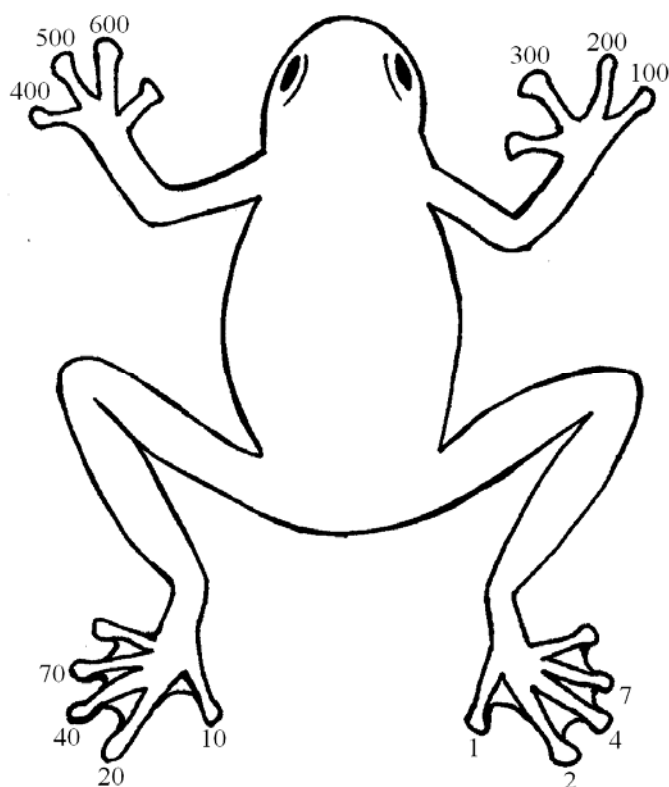
Strict hygiene protocols were followed to ensure pathogens were not introduced into the study sites, or transmitted among captured frogs. We wore shoes dedicated to each site and clean, dry clothing. Frogs were handled while wearing single-use gloves and field instruments (e.g. surgical scissors and callipers) were disinfected with 70% ethanol after each use.

Determination of Infection Status of Frogs

At capture, frogs were swabbed with a Medical Wire and Equipment MW100 Tubed Dryswab®. The swab was run over the ventral surface of the hands, feet and abdomen, twice on each surface. Storage occurred below 5°C while in the field then at -80°C in the laboratory before processing.

Quantitative PCR, using a real-time Taqman assay, determined the presence or absence of Bd. Swabs were processed as described by Boyle et al. (2004) and Hyatt et al. (2007) with the following modifications. The master mix incorporated the TaqMan® Exogenous internal positive control (0.6x Exo IPC Mix, 0.6x Exo IPC DNA) with the addition of 400 ng/μL of bovine serum albumin (BSA). The analysis was performed on the Rotor-Gene™ 6000 (Corbett Research) using Gene-Disc 100 tubes. A 15 μL reaction volume was produced by loading 10 μL of PCR master mix and 5 μL of the diluted sample extract, the diluted negative extraction control (NEC) (extraction of a new swab), or water into Gene-Disc tubes with a CAS-1200™ pipetting robot (Corbett Robotics). The sample extracts and NEC were diluted 1 in 16.7 to obtain the same template/master mix ratio as indicated by Boyle et al. (2004).

For statistical analyses, frogs were considered to be test-positive if the triplicate qPCR assay returned 1-3 wells positive, and test-negative if 0 wells were positive. This ensured conservative estimates of the impact of infection in our analyses. We were unable to quantify the relative amounts of Bd and frog genetic material on the swabs in this study to try to improve standardization of the swabbing method.

Results

Population Structure and Infection Status of Frogs at Murray Upper NP

L. genimaculata, *L. nannotis* and *L. rheocola* were regularly encountered at Murray Upper National Park (see Figures 7B.2-7B.4). Due to dispersal from the stream, adult females, sub-adults and juvenile frogs of any species were rarely captured, so population descriptions are limited to those of adult, male frogs for each species.

Minimum population estimates of adult, male frogs for the November 2005- November 2007 study period are presented in Figure 7B.5. Numbers fluctuated greatly; *L. genimaculata* 15±7 (5-26), *L. nannotis* 7±7 (0-24), *L. rheocola* 13±12 (0-40) (mean ± standard deviation (range)). Our study of climatic conditions influencing frog numbers, prevalence of *B. dendrobatidis* and prevalence of infection (see Objective 2) found that *L. genimaculata* visited the stream after recent high air temperatures and low minimum relative humidity, likely because they were unable to maintain hydration in the rainforest. Dispersal was observed after rainfall when moisture is more readily available away from the stream. Numbers of *L. nannotis* were independent of most climatic conditions, only rainfall resulted in a greater density. *L. rheocola* were most heavily influenced by climatic conditions, numbers increasing with rising air temperatures, rainfall and relative humidity.

Figure 7B.2. Structure of *Litoria genimaculata* Population at Murray Upper National Park
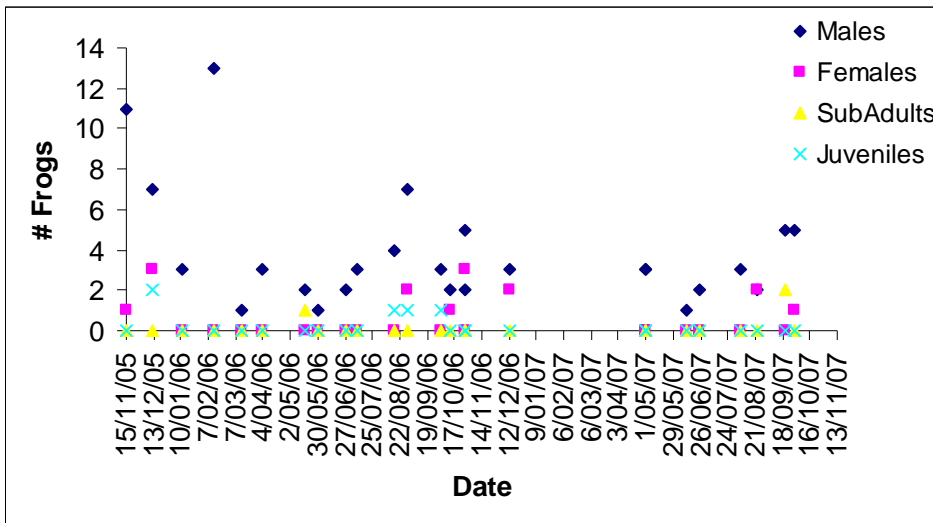
Figure 7B.3. Structure of *Litoria nannotis* Population at Murray Upper National Park
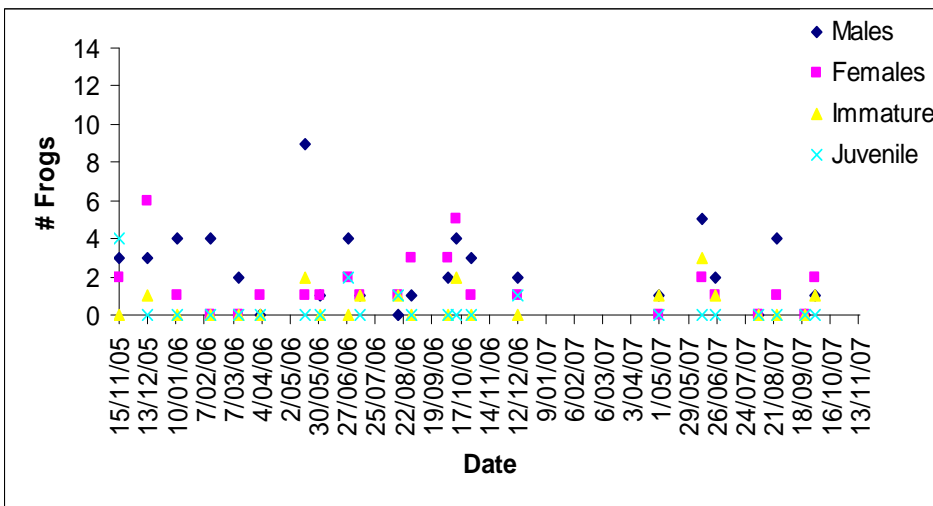
Figure 7B.4. Structure of *Litoria rheocola* Population at Murray Upper National Park
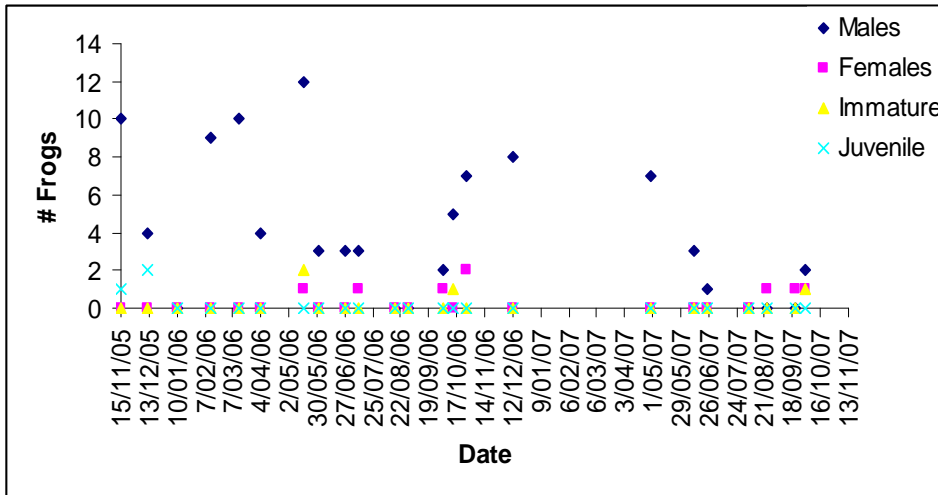
Figure 7B.5. Estimated minimum population sizes of adult, male *Litoria genimaculata*, *L. nannotis* and *L. rheocola* along a 200m stream transect between November 2005 and November 2007 at Murray Upper NP.
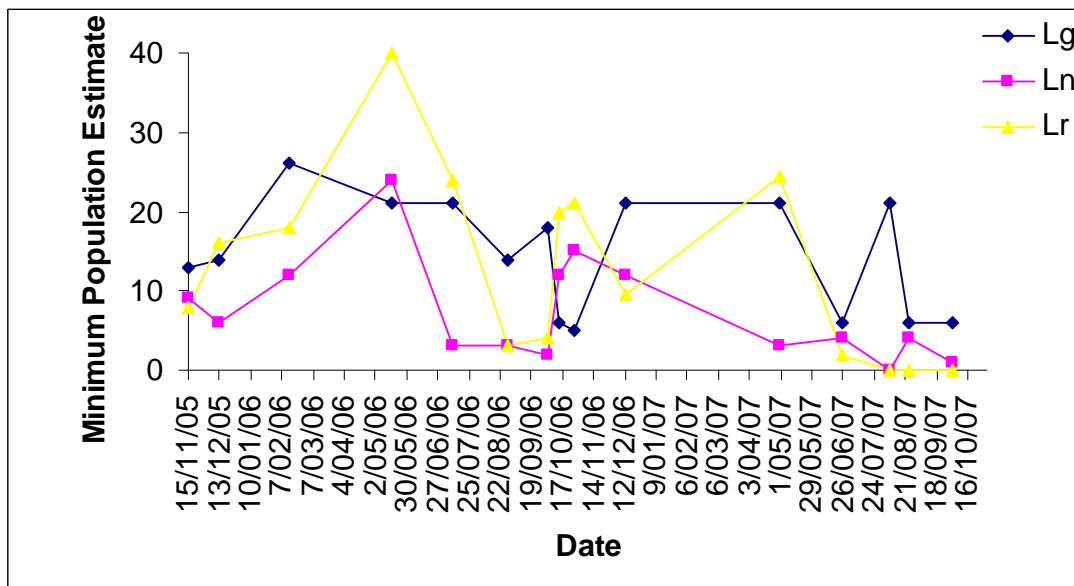
During the 2 year study period, we marked 129 *L. genimaculata*, 58 *L. nannotis* and 78 *L. rheocola* adult, male frogs. Of these marked frogs, *L. rheocola* were recaptured the most frequently, with the highest number of recaptures, and the shortest recapture period. *L. genimaculata* were captured the least frequently, with the least number of recaptures. *L. nannotis* had the longest recapture period, but frogs of all three species demonstrated recapture periods of greater than a year (see Tables 7B.1 and 7B.2). Due to the limited study period, few animals were captured at such long intervals (see Figures 7B.7-7B.9).

Table 7B.1. Numbers of adult, male *Litoria genimaculata*, *L. nannotis* and *L. rheocola* captured and percentage recaptured along a 200m stream transect between November 2005 and November 2007 at Murray Upper NP.

Species	# Frogs Marked	Percentage of Marked Frogs that were Recaptured between 1-5 times				
		1	2	3	4	5
L. genimaculata	129	19%	5%	0%	0%	0%
L. nannotis	58	41%	17%	10%	2%	0%
L. rheocola	78	50%	21%	9%	8%	1%

Table 7B.2. Days between successive captures for adult male *Litoria genimaculata*, *L. nannotis* and *L. rheocola* captured and recaptured along a 200m stream transect between November 2005 and November 2007 at Murray Upper NP.

Species	Mean Recapture Period±StDev(days)	Range (days)
L. genimaculata	102.2±97.6	11-366
L. nannotis	170.4±138.4	10-724
L. rheocola	119.3±95.0	8-449

Figure 7B.7. Frequency of capture by recapture period for *Litoria genimaculata*.
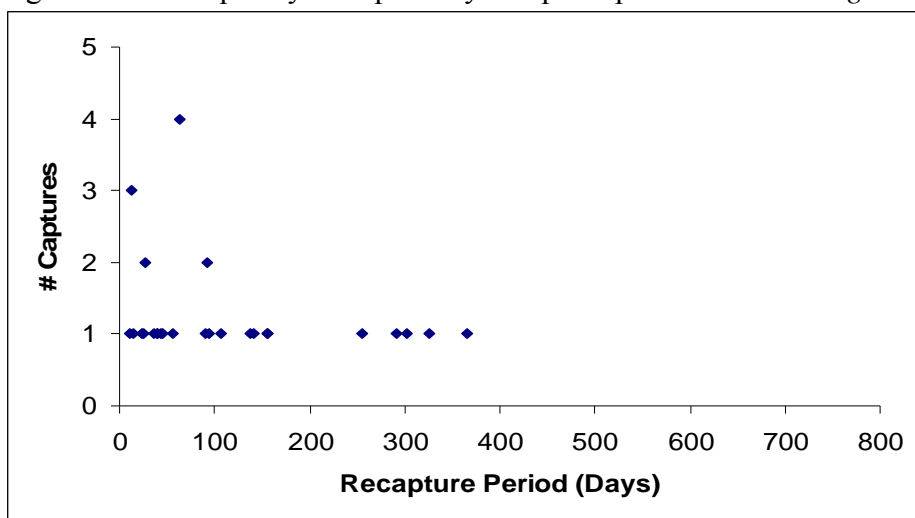
Figure 7B.8. Frequency of capture by recapture period for *Litoria nannotis*.
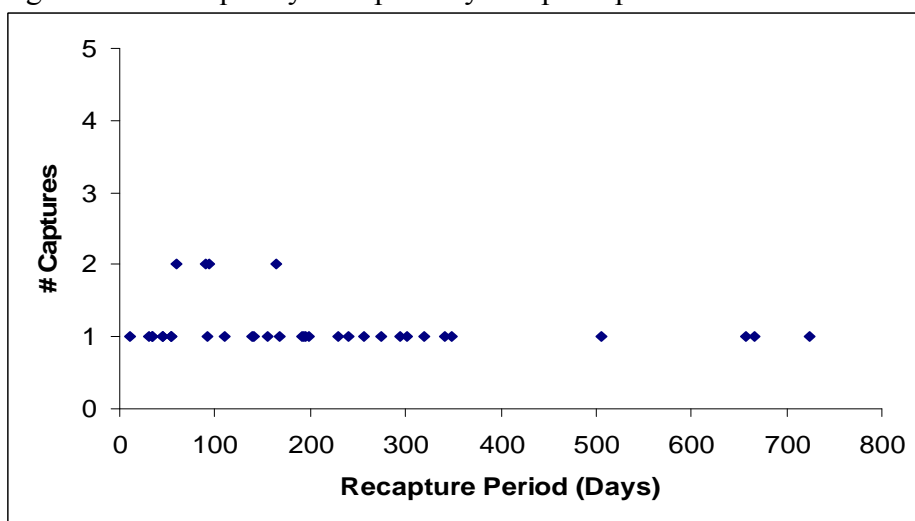
Figure 7.9. Frequency of capture by recapture period for *Litoria rheocola*.
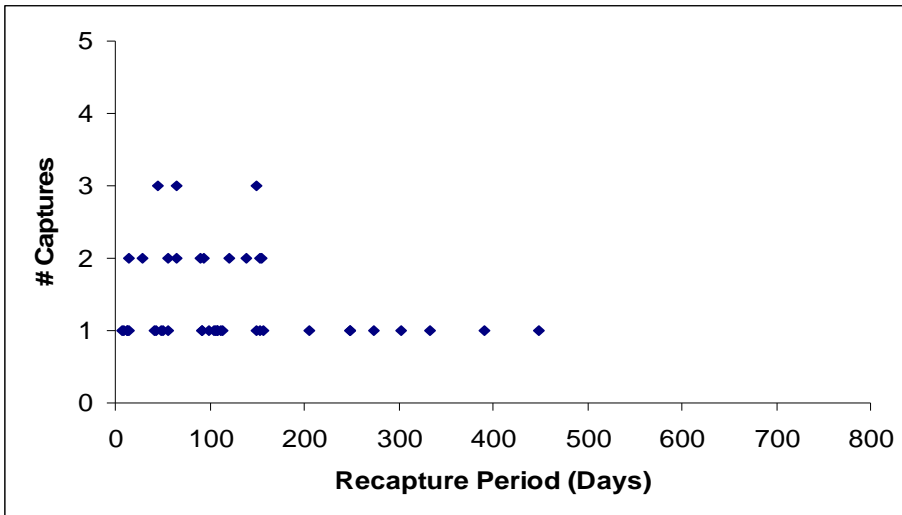
Due to dispersal from the stream, adult females, immature and juvenile frogs of any species were rarely captured (see Figures 7B.2-7B.9). For this reason, subsequent analyses of prevalence, intensity of infection and survivorship are calculated for adult males only. However, all frogs were included in analyses to determine if intensity of infection varied with snout-urostyle length.

Prevalence reached 100% in all three species of frog at Murray Upper NP, temporal variation following trends described for other Australian populations', increasing in the cooler, austral winter and decreasing in the warmer, austral summer (see Figures 7B.10-7B.15). Prevalence of *Bd* could not be predicted from the density of adult, male frogs by species or for all frogs combined although there was some correlation (see Figures 7B.10-7B.15 and 7B.17). Intensity of infection (mean zoospore equivalents calculated by qPCR assay) did not vary consistently with prevalence of *Bd* and did not vary with frog size (see Figure 7B.16), but few juvenile and immature *L. genimaculata* and *L. rheocola* were captured to detect such a trend.

Figure 7B.10. Prevalence (%) of *Batrachochytrium dendrobatidis* and number of adult male *Litoria genimaculata* at Murray Upper NP.
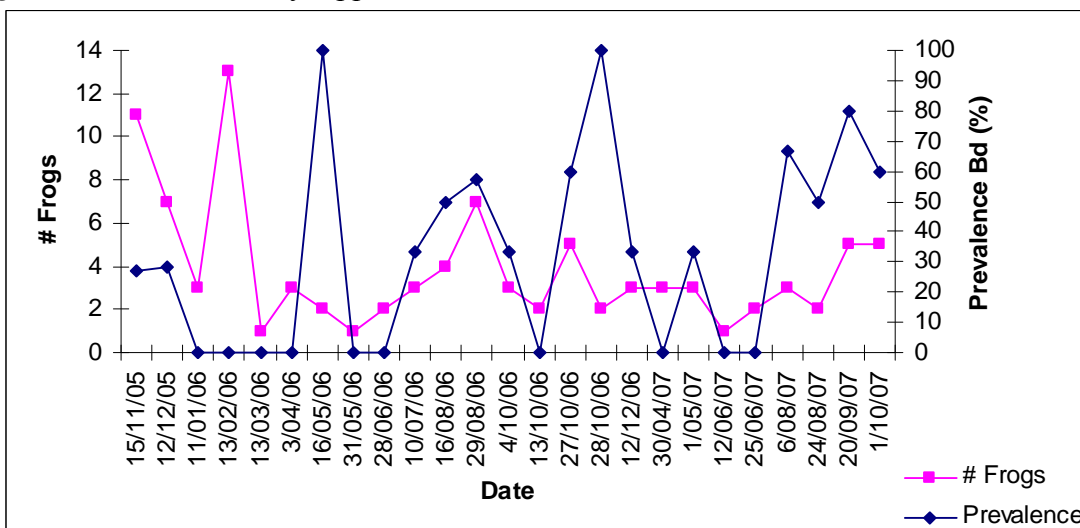
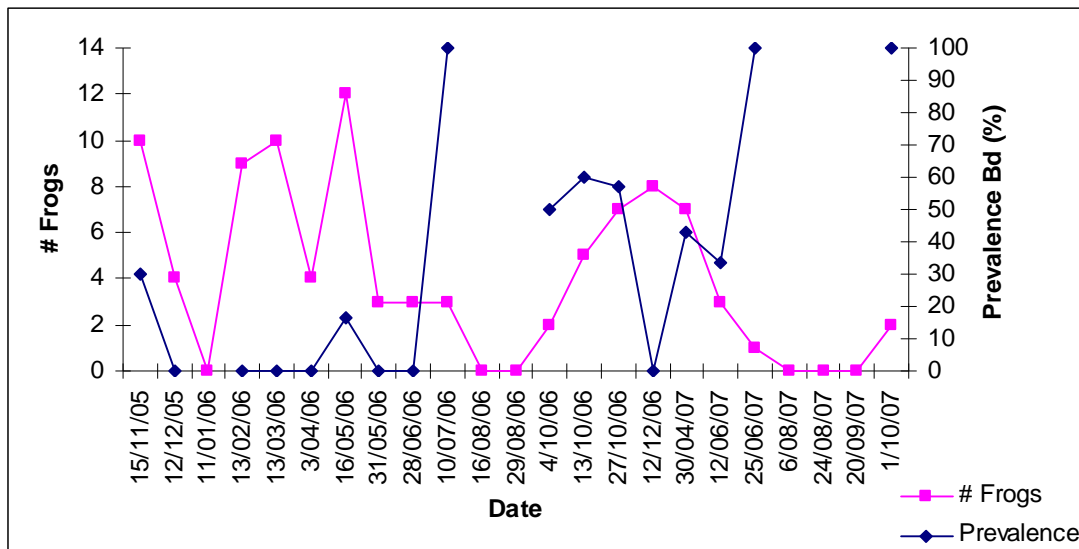Figure 7B.11. Intensity of infection (mean zoospore equivalents ± range) and prevalence (%) of *Batrachochytrium dendrobatidis* among adult male *Litoria genimaculata* at Murray Upper NP.
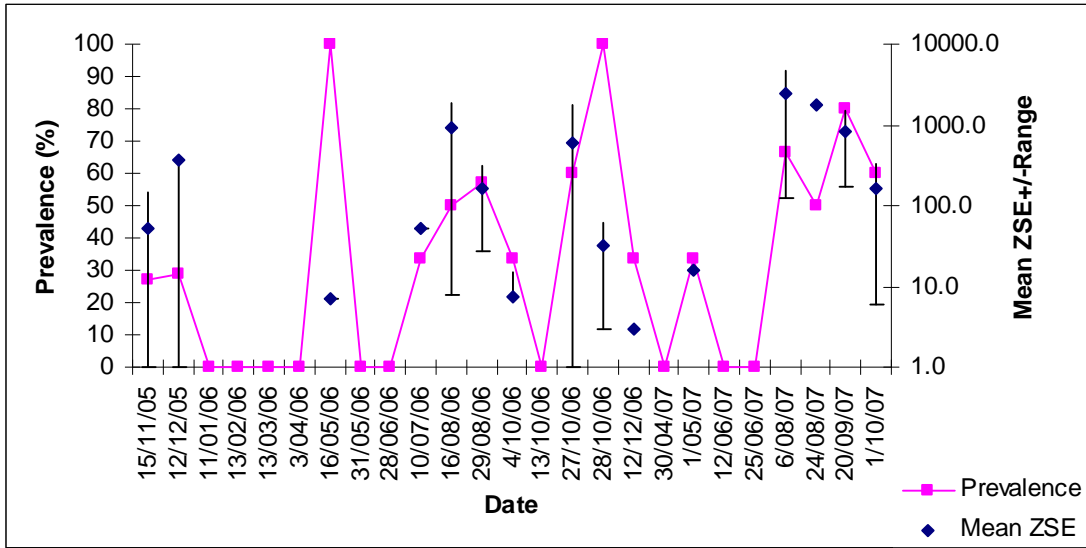
Figure 7B.12. Prevalence (%) of *Batrachochytrium dendrobatidis* and number of adult male *Litoria nannotis* at Murray Upper NP.
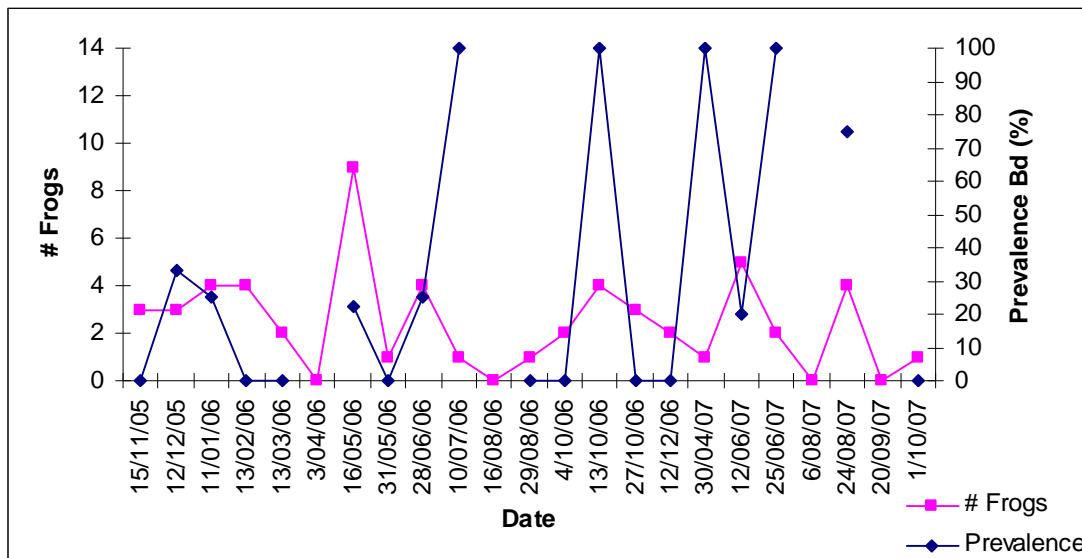
Figure 7B.13. Intensity of infection (mean zoospore equivalents ± range) with prevalence (%) of *Batrachochytrium dendrobatidis* among adult male *Litoria nannotis* at Murray Upper NP.
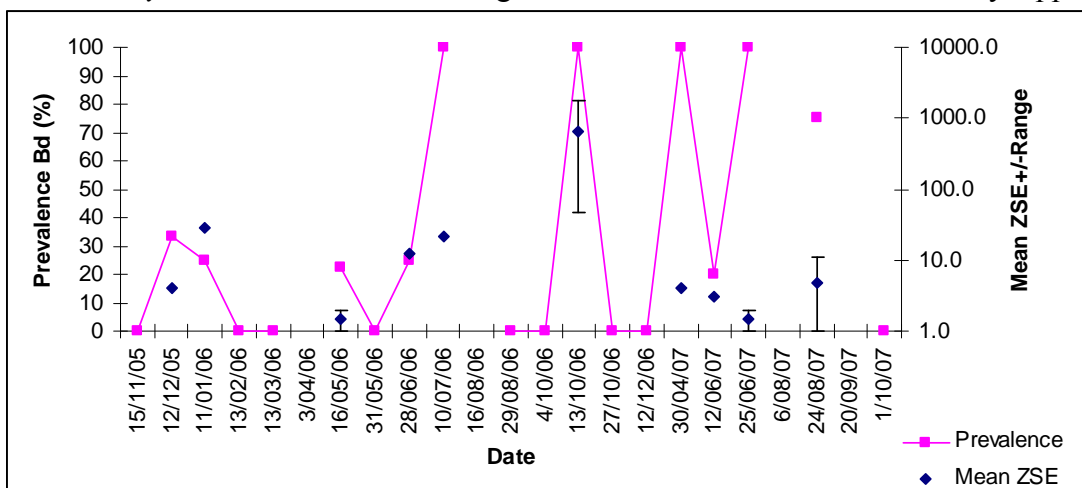
Figure 7B.14. Prevalence (%) of *Batrachochytrium dendrobatidis* with number of adult male *Litoria rheocola* at Murray Upper NP.

Figure 7B.15. Intensity of infection (mean zoospore equivalents ± range) and prevalence (%) of *Batrachochytrium dendrobatidis* among adult male *Litoria rheocola* at Murray Upper NP.
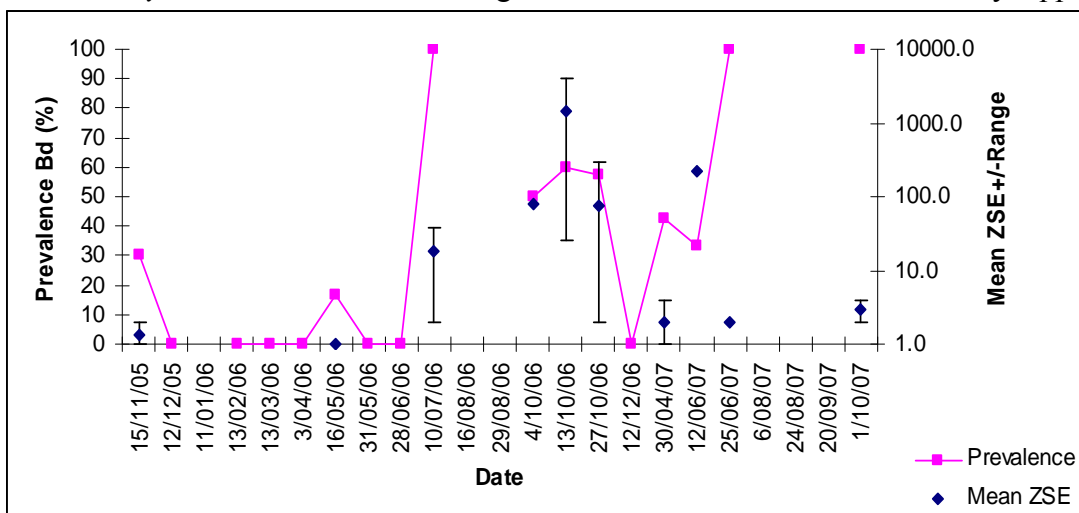
Figure 7B.16. Intensity of infection with *Batrachochytrium dendrobatidis* by snout-urostyle length of frogs at Murray Upper NP

a) infected adult, male *L. genimaculata* (r=-0.079, *P*=0.351, n=143).
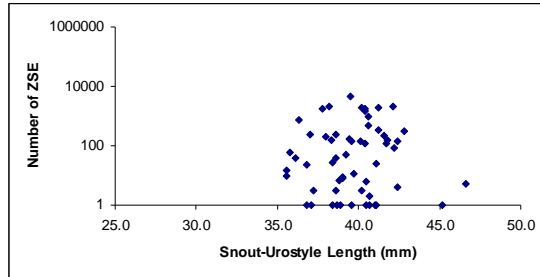
b) infected *L. genimaculata* (all sizes, male and female)
(r=-0.074, *P*=0.312, n=189).
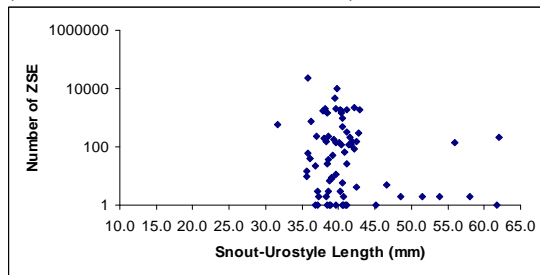
c) infected adult, male *L. nannotis* (r=-0.097, *P*=0.387, n=82).
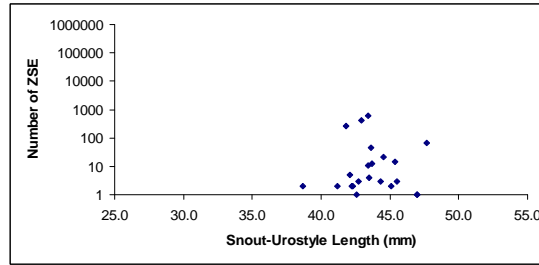
d) infected *L. nannotis* (all sizes, male and female)
(r=-0.101, *P*=0.176, n=180).
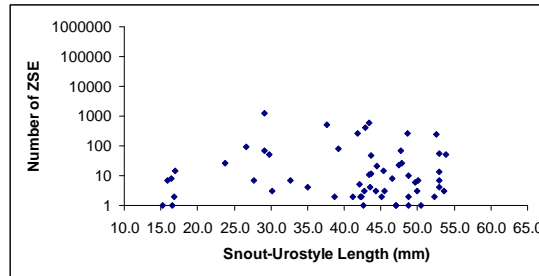
e) infected adult, male *L. rheocola* (r=-0.036, *P*=0.704, n=114).
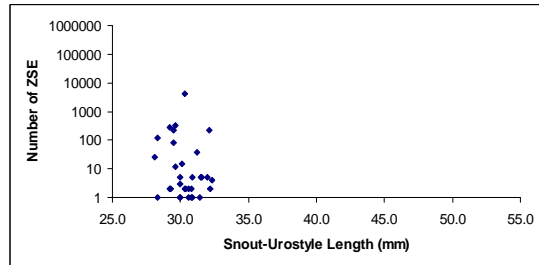
f) infected *L. rheocola* (all sizes, male and female)
(r=-0.086, *P*=0.313, n=139).
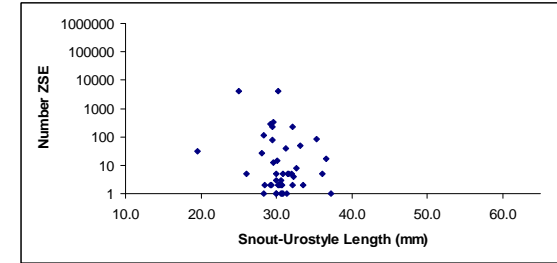
Figure 7B.17. Variation in prevalence (%) of *Batrachochytrium dendrobatidis* with total number of frogs (all species, all age-classes, both sexes) at Murray Upper NP.
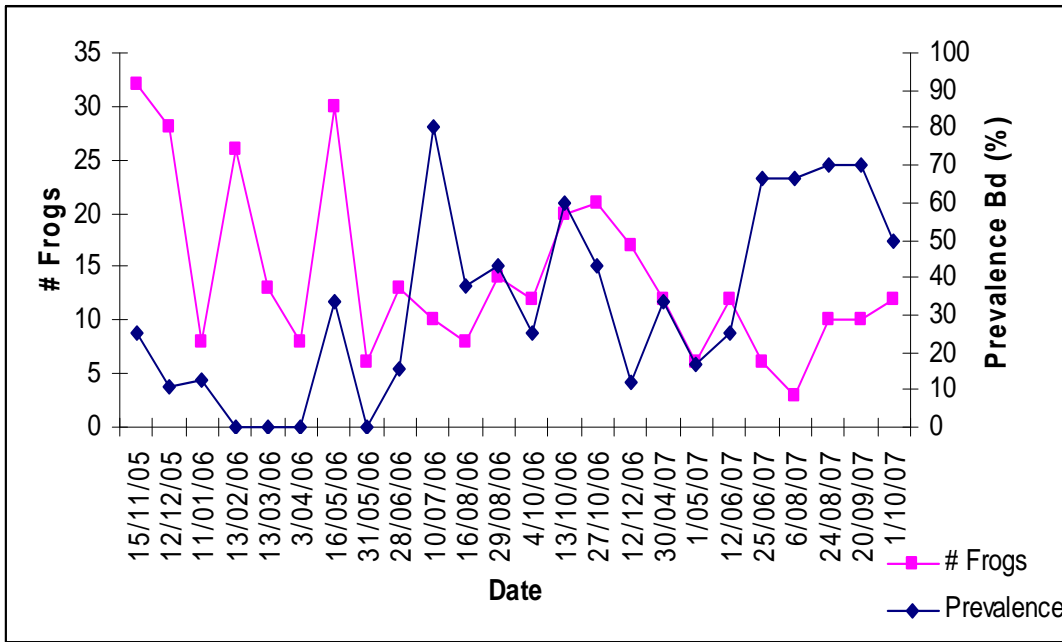
L. genimaculata showed the greatest change in intensity of infection in animals captured on multiple occasions, and *L. rheocola* the least (see Figures 7B.18-7B.23). Temporal variation in change in intensity of infection was observed in all species at Murray Upper NP. Two *L. genimaculata* individuals demonstrated large decreases in the *Bd* load in July-October 2006, while some *L. rheocola* individuals showed a similar trend but at a smaller scale. However, intensity of infection increased in *L. nannotis* individuals during the same period and the greatest recovery from infection occurred in one individual in May 2007.

Figure 7B.18 Change in intensity of infection with length of inter-recapture period in *Litoria genimaculata* at Murray Upper NP.
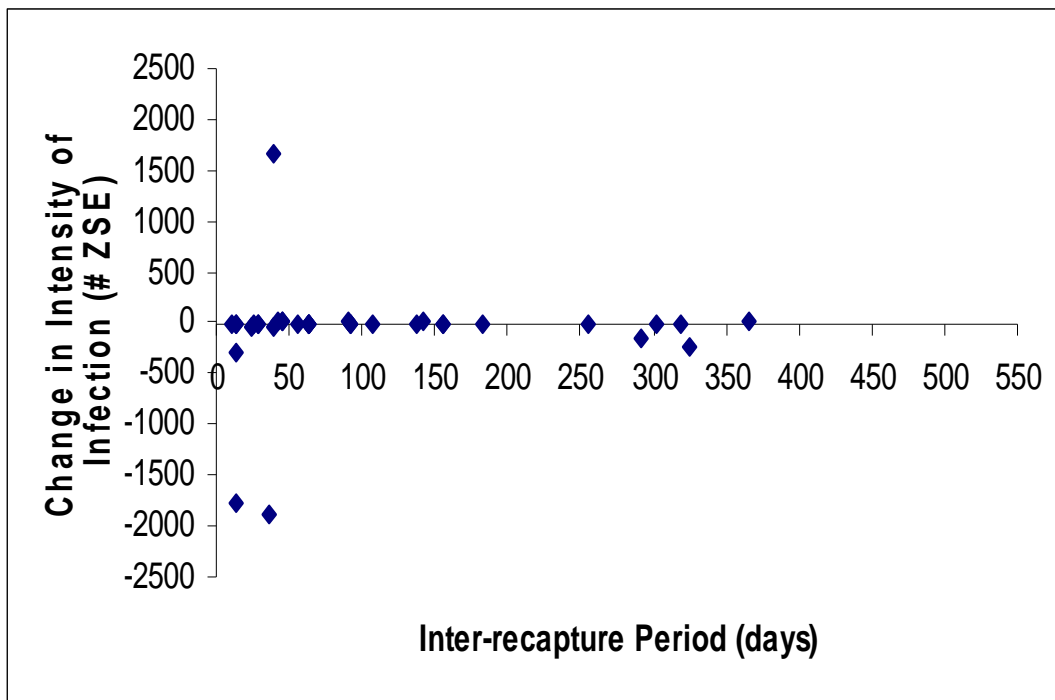

Figure 7B.19 Temporal variation in change in intensity of infection in *Litoria genimaculata* at Murray Upper NP.
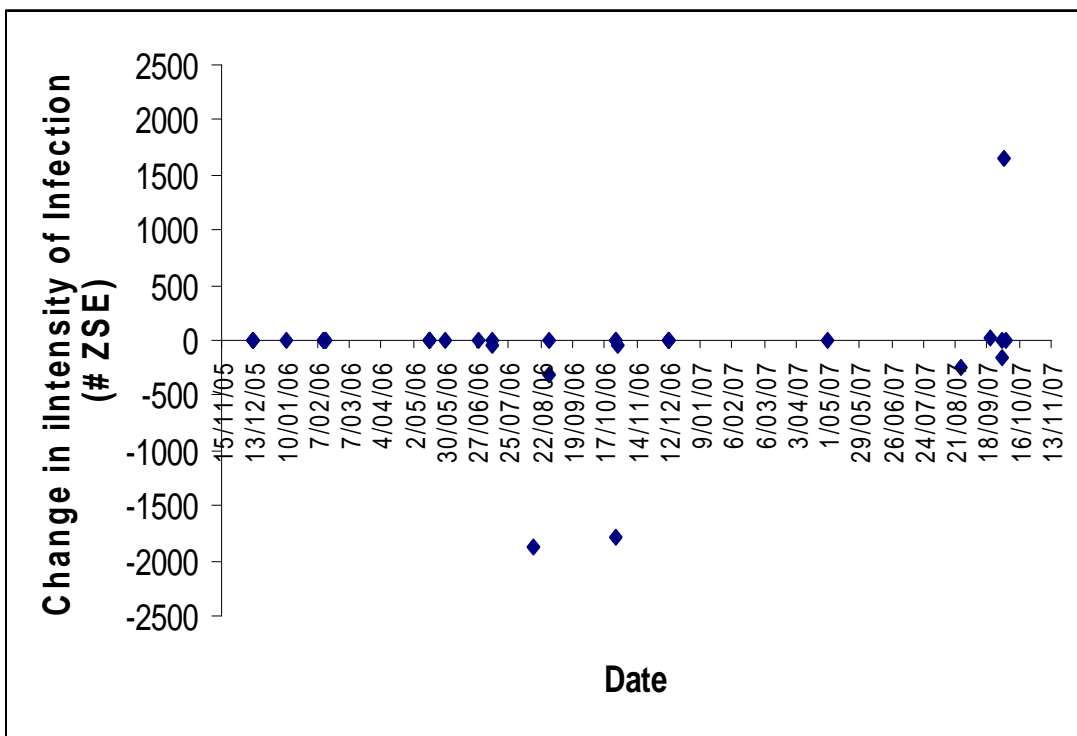
Figure 7B.20 Change in intensity of infection with length of inter-recapture period in *Litoria nannotis* at Murray Upper NP.
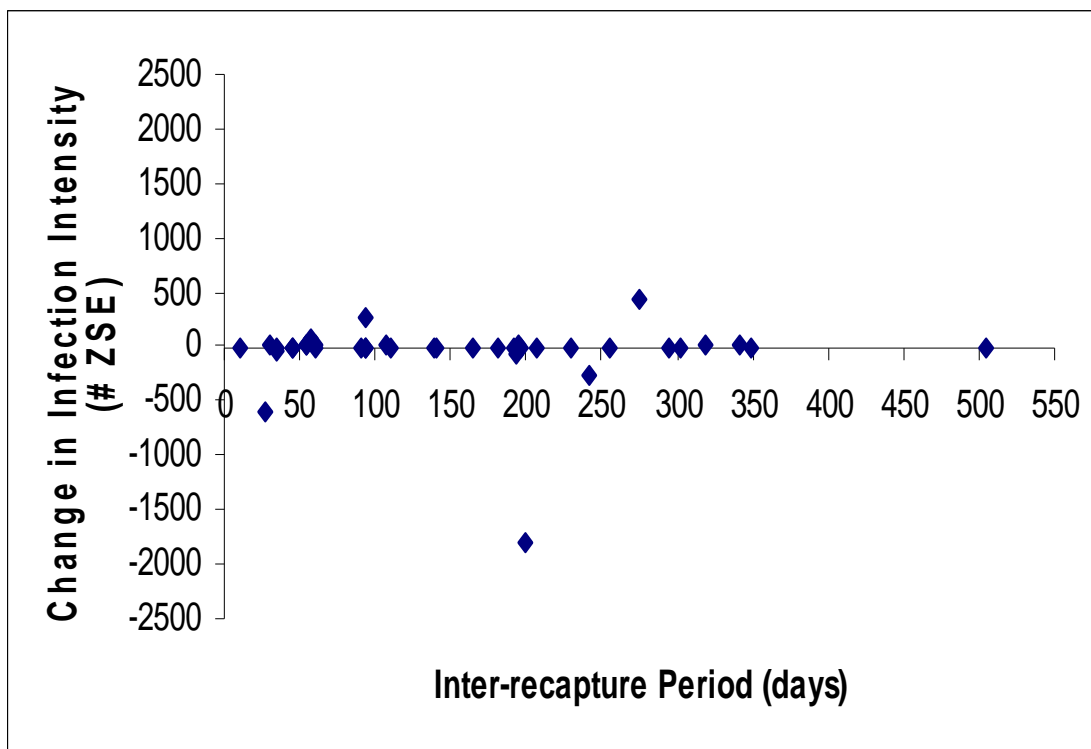

Figure 7B.21 Temporal variation in change in intensity of infection in *Litoria nannotis* at Murray Upper NP.
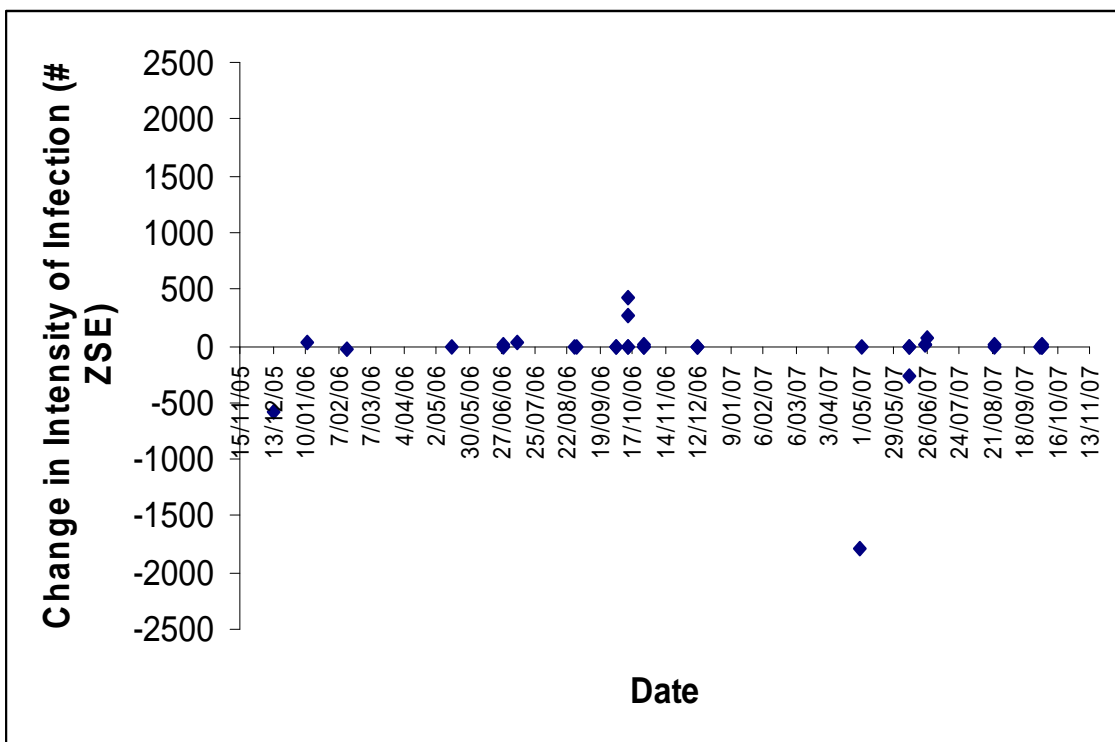
Figure 7B.22 Change in intensity of infection with length of inter-recapture period in *Litoria rheocola* at Murray Upper NP.
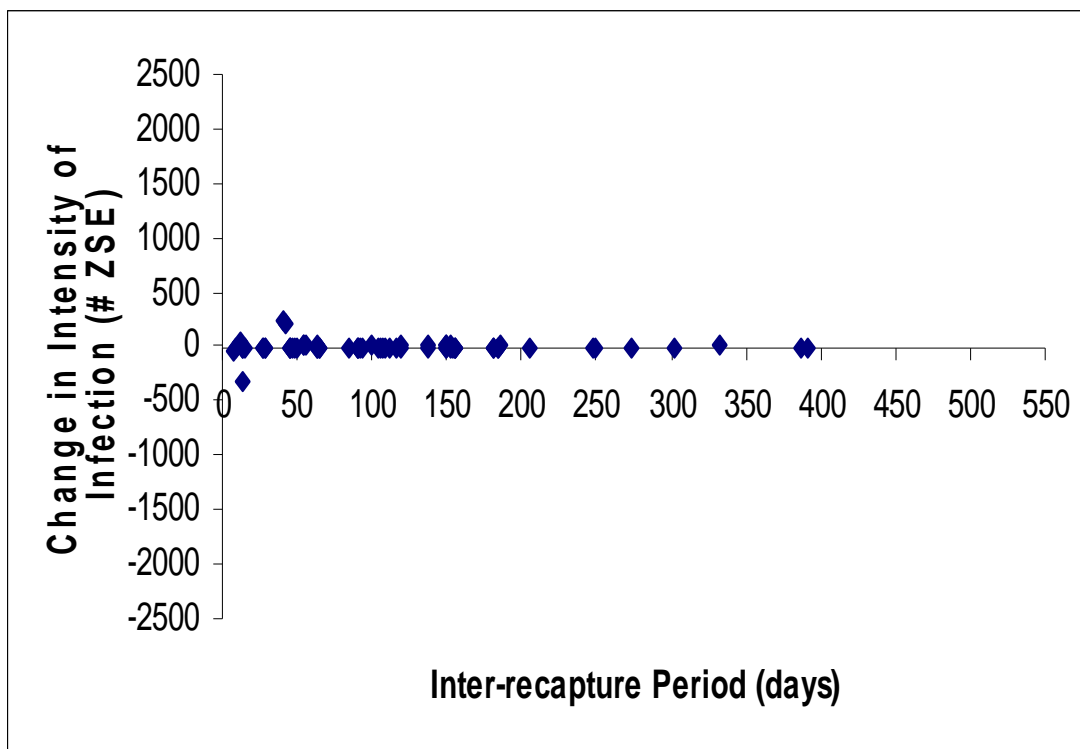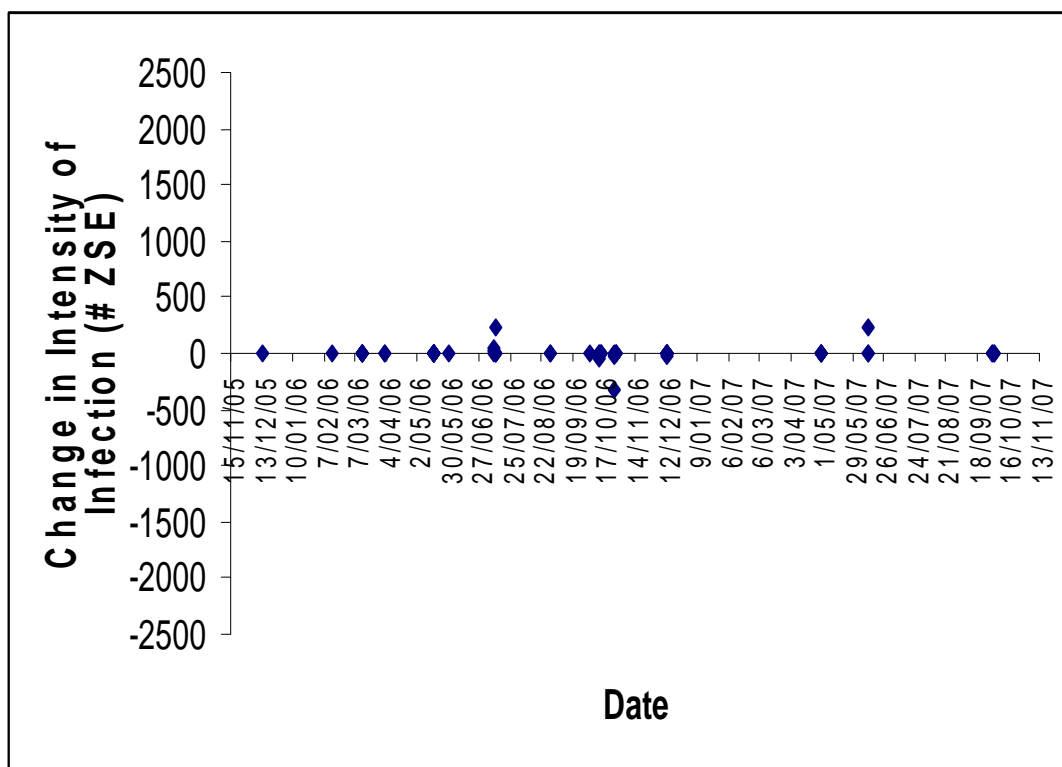

Figure 7B.23 Temporal variation in change in intensity of infection in *Litoria rheocola* at Murray Upper NP.

Population Structure and Infection Status of Frogs at Tully Gorge NP

L. nannotis, *L. rheocola* and *N. dayi* were regularly encountered at Tully Gorge NP; *L. genimaculata* less frequently (see Figures 7B.24-7B.27). Due to dispersal from the stream, adult females, sub-adults and juvenile frogs of any species were rarely captured, so population descriptions are limited to those of adult, male frogs for each species.

Minimum population estimates of adult, male frogs for the November 2005- November 2007 study period are presented in Figure 7B.28. Numbers fluctuated greatly; *L. genimaculata* 1±2 (0-6), *L. nannotis* 20±24 (0-98), *L. rheocola* 84±152 (0-672) and *N. dayi* 53±85 (0-340) (mean ± standard deviation (range)). Our study of climatic conditions influencing frog numbers, prevalence of *B. dendrobatidis* and prevalence of infection (see Objective 2) found that numbers of *L. nannotis* and *N. dayi* both increased with higher mean air temperature, and rising minimum relative humidity and maximum relative humidity respectively. *L. rheocola* numbers appeared independent of climatic conditions. Insufficient *L. genimaculata* were encountered for significant analyses. There was a negative correlation between prevalence and/or intensity of infection with air temperature and maximum relative humidity.

During the 2 year study period, we marked 20 *L. genimaculata*, 106 *L. nannotis,* 277 *L. rheocola* and 234 *N. dayi* adult, male frogs. Of these marked frogs, *L. nannotis* were recaptured the most frequently, with the highest number of recaptures. *L. genimaculata* were captured the least frequently, with the least number of recaptures, but had the shortest mean and range recapture period. *N. dayi* had the longest recapture period, but *L. nannotis*, *L. rheocola* and *N. dayi* all demonstrated recapture periods of greater than a year (see Tables 7B.3 and 7B.4). Due to the limited study period, few animals were captured at such long intervals (see Figures 7B.29-7B.32).

Due to dispersal from the stream, adult females, immature and juvenile frogs of any species were rarely captured (see Figures 7B.24-7B.27). For this reason, subsequent analyses of prevalence, intensity of infection and survivorship are calculated for adult males only. However, all frogs were included in analyses to determine if intensity of infection varied with snout-urostyle length.

Prevalence reached 100% in all four species of frog at Murray Upper NP, temporal variation following trends described for other Australian populations, increasing in the cooler, austral winter and decreasing in the warmer, austral summer (see Figures 7B.35-7B.40). Prevalence of *Bd* could not be predicted from the density of adult, male frogs by species or of all frogs combined however there were patterns of correlation (see Figures 7B.35-7B.40 and 7B.42). Intensity of infection (mean zoospore equivalents calculated by qPCR assay) did not vary consistently with prevalence of *Bd*, but did decrease with increased frog snout-urostyle length in *L. nannotis* and *L. rheocola* (see Figure 7B.41). Insufficient juvenile and immature *L. genimaculata* and *N. dayi* were captured to determine if such a relationship existed in these species also.

Figure 7B.24. Structure of *Litoria genimaculata* Population at Tully Gorge NP
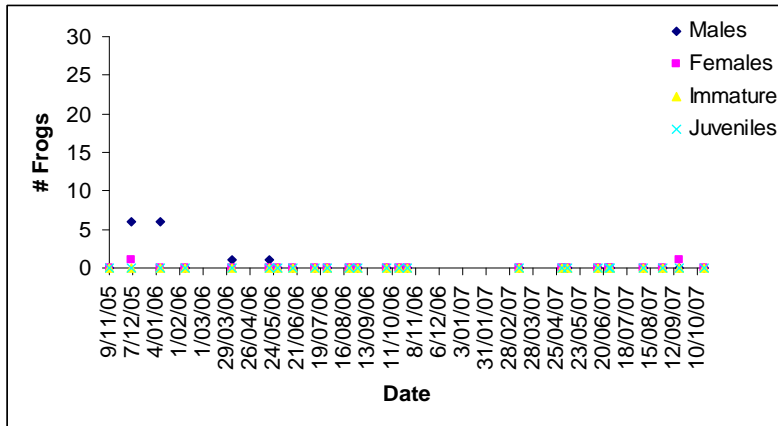

Figure 7B.25. Structure of *Litoria nannotis* Population at Tully Gorge NP
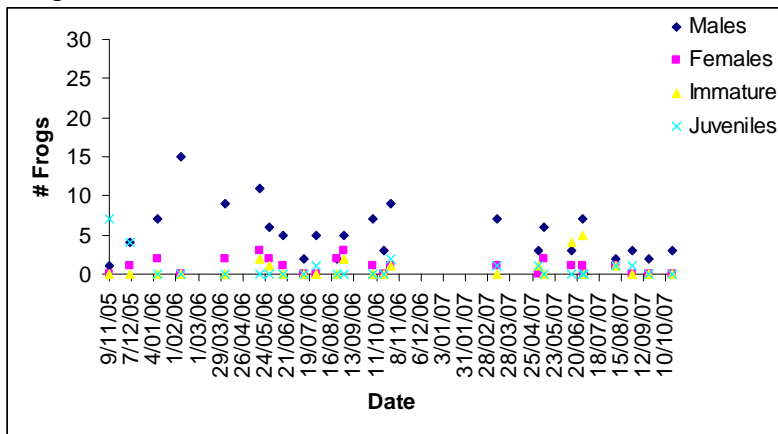

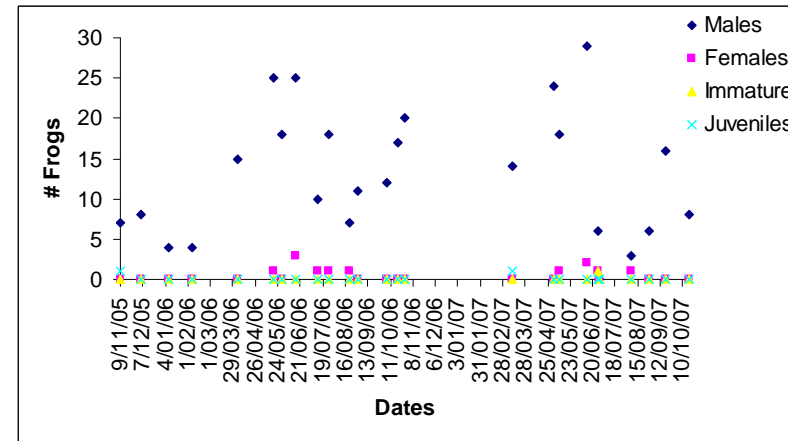Figure 7B.26. Structure of *Litoria rheocola* Population at Tully Gorge NP

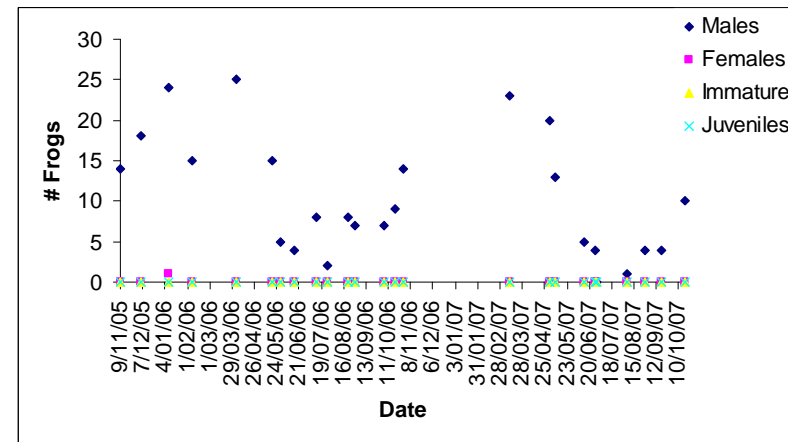Figure 7B.27. Structure of *Nyctimystes dayi* Population at Tully Gorge NP

Figure 7B.28 Estimated minimum population sizes of adult, male *Litoria genimaculata*, *L. nannotis* and *L. rheocola* along a 150m stream transect between November 2005 and November 2007 at Tully Gorge NP.
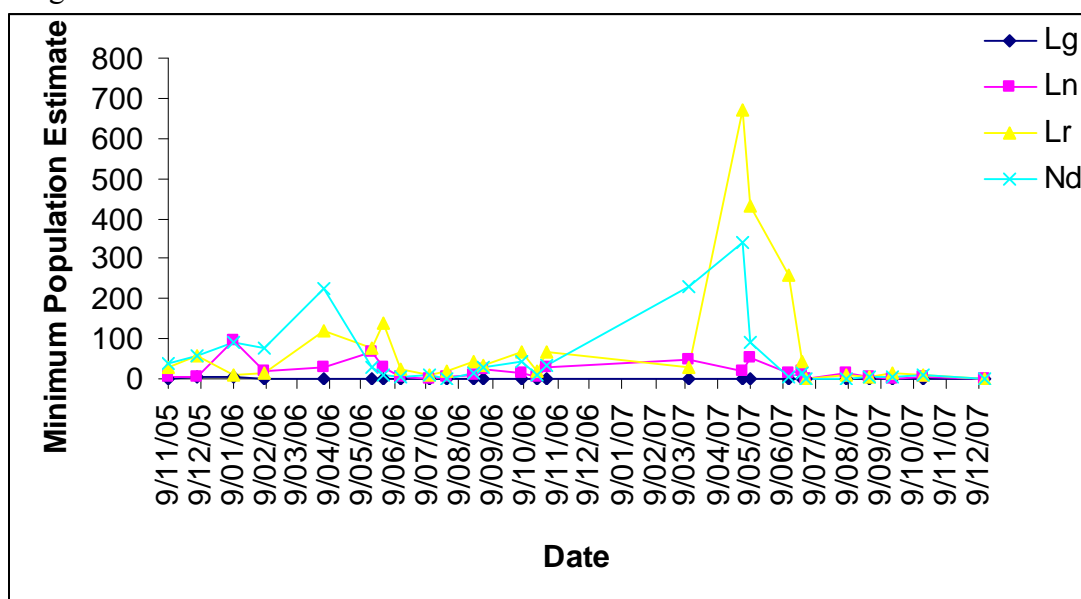

Table 7B.3. Numbers of adult, male *Litoria genimaculata*, *L. nannotis*, *L. rheocola* and *Nyctimystes dayi* marked and recaptured along a 150m stream transect between November 2005 and November 2007 at Tully Gorge NP.

Species	# Frogs Marked	Percentage of Marked Frogs that were Recaptured between 1-9 times									
		1	2	3	4	5	6	7	8	9	Unknown
L. genimaculata	20	25	10	0	0	0	0	0	0	0	0
L. nannotis	106	56	27	15	8	5	3	1	1	1	2
L. rheocola	277	47	21	9	4	1	1	1	1	0	2
N. dayi	234	43	21	10	6	3	2	1	0	0	0

Table 7B.4. Days between successive captures for adult male *Litoria genimaculata*, *L. nannotis* and *L. rheocola* captured and recaptured along a 150m stream transect between November 2005 and November 2007 at Tully Gorge NP.

Species	Mean Recapture Period±StDev(days)	Range (days)
L. genimaculata	40±20	25- 85
L. nannotis	88±90	6-490
L. rheocola	71±74	6-456
N. dayi	71±78	6-566

Figure 7B.29. Recapture Periods for *Litoria genimaculata* at Tully Gorge NP

Figure 7B.30. Recapture Periods for *Litoria nannotis* at Tully Gorge NP

Figure 7B.31. Recapture Periods for *Litoria rheocola* at Tully Gorge NP

Figure 7B.32. Recapture Periods for *Nyctimystes dayi* at Tully Gorge NP

Figure 7B.33. Variation in prevalence (%) of *Batrachochytrium dendrobatidis* with number of adult male *Litoria genimaculata* at Tully Gorge NP.

Figure 7B.34. Variation in intensity of infection (mean zoospore equivalents ± range) with prevalence (%) of *Batrachochytrium dendrobatidis* among adult male *Litoria genimaculata* at Tully Gorge NP.

Figure 7B.35. Variation in prevalence (%) of *Batrachochytrium dendrobatidis* with number of adult male *Litoria nannotis* at Tully Gorge NP.
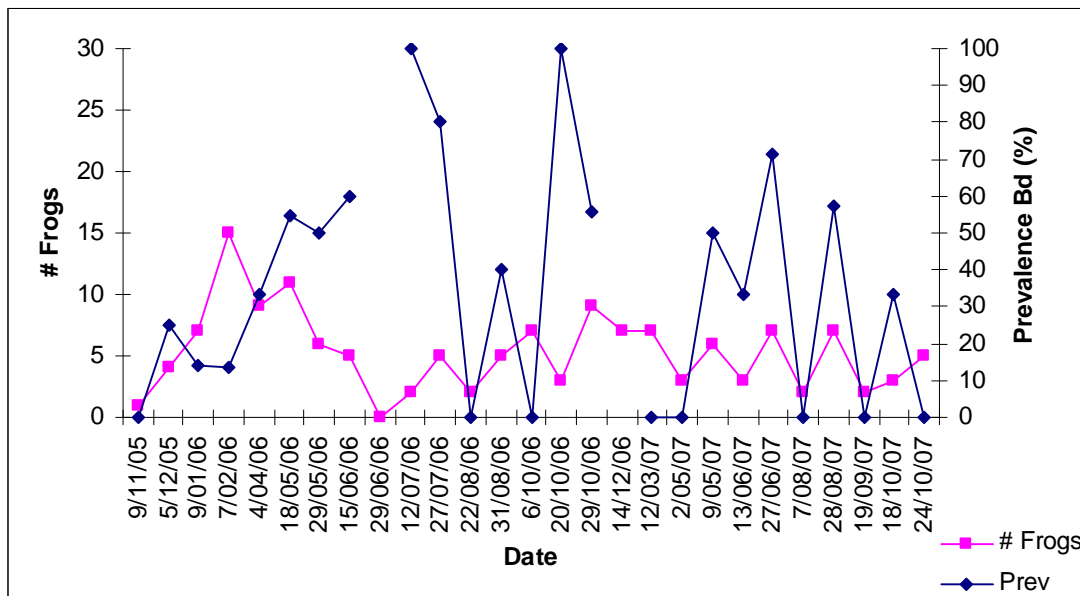
Figure 7B.36. Variation in intensity of infection (mean zoospore equivalents ± range) with prevalence (%) of *Batrachochytrium dendrobatidis* among adult male *Litoria nannotis* at Tully Gorge NP.
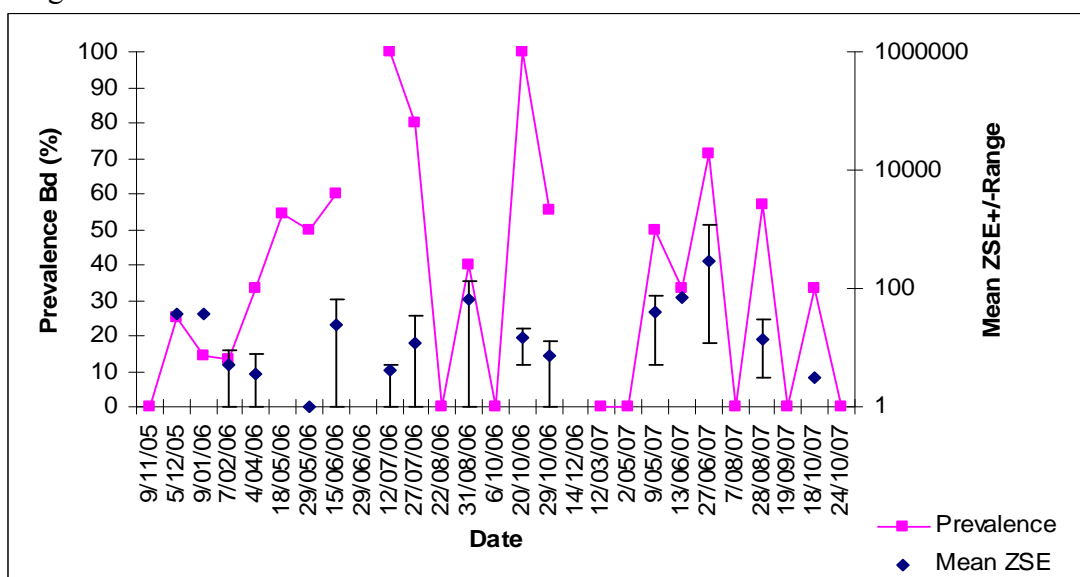
Figure 7B.37. Variation in prevalence (%) of *Batrachochytrium dendrobatidis* with number of adult male *Litoria rheocola* at Tully Gorge NP.
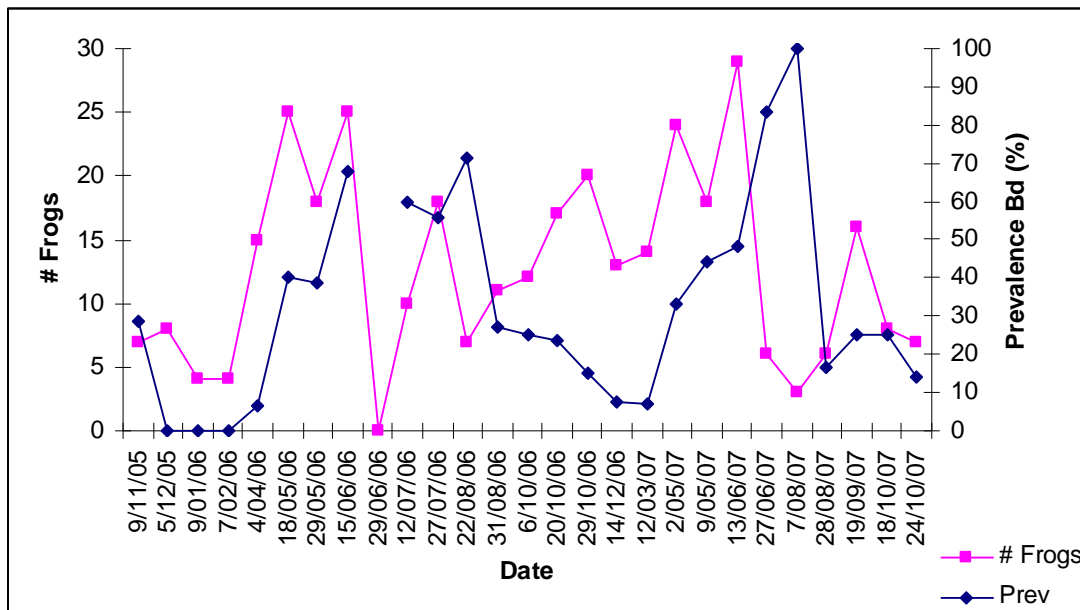
Figure 7B.38. Variation in intensity of infection (mean zoospore equivalents ± range) with prevalence (%) of *Batrachochytrium dendrobatidis* among adult male *Litoria rheocola* at Tully Gorge NP.
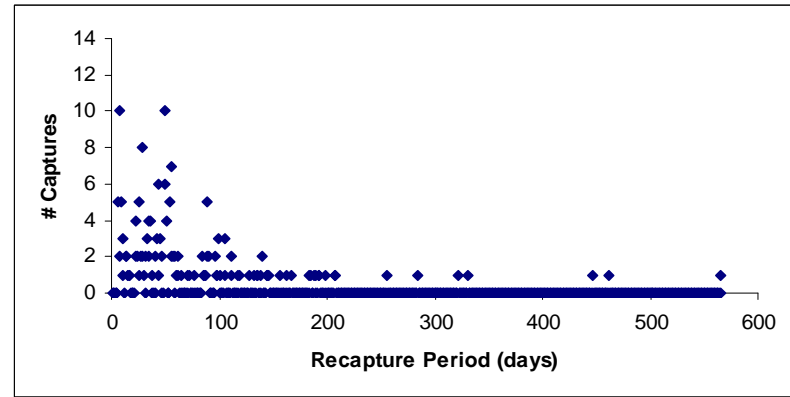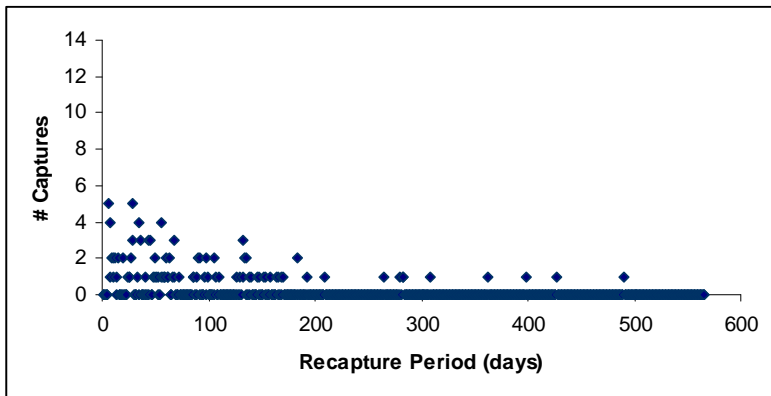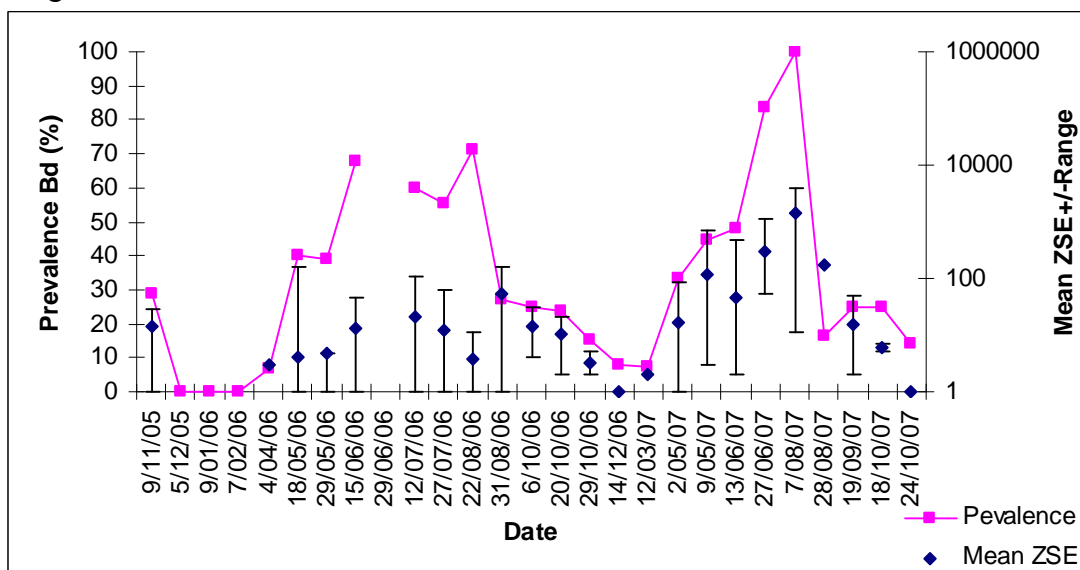
Figure 7B.39. Variation in prevalence (%) of *Batrachochytrium dendrobatidis* with number of adult male *Nyctimystes dayi* at Tully Gorge NP.
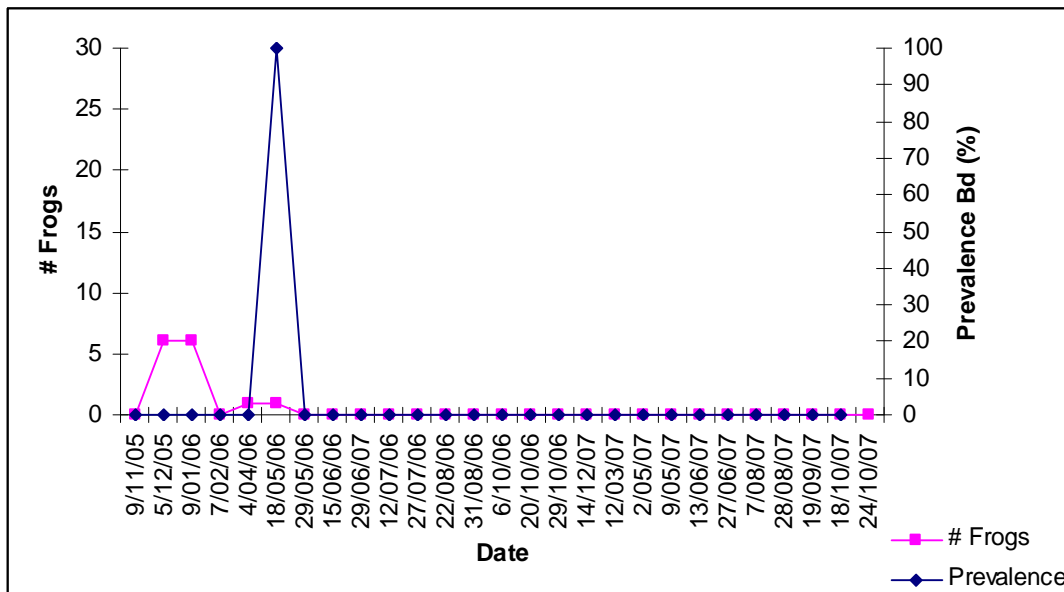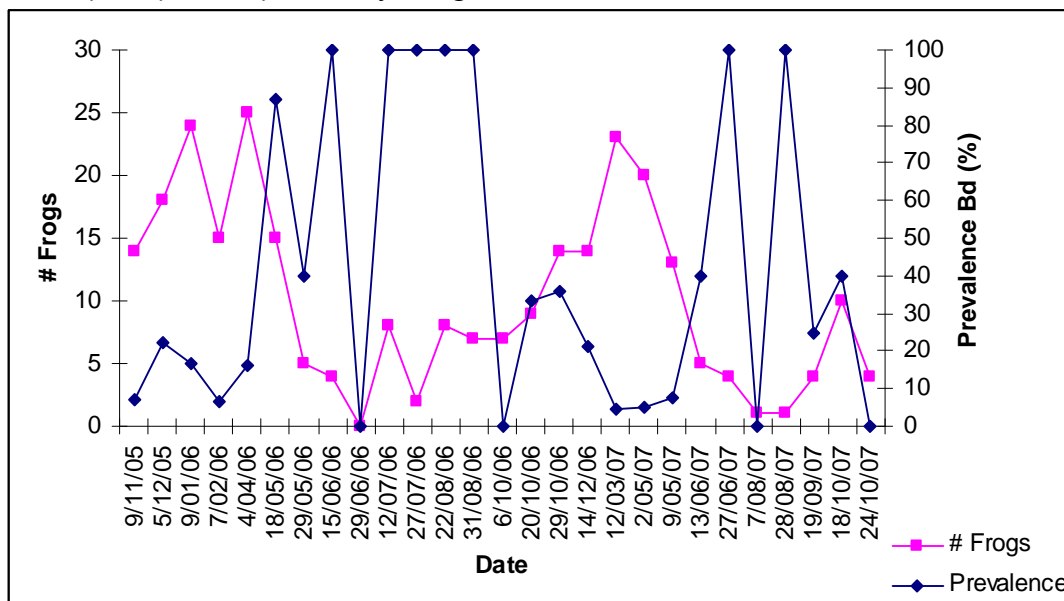

Figure 7B.40. Variation in intensity of infection (mean zoospore equivalents ± range) with prevalence (%) of *Batrachochytrium dendrobatidis* among adult male *Nyctimystes dayi* at Tully Gorge NP.
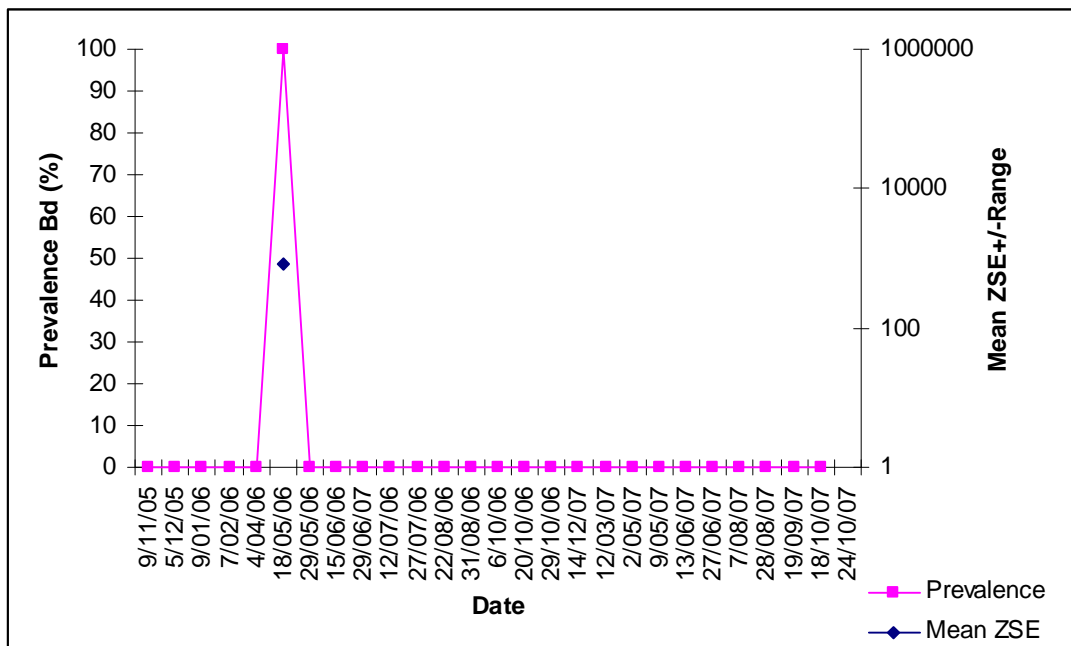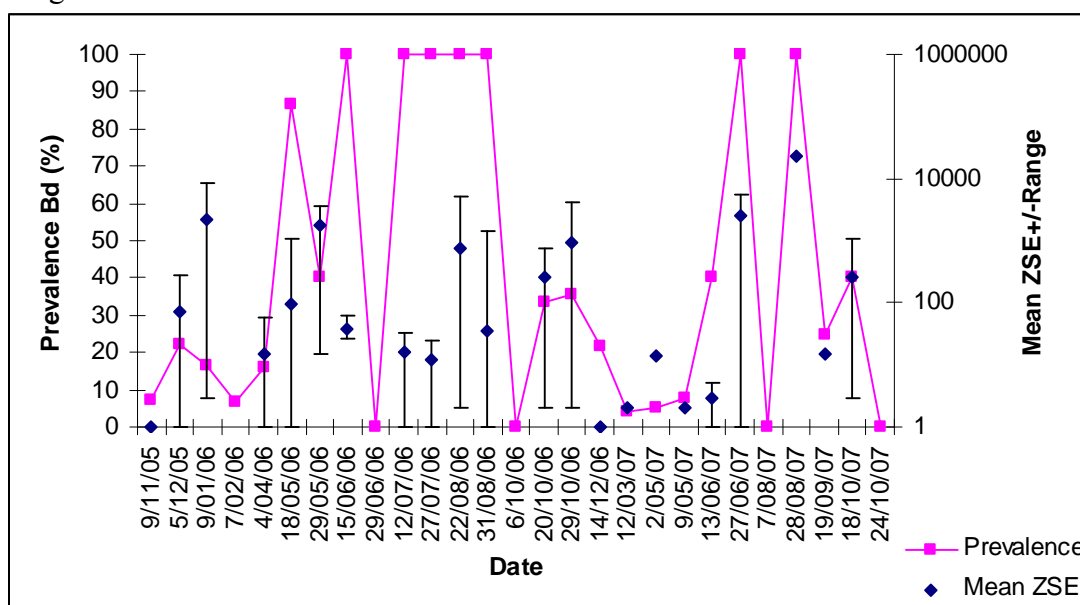
Figure 7B.41. Intensity of infection with *Batrachochytrium dendrobatidis* is not influenced by snout-urostyle length

a) infected adult, male *L. nannotis*
(r=-0.007, P=0.956, n=59).
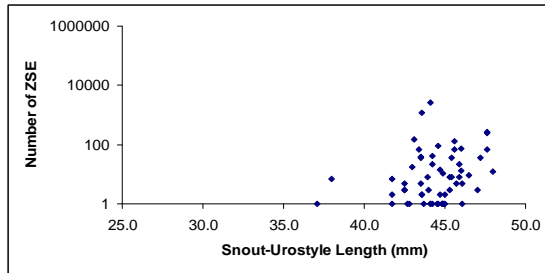
b) infected *L. nannotis* (all sizes, male and female)
(r=-0.258, P=0.011, n=97).
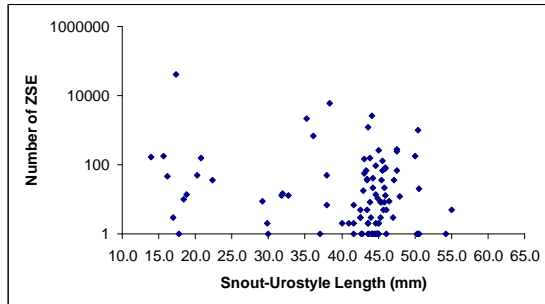
c) infected adult, male *L. rheocola*
(r=-0.101, P=0.230, n=143).
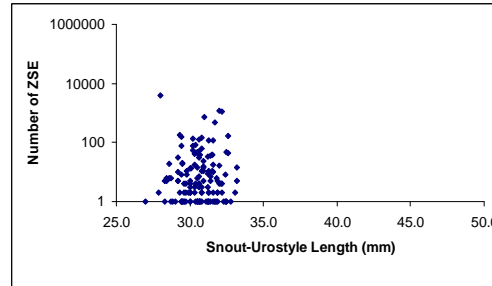
d) infected *L. rheocola* (all sizes, male and female)
(r=-0.218, P=0.006, n=157).
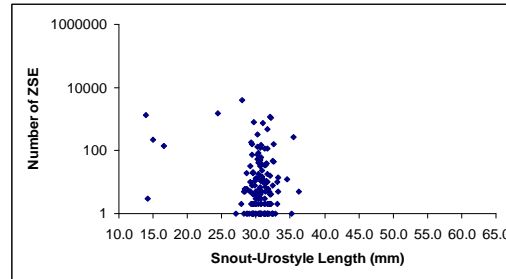
e) infected adult, male *N. dayi*
(r=-0.102, P=0.352, n=85).
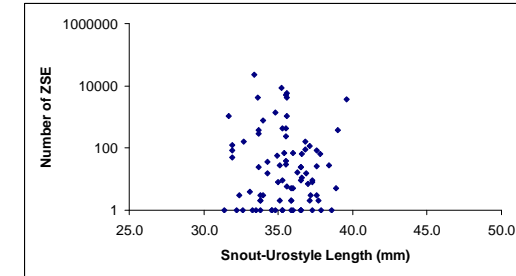
f) infected *L. N. dayi* (all sizes, male and female)
(r=0.068, P=0.528, n=89).
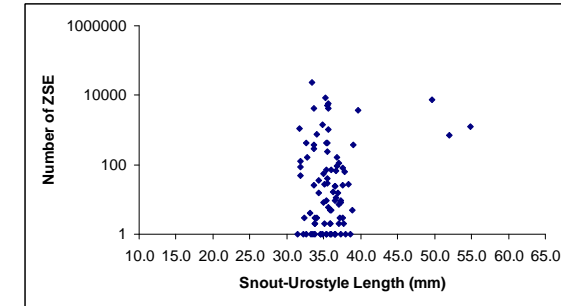
Figure 7B.42. Variation in prevalence (%) of *Batrachochytrium dendrobatidis* with total number of frogs (all species, all age-classes, both sexes) at Tully Gorge NP
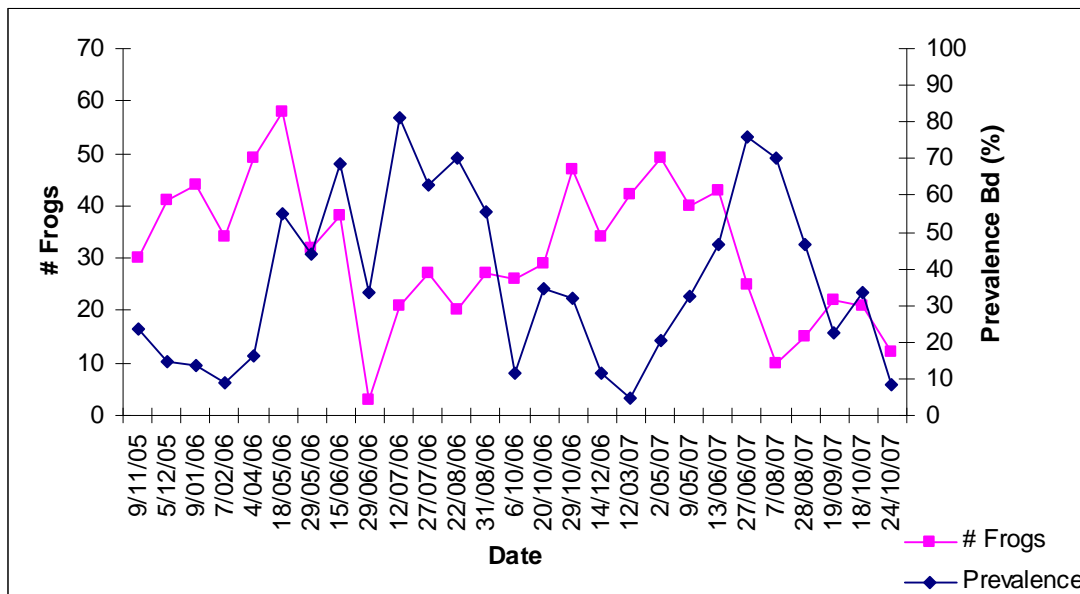
Little change in intensity of infection in infected *L. nannotis* was observed during the study period at Tully Gorge NP. Greater variation was observed in *L. rheocola*, and *N. dayi* showed the greatest increase and decrease in intensity of infection (see Figures 7.43-7B.48). Increases in infection intensity and recovery occurred year round in *N. dayi*, with no clear pattern of temporal variation.

Figure 7B.43 Change in intensity of infection in *Litoria nannotis* at Tully Gorge NP.
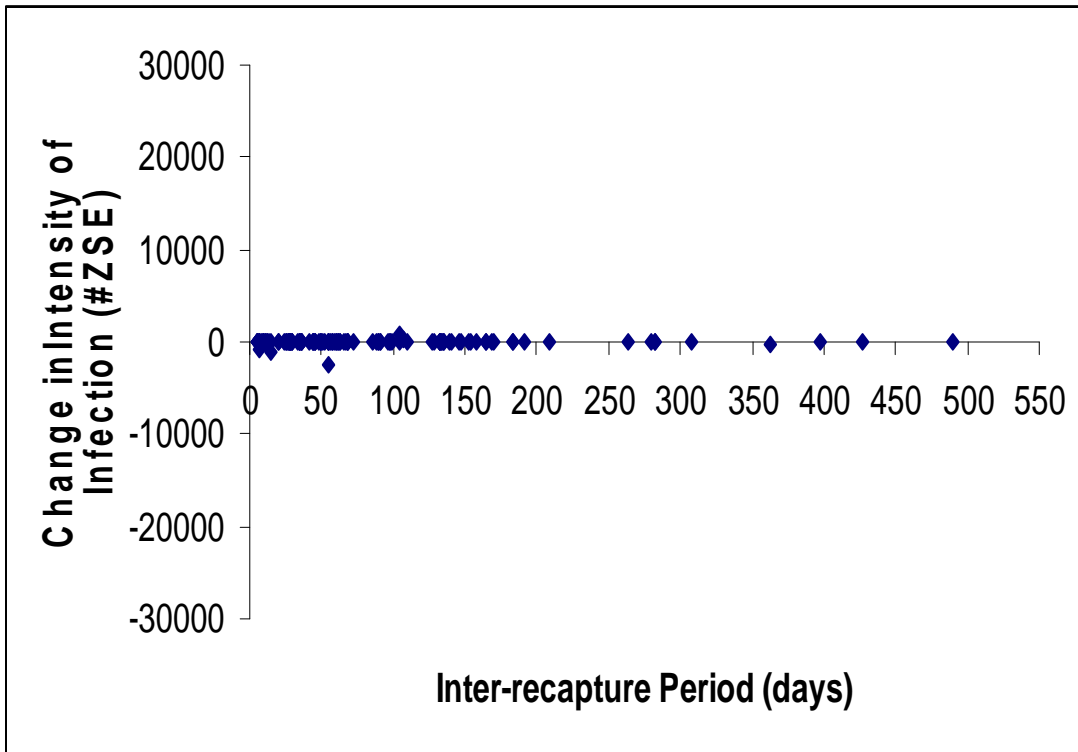
Figure 7B.43 Temporal variation in change in intensity of infection in *Litoria nannotis* at Tully Gorge NP.
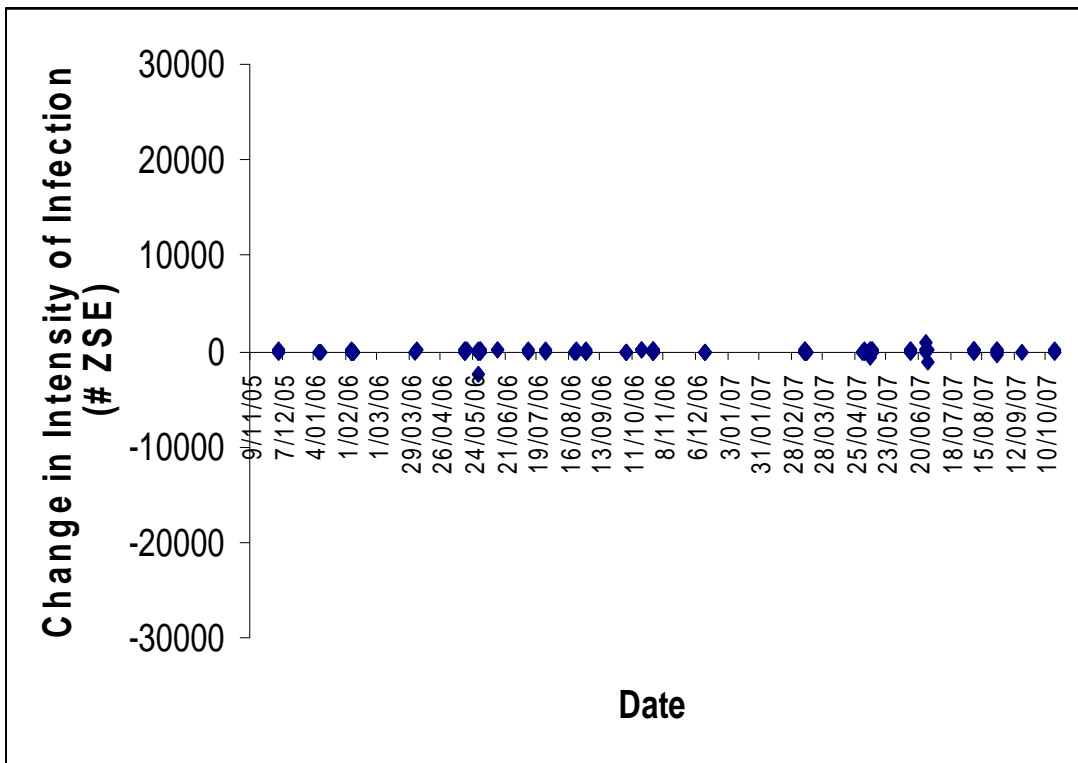
Figure 7B.45 Change in intensity of infection in *Litoria rheocola* at Tully Gorge NP.
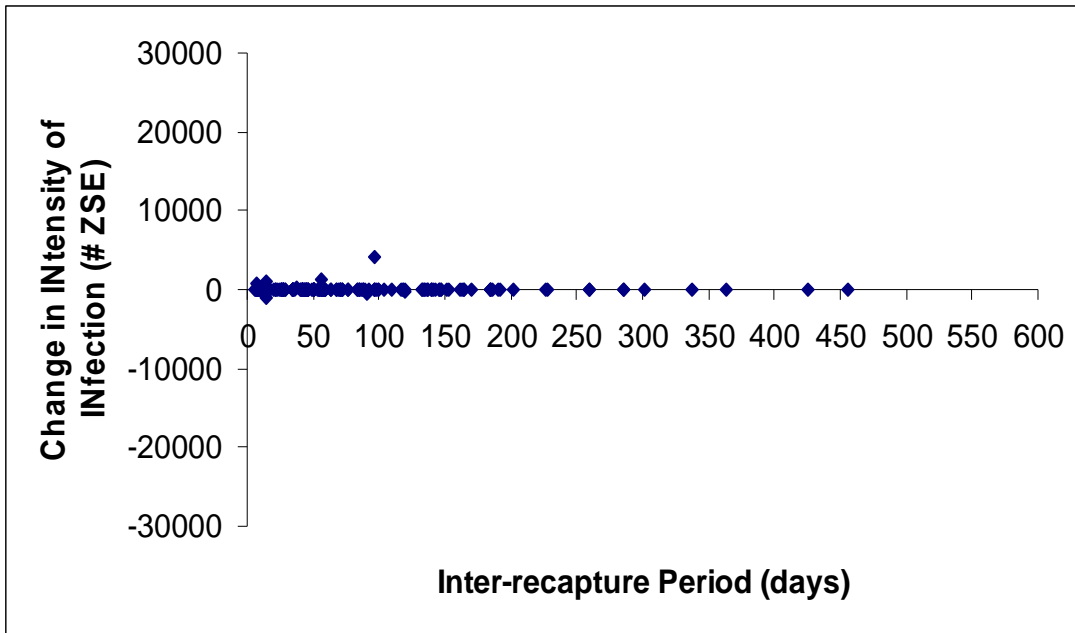
Figure 7B.46 Temporal variation in change in intensity of infection in *Litoria rheocola* at Tully Gorge NP.
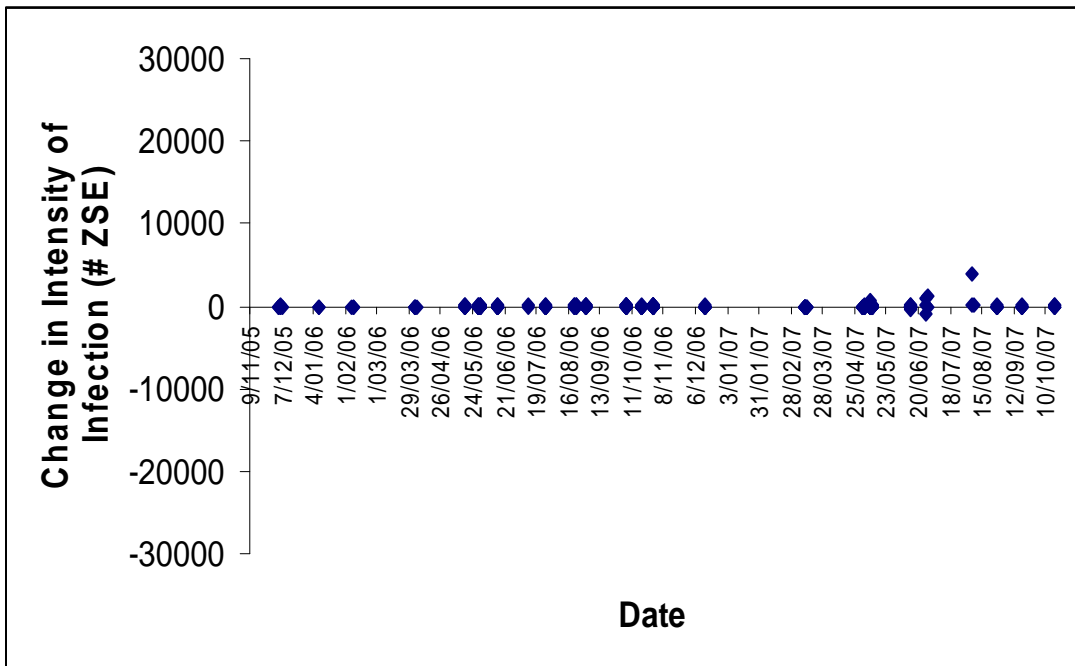
Figure 7B.47 Change in intensity of infection in *Nyctimystes dayi* at Tully Gorge NP.
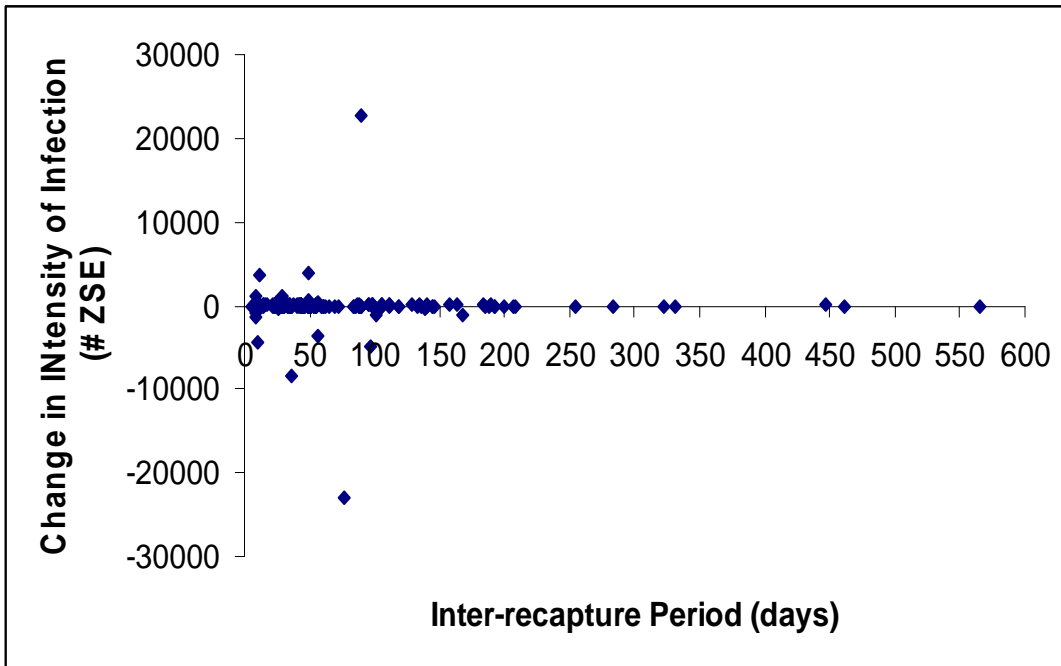
Figure 7B.48. Temporal variation in change in intensity of infection in *Nyctimystes dayi* at Tully Gorge.
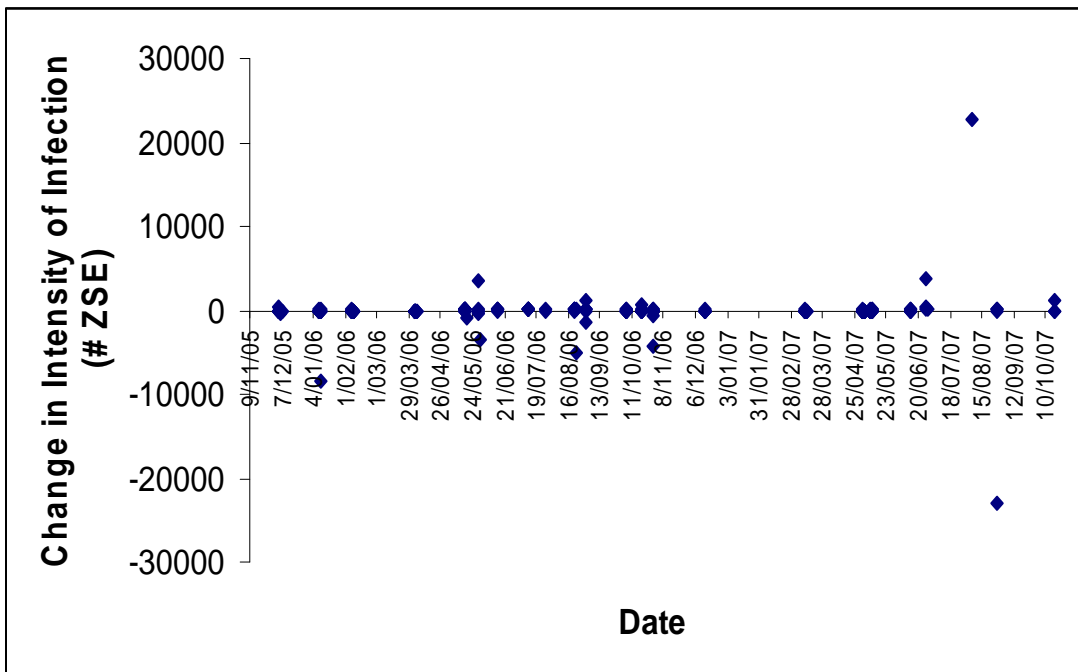
Discussion

The population of *L. genimaculata* at Murray Upper NP was estimated to be far larger than that of Tully Gorge NP, however the minimum population size is estimated from the number of recaptures of marked animals on the second night of each survey. As recaptures at Tully Gorge NP were low, the *L. genimaculata* population at this site may be far larger. Despite the greater stream width and increased habitat to search at Murray Upper NP, numbers of *L. nannotis* and *L. rheocola* marked and recaptured were higher at Tully Gorge NP, so the populations of these species at the latter site were substantially larger. *N. dayi* does not occur at Murray Upper NP.

Numbers of frogs on the stream fluctuated greatly at each location, as a result of climatic conditions. Factors such as rainfall influenced frog numbers and search capability. In heavy rain frogs were difficult to see, and the search speed might have been slower. Surveys were delayed during periods of intense rainfall or flooded streams for our safety.

The long recapture intervals for all species at both locations increased the difficulty of recapturing marked individuals on successive surveys to follow the history of infection. The proportion of marked frogs recaptured on one occasion were similar between the two sites, but were greater at Tully Gorge NP during successive captures. The minimum recapture period was an artefact of the frequency of our surveys. The long maximum recapture period, often greater than a year, was not unexpected. While the life expectancy of these frogs was unknown, capture of a frog requires the simultaneous presence of the frog and researcher on the same section of the stream at the same time. As both frogs and researchers were moving, the chance of encounter was low. Few frogs that were spotted evaded capture.

Prevalence of *Bd* in all species at both sites followed the known pattern for the austral seasons, increasing during the winter months and declining over the summer. Prevalence reached 100% in all combinations of species and site, but fluctuated greatly. Prevalence was not obviously dependent on the number of frogs of each species independently and when combined. This suggested that transmission is largely independent of frog density and the importance of environment to frog transfer of pathogens could be high. Of the four frog species studied, only *L. nannotis* occurs in groups (Hodgkison and Hero 2002) where transmission of pathogens between conspecifics might have been high. Intensity of infection appeared to correlate with prevalence.

It was expected that snout-urostyle length might influence intensity of infection. Larger frogs, with a larger surface area, would be able to host a greater number of zoosporangium and have a corresponding higher load of *Bd*. Low numbers of juveniles and immature frogs were encountered due to their dispersal from the stream and secretive behaviour (males are often caught while sitting in the open and calling). However, intensity of infection was negatively correlated with length in *L. nannotis* and *L. rheocola* at Tully Gorge NP. While not significant, the calculated Pearson correlation was also negative for *L. genimaculata*. This suggested the smaller sized frogs are more vulnerable to infection than the larger adults, or that self-reinfection (see Carey et al. 2006) was easier. Due to the lack of keratin in their body, infection in tadpoles is restricted to the mouthparts and believed to be non-lethal. After metamorphosis, infection quickly spreads to keratinised tissue and leads to fatal infection of susceptible species (Marantelli et al. 2004). The juvenile frogs we encountered were not recent metamorphs, but may have been carrying residual infection from their tadpole stage. High mortality and low detectability of sick individuals in this size-class would have contributed to our low capture rate.

N. dayi was the exception to this trend, with length and intensity of infection increasing together. This might be due to the disparity in size between *N. dayi* adult females and adult males, the size difference is not as great between the mature sexes in other species.

Intensity of infection increased and declined in all species. Infection load would increase after self-reinfection from released zoospores (Carey et al. 2006) or additional exposure to environmental sources of *Bd* (see Objective 2). It is believed a threshold of infection must be reached before mortality occurs (Carey et al. 2006). Recovery from *Bd* infection has been previously described (Kriger and Hero 2006, Retallick and Miera 2007). Frogs may have their *Bd* load decreased by warmer temperatures (Woodhams et al. 2003, Berger et al. 2004, Andre et al. 2008).

N. dayi was the most vulnerable to infection with *Bd*. This species departs the stream in the cooler months to aestivate, returning as mean air temperatures increase. Individuals were occasionally caught on the stream during winter but encounters were infrequent. Their refugia habitat has not been determined but will be subject to investigation by radio telemetry this winter. The number of frogs decline in the early winter months. This may be due to mortality or departure from the stream. Greater understanding of the habitat selection and environmental transmission pathways is needed to determine if this species contracts the infection while present on the stream at the end of autumn, or environmental transmission pathways play an important role.

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Skin surface characteristics and susceptibility to chytridiomycosis in two amphibian species, Crinia signifera and Pseudophryne corroboree

Summary

The fungal pathogen Batrachochytrium dendrobatidis (Bd) causes an epidermal infection in amphibians known as chytridiomycosis that is lethal to a wide range of amphibian species. There are, however, differences in susceptibility to chytridiomycosis among individuals, populations and species of amphibians and the underlying determinants of variability are not well understood. Colonization by Bd and subsequent disease development may be largely influenced by host defense mechanisms, such as glandular secretions or bacterial flora, occurring at the skin surface, which is the point of initial pathogen-host contact. Using scanning electron microscopy on whole frogs and light microscopy of histological sections, we investigated skin surface characteristics of two infected and uninfected Australian frog species, Crinia signifera and Pseudophryne corroboree, that differ in susceptibility to chytridiomycosis. We found that these species have complex cutaneous environments; we observed shed skin, dermal folds glandular pores and serous glands, which produce antimicrobial peptides in other amphibians. In both species we found features of skin that were similar in appearance. Number and distribution of glandular pores varied among individuals but were generally more abundant in dorsal epidermis compared to ventral epidermis. Patchy areas of Bd-infected and hyperkeratotic epidermal cells were observed throughout the pelvic patch, groin regions and on the digits of infected P. corroboree, but not in uninfected frogs or in infected C. signifera despite detection of Bd DNA with PCR analysis. No bacterial microflora was observed on these individuals, and it is therefore unlikely to have influenced fungal infections in this study.

Introduction

The fungal pathogen Batrachochytrium dendrobatidis (Bd) causes an epidermal infection in amphibians known as chytridiomycosis, which is causing catastrophic amphibian die-offs around the world (Berger et al. 1998, Skerratt et al. 2007). There is, however, wide variation among species, populations and individuals in susceptibility to chytridiomycosis. Mortality rates can range from 0% (Rana catesbiana; Daszak et al. 2004) to 100% (Litoria caerulea: Berger et al. 2005b) in rigorous laboratory infection experiments and, furthermore, in the wild amphibian species and populations persist with various levels of infection (Kriger & Hero 2006, McDonald et al. 2005, Retallick et al. 2004, Woodhams & Alford 2005, Hanselmann et al. 2004, Briggs et al. 2005). The determinants of variation in susceptibility to infection and disease are probably multiple and complex. Environmental conditions such, as temperature and humidity, are clearly critical factors in the host-

pathogen dynamic (Berger 2001, Berger et al. 2004, Woodhams & Alford 2005). Therefore, interspecific amphibian behaviours that alter body temperatures, such as aggregating in retreat sites (Rowley 2006, Rowley & Alford 2007) or close association with water bodies (Skerratt et al 2007), may alter transmission rates and influence the outcome of infection. It has also been proposed that colonization by Bd and subsequent disease development may be largely influenced by host defense mechanisms, such as glandular secretions or bacterial flora, occurring at the skin surface, which is the point of initial pathogen-host contact.

Amphibian epidermal defenses
Frog skin, an important, physiologically active organ, is also a nutritive substrate on which microorganisms can flourish (Clark 1997). Cutaneous characterisitics such as secretory glands in the superficial layers of epidermis allow amphibians to maintain proper skin functioning while providing a defense against potential pathogens (Heatwole & Barthalmus 1994, Bowie et al, Clark 1997). Two types of glands are abundant on the surface of frog skin: mucous and serous (also known as granular or poison) glands. Mucous secretions prevent desiccation (Blaylock 1976), facilitate temperature control (Lillywhite 1971) and protect from abrasive damage (Clarke 1997). Serous glands are identified as the primary source of chemicals active against predators (Lenzi-Mattos et al. 2005, Barthalamus 1994, Erspamer 1994) and against invasion by microorganisms (Clarke 1997, Bowie et al. 1998). Just as amphibian integument differs over the body surface of individuals and among species (Heatwole & Barthalamus 1994, Moore & Lofts 1964), serous glands and their secretions are heterogeneous in abundance (Czopek 1965), anatomical distribution (Bevins et al. 1990), morphology (Neuwirth et al. 1979) and functional characteristics (Delphino et al. 2002).

Cutaneous microflora
Bacteria flora found on frog skin may contribute to epidermal defense (Austin 2000, Harris et al. 2006, 2009, Belden & Harris 2007, Brucker et al. 2008, Harris et al. 2009, Lauer et al. 2007, Lauer et al. 2008, Woodhams et al. 2007, Woodhams et al. 2007). Mutually beneficial associations between animals and microbes have been identified in other host-pathogen systems (Currie et al. 2006) and are expected to be important for amphibians. Although little is known about amphibian microflora, some cutaneous bacteria produce compounds with beneficial antimicrobial properties (Austin 2000) and may protect against pathogenic organisms (Harris et al. 2006, 2009).

Pathogen colonization is regarded as a first step in pathogenesis (Cameron and Douglas 1996) and primarily occurs in the ventral epidermis in chytridiomycosis (Berger et al. 2007). We surveyed the skin surface for features associated with foci of Bd-infected epidermis that might aid or prevent Bd colonization. Using scanning cryo-electron microscopy and histological methods we compared cutaneous characteristics such as mucous and cutaneous glands as well as bacterial flora. Cryo-electron microscopy does not use traditional preservation methods such as chemical fixation or dehydration, therefore specimens can be preserved in a state very close to their original one. Advantages of this technique are that entire frogs can be examined and biological materials (bacteria, mucous, debris) adhering to the skin surface are not eliminated during processing. This technique allowed us to better observe the environment that may be encountered by Bd at its point of contact with amphibian skin.

We chose two sympatric species to examine skin surface characteristics and differences in susceptibility to chytridiomycosis. The common froglet (Crinia signifera) and the southern corroboree frog (Pseudophryne corroboree) previously occupied high montane and sub-alpine bog habitats in southeast Australia (Cogger 1992). Crinia signifera is still commonly found and is listed as a species of least concern on the IUCN Red list of threatened species. In contrast, P. corroboree is listed as critically endangered due to a drastic population declines estimated to be more than 80% (IUCN 2008); chytridiomycosis is suspected to be a major factor in these declines (IUCN 2008).

Methods

Animals collected for this study were obtained from a collaborative reintroduction experiment that used rearing mesocosms, 200 L polypropylene tubs, situated at seven sites in the Snowy Mountains Region of New South Wales. Bd occurs in these areas and prevalence in adult C. signifera populations was 86% (119/138) at the time of tadpole collections (Hunter et al. in prep). Tadpoles of both species were collected just prior to metamorphosis and transported to the Amphibian Research Center (Werribee, Australia) for rearing and assessment of post metamorphic survivorship. Tadpoles were held in tanks at 16 °C (± 2 °C), fed fish flakes and frozen endive, and water was changed every two to three days. Metamorphs were removed from the tanks and individually housed in 850 ml plastic containers that contained moist sterile moss. Metamorphs were fed small crickets and moss was continually moistened using reverse osmosis water.

To determine infection status metamorphs were swabbed on their ventral skin and feet (Hyatt et al. 2007). Swabs were processed at CSIRO Australian Animal Health Laboratories (CSIRO-AAHL, Geelong, Australia) using a Taqman real-time PCR assay (Boyle et al. 2004). Once clinical signs of infection (Berger et al 1998, Voyles et al 2007) became obvious in P. corrborree, metamorphs were transported in individual containers to CSIRO-AAHL. Frogs were euthanised by exposure to nitrogen gas in individual plastic bags, then mounted on a stage and cooled by immediate submersion in liquid nitrogen and preserved in liquid nitrogen dewars. For microscopic analysis, specimens were transferred without warming. Ventral and dorsal surfaces were observed for cell structure, presence of mucous, pores indicating glands, and the presence of bacterial microflora. Following scanning electron microscopy (SEM), specimens were preserved in 10% neutral buffered formalin. Skin tissue samples or whole frogs were embedded in paraffin sectioned and stained with haematoxylin and eosin using routine histological techniques (Humason 1967, Berger et al. 2002, Olsen et al. 2004). Sections were examined with a compound microscope to identify gland types.

Results

All but two infected P. corroborree died shortly after metamorphosis. Two infected and twelve uninfected P. corroborree were transported to CSIRO-AAHL for microscopic inspection. Six infected C. signifera were small enough to use for cryoSEM. Both C. signifera and P. corroboree have complex cutaneous environments at the epidermal surface (Figures 7C.1, 7C.2). Using the scanning electron microscope we observed shed skin, glandular pores, mucous secretions anddermal folds in P. corroboree and C. signifera. Although there was substantial variation among individuals in density and anatomical location, mucous and serous glands occured in the dorsal and ventral epidermis in both species. Actively secreting pores were observed in the ventral epidermis of P. corroboree and skin shedding was observed on the ventral forelimb in C. signifera. No bacteria were observed on either species by scanning or light microscopy.

Figure 7C.1 A composite images of Pseudophryne corroboree (ventral view).
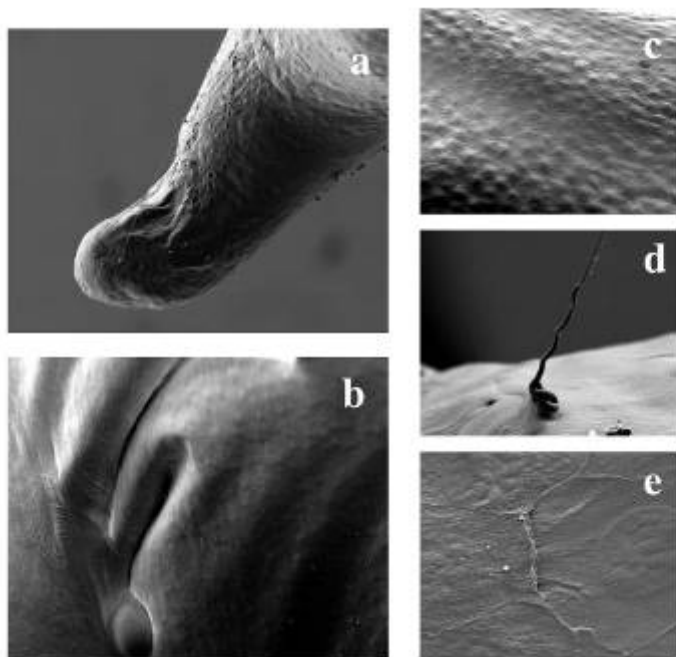
Figure 7C.2 The complex environment of the amphibian skin surface: digit of an upper limb (a), dermal folds (b), squamous epidermal cells in the ventral epidermis (c, e), a possible burrowing worm (d).
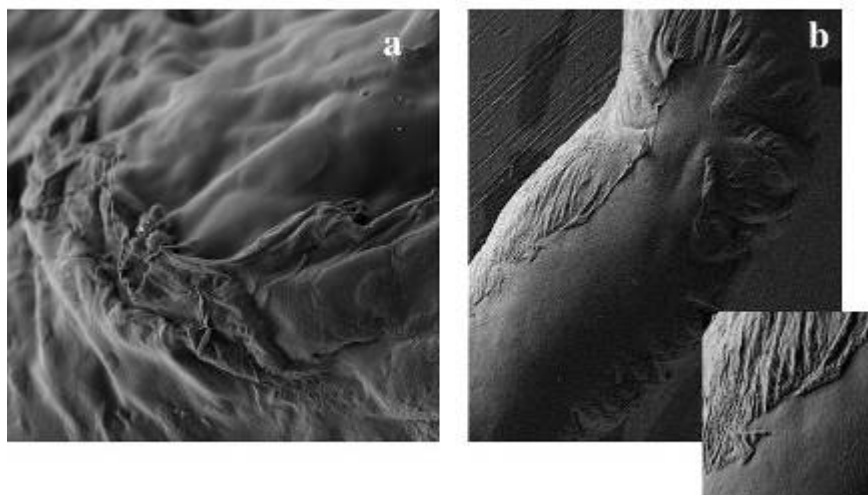
Figure 7C.3 Sloughing skin on the dorsal surface (a) and upper limb (b) of an uninfected Crinia signifera.
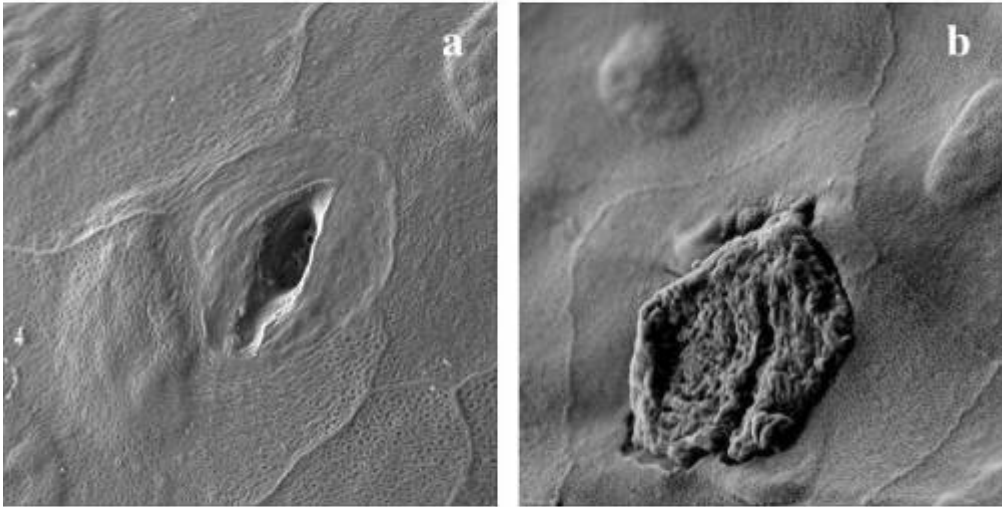
Figure 7C.4 Epidermal pore and visible duct in the ventral skin of an infected Pseudophryne corroboree (a). Epidermal pore with secretion in the ventral skin of an infected Pseudophryne corroboree (b).

Despite detection of Bd DNA by PCR analysis, SEM revealed no evidence of infected hyperkeratotic areas in C. signifera. In contrast, pathology associated with chytridiomycosis was seen in P. corroboree (Figure 7C.5). Patchy areas of hyperkeratosis were observed throughout the pelvic patch, the groin regions, and on the digits of infected P. corroboree (Figure 7C.5). No glandular pores were seen in the immediate vicinity of Bd-infected regions. However, pores secreting mucous substances were observed in ventral skin of infected P. corroboree (Figure 7C.4).
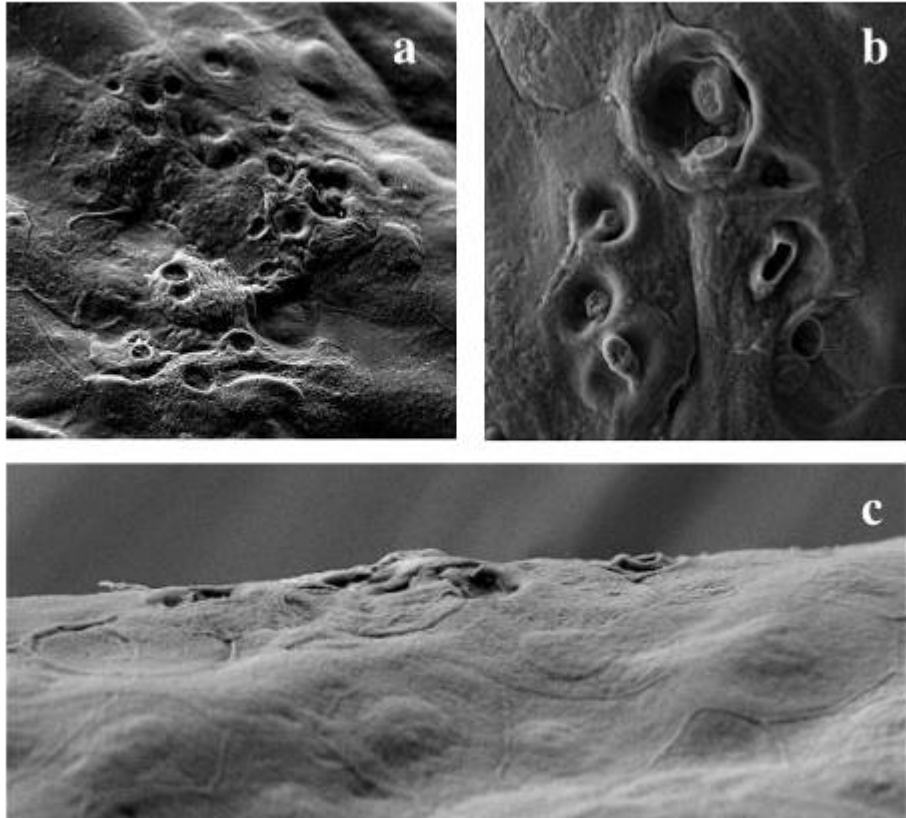
Figure 7C.5 Batrachochytrium dendrobatidis in patchy hyperkeratotic areas in the ventral (a, b) and digital (c) epidermis of an infected Pseudophryne corroborree (comment LS4)

Discussion

Both species investigated in this study had similar complex cutaneous environments at the ventral epidermal surface. Serous glands were present in ventral surfaces where Bd is commonly found and there was no evidence of a cutaneous bacterial flora in any of the frogs we observed with cryo-EM. Much of the microflora of amphibians is acquired from their environment (Clarke 1997) and the composition of microflora can change when amphibians are moved into captivity (personal communication R. Harris). Therefore, our results may not conditions these species would encounter in nature because the frogs were reared throughout their postmetamorphic lives under controlled conditions designed to minimize transmission of infectious agents. Although the lack of cutaneous microflora suggests that bacteria were not an important factor in these Bd outbreaks, our results are far from conclusive.

It is unknown if exposing skin to Bd is sufficient to stimulate secretion of anti-microbial peptides (Rollins-Smith 2001). The type of stimulus may modulate skin secretions (Mills & Prum 1984). For example, the mechanical stimulus of a predator attack may induce the secretion of toxic substances (Lenzi-Mattos et al. 2005) that differ from the substances secreted in the presence of microorganisms (Mangoni et al. 2001). From the images collected in this study it appears that at least some of the glands in the ventral epidermis are actively secreting substances during Bd infection in P. corroboree. We could not determine, however, whether these secretions are effective against Bd. The effectiveness of antimicrobial peptides against Bd may depend on the type and amount of the secretions produced (Rollins-Smith 2001, Woodhams et al. 2006) and perhaps, the timing of the secretions as well. For example, secretions could protect against zoospore colonization but it is unknown if anti-microbial peptides are absorbed inside epidermal cells, where sporangia grow. While we were not able to determine the type of secretions produced by P. corroboree nor quantify the amount of peptides produced during infection, glandular pores were not observed in Bd infected

areas when viewed by SEM. In addition, it did not appear from our study that secretions dispersed far from pores. Our work suggests that infections may be patchy in the skin in order to avoid defensive skin structures. Future experimental work may resolve these questions.

Tadpoles of both of C. signifera and P. corroboree collected from the same areas and maintained in the same environmental conditions became infected with Bd. They were also both infected at the metamorphic stage, however, only P. corroboree had evidence of Bd-infected epidermis and had greater rates of mortality. This is agrees with the effects observed at the population level in the field (Hunter et al. in prep) where it appears that P. corroboree are far more susceptible to chytridiomycosis than C. signifera. However, a controlled infection experiment using a standard dose of a single isolate of Bd is needed to confirm that these species differ in their inherent resistance to disease development at the individual level.

Appendices

Appendix 1

Investigation into the role of the amphibian chytrid fungus (*Batrachochytrium dendrobatidis*) in the decline of the green and golden bell frog (*Litoria aurea*)

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Background

The green and golden bell frog *Litoria aurea* is a large pond breeding Hylid that was once common throughout the tablelands and floodplains of eastern NSW but over the last 40 years have undergone a widespread range contraction with the disappearance of nearly all inland populations (Mahony, 1999, White and Pyke, 1996). *L. aurea* currently persist in a series of highly fragmented populations scattered along the coastline in often highly disturbed habitat with a strong marine influence (White and Pyke, 1996). These remaining populations are small, isolated and very few are in protected conservation reserves (White and Pyke, 1996). As a result, this once common species is now considered to be endangered in NSW under the Threatened Species Conservation Act 1995 and vulnerable nationally under Schedule 1 of the Environment Protection and Biodiversity Conservation Act 1999.

The cause of this decline has historically been attributed to the introduction of a predatory mosquito fish and habitat modification (Weatherley and Lake, 1967) but the role of these threats in *L. aurea*'s range contraction has been difficult to determine because they are often inconsistent with the pattern of decline. Given the inland disappearance of *L. aurea* populations, the causal agent must be less severe in, or absent from, remaining habitat. However, *G. holbrooki* are widespread throughout the waterways of eastern NSW (Hamer, 2002, Van De Mortel and Goldingay, 1998) including the coastal zone where *L. aurea* persist and several remaining *L. aurea* populations occur and breed in waterbodies containing high numbers of *G. holbrooki* (Hamer, Lane and Mahony, 2007, Mahony, 1999). The role of habitat modification is also unclear as *L. aurea* is an opportunistic, colonising species that is highly tolerant of disturbance (Pyke *et al.*, 2002). They have disappeared from inland areas used primarily for agriculture despite readily colonizing agricultural lands (Courtice and Grigg, 1975) and being historically recorded to utilize farm dams , only to persist in the urbanized coastal zone.

Recently, the localized extinction of a reintroduced *L. aurea* population in the Hunter Region of NSW occurred in the presence of the amphibian chytrid fungus *Batrachochytrium dendrobatidis* (Boyle *et al.*, 2004) suggesting that it may have played a role in the *L. aurea* decline. This study aimed to investigate the role of *B. dendrobatidis* in the *L. aurea* decline and did so by addressing a number of research questions.

Research Questions

Question 1 -   1.1. Is *L. aurea* susceptible to *B. dendrobatidis* infection, disease expression and outcome?
1.2. Do susceptibilities differ between the declining *L. aurea* and a co-occurring non-declining species?
1.3. What causes any differences in species susceptibility differences?

Question 2 -   Does *B. dendrobatidis* occur in remaining *L. aurea* populations?

Question 3 -   3.1. Does *B. dendrobatidis* occur throughout a frog community where *L. aurea* persist?
3.2. What effects are these infections having on body condition and survivorship?

Question 4 -   Are there environmental inhibitors of *B. dendrobatidis* that allow *L. aurea* to persist in disturbed coastal sites?

Question 5 -   Does NaCl inhibit the infective capacity of *B. dendrobatidis* and reduce disease expression?

Research Outcomes

Question 1 -   1.1. Is *L. aurea* susceptible to *B. dendrobatidis* infection, disease expression and outcome?
1.2. Do susceptibilities differ between the declining *L. aurea* and a co-occurring non-declining species?
1.3. What causes any differences in species susceptibility differences?

Question 1 was addressed by conducting an artificial infection experiment on *L. aurea* and *Limnodynastes peronii* tadpoles and juveniles. *L. peronii* are a common species that frequently co-occur with *L. aurea* throughout their range but have not undergone a range contraction. Tadpoles and juveniles of both species were bred at the University of Newcastle and either exposed to a *B. dendrobatidis* zoospore suspension (N = 19 tadpoles and 19 juveniles of each species; isolate Gibbo River-Llesueuri-00-LB-1) or a sterile sham suspension (N = 19 tadpoles and 19 juveniles of each species). Animals were then monitored daily for signs of disease and upon observation of lethargy and poor righting reflex were euthanized. Infection severities in individuals were determined by swabbing and using a real-time PCR Taqman assay (Berger *et al.*, 2004) over three swabbing events. These swabbing events were initially intended to be at 2 weeks, 3 months and 6 months after initial exposure to *B. dendrobatidis* but nearly all *L. aurea* metamorphs and juveniles in treatment tanks had been euthanized within 3 months of inoculation. Therefore, the second swabbing event was done at the death of an individual in a tank, rather than 3 months after inoculation. When an individual of one species required euthanasia, it was swabbed along with an individual of the other species so that comparisons between species could be made. The final swabbing event was still done 6 months after inoculations for the *L. peronii* metamorphs and juveniles that remained.

All control individuals exposed to a sterile sham suspension were found to remain negative for the presence of chytrid throughout this experiment. By comparison, all individuals exposed to *B. dendrobatidis* zoospore suspensions became infected, with infections evident as early as 2 weeks following initial exposure, at the first swabbing event (Figure Q1). No differences were found between species in infection severity of juveniles at the first swabbing event but by the second swabbing event the infection severities of *L. aurea* juveniles increased resulting in a significant

difference between the species (Z = 0.11, P =0.001). All *L. aurea* juveniles showed symptoms of chytridiomycosis and were euthanized before the third swabbing event 6 months after initial exposure to *B. dendrobatidis* (Figure Q2). By comparison, nearly all *L. peronii* individuals remained alive and were found to have infection severities similar, if not lower than those from the previous two swabbing events (Figure Q1). All individuals in control tanks were found to remain healthy throughout the experiment and did not require euthanasia (Figure Q2). Log rank Mantel-Cox tests found significant differences in survival rates between control and treatment groups for *L. aurea* juveniles (χ^2=43.48, df =1, P <0.001), with the control group having a greater rate of survival. No difference was found for *L. peronii* juveniles (χ^2=2.01, df =1, P =0.15).
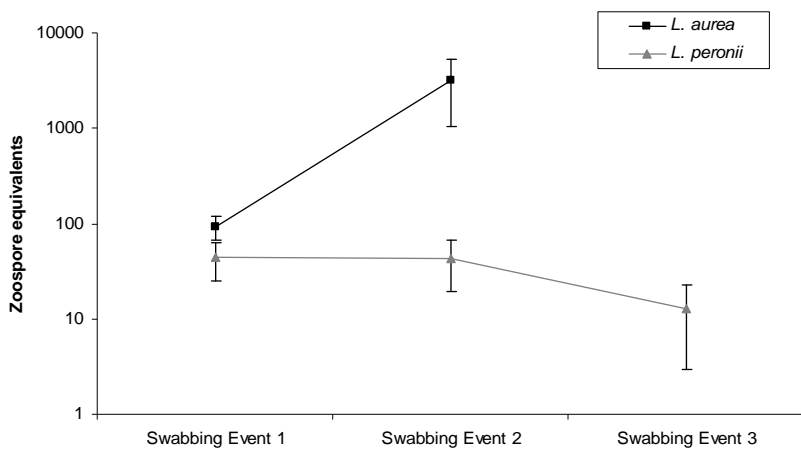

Figure Q1. Infection severities of *Litoria aurea* and *Limnodynastes peronii* juveniles over three swabbing events following exposure to a *Batrachochytrium dendrobatidis* zoospore suspension (± SE).
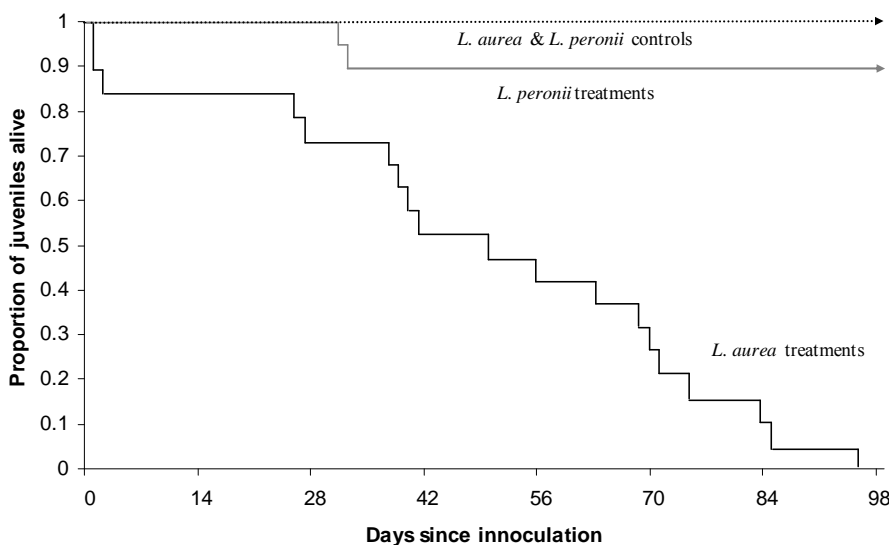

Figure Q2. Survival curves for *L. aurea and L. peronii* juveniles inoculated with *B. dendrobatidis* zoospore suspension (treatments) or sterile sham suspensions (controls).

This study was also repeated in tadpoles and similar results were found. Significant differences were found between species at the first swabbing event after 2 weeks of exposure to *B. dendrobatidis* with *L. aurea* tadpoles carrying significantly more severe infections (Z =2.12, P = 0.03). Following metamorphosis, infection severities continued to be higher in *L. aurea* than *L. peronii* (Z =3.82, P <0.001) and all *L. aurea* showed symptoms of disease and were euthanized before the third swabbing event. Log rank Mantel-Cox tests found significant differences in survival rates between control and treatment groups for *L. aurea* metamorphs (χ^2=36.43, df =1, P <0.001) with the control group having a greater rate of survival. No difference was found for *L. peronii* metamorphs (χ^2=0.20, df =1, P =0.65).

The cause of these differences between species were investigated through the standardization of temperature, zoospore density, pathogen strain, age and life stage and the measurement of encounter rate, skin sloughing and body weight. Linear mixed models found species to be the only significant predictor of infection severity ($F_{(1,31)}$=9.51, P =0.004) and Cox regression models found species to be the only significant predictor of survival rate (Wald =34.36, df = 1, P <0.001). These results suggest that *L. aurea* are highly susceptible to chytrid infection and disease expression with 100% infection and presumed mortality rates. Susceptibility differences between *L. aurea* and *L. peronii* were also found that are consistent with their respective decline histories where *L. peronii* possess a means to inhibit an increase in infection severity over time that is lacking in *L. aurea*.

Question 2 - Does *B. dendrobatidis* occur in remaining *L. aurea* populations?

B. dendrobatidis has been observed to cause the extinction of a reintroduced *L. aurea* population (Stockwell *et al.*, 2008) and cause disease in 100% of juveniles and metamorphs in the previous artificial infection experiment suggesting that it may have played a role in the decline of this species. Given the non-random pattern of the *L. aurea* decline it would be expected that if *B. dendrobatidis* was the cause it would be absent or having little effect in remaining *L. aurea* populations. To investigate this, swabbing surveys were conducted at Kooragang Island, Sydney Olympic Park, Kurnell Peninsular and Broughton Island where four of the largest remaining *L. aurea* populations in NSW persist. At each population spotlight surveys were conducted to locate capture and swab 60 *L. aurea*. The presence and infection severity of *B. dendrobatidis* on swabs was then determined using the real time PCR Taqman assay.

B. dendrobatidis was detected in three of the populations tested, at prevalences of less than 40% (Figure). Broughton Island was the only site where *B. dendrobatidis* was not detected and based on sampling effort must occur within the population at a prevalence of less than 5%. However, given that Broughton Island is an offshore island with minimal human and animal movement between it and the mainland there is a good chance that *B. dendrobatidis* has never reached it. Within the three populations where *B. dendrobatidis* was detected, the severities of infections within individuals were highly variable (Figure). Most infected individuals on Kooragang Island and all from Sydney Olympic Park had infection severities of less than one zoospore equivalent. Kurnell Peninsular individuals had higher infection severities. However, the prevalence and infection severities cannot be compared between populations because they were swabbed at different times of the year and the virulence of chytrid is temperature dependant.
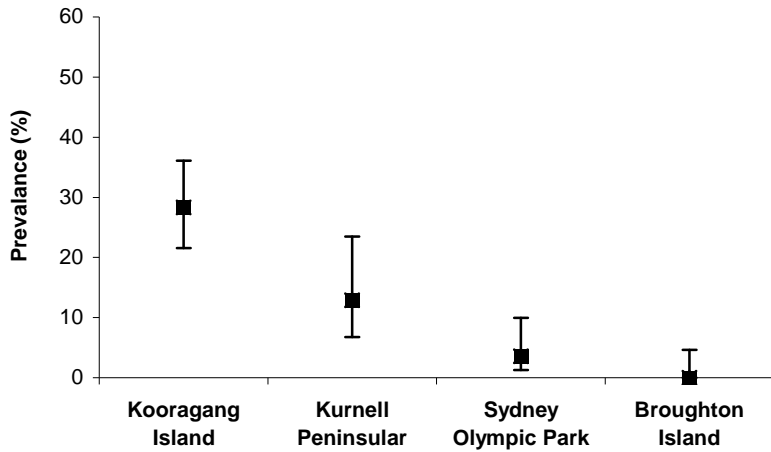
Figure Q3. Prevalence of *B. dendrobatidis* infection in four *L. aurea* populations in NSW (± 95% CI).
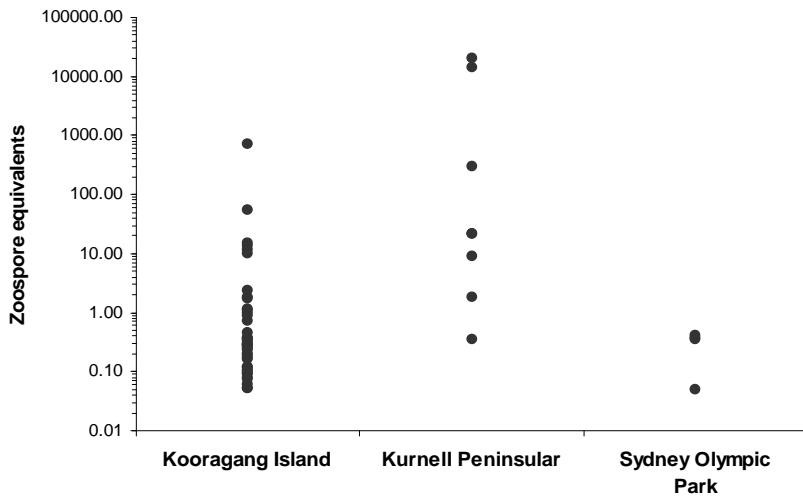
Figure Q4. The number of zoospore equivalents detected on individuals from three infected *Litoria aurea* populations.

These swabbing surveys reveal that *B. dendrobatidis* occurs in the three mainland populations tested at generally low prevalences and infection severities. This result rules out the idea that *L. aurea* persist at these sites because *B. dendrobatidis* is absent but the low levels detected may indicate that the impact on these individuals and populations is low.

Question 3 - **3.1. Does *B. dendrobatidis* occur throughout a frog community where *L. aurea* persist?**
3.2. What effects are these infections having on body condition and survivorship?

During swabbing surveys a greater focus was placed on the Kooragang Island site where co-occurring species were captured and swabbed opportunistically along side *L. aurea*. All individuals captured were also weighed and their length measured to determine a body mass index which was then compared between infected and uninfected individuals to determine if there is an effect of infection on body condition. The effect of infection on survivorship in *L. aurea* was also investigated in three ways. Firstly, all captured *L. aurea* were micro-chipped to infer survivorship from recapture rates. Secondly, the gender of all *L. aurea* individuals were also determined to compare the proportion of males and females within infected and uninfected groups. Differences in the behaviour and biology of the sexes makes the *B. dendrobatidis* encounter rate of males higher than females so if infection was reducing survivorship we would see differences in the proportion of each sex in infected and uninfected groups. Finally, all captured *L. aurea* were placed into age classes based on Bertalanffy growth curves previously generated for the Kooragang Island population. Again, because of differences in the behaviour of different age classes, juveniles would be expected to have a higher *B. dendrobatidis* encounter rate than older individuals and any effect of infection on survivorship will result in different population age structures in infected and uninfected groups.

Four amphibian species that co-occurred with *L. aurea* on Kooragang island were captured and swabbed during surveys and *B. dendrobatidis* was detected in all four species (Figure) at prevalences that were not significantly different to that of *L. aurea* ($\chi^2 = 0.71$, df = 2, P = 0.70). These four species frequently co-occur with *L. aurea* throughout their range but none of them have undergone a decline.
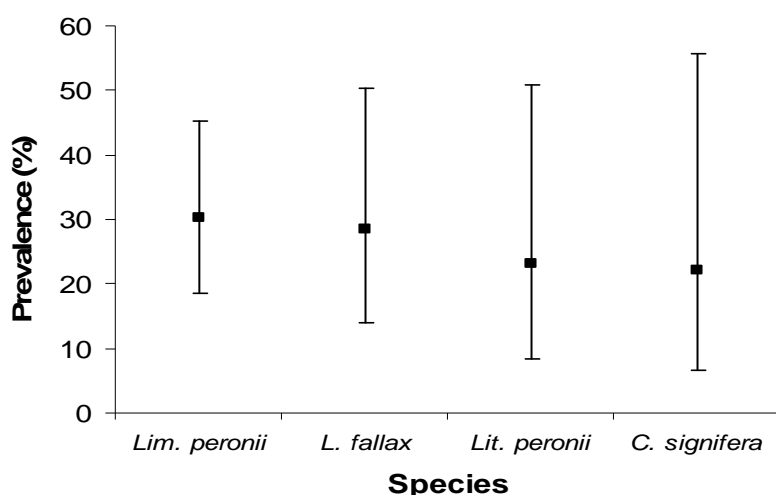
Figure Q5. The prevalence of *Batrachochytrium dendrobatidis* in four frog species co-occurring with *Litoria aurea* on Kooragang Island (± 95% CI).

For each species surveyed on Kooragang Island a body condition index (BCI) was determined and compared between infected and uninfected individuals (Figure). The only difference in body

condition between infected and uninfected individuals was found for *L. aurea* (t = 4.07, df = 142, P < 0.001) with infected individuals having a significantly higher BCI than uninfected (Figure Q6). It is highly unlikely that infection infers an advantage and these results are being investigated further.
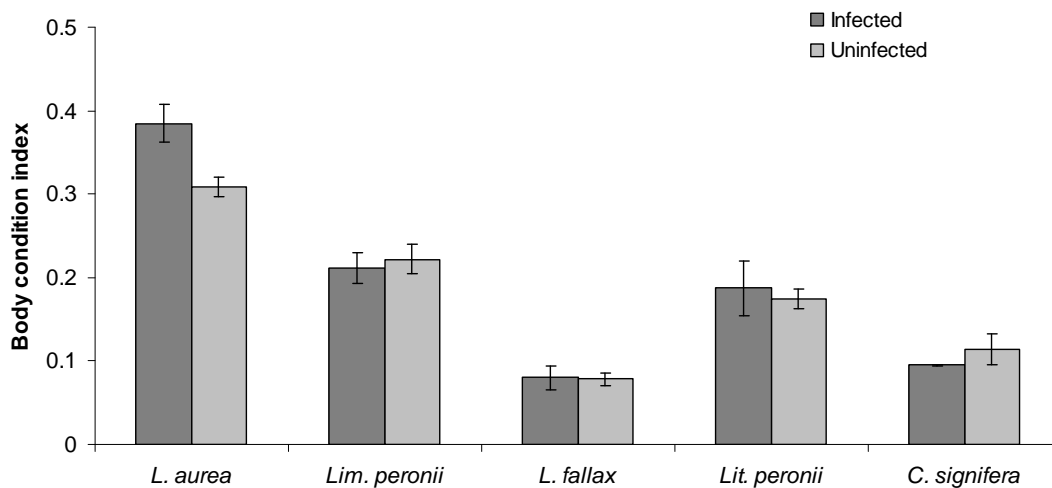
Figure Q6. The mean body condition index of five frog species from Kooragang Island that were either infected or un-infected with *B. dendrobatidis* (± SE).

The proportion of recaptured *L. aurea* that were infected and uninfected were not found to differ suggesting that infection does not reduce survivorship over the time period surveyed (Figure Q7). Similarly, no differences were found in the proportion of infected and uninfected males and females ($\chi 2 = 0.27$, df = 1, P =0.61) suggesting that the higher encounter rates of *L. aurea* males with *B. dendrobatidis* do not result in lower survivorship (Figure Q8).
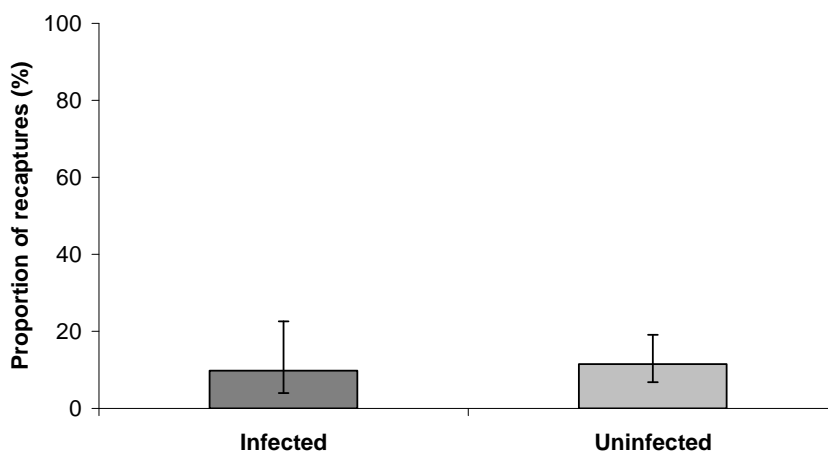
Figure Q7. Proportion (± 95% CI) of infected and uninfected *Litoria aurea* recaptured on Kooragang Island.
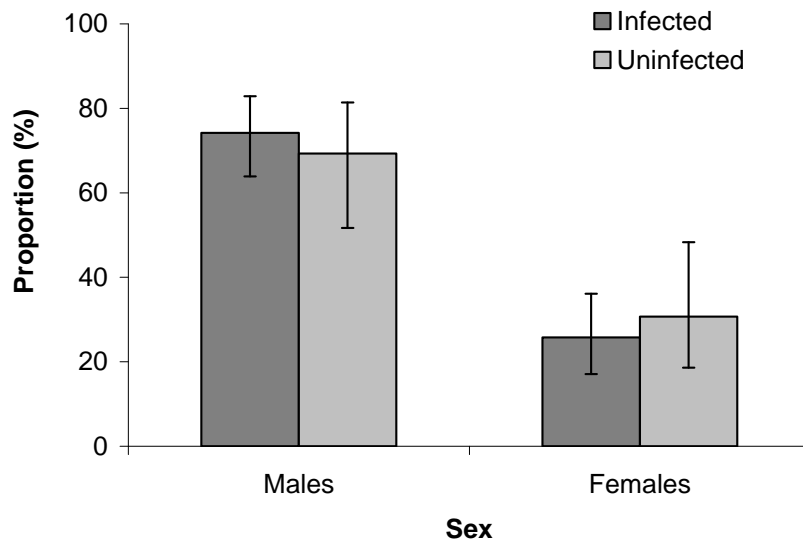
Figure Q8. Proprtion (± 95% CI) of infected and uninfected male and female *Litoria aurea* captured on Kooragang Island.

Question 4 - Are there environmental inhibitors of *B. dendrobatidis* that allow *L. aurea* to persist in disturbed coastal sites?

The contradictory findings that *L. aurea* was highly susceptible to chytridiomycosis and had been responsible for a population extinction (Stockwell *et al.*, 2008) and yet four of the largest remaining populations in NSW persist with low level infections that appear to cause minimal effect on body condition and survivorship, led to investigations into how this co-existence occurs. The role of co-evolution following the introduction of *B. dendrobatidis* into naïve populations was inconsistent with the non-random pattern of *L. aurea* decline. Given the distinct coastal contraction of this species, an environmental inhibitor of *B. dendrobatidis* was considered.

The presence of an environmental inhibitor was investigated by determining the infection severities in 30 individuals of a common non-declining species *Litoria fallax* at 10 ponds in the Hunter Region, 5 ponds where bell frogs persist and 5 where bell frogs have disappeared. At each of these ponds a range of habitat variables were also measured including pond area and distance to nearest ponds, average monthly water temperature, average monthly water quality, ephemerality and the frog, tadpole and fish density. A principle components regression was then used to identify correlations between infection severities and these environmental variables.

Relationships with three environmental variables were identified (Figure 9). Ponds with higher infection severities in their *L. fallax* population had higher fish densities (B=3.0; β=0.63; P=0.015) as well as lower proportion of drying and lower total dissolves solids (B=2.7; β=0.57; P=0.023). Given these correlations, the relationships between infection and each of these three variables were then investigated through a series of controlled laboratory based experiments.
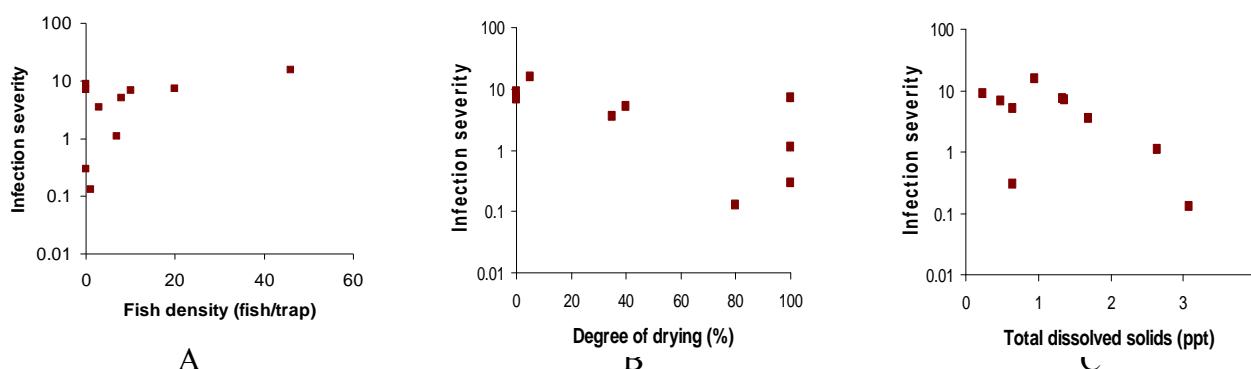
Figure Q9. Relationships between the infection severity (number of zoospore genomic equivalents) in *Litoria fallax* populations at 10 ponds in the Hunter Region and (A) the fish density in each pond, (B) the degree of drying of each pond and (C) the total dissolved solids in each pond.

The relationship between infection severity and fish density suggests that fish may be acting as a reservoir of the fungus. The outer epidermal layer of fish contains some keratin (Moyle and Cech, 2000) so an investigation into whether *B. dendrobatidis* could grow on the epidermis of fish was conducted. However, no attachment or growth was observed.

The relationship between infection severity and the degree to which a ponds dries suggests that drying may act to lower the pathogen load as *B. dendrobatidis* is sensitive to desiccation. To investigate this, an experiment was conducted where models of ponds (tubs containing soil substrate and water) were inoculated with *B. dendrobatidis* and then dried to various degrees by evaporation. The water level was reduced to either 100 % of the soil dry, 50 % of the soil dry and 0 % of the soil dry. These pond models were then refilled with water and a tadpole added. The tadpoles mouthparts were then swabbed several weeks later to determine whether the *B. dendrobatidis* had survived. The

results show that *B. dendrobatidis* had survived all drying events and remained capable of infecting tadpoles (Figure Q10) so the relationship between drying and infection severities could also not be confirmed.
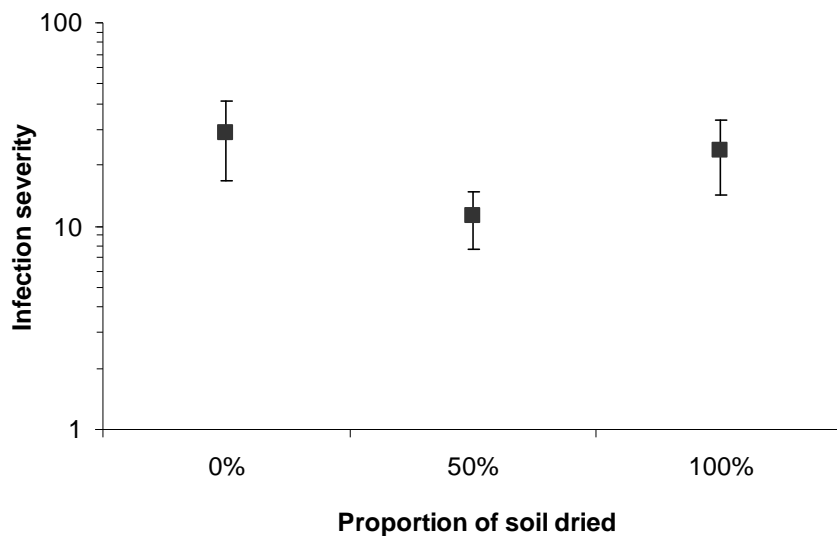
Figure Q10. The infection severity (number of zoospore genomic equivalents ± SE) in tadpole mouthparts after being placed in models of ponds that were dried to different degrees.

Finally, the relationship between infection severity and the total dissolved solids in the waterbody was investigated by growing *B. dendrobatidis* in TGhL culture media made with water from each of the 10 ponds. After 10 days the zoospore density was then determined microscopically. In addition to this culture experiment the concentration of a range of elements in each water sample was determined using inductively coupled plasma mass spectrometry. Relationships between zoospore density and elemental concentrations were then investigated using principle components regression. The strongest relationship found was with sodium (Na) (B=0.05, β=0.86, P=0.002) where higher Na concentrations correlated with lower zoospore densities (Figure Q11). It was also found that the ponds with the highest Na concentrations and the lowest *B. dendrobatidis* density were generally those where bell frogs persist (Figure Q11).
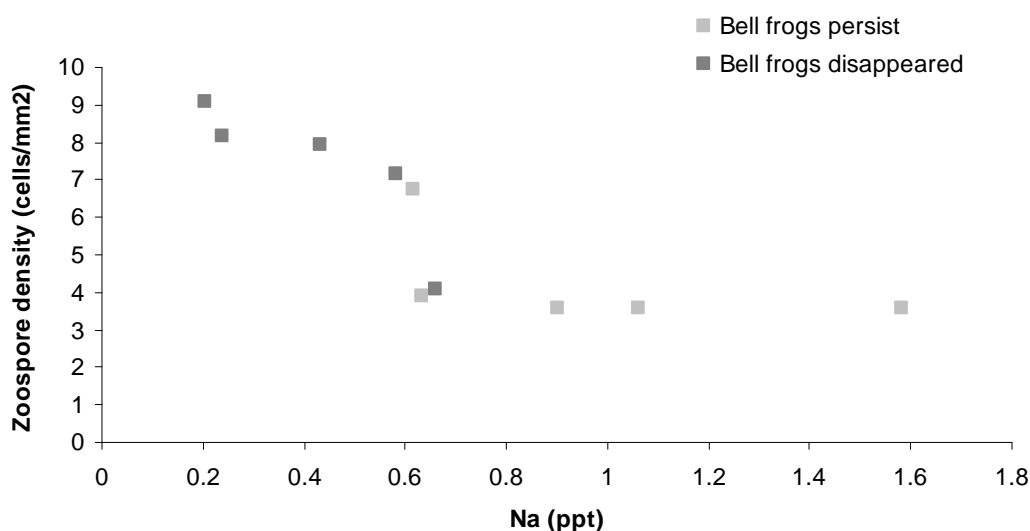
Figure Q11. The density of *Batrachochytrium dendrobatidis* zoospores when grown in culture media made with pond water with varying Na concentrations.

The relationship between Na and the growth of *B. dendrobatidis* was further investigated by repeating the previous culture experiment using culture media made with double distilled water with NaCl added ranging from 0 – 5 ppt. These NaCl concentrations are observed in natural *L. aurea* habitat. The results of this experiment confirmed that NaCl inhibits the growth of *B. dendrobatidis* (Figure Q12) with significantly less growth occurring at 1, 2 and 4 ppt ($\chi2 = 128.7$, df = 7, P < 0.001). These results support the hypothesis that an environmental inhibitor of *B. dendrobatidis* may be responsible for the coastal range contraction of the green and golden bell frog.
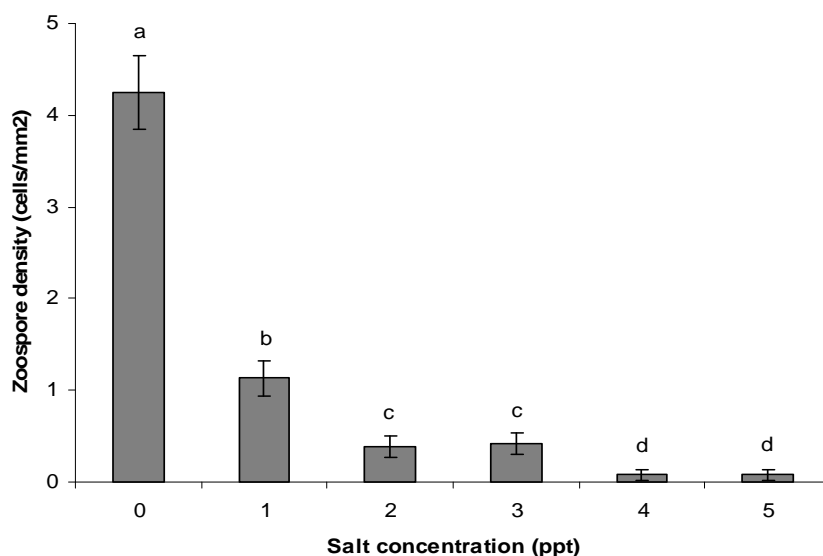
Figure Q12. The density of *Batrachochytrium dendrobatidis* zoospores when grown in culture media with NaCl added.

Question 5 - Does NaCl inhibit the infective capacity of *B. dendrobatidis* and reduce disease expression?

To determine whether the growth inhibition of *B. dendrobatidis* in NaCl translated into reduced infective capacity a number of artificial infection experiments were conducted. Tadpoles of two model species (*Limnodynastes peronii* and *Litoria peronii*) were housed in waterbodies that ranged in NaCl concentration from 0 – 5 ppt. These waterbodies were inoculated with *B. dendrobatidis* and infection levels in these animals determined by swabbing. In both species infection levels were found to be significantly lower in animals grown in 2 ppt and higher than those grown in 0 and 1 ppt NaCl. Controls were included and all remained negative for chytrid.

This same experiment was repeated with juvenile *Litoria peronii*, being housed in a tank with half water and half gravel substrate. The waterbodies ranged from 0-5 ppt and were inoculated with *B. dendrobatidis*. Infection levels in these juveniles were also found to be significantly lower in animals housed in 1 ppt or more than those in 0 ppt. The survival rates of animals housed in 3 and 4 ppt were

significantly higher than those in the other treatments. The survival rates of animals housed in 2 and 5 ppt were significantly higher than those in 0 and 1 ppt. Controls were included and all remained negative.

Although this study found a significant effect of NaCl on infection in tadpoles and juveniles, the survival results in the juvenile experiment were inconsistent with the infection results (ie – significantly lower infection levels in a treatment group did not necessarily correspond with higher survival rate). This is currently being investigated and is likely to be repeated. This study also needs to be repeated with the target species *L. aurea.*

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Appendix 2

Environmental Sampling for *Batrachochytrium dendrobatidis*

(Excerpt from Cashins, S. 2010. Epidemiology of Chytridiomycosis in Rainforest Stream Tadpoles. Ph.D. thesis, James Cook University, Townsville, Australia. 204 p)

Introduction

Although many free-living animal populations are regulated, in part, by disease (McCallum & Dobson 1995, Daszak et al. 2000, Dobson & Foufopoulos 2001), the ability of a pathogen, acting alone, to drive species extinction is very rare. Less than 4% of all known plant and animal extinctions since 1500 are linked, even in part, to disease (Smith et al. 2006). Apart from amphibians, the evidence for disease as the primary causative agent of extinction in the wild is suggestive, but not conclusive in only 3 cases; Hawaiian birds, Australian thylacine and the Christmas Island rat (Guiler 1961, Van Riper et al. 1986, Daszak & Cunningham 1999, Wyatt et al. 2008). In contrast, the recently emerged fungal pathogen of amphibians, *Batrachochytrium dendrobatidis* (*Bd*), is believed to be the primary cause of hundreds of rapid, enigmatic declines and extinctions around the world (Stuart et al. 2004, Bielby et al. 2008), with strong supportive evidence for the role of *Bd* in a few well-studied representative cases (Berger et al. 1998, Lips et al. 2006, Schloegel et al. 2006). This arguably makes *Bd* the most destructive infectious disease of biodiversity ever recorded (Skerratt et al. 2007, Mitchell et al. 2008) and raises the question of what separates this disease from others in history.

Theoretically, a pathogen is most likely to drive species extinction when the pre-epidemic host population is small, a reservoir host is present, or there is a long-lived propagule or saprobic life stage (Boots & Sasaki 2002, de Castro & Bolker 2005). Small populations are more vulnerable to stochastic events, and this vulnerability could be increased following a disease outbreak. Long-lived or saprobic life stages can maintain a positive force of infection on susceptible individuals, reducing the chance of pathogen "fadeout", even as the number of susceptible hosts decline (Anderson & May 1981, Swinton et al. 2002). In some amphibian assemblages, *Bd* tolerant adults and infected tadpoles may act as reservoir hosts while sympatric and more susceptible species decline to extinction. Less clear, however, is the role of long-lived or saprobic life stages of *Bd*. Here, I investigate the presence of *Bd* in the environment by sampling stream water and rock surfaces for *Bd* over time.

Aspects of *Bd*'s reproductive physiology support the hypothesis that it is an amphibian-specific pathogen (Berger et al. 2005). However, its ability to reproduce on a broad range of nutrient sources in-vitro (Piotrowski et al. 2004, James 2007) suggests it may be able to reproduce or survive for extended periods of time in the environment. Di Rosa et al. (2007) presented evidence of a form of *Bd* that was neither a zoospore nor a sporangia but that was positively identified as *Bd* by PCR and immuno-histology using a polyclonal antibody. This preliminary study, however, did not determine the organisms life stage (eg; resting stage, saprobic stage) or its ability to infect amphibians. However, these initial findings raise the possibility that *Bd* may persist in the environment for longer periods of time apart from amphibian hosts. Even in the absence of saprobic growth or a resting stage, prolonged survival of *Bd* zoospores in the environment could alter disease dynamics. For example, mathematical models predict the risk of extinction for *Bufo bufo* to be significantly greater the longer *Bd* can survive in the environment (Mitchell et al. 2008). The survival time of *Bd* within natural water bodies is currently unknown. However, in sterile moist river sand, zoospores survived without reproduction for up to twelve weeks (Johnson & Speare 2005). The survival time of *Bd* in nature is likely shorter and likely influenced by abiotic factors such as temperature and pH (Piotrowski et al. 2004) and biotic factors such as predation (eg: zooplankton; Kagami et al. 2004) and interactions with other microbiota (Chapter 7; Harris et al. 2006). Seasonal or spatial variation in survival of *Bd* persisting in the environment could contribute to the observed variation in prevalence among seasons and species (Chapter 6 and 7).

The primary obstacle in addressing these questions has been the absence of a reliable method to sample the environment for *Bd*. Dynamics of other Chytridiomycetes in water have been studied through microscopy of preserved water samples (Holfeld 1998, Kagami et al. 2007). However, I have found *Bd* difficult to count in field collected samples as it is grossly morphologically similar to other abundant organisms yet is apparently present at a much lower density. It is possible the samples may have been collected at times when environmental zoospore numbers were low. Other Chytridiomycetes are abundant for only a very short time of the year in response to blooms of their host plankton species (Ibelings et al. 2004). The recent development of a diagnostic quantitative PCR assay (Boyle et al. 2004) has created the opportunity to develop novel environmental sampling techniques (Kirshtein et al. 2007, Walker et al. 2007). In Chapter 3 I tested the efficiency of various filters in trapping and detecting *Bd* zoospores via PCR. Here, I incorporate these results into the development of a sampling device that is portable, can be operated in remote and wet conditions and filter reasonably large (>1L) volumes of stream water to increase the chances of detecting low zoospore densities in the environment. I then use this method to test water and rock surfaces at Tully Gorge NP (2006 – 2007), at the same time and transect as the tadpole surveys described in Chapters 6-7. Sampling the environment over time minimizes the possibility that a *Bd* "bloom" event goes unnoticed and sampling the environment and tadpoles at the same time permits comparison of prevalence and intensity in tadpoles with levels of *Bd* in the environment, allowing greater insight into *Bd* dynamics within the stream. For instance, large peaks in levels of environmental *Bd* preceding peaks in tadpole prevalence or intensity would suggest reproduction on an alternate host followed by transmission to tadpoles. Spatial variation in abundance of environmental *Bd* may also provide clues into factors limiting its distribution.

The inclusion of an internal positive control during initial analysis of environmental filter samples revealed widespread inhibition of the PCR reaction. Inhibition is most likely caused by dirt and detritus on the sample that prevent target DNA replication (Hyatt et al. 2007). The use of DNA soil extraction kits and simple dilution of extraction products are two methods commonly used to reduce the effect of inhibitors. To determine the most appropriate method to process inhibited filter samples I compared (in collaboration with the Australian Animal Health Laboratory; AAHL) the effect of a commercially available DNA soil extraction kit (Dneasy™) versus dilution of the extraction product on the sensitivity of the PCR reaction.

Methods

Effect of DNA Soil Extraction Kit and Dilution on Inhibition and Sensitivity of B. dendrobatidis PCR

Batrachochytrium dendrobatidis cultures were grown on 4 TGhL agar plates at 23ºC for 8 days. Plates were flooded with 10 ml dilute salts solution (DS; 10^{-3}M KH_2PO_4, 10^{-4} M $MgCl_2$ and 2 x 10^{-5} M $CaCl_2$)) for 10 minutes to harvest zoospores. The 4 solutions were then combined and centrifuged at 500 RCF at 15ºC for 5 minutes. Following centrifugation, the supernatant was removed and replaced with 2ml DS to rinse and concentrate the zoospores for counting. Four independent aliquots were counted using a haemocytometer. The zoospore solution was then serially diluted to produce solutions of 1,000; 10,000 and 100,000 zoospores/ml. 10 µl from these dilutions (for total zoospore counts of 10, 100 and 1,000) were then added directly to each of the following treatments, replicated 6 times; 1) Millipore Isopore 2µm filter membranes that had previously filtered ~1 L of ultrapure water, 2) Millipore Isopore 2µm filter membranes that had previously filtered ~500 ml of stream water collected from Tully Gorge NP (stream water was passed through the membrane until clogged), 3) Eppendorf tubes containing 30-40 mg zirconium/silica beads for DNA extraction. These tubes were immediately centrifuged and stored at -30ºC until extraction and PCR. Bd inoculated filter membranes were allowed to dry within a laminar flow cabinet and then placed whole within individual Eppendorf tubes with beads (identical to treatment 3 above) for subsequent DNA

extraction and PCR. Stream filtered membranes with no inoculated zoospores were included to control for zoospores potentially naturally present in the water.

Two replicates from each of the above treatments were then extracted with one of the following DNA extraction methods; 1) Standard extraction with Prepman Ultra, 2) Dneasy DNA soil extraction kit as per instructions, 3) Two filters from each "stream water" treatment were diluted a) 1/100 and b) 1/1000 following standard extraction in Prepman Ultra. Replicates for each treatment were kept low (n=2) due to cost considerations.

Field Sampling

The filtration apparatus was constructed from a 45 cm length of 25 mm PVC pipe, serving as a manifold. Into this, 5 valves were fit watertight. At the outflow of each valve, a reducer connected a modified 25 ml syringe with Luer-lock fitting. Five 25 mm Swinnex (Millipore) reusable plastic in-line filter casings containing 2 μm Millipore Isopore membranes (Chapter 3) were attached to each syringe via the Luer lock. A pressure gauge was fitted to the PVC manifold to indicate when to turn the pump off before internal pressure elevated to a level that would damage the filter membranes. A waterproof, battery-operated peristaltic pump delivered water from the stream to the filters. Peristaltic pumps can function against high pressures, thereby maximizing the volume of liquid filtered. In addition, the pumped liquid never touches internal machinery, but is instead pushed through a silicone tube by external rollers, making decontamination of the pump straightforward as tubing can be exchanged between sites. Each valve works independently so that individual membranes can be replaced in mid-filtration without disturbing the other filters and allowing flexibility in the number of filters run in one sample.

Water samples were filtered between September 7, 2006 and September 12, 2007 in Tully Gorge National Park from the same stream transect and at the same time that tadpoles were sampled. During each visit water was filtered from 16 different stream locations, with four replicates within the following 4 habitat types; 1) water column within pools, 2) water column within torrents, 3) rock surfaces within pools, 4) rock surfaces within torrents.

Water from the water column was sampled by gently dipping a 5 litre bucket into the surface of the water and filling it with approximately 4 litres of water. Water was then filtered directly from the bucket. For the rock surface sampling, enough rocks (each less than 15 cm long) to displace 1 litre of water were placed into a bucket containing 4 litres of stream water collected as described above. Rock surfaces were comprehensively scoured with a stiff brush to remove as much material from the rock surface as possible. The water within the bucket was then filtered. Before each sample, 5 litres of water from the water column of the habitat to be sampled were flushed through the system to remove any water from the previous sampling location. Between uses, brushes and filter casings were soaked in 12% NaOCl for one hour and thoroughly rinsed to eliminate possible cross contamination of samples (Cashins et al. 2008).

For PCR analysis, individual filters from each sample were extracted independently (Chapter 3, Boyle et al. 2004)) and diluted 1/1000 if inhibited. A Runs test (Gibbons & Chakraborti 2003) was used to detect lack of independence of *Bd* positive samples by sampling date. A lack of independence can be used to indicate seasonality (Levey 1988), in this case of *Bd* in the environment.

Results

Effect of DNA Soil Extraction Kit and Dilution on Inhibition and Sensitivity of B. dendrobatidis PCR

Due to the low number of replicates for each treatment (n=2) statistical analyses were not attempted. However, the results show very clear patterns (Fig. 8.1). In comparison to the standard extraction procedure using Prepman Ultra, use of the Dneasy soil extraction kit reduced the sensitivity of the PCR reaction, resulting in the detection of fewer zoospore equivalents within both the "pure culture" and "ultrapure water membrane" samples. Extraction from filter membranes using Dneasy appears to reduce PCR sensitivity further as compared to the pure culture controls (Fig. 8.1). The "stream water" membranes were completely inhibited at all zoospore densities when extracted with Prepman Ultra, however, the Dneasy extraction kit removed this inhibition (Fig 8.1). As was the case with the "pure culture" and "ultrapure water" membrane treatments, there was a decrease in sensitivity and fewer zoospores were detected than were present (Fig. 8.1). No positive samples were identified when the "stream water" extraction product was diluted 1/100 following extraction. However, a 1/1000 dilution sufficiently reduced inhibition allowing amplification of target DNA and successful identification of positive samples. Mean zoospore equivalents of the 1/1000 dilution treatments were greater than those estimated from the Dneasy treatments, however due to the high dilution factor, the accuracy of zoospore equivalent estimates is unreliable.
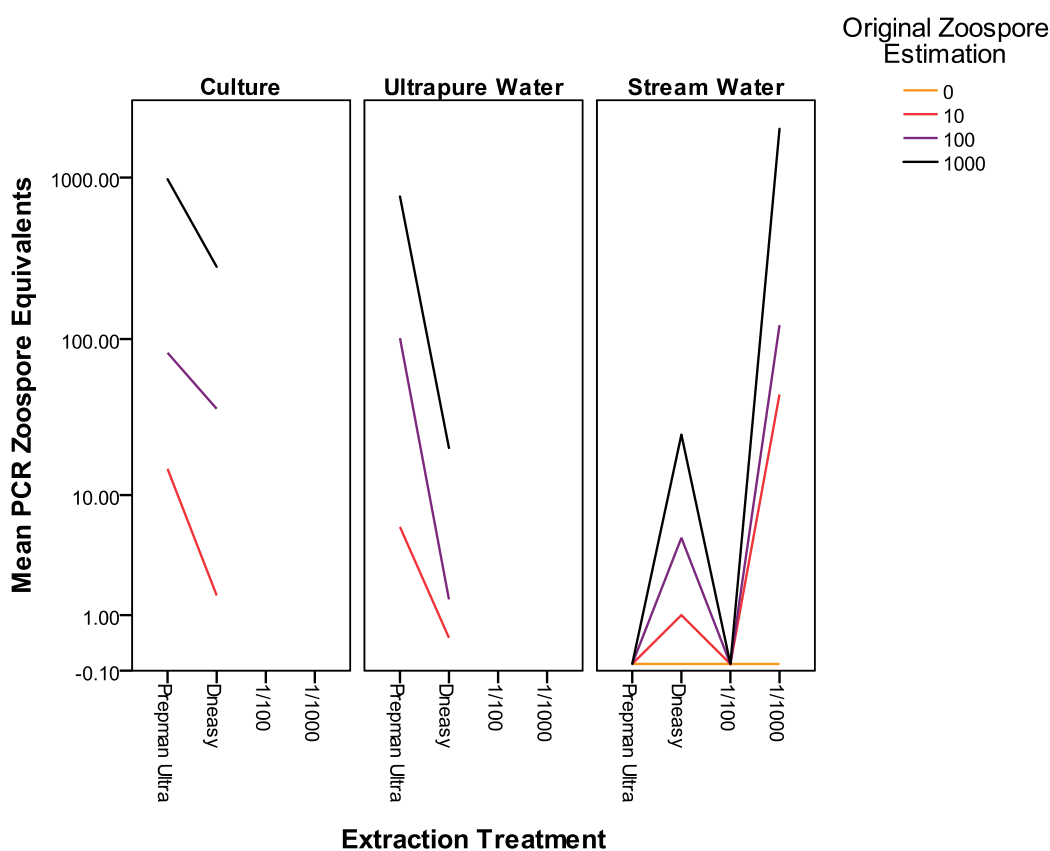
Figure 8.1. Comparison of mean *Bd* zoospore equivalents as determined by qPCR for each treatment. Each box is a separate treatment (pure culture control, 2 μm membranes that filtered ultrapure water and 2 μm membranes that filtered stream water. X axis represents the various extraction protocols trialed (standard Prepman ultra extraction, Dneasy soil extraction kit, 1/100 dilution, and 1/1000 dilution) and Y axis represent mean zoospore equivalents. Connecting lines are present to illustrate patterns more clearly.

Field Sampling

Initially, 53 samples with 2 replicate filters per sample from throughout the year were extracted in Prepman Ultra and diluted 1/100 prior to PCR. Twenty three of these samples had no inhibition in either of the 2 replicate filters, 10 samples had 1 of 2 filters with inhibition and 20 samples had inhibition in both filters (Table 8.1). Overall, 47% (50/106) of filters were inhibited. Inhibition was

present in all habitats sampled (Table 8.1), however water column samples were more frequently inhibited (≥1 inhibited filter) compared to rock surface samples in both pools and torrents.

Table 8.1. Inhibtion of environmental samples at Tully Gorge NP 2006-2007. Two replicate filters from each sample were run individually. Percentages in parentheses are read horizontally.

# Filters Inhibited		0/2	1/2	2/2
Pool	**Water Column**	**3** (23%)	**4** (31%)	**6** (46%)
	Rock Surface	**7** (58%)	**0** (0%)	**5** (42%)
Torrent	**Water Column**	**2** (14%)	**4** (29%)	**8** (57%)
	Rock Surface	**11** (79%)	**2** (14%)	**1** (7%)
Totals		23	10	20

Given the previous results indicating 1/1000 dilution was effective at reducing inhibition (Fig. 8.1), new replicate filters from many of the inhibited samples were extracted and diluted 1/1000 to generate interpretable results. All additional samples were also processed in this way. In total 106 filters from 64 samples were processed that did not contain inhibitors. From these, 4 were positive for *Bd*. Positive samples were detected between July 4, 2007 and August 2, 2007 (Table 8.2). All positive reactions revealed low zoospore equivalents. In 3 of 4 *Bd* positive samples more than one replicate filter was tested, however in each case only 1 filter returned a positive reaction. Three of 4 positive samples showed a positive reaction in 1 out of 3 replicate PCR wells and the fourth *Bd* positive sample returned a positive reaction in all three wells. Two *Bd* positive samples were from rock surface samples in torrents, another *Bd* positive sample was from a rock surface sample in a pool and the final *Bd* positive was from a water column sample in a torrent.

The cluster of 3 consecutive sampling periods returning at least 1 positive sample (data vector: 00000011100) is suggestive of seasonality of environmental levels of *Bd*, however the result is not significant (Runs test, P = 0.124).

Table 8.2 Dates and sampling locations for non-inhibited environmental samples at Tully Gorge NP, 2006-2007.

Date	Sampling Location		Bd+ Filters/ Total	Zoospore Equivalents
7-Sep-06	Pool	Water Column		

		Rock Surface		
	Torrent	Water Column	0/2	
		Rock Surface	0/2	
			0/2	
21-Apr-07	Pool	Water Column		
		Rock Surface		
	Torrent	Water Column	0/2	
		Rock Surface	0/2	
7-May-07	Pool	Water Column	0/1	
		Rock Surface	0/2	
	Torrent	Water Column	0/1	
		Rock Surface	0/2	
18-May-07	Pool	Water Column	0/2	
		Rock Surface	0/3	
			0/2	
	Torrent	Water Column	0/2	
		Rock Surface	0/2	
			0/2	
5-Jun-07	Pool	Water Column	0/2	
			0/2	
		Rock Surface	0/2	
			0/2	
	Torrent	Water Column	0/2	
			0/2	
		Rock Surface	0/2	
			0/2	
21-Jun-07	Pool	Water Column	0/1	
			0/1	
		Rock Surface	0/1	
			0/2	
	Torrent	Water Column	0/1	
			0/1	
		Rock Surface	0/1	
			0/2	
4-Jul-07	Pool	Water Column	0/1	
			0/1	
		Rock Surface	0/1	
			0/2	
	Torrent	Water Column	**1/2**	.5*
			0/1	
		Rock Surface	0/2	
			0/2	
19-Jul-07	Pool	Water Column	0/2	
			0/2	
		Rock Surface	**1/1**	<100*
			0/1	
	Torrent	Water Column	0/2	
			0/1	
		Rock Surface	**1/3**	3***
			0/2	
2-Aug-07	Pool	Water Column	0/2	
			0/2	
		Rock Surface	0/2	
			0/1	
	Torrent	Water Column	0/2	
			0/1	
		Rock Surface	**1/3**	.8*

			0/2	
15-Aug-07	Pool	Water Column	0/1	
			0/1	
		Rock Surface	0/1	
			0/1	
	Torrent	Water Column	0/2	
		Rock Surface	0/1	
12-Sep-07	Pool	Water Column		
		Rock Surface		
	Torrent	Water Column	0/1	
			0/1	
		Rock Surface	0/1	
Total			**4/106**	**(3.7%)**

* = number of positive wells in PCR reaction

Discussion

In Chapter 3, I determined that 2 µm Millipore Isopore filter membranes were an efficient option for extracting *Bd* DNA. Here I described the construction of a simple, portable and robust filtration apparatus, using these filters, for field sampling of *Bd*. I demonstrate that both a DNA soil extraction kit (Dneasy) and 1/1000 dilution of the extraction product are suitable to remove inhibitors from filter membranes clogged with organic stream material (Fig. 8.1). Reducing inhibition enabled the qPCR assay to positively identify *Bd* inoculated samples containing as few as 10 zoospores. These combined techniques were then used to sample the water column and rock surfaces for *Bd* in a stream environment for the first time. From this sampling, four *Bd* positive samples were found over a one year period (Table 8.2).

All *Bd* positive samples were collected in mid-winter over three sampling events within a four-week period between July 4 and August 2. The temporal clustering of positive samples is suggestive of seasonality, however it is not significantly different from random. This time period, however, coincides with the observed maxima of *Bd* prevalence in torrent-adapted tadpoles (Chapter 6). Prevalence of infection in these tadpoles increased steadily over the months leading up to this time period suggesting that elevated levels of *Bd* in the environment were the result of, and not the cause of highly and heavily infected tadpoles.

The number of *Bd* zoospore equivalents recovered from the water and rock surfaces was very low (Table 8.2), however these low levels may in fact, be biologically significant. Each water sample was only a small percentage of the total volume of water passing through the high energy stream and each sample of rocks represented only a small fraction of the total surface area of the complex and rocky stream bed. Therefore, the presence of 1-10 zoospores within 1000 ml of stream water or on the surface of 4-7 small (<15 cm) rocks quickly expands to a much larger cumulative exposure risk to the amphibian population.

Two of the positive filters were from rock surface samples within torrents, one was from a rock surface sample within a pool and another was from a water column sample within a torrent. Because rock surfaces were scrubbed within water collected from the water column it is possible the detected *Bd* zoospores were from the water column. However, the increased volume of organic material removed from the scrubbed rocks resulted in a faster filter clogging time and a lower volume of water filtered. Therefore, the density of zoospore equivalents from rock surface samples is more than double that of the water column samples.

The apparent presence of *Bd* on rock surfaces may represent important avenues for disease transmission. Torrent tadpoles (*Litoria nannotis, L. rheocola, and Nyctimystes dayi*) are grazers that disperse readily (*L. nannotis*; Chapter 5) and are in near constant contact with rock surfaces in search of food (Chapter 5). As a result, the mouthparts (where infection occurs) of torrent tadpoles are likely to come into contact with a very large total substrate surface area over the course of development. The observed cumulative increase in infection risk with size class (Chapter 6) is consistent with transmission via a prevalent, but widely dispersed pathogen. Tadpoles with a shorter larval duration would thus be predicted to have a lower cumulative risk of infection and exhibit a lower prevalence of infection prior to metamorphosis. *Litoria genimaculata* tadpoles have a shorter developmental time than the torrent tadpoles (Chapter 5), and have a significantly lower overall prevalence (Chapter 6). Similar to the torrent tadpoles, risk of infection increased with body size for *L. genimaculata* indicating exposure risk increased with time in water. Host behaviour and habitat may also play a role in the observed lower prevalence as discussed in Chapter 6.

The detection of low levels of *Bd* in the environment occurred only at the end of a steady increase up to 100% prevalence in torrent tadpoles (Chapter 6) suggesting the detected environmental zoospores were shed from infected amphibian hosts. This, combined with the evidence that seasonal patterns in torrent tadpole prevalence are best explained by time of exposure combined with metamorphosis and recruitment (Chapter 6) strongly suggests transmission and seasonal infection dynamics within tadpoles are primarily tadpole driven. I gathered no data to suggest the presence of a widespread saprobic life stage or a "*Bd* bloom" event. The qPCR assay used here does not differentiate between living and dead zoospores and I did not seek to estimate zoospore survival time in vivo. However, the low levels of *Bd* observed even with concurrent high prevalence and intensity of infection in tadpoles suggests that *Bd* does not survive long enough to accumulate and become ubiquitous in the environment. This may be in part due to dynamics of the stream environment. Levels of *Bd* found in ponds of the Sierra de Guadarrama mountain range in Spain ($0.5 - 262$ zoospores l^{-1}) and ponds of Montana and Colorado, USA ($19 - 454$ zoospores l^{-1}) were higher than the zoospore levels I detected here (Kirshtein et al. 2007, Walker et al. 2007). The absence of continuous water turnover in ponds prevents flushing of zoospores and likely allows higher densities of *Bd* to accumulate in the environment. In Spain, unusually low water levels were speculated to have further increased density of *Bd* (Walker et al. 2007). Assuming detected zoospores are in fact viable, the elevated density of zoospores within ponds may increase the risk of transmission via water contact in these habitats.

The ability of *Bd* to survive for long periods of time or to grow saprobically in the environment has the potential to increase the risk of species extinction by providing a positive force of infection on susceptible individuals even as amphibian hosts decline (Mitchell et al. 2008). This study, however, provided no evidence to support the possibility of either a saprobic life stage or a long-lived life stage. Although transmission among tadpoles is likely due to released zoospores on shared substrate surfaces, the lifespan of the zoospores appears short-lived. Study of *Bd* survival away from amphibian hosts will make important contributions to further understanding transmission in streams.

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