Epidemiology of Chytridiomycosis in Rainforest Stream Tadpoles

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STATEMENT ON THE CONTRIBUTION OF OTHERS

This thesis was co-supervised by Prof. Ross Alford and Dr. Lee Skerratt, but received valuable input from a number of other people. Ross Alford and Lee Skerratt contributed in the form of ideas, experimental design, editorial assistance, statistical advice and provided the majority of funding. Dr. Bryan Windmiller, Robert Puschendorf, Jamie Voyles and anonymous reviewers provided useful advice and suggestions on individual chapters. Dr. Stephen Garland and Ruth Campbell at the School of Public Health and Tropical Medicine, JCU and Veronica Olsen and Dr. Alex Hyatt at the Australian Animal Health Laboratory, CSIRO performed most of the diagnostic PCR tests for *Batrachochytrium dendrobatidis* and contributed experimental design and sampling advice. Bryan Windmiller provided valuable advice on the design of field work and together with Hayley Ricardo and 15 volunteers (listed by name in the acknowledgements) provided logistical and field assistance throughout this project.

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ABSTRACT

Amphibians are declining at an alarming rate and approximately one third of species are currently threatened with extinction. A primary cause of this decline has been the emergence of the disease chytridiomycosis caused by the pathogen, *Batrachochytrium dendrobatidis* (*Bd*). Historically, the extinction of free-living species due to disease is exceedingly rare; however, dozens of amphibians in recent years are feared gone due to *Bd*. For disease to drive extinction, theory indicates a reservoir host is needed to maintain a positive force of infection on susceptible individuals to prevent pathogen "fade out" as the doomed species decline. Accordingly, understanding pathogen dynamics (e.g. prevalence, intensity, transmission, seasonality) within reservoir hosts is critical to properly understand and mitigate species declines and prevent extinction. In the case of chytridiomycosis, no non-amphibian hosts have been found, however, less susceptible adults and amphibian larvae can serve as reservoirs.

While most research has focused on infection in adults, tadpoles probably are important reservoirs; they carry the pathogen and are thought to suffer few negative effects, and most species that have declined are associated with aquatic habitats. To better understand the role tadpoles play in pathogen dynamics I investigated the epidemiology of *Bd* in a tadpole assemblage (consisting of five species) within two rainforest streams over two years. I studied changes in prevalence and intensity of infection over time and how their values were affected by abiotic factors such as temperature and water flow rate, as well as by biotic factors such as the ecology, behaviour and developmental rate of each species. In species with a high prevalence of infection, I studied the response of tadpoles to infection and the effects these responses had on the infection and on their physical condition. A saprobic or long-lived life stage of *Bd* could significantly alter pathogen dynamics among hosts. To investigate this possibility, I developed a method to detect *Bd* in the environment and I used this to sample the stream over the course of one year.

I found significant species-specific variation in space and resource use within the tadpole assemblage; these differences appear to affect susceptibility to infection. Torrent-adapted tadpoles were significantly more likely to be infected than pool-adapted

tadpoles. This is likely due to differences in rates of development that affect duration of exposure to *Bd* and differences in behaviour that affect pathogen transmission. Prevalence of infection in torrent tadpoles increased with body size (proxy for duration of exposure) indicating that transmission occurred throughout the year. Prevalence varied seasonally between ~ 25-100% and was driven by a combination of duration of exposure, recruitment of small tadpoles and metamorphosis of large tadpoles. Drivers of infection intensity are less clear, however, body size and water flow are important and in fast-flowing habitats repeat transmission from the external environment appears to be more important than self-reinfection in determining individual infection intensities.

After infection most torrent tadpoles suffered significant tooth loss. This loss severely decreased their ability to feed (in some cases causing apparent starvation), which led to significant decreases in body condition for many individuals. Most tadpoles, however, regrew mouthparts despite continued infection, resumed feeding, and metamorphosed. The relationships between infection intensity, prevalence, tooth loss and body condition indicate that these tadpoles have a measure of tolerance or increased resistance, which may be a result of strong selection pressure exerted by chytridiomycosis.

Environmental sampling for *Bd* revealed that environmental levels are low throughout the year, but may increase when prevalence in tadpoles is highest, suggesting that tadpoles are the major source of *Bd* zoospores in the environment.

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Chapter 1 : General Introduction

Global biodiversity is in decline and it is widely believed that that we are in the midst of the sixth great mass extinction event (Wake and Vredenburg, 2008). This recent extinction event is thought to be human driven, with the overall extinction rate estimated to exceed pre-human levels by 100-1,000 times (Pimm et al., 1995). Within this trend of decreasing biodiversity, amphibians are disproportionately represented. At least one third of the 6,300 described amphibian species are threatened with extinction (Sodhi et al., 2008, Stuart et al., 2004). The negative impact of humans on amphibians is evident. Threats such as over-exploitation for food, introduced predators and climate change currently impact a range of species; however the primary causes of global amphibian declines and disappearances appear to be habitat destruction and susceptibility to the pathogen *Batrachochytrium dendrobatidis* (hereafter referred to as Bd; Bielby et al., 2008, Alford and Richards, 1999, Berger et al., 1998, Collins and Storfer, 2003, Lips et al., 2006, Skerratt et al., 2007, Sodhi et al., 2008, Stuart et al., 2004).

The negative effects of habitat destruction are predictable and evident. Species, particularly those with restricted distributions, cannot survive without suitable habitat intact (Blaustein et al., 1994, Stuart et al., 2004). The response of conservationists to this threat is equally straightforward; critical habitat must be protected for species survival. The negative effects of a pathogen such as *Bd*, however, are far less intuitive. Very rarely has a pathogen of any taxon been known to cause widespread declines, let alone extinctions. Avian malaria and rinderpest are examples of multihost pathogens negatively impacting populations on a broad scale (Van Riper et al., 1986, Kock et al., 1999); however, a pathogen that causes multiple extinctions in otherwise healthy, free-living populations is almost unprecedented. To mitigate this threat to amphibian biodiversity it is important to understand how disease-driven declines occur. To contribute to this objective, I investigated a currently under-studied, but important

aspect of this multi-host disease: the epidemiology of *Batrachochytrium dendrobatidis* in tadpole hosts.

When amphibian populations were disappearing across eastern Queensland in the 1990's some researchers suspected an infectious agent because many of the declines were occurring in otherwise undisturbed, unpolluted and protected habitat, and there was a seemingly temporal progression of declines across the landscape, suggestive of spread (Laurance et al., 1996). In 1997, about 10 years after the collective realization that amphibians were in decline and approximately 20 yrs after the time when the declines had actually begun, the infectious agent in question (*Bd*) was discovered and described from sick and dying frogs (Berger et al., 1998, Longcore et al., 1999).

Batrachochytrium dendrobatidis is an aquatic fungus that infects and produces propagules within the keratinized epidermal cells of postmetamorphic amphibians and the mouthparts of tadpoles (Berger et al., 2005a, Longcore et al., 1999). The fungus has a motile, uniflagellate infectious zoospore (2-4 µm diameter) that enters the target host cell before asexually reproducing through the formation of a zoosporangium (10-40 µm diameter; Longcore et al., 1999, James et al., 2006a, James et al., 2006b). Zoospores are released via a discharge tube into the external aquatic environment for transmission to sympatric hosts or to adjacent epidermal cells, which leads to host reinfection and possible increases in pathogen load (Berger et al., 2005a, Longcore et al., 1999). Once a threshold of infection intensity is crossed within an individual (Carey et al., 2006), electrolyte levels that are crucial to muscle function become disrupted, leading to cardiac standstill and death (Voyles et al., 2007, Voyles et al., 2009b).

Epidemiological and genetic evidence indicate that it is likely that *Bd* recently emerged from a single location where it existed as an endemic pathogen, and radiated across the globe causing declines and extinctions in naïve and susceptible populations (James et al., 2009, Rohr et al., 2008, Skerratt et al., 2007). Where the first appearance of *Bd* at a site has been documented, prevalence of infection increased rapidly as the density of frogs drastically declined due to disease (Brem and Lips, 2008, Lips et al., 2006).

Currently, *Bd* is linked to the decline of hundreds of species (Skerratt et al., 2007, Stuart et al., 2004) and the extinction of dozens; including approximately 30 species of *Atelopus* (including the Panamanian golden frog; La Marca et al., 2005), the gastric brooding frogs of Australia (McDonald and Alford, 1999) and the sharp-snouted day frog of Australia (McDonald and Alford, 1999, Schloegel et al., 2006).

Simple theories of host-pathogen interactions suggest that disease is unlikely to cause extinction because the pathogen should fade out as host density declines below the minimum threshold needed to maintain a positive force of infection (Anderson and May, 1981, Swinton et al., 2002). However, the availability of multiple hosts is predicted to decrease the likelihood of pathogen "fade out" within a population (Power and Flecker, 2008). *Batrachochytrium dendrobatidis*, like many pathogens, can infect multiple host species. In fact, *Bd* is likely able to infect nearly every amphibian species, as well as their larvae, if environmental conditions are suitable (Berger et al., in press); however, the susceptibility of adults to chytridiomycosis varies widely among species, and most larvae do not die of infection (Woodhams and Alford, 2005). This diversity in hosts and their susceptibilities combined with the unusually high virulence of *Bd* in some species may be what allows disease-driven amphibian declines to occur. Theoretically, the less susceptible hosts and life stages can maintain infection as the more susceptible species decline, sometimes to extinction (Woolhouse et al., 2001). The infection dynamics in these less susceptible reservoir hosts thus become critically important in understanding disease dynamics and decline.

Tadpoles, in particular, are a potentially very effective reservoir host as they are often abundant in nature, they develop within water where *Bd* is readily transmitted (Rachowicz and Vredenburg, 2004), and (in permanent water bodies) they are often present throughout the year. In fact, tadpoles have been linked to species decline in a variety of ways. Species with tadpoles, particularly stream dwellers, are at greater risk of decline (Bielby et al., 2008, Todd, 2007, Hero et al., 2005, Lips et al., 2003, Sodhi et al., 2008, Williams and Hero, 1998). Mathematical models predict that increased densities of infected tadpoles will increase the risk of species extinction (Mitchell et al.,

2008, Briggs et al., 2005) and the only amphibians to suffer declines in Peñalara National Park in Spain were noted to be the only species with a long-lived aquatic larval stage (Bosch and Martinez-Solano, 2006, Bosch et al., 2001). Frogs appear most susceptible to mortality immediately following metamorphosis, therefore maintenance of infection through metamorphosis or transmission from less-developed conspecific tadpoles may have critical effects on species survival and recruitment (Rachowicz and Vredenburg, 2004, Carey et al., 1999, Berger et al., 1998). Infection dynamics in tadpoles, however, have received minimal research attention.

The general aim of this study was to study the epidemiology of *Bd* in an assemblage of rainforest stream tadpoles to better understand infection dynamics within and among species over time and to better understand the significance of larvae in species declines and extinctions. To achieve this I tracked dynamics of *Bd* and the response to infection in tadpoles for one year at one rainforest stream site, and for two years at a second stream site, in north Queensland. The assemblage studied consisted of three species with tadpoles adapted to fast-flowing water (*Litoria nannotis*, *L. rheocola* and *Nyctimystes dayi*), one species with tadpoles found in isolated stream-side pools (*L. xanthomera*) and one species with tadpoles found in stream-connected pools (*L. genimaculata*). With the exception of *L. xanthomera*, all of these species declined throughout their range beginning in the late 1980's to early 1990's (McDonald and Alford, 1999). *Litoria nannotis*, *L. rheocola* and *N. dayi* disappeared above 400 meters, but persisted below this elevation. All are currently listed as endangered (IUCN). *Litoria genimaculata* declined at the time of the other species, but has rebounded throughout its range and is currently listed as of least concern (IUCN). Another species, *Taudactylus acutirostris*, has not been seen since 1994 and is currently listed as critically endangered (Schloegel et al., 2006; IUCN). Specifically, my aims were to:

- Describe how tadpoles differ among species in behaviour, space, time, resource use and developmental patterns
- Determine how these differences affect risk of infection

- Determine the other environmental and biological factors that drive risk and intensity of *Bd* infection in tadpoles over time
- Describe the pathophysiological effects of and responses to infection in susceptible tadpoles of each species

The presence of a saprobic or long-lived life stage in the environment would significantly alter transmission dynamics among larvae and adults (Mitchell et al., 2008). Therefore, in order to examine this potential effect I aimed to:

- Develop techniques to sample *Bd* in the environment using PCR
- Describe seasonal and spatial variation in the number of *Bd* zoospores in the stream environment

This thesis is written as a series of stand alone, but connected manuscripts or chapters. Chapters two, three and four are published or submitted manuscripts describing various methods developed. Chapter two investigates the ability of sodium hypochlorite (NaOCl) to denature *Bd* DNA so that it is unrecognizable by PCR. This is important to allow decontamination of equipment, particularly during filtration, without the possibility of cross-contamination of samples with *Bd*. Chapter three compares the sensitivity of various methods of *Bd* DNA collection in order to determine the best filter membrane to use during environmental sampling and to compare this sensitivity to that of standard swabs. Chapter four investigates the lethal effects of disposable gloves on tadpoles. At the beginning of this study it was discovered that some gloves were apparently lethal. This investigation was undertaken to determine gloves that were safe to carry out the study. Chapter five describes the field sites, the general methods used, and aspects of the ecology, behaviour and development of the tadpole assemblage studied. Chapter six examines the differences among species in the intensity and prevalence of *Bd* over time and investigates the biotic and abiotic drivers of species and seasonal variation, taking into account the results of chapter five. Chapter seven investigates the pathophysiological effects of infection, particularly mouthpart loss on

tadpoles of *L. nannotis and L. rheocola*, and how these changes can lend insight into transmission of *Bd* within streams. Chapter eight describes the sampling of the environment for *Bd* across habitat types within a stream over a one year period. Chapter nine summarizes my findings and discusses their conservation implications.

Chapter 2 : Sodium hypochlorite denatures the DNA of the amphibian chytrid fungus *Batrachochytrium dendrobatidis*[*]

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

Abstract

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

Introduction

Chytridiomycosis, caused by the highly virulent fungus *Batrachochytrium dendrobatidis* (*Bd*), has caused declines of natural amphibian populations and deaths in captive populations around the world (Berger et al., 1998, Lips et al., 2006, Rachowicz

et al., 2006, Pessier et al., 1999, Skerratt et al., 2007). *Bd* is a highly transmissible pathogen requiring proper hygiene protocols, including disinfection of equipment, to prevent its spread (Speare et al., 2004). Many disinfectants are very effective at killing *Bd* on a range of substrates (Johnson et al., 2003, Webb et al., 2007). However, for researchers collecting and processing samples for diagnostic PCR, killing *Bd* is not enough; it is necessary to destroy its DNA to prevent cross contamination of samples that may lead to false positives.

DNA-based PCR tests are now commonly used diagnostic tools to detect *Bd* due to their sensitivity and specificity (Boyle et al., 2004, Hyatt et al., 2007, Annis et al., 2004). When using diagnostic PCR it is important to prevent contamination of samples. Even very low levels of DNA contamination on sampling equipment can create false positives. Recent papers have raised concerns regarding the generation of false positives via shared equipment (Woodhams et al., 2007, Kirshtein et al., 2007). While it is now standard practice to disinfect field equipment with bleach, > 70% ethanol or other treatments to prevent the transfer of live *Bd* between individual amphibians and sites (Johnson et al., 2003, Speare et al., 2004, Webb et al., 2007), these protocols may not denature *Bd* DNA. This would make the results of diagnostic PCR unreliable. Dipping instruments in alcohol and burning off the residue is an effective sterilisation technique and may render *Bd* DNA undetectable by PCR; however, this has not yet been tested and flaming is an impractical solution for larger field tools such as collection trays and animal enclosures and equipment that would be destroyed by flames such as fabrics and plastics.

The antimicrobial properties of NaOCl are well known and it is widely used as a disinfectant in medicine (Eventov et al., 1998), endodontics (Gomes et al., 2001), water treatment, and around the home. Archaeologists and forensic scientists have discovered its usefulness for destroying contaminating DNA prior to PCR amplification of target DNA from teeth and bones (Kemp and Smith, 2005). NaOCl has also been used to denature pathogens and allergens. Sehulster et al. (1981) found that NaOCl eliminates Hepatitis B antigenicity, Matsui et al. (2003) reduced the immunogenicity of a cat

allergen, and Martyny et al. (2005) denatured the fungal allergen *Aspergillus fumigatus* on environmental substrates, significantly reducing its recognition by ELISA. We experimentally tested three concentrations of NaOCl over four exposure periods to determine its effectiveness at denaturing *Batrachochytrium dendrobatidis* DNA as determined through a real-time PCR assay.

Materials and Methods

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

After 3 d, microscopic examination confirmed that there was considerable growth in each well, with mature zoosporangia and active zoospores. We removed the broth from each well and replaced it with 200 µl of one of three NaOCl concentrations; 1%, 6%, 12%, or a control (TGHL). Each NaOCl dilution at each exposure period had seven replicates and the control group had ten replicates.

In order to prevent *Bd* in the control group from continuing reproduction, controls were processed immediately after addition of the TGHL. The 200 µl of TGHL was removed and 200 µl of a dilute saline solution added (DS;Boyle et al., 2003). The bottom and sides of each control well were scraped 16 times, and the corner where the sides meet the bottom of the well was scraped four times with a sterile wooden dowel. The 200 µl aliquot of DS was then removed and placed in a 1.5 ml Micro tube (Sarstedt Inc. USA). A second 200 µl aliquot of DS was then added to the well and the process was repeated. All samples were immediately centrifuged at 16,100 g for 3 m to form a pellet of *Bd*. Most of the supernatant (280 µl) was removed and discarded and the tube placed in a -60 °C freezer.

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

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

significantly from controls. All statistical analyses were performed using Statistica 7.1 (StatSoft Inc.).

Results

The 1% NaOCl solution did not differ significantly in effectiveness across the four exposure periods (ANOVA, $F = 0.777$, $df = 3, 27$ $p = 0.519$), and comparison with the control treatment indicated that it did not denature *Batrachochytrium dendrobatidis* DNA effectively enough over any of the four exposure periods for use as a denaturing agent ($t = -0.340$, $df = 36$, $p = 0.736$; Fig. 2.1). Only very low numbers of zoospores were detected in the 6% NaOCl treatment (Fig. 2.1). These did not differ among exposure period treatments (ANOVA, $F = 0.531$, $df = 3, 27$ $p = 0.665$), and were significantly reduced compared to controls ($t = 3.088$, $df = 36$, $p = 0.004$). The 12% NaOCl treatment was 100% effective and denatured all *Bd* DNA across all exposure periods (Fig. 2.1). No inhibition from the 12% NaOCl was detected with the internal positive controls (mean Ct value 12% NaOCl = 28.12, mean Ct value control = 28.61, $t = -0.985$, $df = 6$, $p = 0.363$). Visual inspection of each well prior to the removal of NaOCl revealed that zoosporangia and zoospores from the 6% and 12% NaOCl treatments were severely fragmented and completely unrecognisable. The 1% NaOCl treatment wells contained shrivelled and shrunken but recognizable zoospores and zoosporangia.

Discussion

We found that 12% NaOCl can be effectively used to denature 100% of even very high densities of *Bd* within 1 hr. Similarly, 6% NaOCl was extremely effective, with only 6-13 zoospore equivalents detected across all exposure periods (Fig.1). This represents a mean reduction from the controls of 99.999%. As the PCR assay can detect as little as one zoospore (Boyle et al., 2004), a minimum of approximately 100,000 contaminant zoospores would need on average to be present for a false positive result following treatment with 6% NaOCl. In practice, the number of *Bd* zoospores contaminating equipment is likely to be a fraction of the levels examined in this study. For example, the highest level of *Bd* reported on a swab sample from an infected animal is 545,000

zoospores on a *Mixophyes fasciolatus* (Hyatt et al., 2007). Any contamination of equipment is likely to be at levels far below those of severely infected animals. It is possible then,

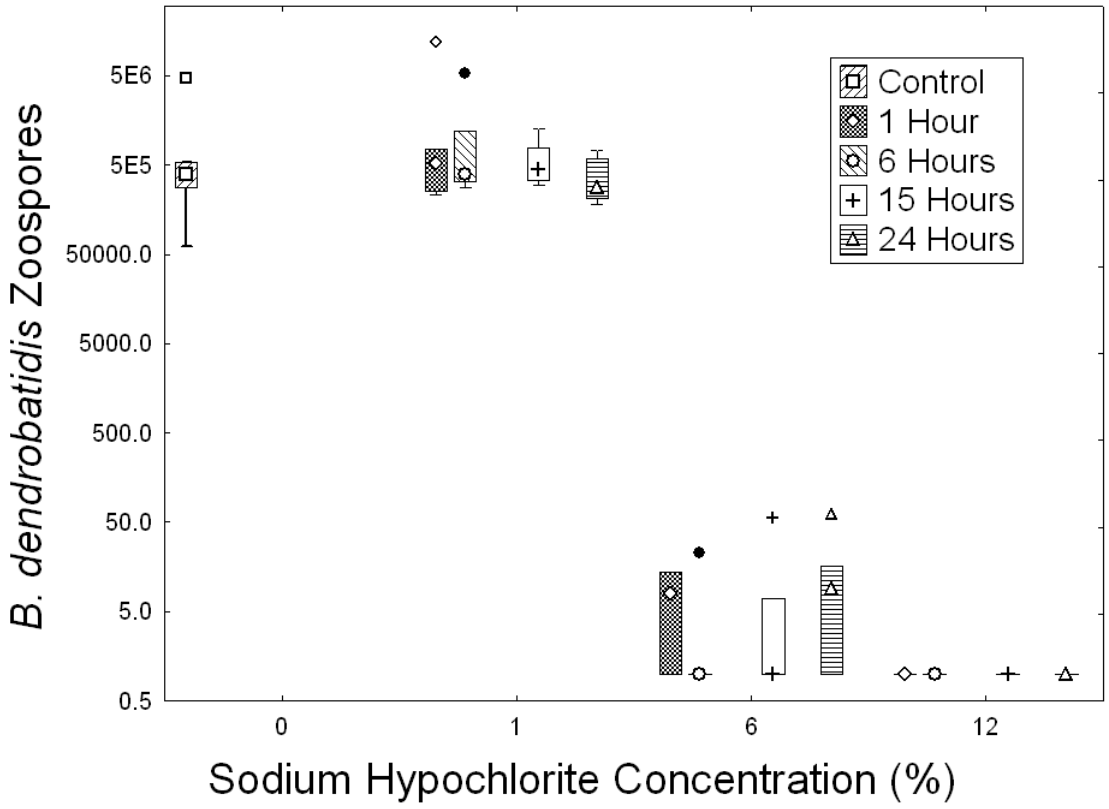


Figure 2.1. Numbers of Batrachochytryium dendrobatidis zoospores detected in controls and at three concentrations of sodium hypochlorite after varying periods of exposure. Symbols within boxes represent the median, boxes represent the upper and lower quartiles, whiskers represent the non-outlier spread and symbols above boxes represent the outliers.

that a 6% NaOCl solution can be used to denature DNA on clean equipment sufficiently to prevent false positives through PCR; however, as field equipment often contains soil, plant material and other particulates which may either shield *Bd* or decrease the efficiency of NaOCl (LeChevallier et al., 1988) it is far safer and advisable to use 12% NaOCl to prevent cross contamination. Prior to soaking in NaOCl equipment should be cleaned to reduce the negative impact of any attached particles. The 1% NaOCl solution did not effectively denature *Bd* zoospores at any of the exposure periods and should not be used to prevent sample contamination.

Most household bleach products contain between 4.00-6.15 % NaOCl and the most common concentration of commercially available bulk NaOCl is 12%, making the concentrations tested here readily available to most researchers. Exactly how NaOCl deactivates micro-organisms has never been experimentally shown (Gomes et al., 2001),however(Gomes et al., 2001). However, it is believed that cell death is a result of oxidation of sulfhydryl groups and amino acids on the exterior of the cell by OCl^- (Eventov et al., 1998). NaoCl(Eventov et al., 1998). Sodium hypochlorite destroys DNA in a similar manner through oxidative damage (Kemp and Smith, 2005, Ohnishi et al., 2002),(Kemp and Smith, 2005, Ohnishi et al., 2002), resulting in the breakdown of DNA into segments shorter than that recognized by the PCR assay (Prince and Andrus, 1992).(Prince and Andrus, 1992).

Because PCR is now the preferred diagnostic test for chytridiomycosis (Hyatt et al., 2007) it is crucial to minimise contamination so that results are comparable and reliable. Just as great care should be taken in the laboratory during processing of samples to prevent contamination, equal care must be taken in the field during the collection of samples. When sampling amphibians for infection, each individual should be captured with a separate pair of gloves or plastic bag. If animals are captured in a bag, a fresh pair of gloves should be worn during each swabbing and each swab should be housed individually. These precautions should be sufficient to prevent cross contamination of DNA during field surveys where the animals are captured by hand and returned to their natural habitat following swabbing; however, as researchers move from documenting occurrence and prevalence of chytridiomycosis into more manipulative experimental work, the risk of contaminating samples through shared contact presents an important problem. For example, reusing experimental equipment such as containers, enclosures, sorting trays and nets in conjunction with PCR can result in contamination of samples. Also, equipment used to search for *Bd* in the environment such as filter holders and tubing could cross contaminate samples (Kirshtein et al., 2007).(Kirshtein et al., 2007). As NaOCl is toxic, great care should be taken to avoid release or spillage of bleach into water bodies, drains or drainages when in the field. Used bleach that needs to be

disposed of should always be carried back to the lab or, if absolutely necessary, spread onto a flat surface such as a paved road to evaporate. NaOCl evaporates quickly and breaks down to water, oxygen and table salt (NaCl); however, other chemicals may be added during production, particularly in household cleaning products (Clarkson and Moule, 1998).(Clarkson and Moule, 1998).

Bleach already forms a part of many researchers' tool kit for the purposes of disinfection. Our experiment shows that NaOCl can also be used to denature *Bd* DNA to prevent its recognition by PCR and reduce the likelihood of cross contamination of samples.

Chapter 3 : Effect of Sample Collection Techniques on Sensitivity of a Real Time PCR Assay for Detecting the Amphibian Pathogen *Batrachochytrium dendrobatidis*

Abstract

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

Introduction

The disease chytridiomycosis, caused by the amphibian fungal pathogen *Batrachochytrium dendrobatidis* [*Bd;* Phylum Chytridiomycota, Class Chytridiomycetes, Order Rhyzophydiales, Family not yet placed; (James et al., 2006, Longcore et al., 1999) has caused an unprecedented, global loss of amphibian biodiversity (Skerratt et al., 2007). *Batrachochytrium dendrobatidis* zoosporangia produce aquatic zoospores (2-4 μm diameter) that invade the superficial epidermis of the stratum corneum and stratum granulosum of amphibians, resulting in mild skin lesions including hyperplasia, hyperkeratosis and excessive skin sloughing (Berger et

al., 1998, Berger et al., 2005c, Pessier et al., 1999). Heavy infections appear to disrupt normal cell function, causing osmotic imbalance from loss of electrolytes. A decrease in electrolytes, such as seen in severely infected frogs, can impair cardiac function resulting in death (Voyles et al., 2007)

This pathogen has emerged globally over the past 30 years (Skerratt et al., 2007) and evidence implicates human mediated transport of infected hosts as a likely significant driver of spread into new areas (Fisher and Garner, 2007).(Fisher and Garner, 2007). While many species are highly susceptible to chytridiomycosis, individual hosts can carry sub-lethal infections for long periods before death or even indefinitely with no overt symptoms rendering any amphibian a potential carrier (Daszak et al., 2004).(Daszak et al., 2004). At least 28 species of introduced amphibians have been known to carry *Bd*, many asymptomatically (Fisher and Garner, 2007). Even heavily infected frogs can appear behaviorally and physiologically normal until just a few days or hours before death (Berger et al., 2004, Voyles et al., 2007).(Berger et al., 2004, Voyles et al., 2007). As a result, infected frogs have been found within every major industry that moves amphibians, including the pet (Mutschmann et al., 2000, Speare, 2000), bait (Picco and Collins, 2007), zoological (Pessier et al., 1999, Raverty and Reynolds, 2001), scientific (Parker et al., 2002, Weldon et al., 2004) food stocking (Hanselmann et al., 2004) and food trading industries (Mazzoni et al., 2003, Daszak et al., 2006).(Daszak et al., 2006, Mazzoni et al., 2003). Often, transported amphibians escape and establish viable populations. Infected feral bullfrogs (*Rana catesbeiana*) have been found on three continents (Garner et al., 2006) and were the first wild amphibians found carrying the pathogen in Britain (Cunningham et al., 2005).(Cunningham et al., 2005).

Despite tens of millions of frogs being transported annually (OIE, 2006), the amphibian trade has remained largely unregulated. More stringent inspection of this global market is important to prevent further spread of this and other amphibian diseases. Experts in the field, national threat abatement plans and international action plans urgently recommend screening of transported amphibians at the national and international level

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

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

Filtration can also be used to sample amphibians (Hyatt et al., 2007) and has recently emerged as an important technique to detect *Bd* in the aquatic environment (Kirshtein et al., 2007, Walker et al., 2007). While there are a variety of possible filtration methods, the quickest and least complicated method is to simply push water samples through a

filter membrane. The trapped zoospores are extracted and processed through PCR in the same manner as swab samples. An important application of filtration may be to screen samples of water transported within and between regions. *Bd* can survive up to six weeks in sterile water and moist sand (Johnson and Speare, 2003). Therefore, transported water previously in contact with an infected amphibian, even weeks prior, could harbor the pathogen and should be screened or disinfected (for treatments see: Johnson et al., 2003, Webb et al., 2007).

Here we compare five different sampling materials used to collect *Bd*; one swab, and four filters (two filter types x two pore sizes) across four dilutions of zoospores to determine how efficiently DNA is extracted from each and to provide recommendations for the best filtration materials.

Materials and Methods

We established that swabs validated for sampling amphibians (Hyatt et al., 2007; tubed dryswab, Medical Wire & Equipment Co (Bath) Ltd.) could absorb 10 µl of fluid with no loss. Therefore all treatments (swabs and filters) and controls were inoculated with 10 µl of *B. dendrobatidis* culture. Innoculations of *B. dendrobatidis* zoospores were prepared by seeding 2 ml of actively growing culture in TGhL broth (16 g tryptone, 4 g gelatin hydrolysate, 2 g lactose, 1000 ml ultrapure water) onto two TGhL agar plates. Plates were sealed with Parafilm® and incubated at 21 °C. Following five d, zoospores were harvested by flooding each plate twice with 2 ml DS (dilute salts solution; 10^{-3}M KH_2PO_4, 10^{-4} M $MgCl_2$ and 2 x 10^{-5} M $CaCl_2$) and leaving for 2 min. The 8 ml zoospore suspension was centrifuged at 4500 x g for 5 min to pellet the zoospores. The supernatant was removed and DS added to create a concentrated suspension. Four independent zoospore counts were made with a haemocytometer. The final concentration was 5.72 x 10^6 zoospores/ml. This was serially diluted 1:10 three times to form four concentrations. Ten µl from the four dilutions (for total zoospore counts of: 57,200; 5,720; 572 and 57) were subjected to each of the following five sample collection treatments: "Swab" and four different membrane "Filters" (two filter types x two pore sizes). Each treatment was replicated three times prior to qPCR processing. There was also a group (designated “Control”) in which the above dilutions were

directly processed for qPCR without application of a sample collection treatment and served as a comparison for the treatments. All samples were stored at -30 °C until DNA extraction and qPCR processing.

Swab: Ten µl were applied directly to each swab. Swab tips were then snapped off into an Eppendorf tube for DNA extraction.
Filters: The following four 25 mm filter membranes were tested: MF 1.2 and 5.0 µm Millipore™ membranes (mixed cellulose esters) and IP 2.0 and 5.0 µm Isopore™ Millipore membranes (polycarbonate). Each membrane was held in a Swinnex ® filter holder (Millipore). Five ml DS was added to 5 ml syringes. The filter holders were attached to the syringe tip and 10 µl of zoospore dilution was added to the DS. The syringe plunger was inserted and the syringe inverted five times to evenly distribute zoospores before pushing the liquid through the filter. This was repeated once with clean DS to rinse. The membranes were removed with sterile forceps, allowed to dry and placed in Eppendorf tubes for DNA extraction.
Control: Ten microlitres of pure culture at the four dilutions above were added directly to an Eppendorf tube for DNA extraction.

All samples were bead-beaten with 30-40 mg zirconium/silica beads. DNA was extracted in PrepMan Ultra and processed with real-time TaqMan® (Applied Biosystems) quantitative PCR assay following the protocol described by Boyle *et al*. (2004). The analysis was performed on the Rotor-Gene™ 6000 (Corbett Research) using Gene-Disc 100 tubes. The sample extract and negative control were diluted 1 in 10. Triplicate analyses were performed for each sample, negative control and no-template-control, and quadruplicate analyses were undertaken for each standard (100, 10, 1, 0.1 zoospore equivalents). For statistical analyses we use the cycle threshold (Ct) value at a threshold of 0.01. If a sample contains more initial DNA it will reach the threshold of detection at a lower Ct value. Hypotheses were tested using two-way ANOVA to compare differences in Ct values among collection materials and zoospore concentrations. *Post hoc* tests (Tukey's HSD) were used to identify which treatments

differed. All statistical analyses were performed with Statistica 7 (Statsoft, Tulsa OK, USA).

Results

There was no significant difference in extraction efficiency and therefore sensitivity between the swab samples and the pure culture controls at any of the four concentrations of *Bd* (Fig. 3.1; df = 48. 57 zoospores, p = 1.000; 572 zoospores, p = 0.926; 5,720 zoospores, p = 1.000; 57,200 zoospores, p = 1.000). The same was not true of the filters. When averaged across dilution treatments, less *Bd* was detected from each of the four filters than from the pure culture controls (Fig. 3.2; df = 48. IP 2.0, p = 0.0008; IP 5.0, p = 0.0003; MF 1.2, p = 0.0001; MF 5.0, p = 0.0003). However, when we looked at the interaction between filter type and zoospore concentration separately we found that only at the lower concentrations were fewer zoospores detected compared with pure culture controls. All four filter types from the lowest concentration (57 zoospores) had Ct values significantly higher than controls (df = 48. IP 2.0, p = 0.0008; IP 5.0, p = 0.0273; MF 1.2, p = 0.0049; MF 5.0, p = 0.0126). The only other filter treatment with a Ct value significantly higher than the controls was the MF 1.2 filter at the 572 zoospore concentration (df = 48, p = .0040). There was no difference among filters at any concentration level.

Discussion

The Ct value of swab samples was not significantly different from the controls indicating a very high percentage of *Bd* DNA present on swabs was recovered during extraction. Therefore, when *Bd* is successfully collected on a swab and processed following the protocol of Boyle et al (2004), the end result will be efficient extraction and amplification of DNA resulting in a highly sensitive test. Therefore, a negative qPCR assay accurately reports the absence of *Bd* DNA on the swab in the absence of other compounds such as complex polysaccarides, humic acids and proteinases that can inhibit PCR (Lantz et al., 1997, Monteiro et al., 1997). This high level of DNA recoverability, in addition to the demonstrated high level of sensitivity, specificity, repeatability and reproducibility (Hyatt et al., 2007) make this PCR-based swab assay

an ideal diagnostic tool to screen animals involved in the global amphibian trade and for research efforts to study this disease.

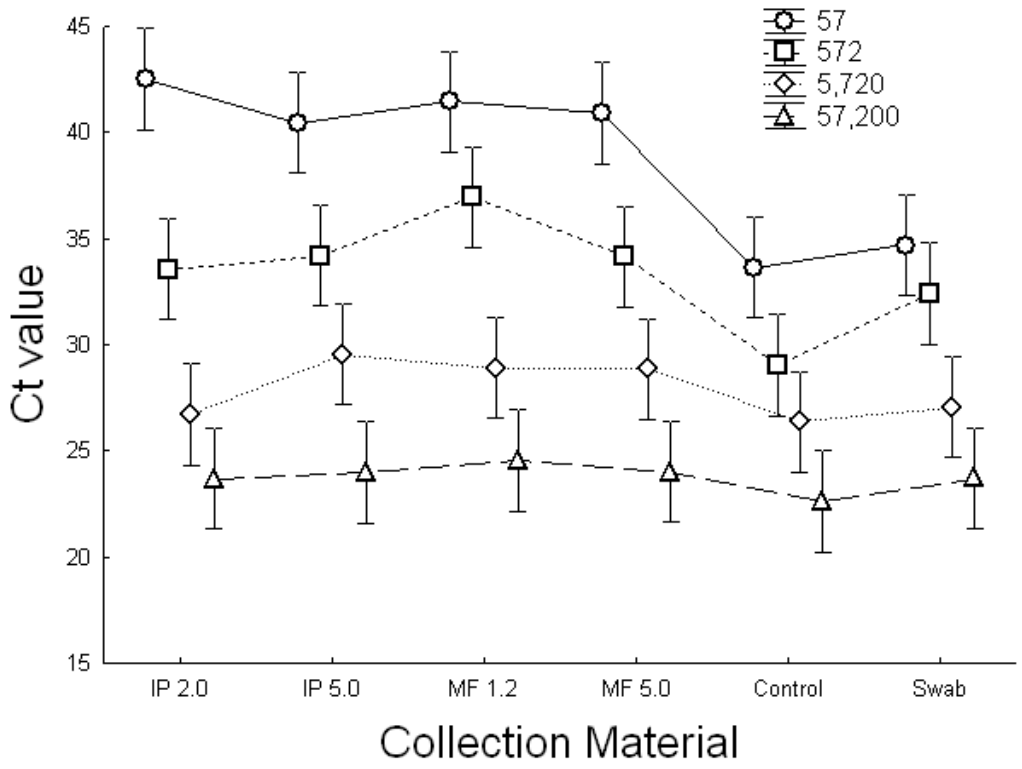


Figure 3.1. Interaction diagram between sample material and zoospore concentration. Symbols represent the mean cycle threshold value (Ct) and whiskers represent the 95% confidence interval. Connecting lines are included to aid in visualization. IP 2.0 = Isopore 2.0 µm, IP 5.0 = Isopore 5.0 µm, MF 1.2 = Mixed Cellulose Esters 1.2 µm, MF 5.0 = Mixed Cellulose Esters 5.0 µm.

We found some loss of DNA during filtration, extraction and qPCR assay and, predictably, this loss had a greater relative impact when *Bd* density was low. This loss resulted in, on average, 7.7 additional amplification cycles before reaching the Ct threshold at the 57 zoospore concentration, 5.7 additional cycles at 572 zoospores, 2.2 cycles at 5,720 zoospores and 1.4 cycles at 57,200 zoospores. This makes the lowest consistently detectable number of zoospores (a positive reaction in each of three wells) with these filters, approximately 40 zoospores under clean conditions (i.e. no inhibition). Organic compounds, such as humic acids, are commonly found in soils and are strong PCR inhibitors (Braid et al., 2003). Therefore, in turbid or sediment-laden

water more zoospores are likely needed in the sample to achieve consistent positive results (Kirshtein et al., 2007, Walker et al., 2007). DNA soil extraction kits such as

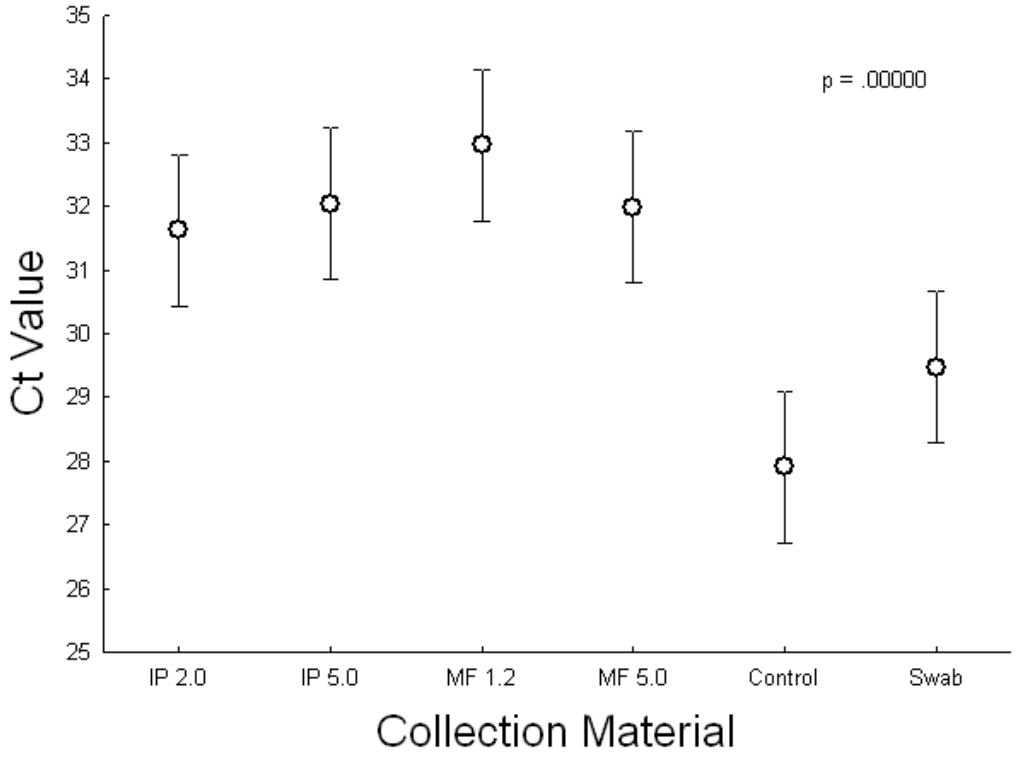


Figure 3.2. Mean cycle threshold value (Ct) by sample material averaged across zoospore concentration. Symbols represent the mean Ct value and whiskers represent the 95% confidence interval. IP 2.0 = Isopore 2.0 µm, IP 5.0 = Isopore 5.0 µm, MF 1.2 = Mixed Cellulose Esters 1.2 µm, MF 5.0 = Mixed Cellulose Esters 5.0 µm.

MoBio Power Soil can be used to purify the sample and reduce inhibition (Walker et al., 2007). Alternatively, increasing extraction dilutions from 1 in 10 can also remove inhibition, however, both techniques will decrease sensitivity requiring more initial target DNA to be present.

The number of *Bd* zoospores detected in studied pond sites has been relatively high (between 0.5 to 454 zoospore equivalents l-1Walker et al., 2007, Kirshtein et al., 2007), however, at many sites, such as fast flowing streams, numbers of *Bd* are likely to be much lower as a result of the constant flushing and dispersal of zoospores in the current. Increasing the volume of water sampled will improve the chances of detecting low

numbers of *Bd*. Accordingly, we tested filters with pore sizes above and below either end of the zoospore diameter spectrum (2-4 μm), in order to maximize the volume of liquid sampled. We detected no difference between filter types or pore sizes. Millipore Isopore membranes (polycarbonate) contain a uniform pore size, trapping all particles larger than the pores on the surface of the membrane. This allows easier extraction of sample material for microscopic assessment. These filters are non-absorbent, resulting in faster drying time following filtration and less absorption of reagents during processing. In comparison, the Millipore MF (mixed cellulose esters) membranes do not have a uniform pore size, instead particles are trapped in a matrix of interwoven fibers rendering most sample material inaccessible for microscopic assessment. These filters are absorbent, resulting in longer drying times and increased adsorption of reagents. As all filters were found to be equally efficient, we recommend the Millipore Isopore 5 μm membrane for *Bd* filtration to maximize volume of water sampled and reduce drying time in the field and absorption of reagents in the laboratory.

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

Chapter 4 : Lethal Effect of Latex, Vinyl and Nitrile Gloves on Tadpoles*

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

Introduction

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

Case study 1: laboratory

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

mouthparts repeatedly to collect *Bd* DNA on the cotton fibers. Each tadpole was in hand for approximately 30-90 s before being returned to its container.

Thirty-six *L. genimaculata* had been processed in this way when we observed that some of the earliest handled tadpoles appeared listless, could not remain upright and had difficulty using their tails for locomotion. Upon closer inspection the tails of the listless tadpoles were gray and dead in appearance at the locations where gloved fingers held them in place during swabbing. At that time, we suspended tadpole handling. Within 24 h, 26/36 tadpoles died. The surviving ten tadpoles did not appear listless, showed no overt adverse effects and survived 4-6 wk to metamorphosis (Fig. 4.1A). Although care was taken to handle tadpoles gently, the observed mortality could possibly have been due to mechanical damage, so we initiated a series of experiments. Based on the results of these experiments (see below) we switched from latex to vinyl gloves for the remainder of the lab study. Ten unhandled *L. genimaculata* and *L. nannotis* tadpoles were processed as previously described except with vinyl instead of latex gloves. Following 24 h of observation no mortality or ill effects were noted. Satisfied that vinyl gloves were safe for tadpoles of these species, the remaining 13 *L. genimaculata* and 22 *L. nannotis* tadpoles were processed using vinyl gloves (Fig. 4.1A). All tadpoles appeared unaffected after handling. In total, 26/36 *L. genimaculata* died following handling with latex gloves, while 0/23 *L. genimaculata* and 0/32 *L. nannotis* died following contact with vinyl gloves.

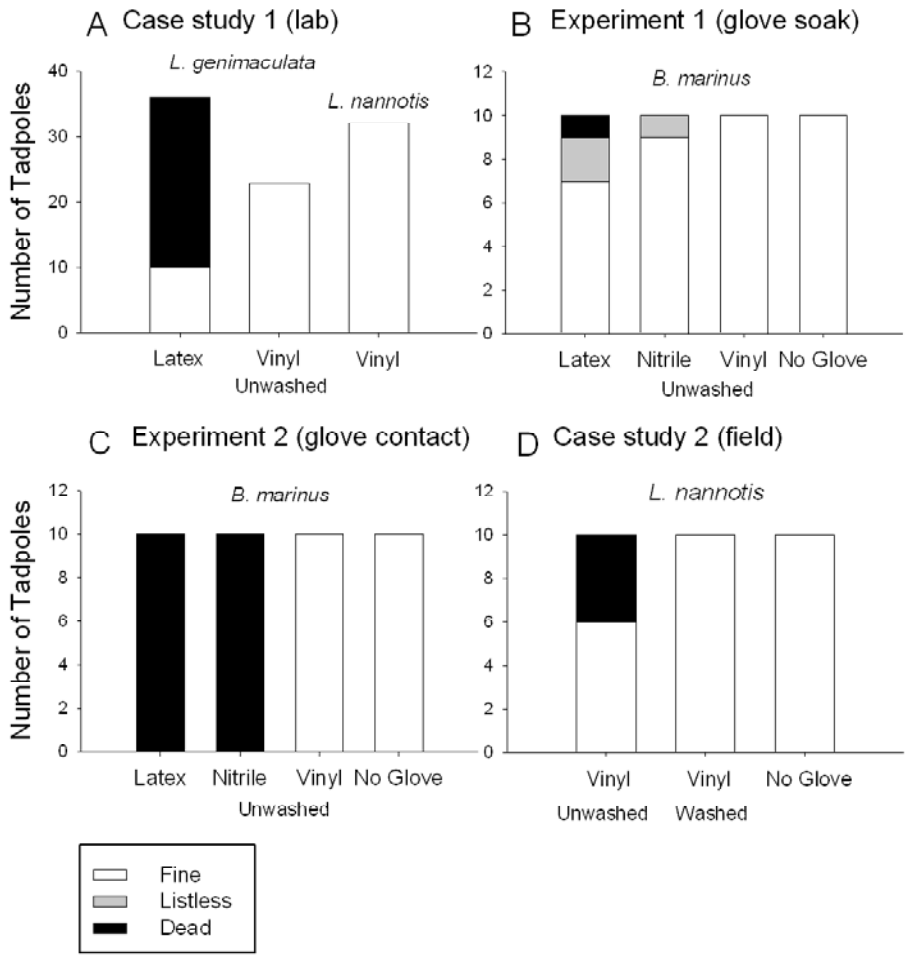


Figure 4.1. Tadpole mortality in response to disposable glove exposure. (A) Case study 1: laboratory; Number of *Litoria genimaculata* and *Litoria nannotis* tadpole deaths within 24 h following contact with latex or vinyl gloves for 30-90 s. (B) Experiment 1: glove soak; Number of *Bufo marinus* tadpole deaths within 72 h following contact with water containing latex, vinyl and nitrile gloves soaked for 5 m. (C) Experiment 2: glove contact; Number of *B. marinus* tadpole deaths within 24 h following direct contact with latex, vinyl, and nitrile gloves and bare hands for 60 s. (D) Case study 2: field; Number of *L. nannotis* deaths within 24 h following direct contact with unwashed vinyl gloves, washed vinyl gloves and bare hands for 30-90 s.

Experiment 1: glove soak

In order to determine the best gloves for handling tadpoles we conducted an experiment testing the three most common glove types; latex (SuperMax, low powder), vinyl (Livingstone, clear, low powder) and nitrile (Livingstone, low powder). Forty non-native *Bufo marinus* tadpoles were captured from a local pond and allowed to rest in individual 1000 ml containers with 500 ml of collected rainwater and a pinch of powdered tadpole chow (3:1 alfalfa pellets:fish food, ground and passed through a 250 μm sieve). After 24 h one of each glove type was draped over the edge of the container for 5 min so that the five fingers of each glove were submerged. Ten control containers had no contact with a glove. The condition of each tadpole was recorded at 2, 12, 24, and 72 h following removal of the glove, however, tadpole condition did not change beyond the 2 h post-exposure point. One of ten tadpoles exposed to the latex gloves died and two more were listless and floating awkwardly within two hours of glove exposure. One of the tadpoles exposed to nitrile gloves was listless while all of the vinyl and control treatments appeared unaffected (Fig. 4.1B). The listless tadpoles remained in an impaired state for the full 72 h of observation and appeared permanently affected. These listless tadpoles were euthanized and preserved in 70% ethanol. Although 3/10 *B. marinus* tadpoles exposed to latex gloves experienced deleterious effects, the rate of mortality was lower than we expected given the high level of mortality we previously observed in *L. genimaculata*. We hypothesized this difference was related to the different methods of glove exposure. The *L. genimaculata* in "Case study 1" were in direct physical contact with the gloves during measuring and swabbing while the *B. marinus* in "Experiment 1" were in water in which gloves were soaked. This indirect glove contact may have yielded a lower dose of the toxic compound(s).

Experiment 2: glove contact

To determine whether direct glove contact increases mortality we ran a second experiment in which *B. marinus* tadpoles were handled in the same manner as previously described for *L. genimaculata* and *L. nannotis*. Each tadpole was gently held in place at the base of the tail between the thumb and index finger for 60 s with one of

the three types of gloves as treatments or with an ungloved hand as a control. A new glove was worn for each tadpole and the treatments were interspersed, with each glove type and the bare hand treatment applied in succession. Prior to the no glove treatment, hands were rinsed in tap water and dried with a paper towel to remove any residual powder from the previous glove treatment.

Within 2 h of handling, all tadpoles that had been in contact with latex or nitrile gloves were dead or listless. Those that were listless died within 24 h (Fig. 4.1C). Listless tadpoles had little to no tail function and the usually dark black tail had a discolored, dead-looking, gray appearance. This discoloration was most pronounced where direct contact with the gloves occurred. Particles in the water soon began to attach to the epidermis of the dying tail, giving it a fuzzy appearance. None of the tadpoles handled with either vinyl gloves or bare hands suffered noticeable ill effects and all survived to metamorphosis (Fig. 4.1C).

Case Study 2: field

We applied our conclusion that vinyl is the safest glove material to "Case Study 1: lab" (described above) as well as a field study monitoring *Bd* in the wild. Individual tadpoles were to be captured, handled with vinyl gloves, measured, swabbed for *Bd* infection and returned to the stream unharmed. During initial field sampling, individuals were processed and kept temporarily in a holding tray to monitor condition following swabbing. Unexpectedly, of the first ten *L. nannotis* tadpoles processed, four became listless and died within 1 h (Fig. 4.1D). The remaining six tadpoles appeared normal and did not develop signs over the following 24 h. As a test, the next ten captured tadpoles were processed with bare hands and suffered no ill effects, suggesting the gloves and not the handling were the cause of mortality. The next ten captured tadpoles were processed with vinyl gloves that were rinsed in a bucket of water prior to handling. All of these tadpoles survived and appeared normal suggesting that a substance on the outside of the vinyl glove was toxic and that rinsing successfully removed it. All tadpoles were held for 24 h for observation. From this point on we incorporated the

rinsing of vinyl gloves into the standard field protocol. Vinyl gloves were rinsed in a 10 L bucket of water which was changed after at most ten tadpoles. This was adequate to ensure the glove-wash residue did not attain a high enough concentration to cause harm. To date over 2500 tadpoles have been handled with washed vinyl gloves with no ill effects. On a few occasions, the rinsing step was accidentally skipped and many of these tadpoles became listless and died.

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

Our results show that unwashed latex, nitrile and vinyl gloves can be toxic to tadpoles. Unwashed latex and nitrile gloves caused up to 100% tadpole mortality following only 30-90 s of direct contact (Fig. 4.1C). Rapid, localized necrosis of tissue at the point of contact was observed grossly. Even 5 min of partial glove submersion was sufficient to cause mortality in the latex and nitrile treatments (Fig. 4.1B).

Despite a thorough literature search, only two references to the toxic effects of gloves on tadpoles were found and both of these were published in toxicological journals, likely to have low readership by herpetologists. In a letter to the editor, Sobotka and Rahwan (1999) reported that water from unwashed latex gloves (American Dental Association, Safeskin brand) and washed latex gloves (Baxter Pharmaseal Flexam) soaked for 24 h caused mortality in *Xenopus laevis* tadpoles. However, water from washed vinyl gloves (Baxter Triflex) did not. Gutleb et al. (2001) reported 100% mortality in *Xenopus laevis and Rana temporaria* tadpoles exposed to water from

unwashed latex gloves (Becton-Dickinson) soaked for 24 h. Even very dilute solutions of glove- soaked water (0.29% for *X. laevis* and 0.15% for *R. temporaria*) caused 100% mortality. Gutleb et al. (2001) found that vinyl gloves (Becton-Dickinson) soaked for 24 h also killed tadpoles, but only at relatively high concentrations: 33% and above. Mortality was 100% at or above this concentration but 0% below this concentration.

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

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

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

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

Chapter 5 : Sites, Tadpole Ecology

Introduction

Single host pathogen systems are easier studied and are thus better understood; however, most pathogens are capable of infecting multiple species (Power and Flecker, 2008). The interactions of multiple-host pathogens with their hosts are complex and depend on the ecology and behavior of all host species. Differences in these factors among host species can affect the prevalence, intensity, persistence, transmission and seasonality of infection in any one species, as well as the evolution of pathogen virulence and host resistance (Woolhouse et al., 2001, Keesing et al., 2006). As a result, generalist pathogens tend to have less predictable epidemiologies, and to properly interpret data on disease processes requires an understanding of the ecology of each host species.

The amphibian pathogen *Batrachochytrium dendrobatidis* (*Bd*) is a generalist that is known to infect over 350 species around the world (Speare and Berger, 2005, Kenyon, 2008, Fisher et al., 2009) and, under suitable conditions it may be capable of infecting all frogs, salamanders and larvae with keratinized cells (Berger et al., in press). Consistent with other multi-host systems, the ecology and behaviour of adult frogs in Australian rainforest streams is believed to affect the prevalence of infection and susceptibility to chytridiomycosis (Rowley and Alford, 2007, Woodhams and Alford, 2005). Adult frogs, however, are not the only hosts harbouring *Bd* in stream systems. Tadpoles of these species can also become infected and are abundant throughout the year, however, little is known of their role within the multi-host system.

Tadpoles are a strictly growth and energy-gathering life-history stage of frogs (Wassersug, 1975). The primary aim of tadpoles is to consume resources to grow, and metamorphose at as large a size and as quickly as possible within the physical and physiological limits of the species (Wilbur, 1980). A tadpole's fitness is determined by its metamorphic parameters. Individuals that metamorphose at a size, time and in a condition that increases reproductive success as adults have a selective advantage. A

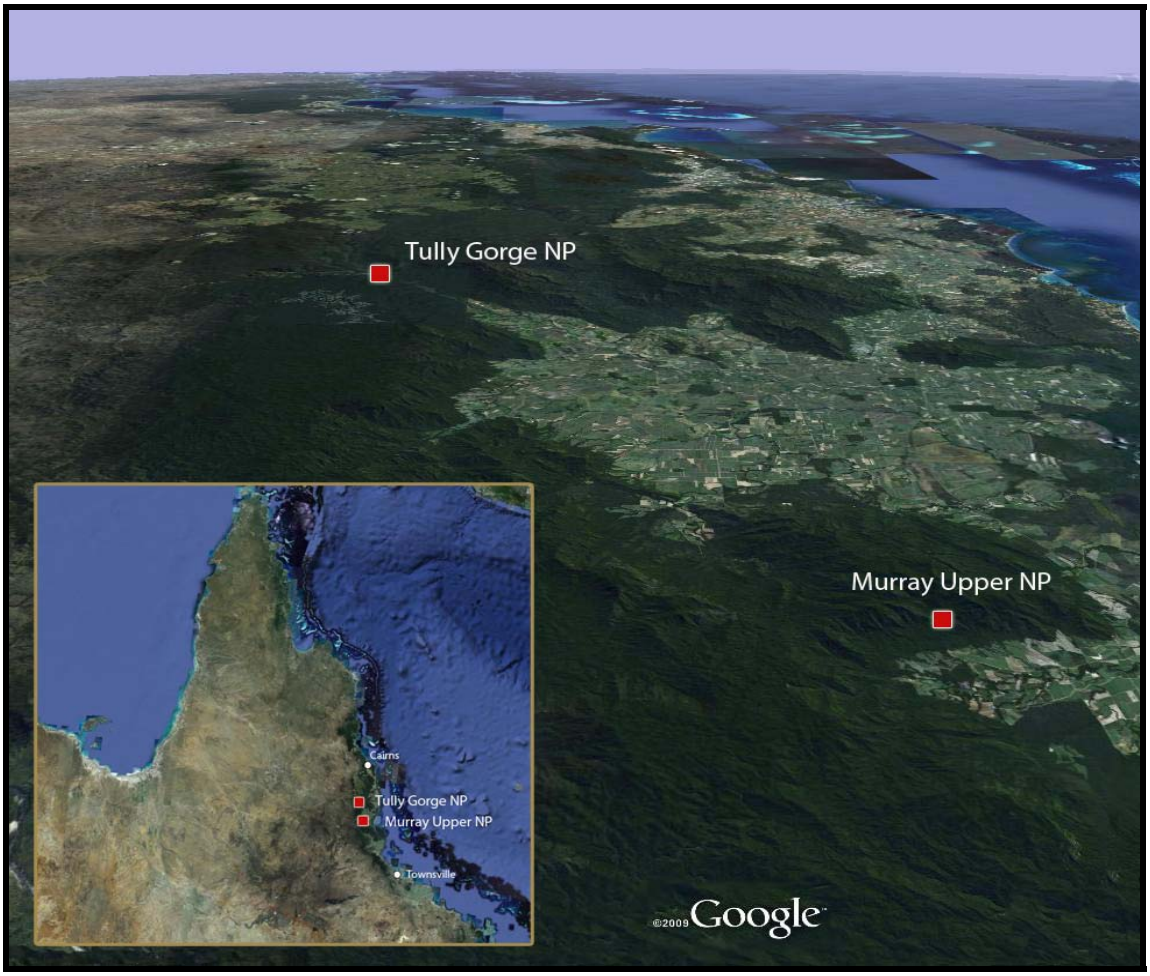
larger size and early season emergence can lead to a larger size and increased survival-rate at first breeding, a decreased time to reach reproductive maturity, and larger egg clutches (Chelgren et al., 2006, Altwegg and Reyer, 2003, Scott, 1994). The selective pressure on tadpoles to maximize condition at metamorphosis has led to an impressive diversity of larval body forms and strategies to exploit the spectrum of aquatic microhabitats (Altig and Johnston, 1989). These divergent strategies may result in differential risk of exposure to, and differential response to infection by, *Bd*.

In this chapter I investigate the ecology of these larval hosts. I sampled tadpoles at two rainforest stream sites over two years to uncover seasonal abundance patterns and habitat preferences. I also conducted a mark-recapture study of the torrent tadpole *Litoria nannotis* to understand site fidelity and dispersal ability. In the remaining chapters, these data are used to interpret patterns of *Bd* infection to better understand *Bd* in streams and the epidemiology of chytridiomycosis in tadpoles.

Site and Species Descriptions

HabitatSampling occurred between February 2006 and February 2008 at two lowland rainforest streams in northeast Queensland, Australia: the creek at bridge 7 in Murray Upper National Park (145° 52.116 E 18° 11.750 S, 210 m asl) and an unnamed creek in Tully Gorge National Park (145° 38.747 E 17°46.340 S, 130 m asl, Figure 5.1-5.2). Both sites are surrounded by simple notophyll vine forest (Tracey and Webb, 1982). The creeks are generally fast flowing and creek beds are composed of granite rocks ranging from small pebbles to large boulders (>10 m diameter). Small waterfalls, riffles and runs (> 5cm/s^2 flow rate; Fig. 5.4, 5.6) are the dominant stream habitat. Climate is highly seasonal, allowing in-stream "connected" pools (< 5 cm/s^2 flow rate; Fig. 5.5) to form in the deeper sections, particularly during the cool/drier winter months (May-November) when water levels drop. The warm/wet summer (December-April) brings monsoonal rains, which increase water flows, and cause periodic spates where water levels rise dramatically, scouring rock surfaces and redistributing boulders and debris.

Because streambed topography includes both steep and flat sections (Fig. 5.3), current velocity is highly variable among sections of the creeks, resulting in a range of microhabitats and creek bed substrates. In the higher velocity sections, currents prevent leaf litter and detritus from settling. The rock surfaces in these habitats appear bare, but support a community of microorganisms (biofilm), including plankton, diatoms, protozoa, bacteria and fungi (Lear et al., 2008). The material that remains suspended and passes over the runs and riffles settle in the pools. Here, sand, leaf litter and detritus accumulate. At Tully, the recession of stream water after heavy rain left stream side rock depressions filled with water. Leaves and other plant material can fall into these isolated pools following formation, introducing nutrients. At times, however, they can remain free of external organic inputs (pers. obs.).



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

Figure 5.2. Closer views of field sites. Left: Murray Upper National Park, Right: Tully Gorge National Park. Blue lines indicate location of transects.

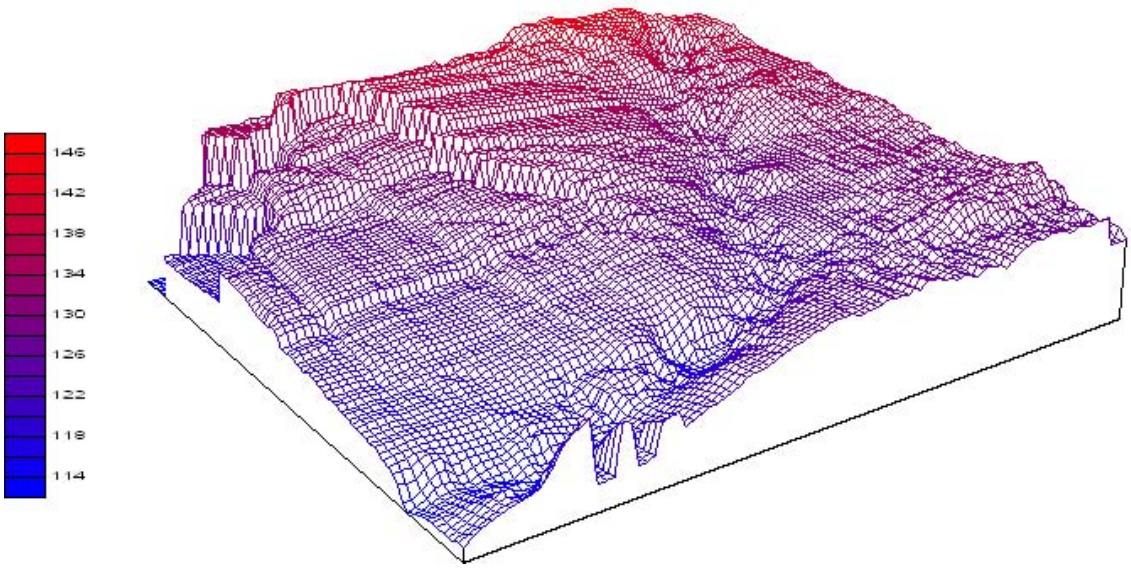


Figure 5.3. Topography of Tully Gorge National Park Site. Graph key represents elevation in meters.

Figure 5.4. Tully Gorge National Park. A series of riffles and small cascades.

Figure 5.5. Murray Upper National Park. A series of riffles ending with a connected pool.

Figure 5.6. Tully Gorge National Park following heavy rain.

Species

The amphibian assemblage at these two creeks is composed of four species that use the stream throughout the year for habitat, foraging, breeding and egg deposition. These are the Waterfall Frog (*Litoria nannotis*), the Common Mistfrog (*L. rheocola*), the Green-eyed Treefrog (*L. genimaculata*), and the Australian Lacelid (*N. dayi*). These species declined throughout their range when *Bd* first emerged and all, except *L. genimaculata,* disappeared above 400 m elevation (McDonald and Alford, 1999, Berger et al., 1999). Below 400 m, these species now persist with *Bd* endemically, while suffering seasonal mortality caused by chytridiomycosis (Andrea Phillott pers. com.). A fifth species, *Taudactylus acutirostris*, has not been seen at these sites since the early 1990's. Evidence indicates their disappearance was due to the emergence of chytridiomycosis (Schloegel et al., 2006, McDonald and Alford, 1999).

Additional species that have a looser stream affiliation and were only seen occasionally include *Litoria junguy/ L. wilcoxii, L. xanthomera* and *Mixophyes schevilli*. These frogs are not known to have declined (McDonald and Alford, 1999). The non-native

Chaunus marinus can breed in slow streams, but typically deposits eggs in shallow puddles and ephemeral pools. No *C. marinus* tadpoles were found during this study. Terrestrial and direct developing microhylids occur in the surrounding forest and do not appear to have declined, however, little is known of this group (McDonald and Alford, 1999).

Tadpoles of *L. nannotis, L. rheocola, N. dayi* and the extinct *T. acutirostris* can be classified as members in the "lotic-suctorial" ecomorphological guild (Altig and Johnston, 1989). They are considered to be "torrent-adapted" because they inhabit the faster flowing (>5 cm/s), turbulent sections of the stream (Liem and Hosmer, 1973, Richards, 1992). Torrent-adapted tadpoles are characterized by a hydrodynamic, depressed body shape, large ventral oral disc, and muscular tail with low fins (Richards, 2002; Fig. 5.7 A-C). These adaptations allow them to adhere to boulder surfaces in fast-flowing habitat, exploit the resources present, and avoid being swept downstream during flood events (Fig. 5.6).

Torrent tadpoles adhere to substrates in fast flowing water, and use their keratinized jaw sheaths and tooth rows (Figure 5.7 D) to scrape and extract the food present on rock surfaces. Studies examining specifically what food source suctorial tadpoles derive most of their nutrition from are lacking, however, tadpoles are believed to assimilate not only the algae and diatoms ubiquitous in gut content analyses, but also the associated protozoa, bacteria, and fungi (Altig et al., 2007, Whiles et al., 2006). During the course of this study, *N. dayi* tadpoles were difficult to capture and even more difficult to observe. Therefore, most observations and data on torrent tadpoles focused on the more abundant *L. nannotis* and *L. rheocola*.

Litoria genimaculata tadpoles are members of the "clasping" ecomorphological guild and are adapted to the slow-flowing (< 5 cm/s) connected-pool stream environment (Altig and Johnston, 1989, Richards, 2002; Fig. 5.5, Davies, 1989). They are less streamlined in shape than the torrent-adapted tadpoles, have smaller mouthparts and deeper tail fins. They have little or no ability to maintain position using their oral discs.

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

Tadpoles of *M. schevilli*, and *L. junguy* & *L. wilcoxii* can be found in connected or streamside pools (Hoskin and Hero, 2008), however, over the course of this study only one tadpole of these species was found (a single *M. schevilli* at Murray Upper National Park). As a result, I will not discuss these, or *C. marinus* tadpoles.

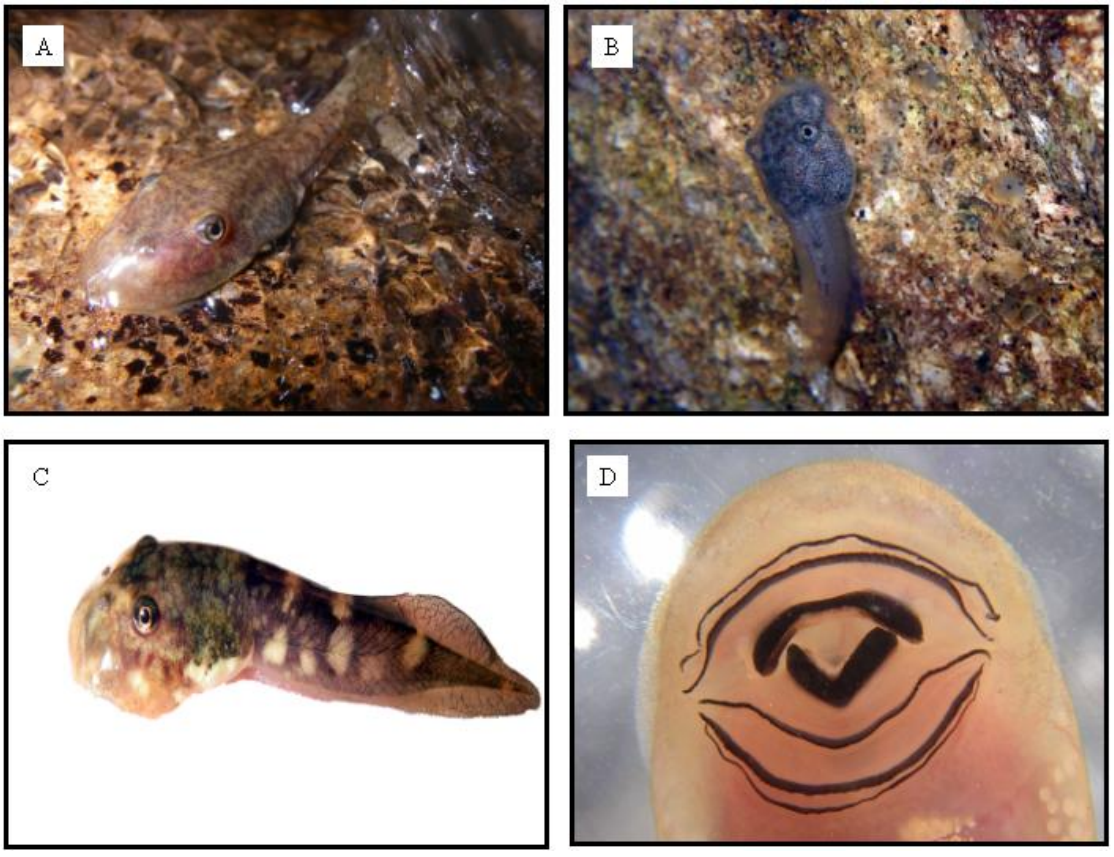


Figure 5.7. Torrent adapted tadpoles. A) *L. nannotis* B) *L. rheocola* C) *N. dayi* D) Ventral view of the large, suctorial mouthparts of *L. nannotis* consisting of two anterior and three posterior keratinized tooth rows and a keratinized jaw sheath.

Materials and Methods

Tadpole Behavior Observations

At the Tully Gorge National Park site, individual tadpoles were visually identified through the water column and observed for 5 min periods. The number of times the individual tadpole broke contact with the substrate and entered the water column was recorded. In total, 50 *Litoria nannotis*, 50 *L. rheocola* and 25 *L. genimaculata* tadpoles were observed

Tadpole Population Surveys

Sampling occurred approximately every four weeks beginning in February 2006 at both sites and then approximately every 2 wk beginning April 2007, to increase sampling precision, at the Tully Gorge National Park site only.

At both sites a 200 m transect was established along the creek. The transects were mapped and then stratified by habitat type into 18 possible mesohabits as proposed by Hawkins et al. (1993). These 18 mesohabitats were then condensed into two broad habitats for sampling purposes, termed "riffle" (>5 cm/s) and "pool" (<5 cm/s) and numbered sequentially (Hawkins et al., 1993). Habitats were sampled proportionally to their abundance. For each visit, a random integer between one and four was selected to choose the first habitat to be sampled. Every fourth habitat patch was then sampled until all habitats of each type were exhausted (Hartwell et al., 1997). This method ensures valid statistical comparisons between habitat types and sampling periods by ensuring ≥ 1 sampled habitat of each type, approximately equal sampling effort within each habitat, good spatial coverage across the transect, and independence of samples among habitats (Hartwell et al., 1997). It was not feasible to capture every tadpole within a selected habitat due to their high density, cryptic nature and ability to move into and out of adjacent habitat patches. Therefore, each habitat was sub-sampled via three 1min dip-net sweeps interspersed with at least a 5 min break between sweeps. Due to differences between habitat types (e.g. flow rate) and the behavior of tadpoles in those habitat types, slightly different sweep techniques were employed. In pools, the-

dip net was swiftly, but gently "bounced" between the substrate and the water column, causing tadpoles within or on the substrate to emerge into the water column for capture. In riffles, the dip-net was quickly scraped across the rock surfaces. When appropriate, upstream rocks were moved to dislodge hidden tadpoles into the downstream net. Captured tadpoles were held for counting and measurement, and were released at the upstream portion of the sampling area following the third sweep. Along the Tully transect, two stream-side isolated pools, suitable for *L. xanthomera* tadpoles, formed following rainfall. As there were only two isolated pools available, both were sampled during each visit. Isolated pools were surveyed for the first year only.

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

Mouthpart and Jaw Loss

Torrent tadpole mouthpart condition was assessed visually through the click-seal bags (Fig. 5.8). Pool tadpoles were examined in hand, with a 10X lens if necessary. The loss of keratinized tooth structures and jaw sheath were ranked from 0-5 using individual scales as follows. **Tooth loss scale**. 0: No loss. 1: Discoloration. 2: Less than 25% loss. 3: 25-50% loss. 4: 51-75% loss. 5: Greater than 75% loss. **Jaw loss scale**. 0: No loss. 1: Thinning of jaw. 2: Small gap in jaw. 3: Medium gap in jaw. 4: Large gap in jaw. 5: Jaw completely missing.

Figure 5.8. *Litoria nannotis* tadpole in a clickseal bag for mouthpart loss analysis

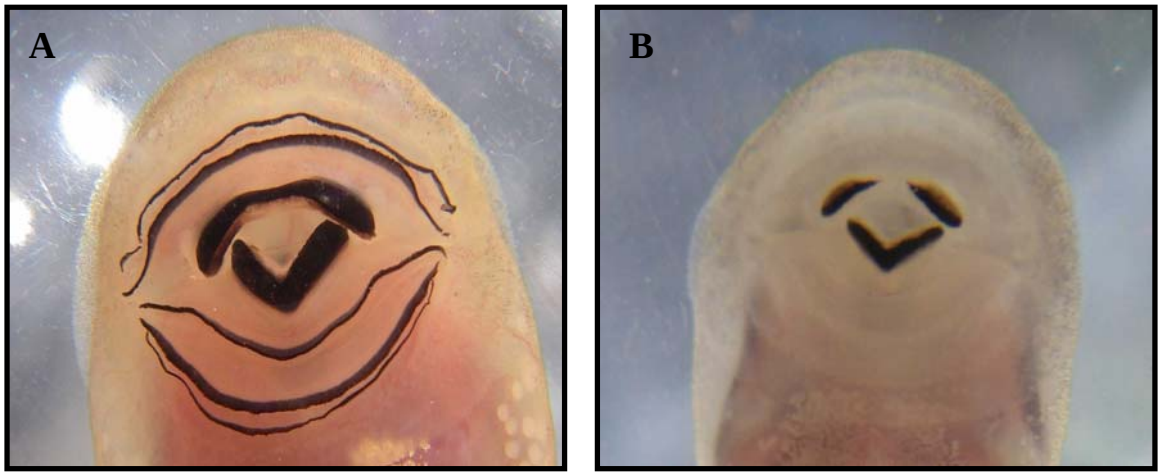


Figure 5.9. Mouthpart Loss. A) *Litoria nannotis* tadpole with mouthpart loss score of 0 (no tooth loss) and a jaw loss score of 0 (no jaw loss). B) *L. nannotis* tadpole with a mouthpart loss score of 5 (≥ 75% tooth loss) and a jaw loss of 4 (large gap in jaw).

Measurements

Body length was measured from the tip of the snout to the base of the tail where the axis of the tail myotomes contacts the body wall (Altig, 2007b) to 0.1 mm precision using vernier calipers. Body lengths were condensed into 9 size classes for some analyses: 1: 0-2.9 mm, 2: 3-4.9 mm, 3: 5-6.9 mm, 4: 7-8.9 mm, 5: 9-10.9 mm, 6: 11-12.9 mm, 7: 13-14.9 mm, 8: 15-16.9 mm, 9: 17 mm +). These size classes were used for tadpoles of all species. Developmental stage (Gosner, 1960) was not recorded as the hind limbs of *L. nannotis*, *L. rheocola*, and *N. dayi* develop in sheaths beneath the epidermis until late in development and cannot be examined without dissection. Mass was measured using a digital balance accurate to 0.01 g.

Environmental Measurements

Water flow

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

Water temperature

Thermochron iButton™ temperature dataloggers (DS1921Z-F5, Dallas Semiconductor, Dallas, Texas USA) were used to record water temperature (Johnson et al., 2005). Dataloggers were individually placed in click-seal bags within water tight metal canisters and placed underwater. Canisters were anchored to streamside supports with wire cable to prevent dislodgement downstream. The iButtons™ were programmed to record temperature every hour. Two iButtons™ were placed in pools and two were placed in riffles at different locations along the transect.

Air temperature and relative humidity

Two temperature and humidity datalogging stations (Tinytag™ Plus, Gemini Data Loggers Ltd.) were set up at the 50 m and 150 m marks of each transect to record every hour.

Additional Environmental Variables

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

Batrachochytrium dendrobatidis real time Taqman PCR assay

Real time Taqman PCR assays were carried out using the methods discussed in Chapter 6.

Torrent tadpole mark-recapture

Very little is known regarding dispersal or survival of torrent adapted tadpoles. The objectives of this mark recapture study were to 1) gain a greater understanding of site fidelity and dispersal of *L. nannotis* within the stream and 2) determine if widespread mortality occurs in association with *Bd* infection and associated mouthpart loss (discussed in Chapter 7).

On 15 May 2007, a 5 m stretch of torrent habitat (50-55 meter mark) was exhaustively sampled using the removal method until the rate of capture was very low, indicating that most of the tadpoles had been collected. Two researchers simultaneously conducted seven 15 min dip net sampling sessions. Each sampling session was separated by a 5 min break. The number of tadpoles captured during each sampling period was regressed on the total number of tadpoles previously captured. From this the position of the x-intercept was extrapolated and used as the estimate of total population size (Heyer et al., 1994). Upon capture, tadpoles were allocated to individual bags, measured, weighed, swabbed and mouthpart loss was recorded as previously described. In addition, tadpoles were marked via visible implant elastomer (VIE; Northwest Marine Technology Inc., Fig. 5.10). Elastomers are a colored, silicone based polymer that is injected under the skin as a liquid and then cures as a biocompatible solid. VIE has been used effectively to mark a range of taxa including adult and larval amphibians (Anholt et al., 1998, Nauwelaerts et al., 2000). A 0.3 cc syringe was used to apply a small volume of elastomer beneath the skin on the dorsal side at the base of the tail (Fig. 5.10). To reduce possible transmission of disease, the syringe tip was immersed in 70% ethanol between tadpoles (Speare et al., 2004).

Figure 5.10. ***Litoria nannotis*** **tadpole marked with an orange visible implant elastomer (VIE) mark.**

Of 132 captured *L. nannotis* tadpoles, one died following dipnetting, and one died following marking. The remaining 130 tadpoles appeared in good health following marking and were released at the upstream section of the 5 m site of capture. Sampling for tadpoles was carried out above, below, and within this 5 m stretch 1 d and 3 wk post-release. This was done to allow mark-recapture estimates of population size within the 5 m stretch, and provide a picture of rates of emigration from the sampled area. On August 16, 2007, 12 wk post-release, a second removal sampling census was carried out in the same 5 m stretch of stream. The recapture rate was measured and a second population estimate was calculated, as before, by regressing the number of tadpoles captured at each sampling period on the total tadpoles and extrapolating the position of the x-intercept.

Results

Tadpole Behavior

Litoria genimaculata tadpoles left the substrate and entered the water column significantly more times than either *L. nannotis* or *L. rheocola* over a 5 min period (ANOVA, $F_{2,120} = 490.35$, $p < 0.00005$; Fig.5.11). *Litoria nannotis* tadpoles broke contact with the substrate, on average, 0.06 times/ 5 min, *L. rheocola* 0.2 times/5 min, and *L. genimaculata* 11.4 times/5 mins. Only 6% (3/50) of *L. nannotis* left the substrate and all three did so only briefly, one time. 12% (6/50) of *L. rheocola* moved from the substrate with only three individuals doing so more than once and none more than three times. All 25 *L. genimaculata* tadpoles left the substrate and did so frequently (5-19 times).

Spatial Organization

Tadpoles of different species occupied habitats with significantly different mean water velocities (ANOVA; $F_{4, 2562} = 655.91$, $p<0.00005$. Fig.5.12). Tukey's Unequal N HSD post hoc test shows tadpoles of all species pairs occupied significantly different water velocity profiles except *L. genimaculata* and *L. xanthomera*. *Nyctimystes dayi* tadpoles were captured in the fastest flowing water (mean flow rate: 65 cm/s), followed by *L.*

nannotis (40 cm/s), *L. rheocola* (30 cm/s), *L. genimaculata* (1cm/sec) and *L. xanthomera* (0 cm/s).

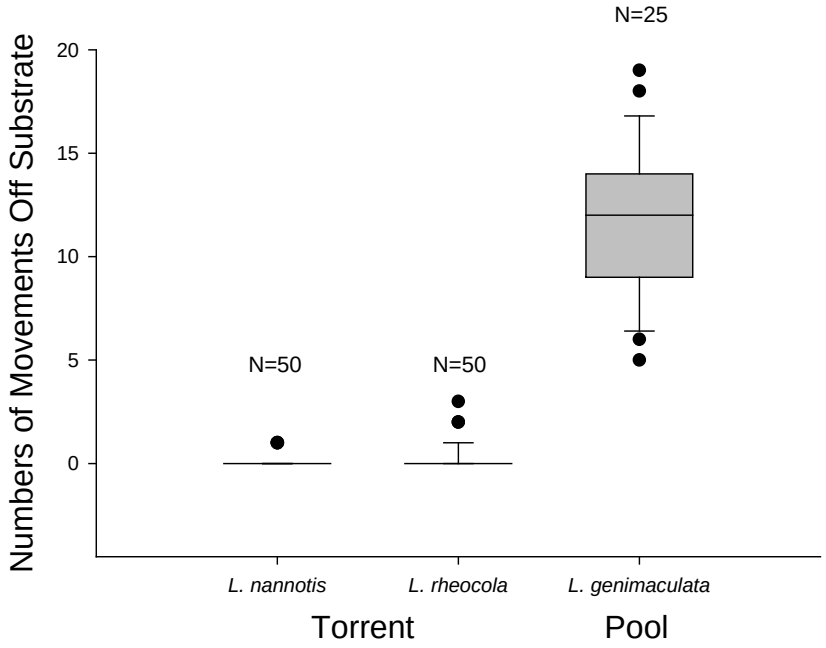


Figure 5.11. Boxplot of numbers of movements off the substrate of *L. nannotis*, *L. rheocola* and *L. genimaculata* over 5 minute observation periods. N = the number of tadpoles observed.

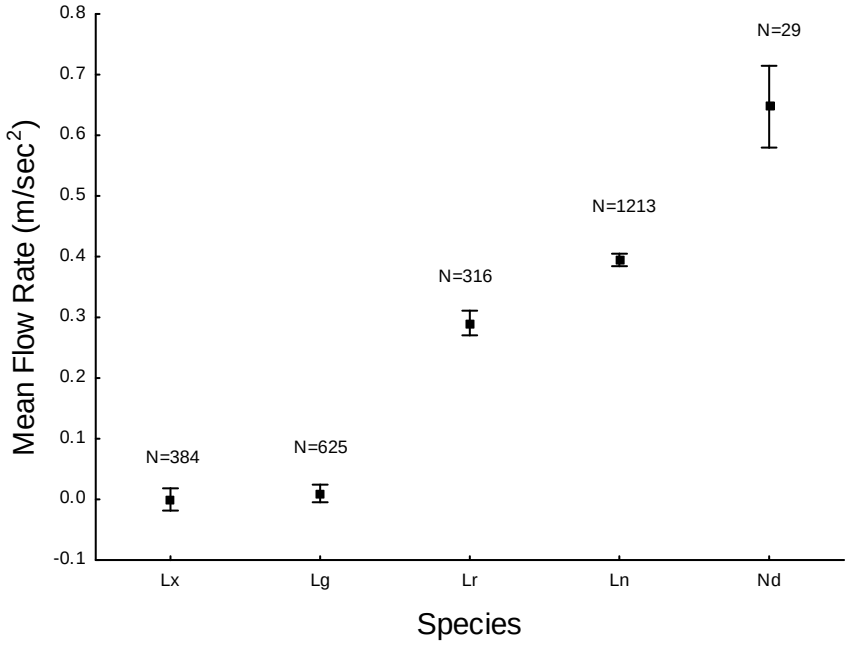


Figure 5.12. Mean water flow rate profile of tadpoles of each species at Tully Gorge National Park and Murray Upper National Park. Boxes represent the mean and whiskers represent the upper and lower 95% confidence intervals. ANOVA, $F_{4,2562} = 655.91$, $p<0.00005$

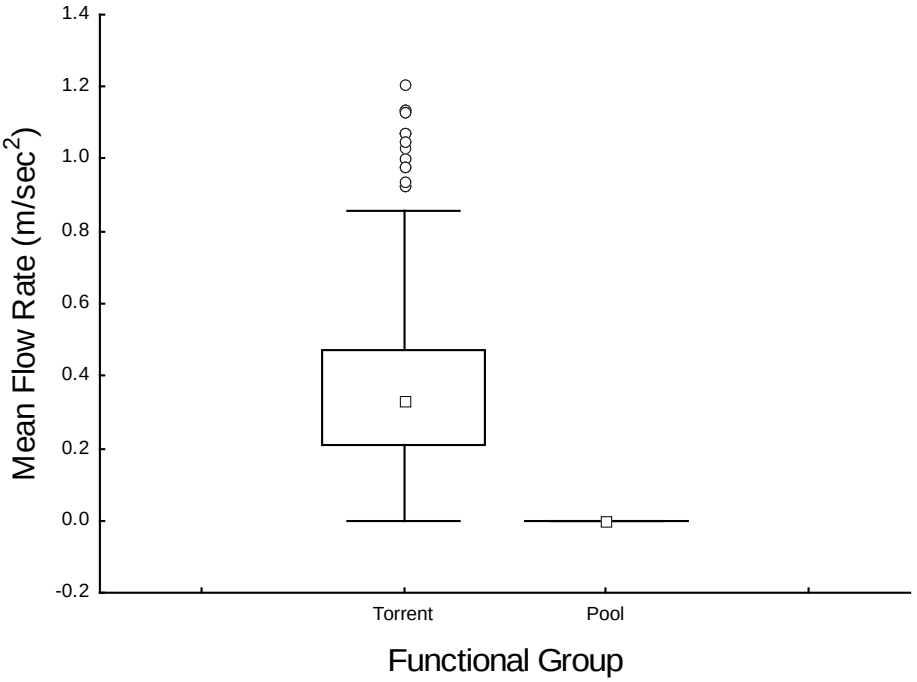


Figure 5.13. Boxplot of mean water flow rate by tadpole functional group, torrent and pool tadpoles at Tully Gorge National Park and Murray Upper National Park. Independent samples T-test, t_{2649} = 49.69, p<0.0005

When combined, tadpoles in the "pool" functional group occupied habitat with significantly slower mean water flow than tadpoles in the "torrent" group (pool: 0.6 cm/s, torrent: 37.8 cm/s; t_{2649} = 49.69, p<0.0005; Fig.5.13). Flow rates occupied by tadpoles did not differ significantly between sites (ANOVA; $F_{1,2148}$ = 0.138, p=0.71), however, there was a significant species X site interaction (ANOVA; $F_{2, 2148}$ = 8.1266, p=0.0003; Fig.5.14). *Litoria nannotis* tadpoles occupied slightly slower flowing water at Tully than at Murray Upper (Tully NP: 39 cm/s, Murray Upper NP: 45 cm/sec, Tukey's HSD; p=0.035). Occupied flow rates did not differ significantly between sites for *L. rheocola* (Tukey's HSD; p=0.627) or *L. genimaculata* (Tukey's HSD; p=0.999). *Nyctimystes dayi* was not included in the species X site interaction analysis as there were too few tadpoles captured at Murray Upper for a valid statistical comparison. At Tully, there was no significant difference in the velocity of water occupied across size classes of *L. genimaculata* (ANOVA; $F_{5,186}$ = 0.4997, p=0.776), L. *rheocola* (ANOVA; $F_{5,272}$ = 0.7234, p=0.606) or *L. nannotis* (ANOVA; $F_{7,1019}$ = 1.0481, p=0.396; Fig 5.15). High variability in the two smallest size classes is due to small sample sizes.

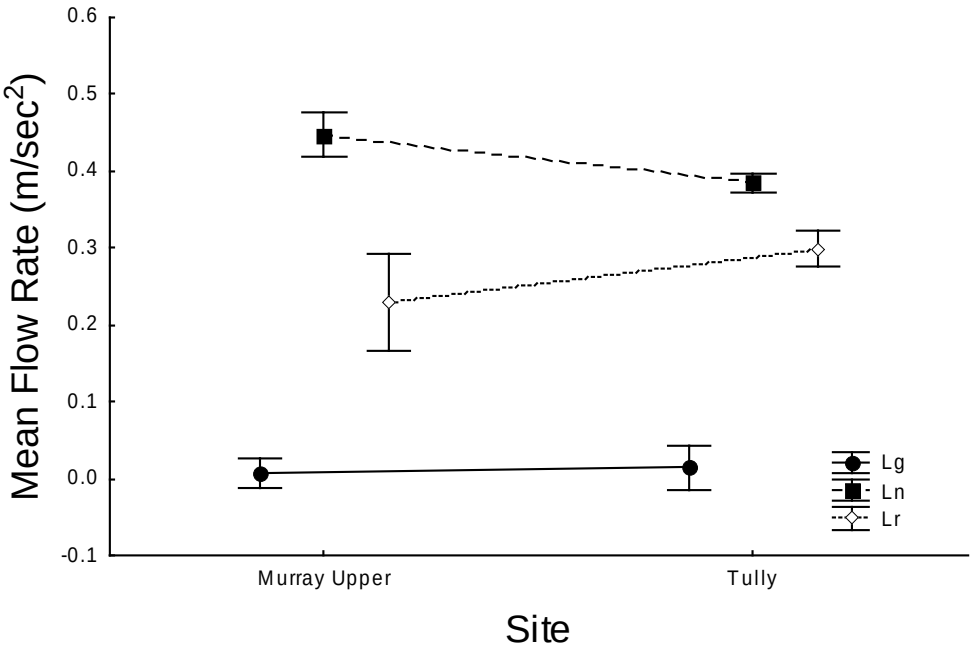


Figure 5.14. Mean water flow profile by site and species. Connecting lines are a visual aid only. Symbols represent the mean and whiskers represent the upper and lower 95% confidence intervals. ANOVA; $F_{2, 2148} = 8.1266$, p=0.0003

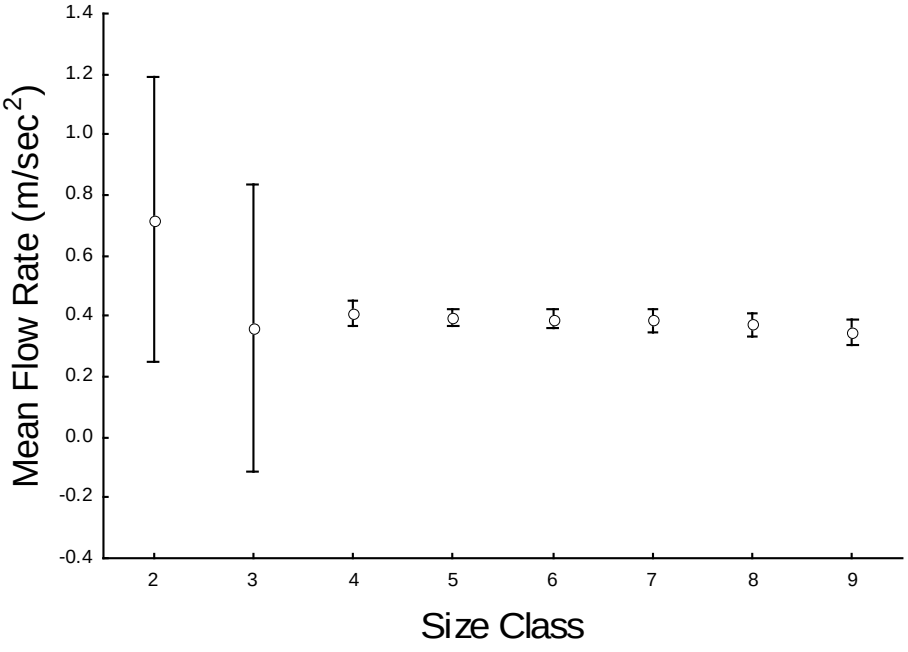


Figure 5.15. Mean water flow rate occupied by *Litoria nannotis* in size classes 2 through 9 at Tully Gorge National Park. Circles represent the mean and whiskers represent 95% confidence intervals. ANOVA; $F_{7,1019} = 1.0481$, p=0.396

Seasonal Size Structure

Litoria nannotis

Recruitment of small *L. nannotis* tadpoles into the population occurred throughout the year, with seasonal peaks over summer and early autumn, between the months of January and April (Fig. 5.14 A). Large tadpoles were present throughout the year, with peaks in relative abundance over spring, between September and November (Fig. 5.16 C). Metamorphosing individuals or recent metamorphs were observed only in October through April of both years (Fig. 5.16 C). Modal progression of size classes suggests that tadpoles that hatched in early summer grew and developed over the winter, and metamorphosed the following spring and summer, taking approximately 9-12 months to develop through metamorphosis. Large tadpoles, at an appropriate size for metamorphosis, but without emergent limbs, were present following the end of the metamorphosis period in April. These large tadpoles would likely overwinter and presumably be among the first to metamorphose the following spring and summer. Overwintering individuals could therefore remain as larvae for 16 months or more. In June, in both 2006 and 2007, there was a slightly bimodal distribution of large and small size classes (Fig. 5.17), possibly representing the larger tadpoles that failed to metamorphose and those resulting from the most recent recruitment of small tadpoles into the population.

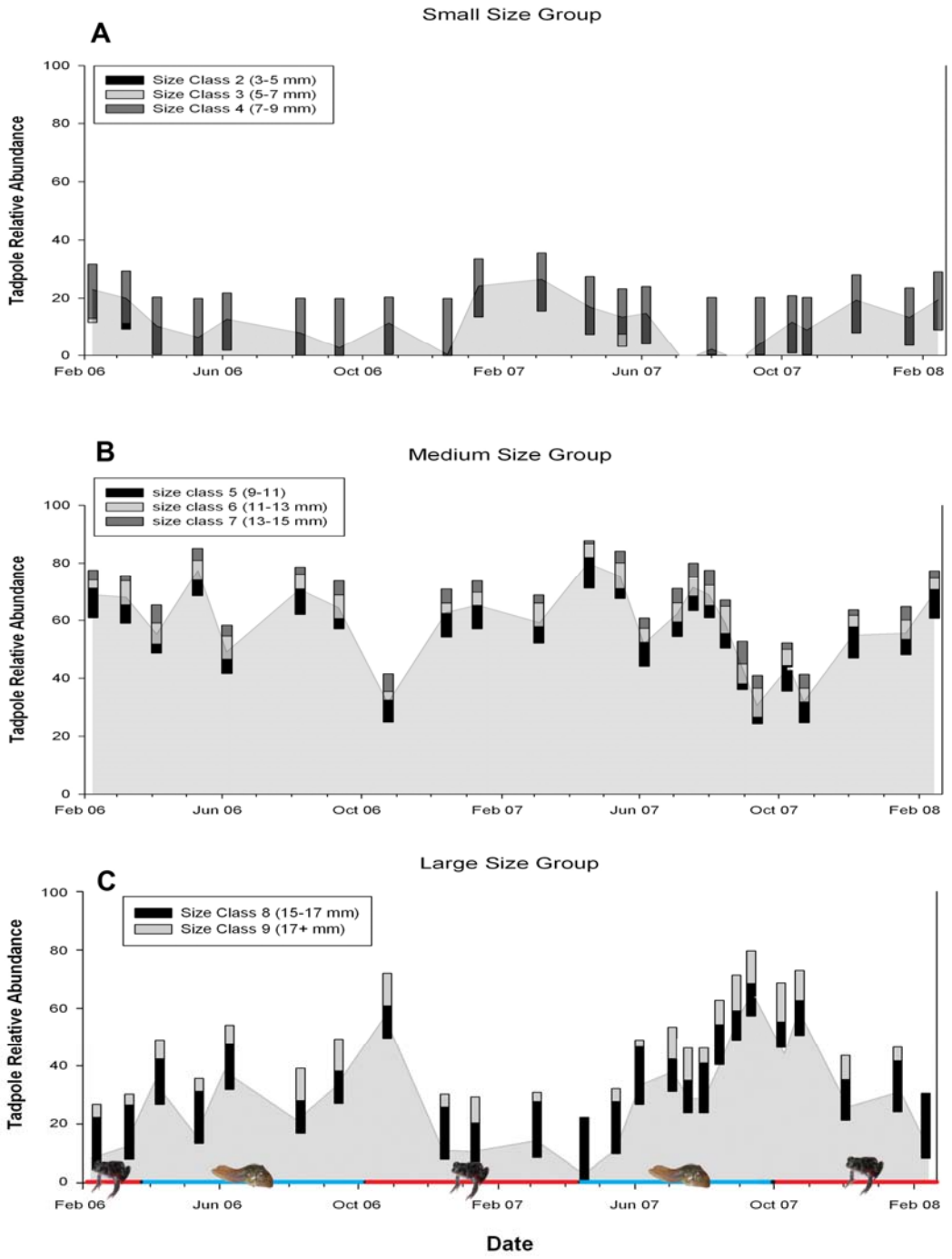


Figure 5.16. Relative abundances of size classes of *Litoria nannotis* tadpoles at Tully Gorge National Park, February 2006 – February 2008. Shaded gray areas represent the relative abundance of each size-group. The stacked bars represent the relative abundance of the individual size classes that comprise the size-group. The horizontal blue bars signify periods of tadpole growth without metamorphosis and the red bars signify periods when metamorphosis occurs.

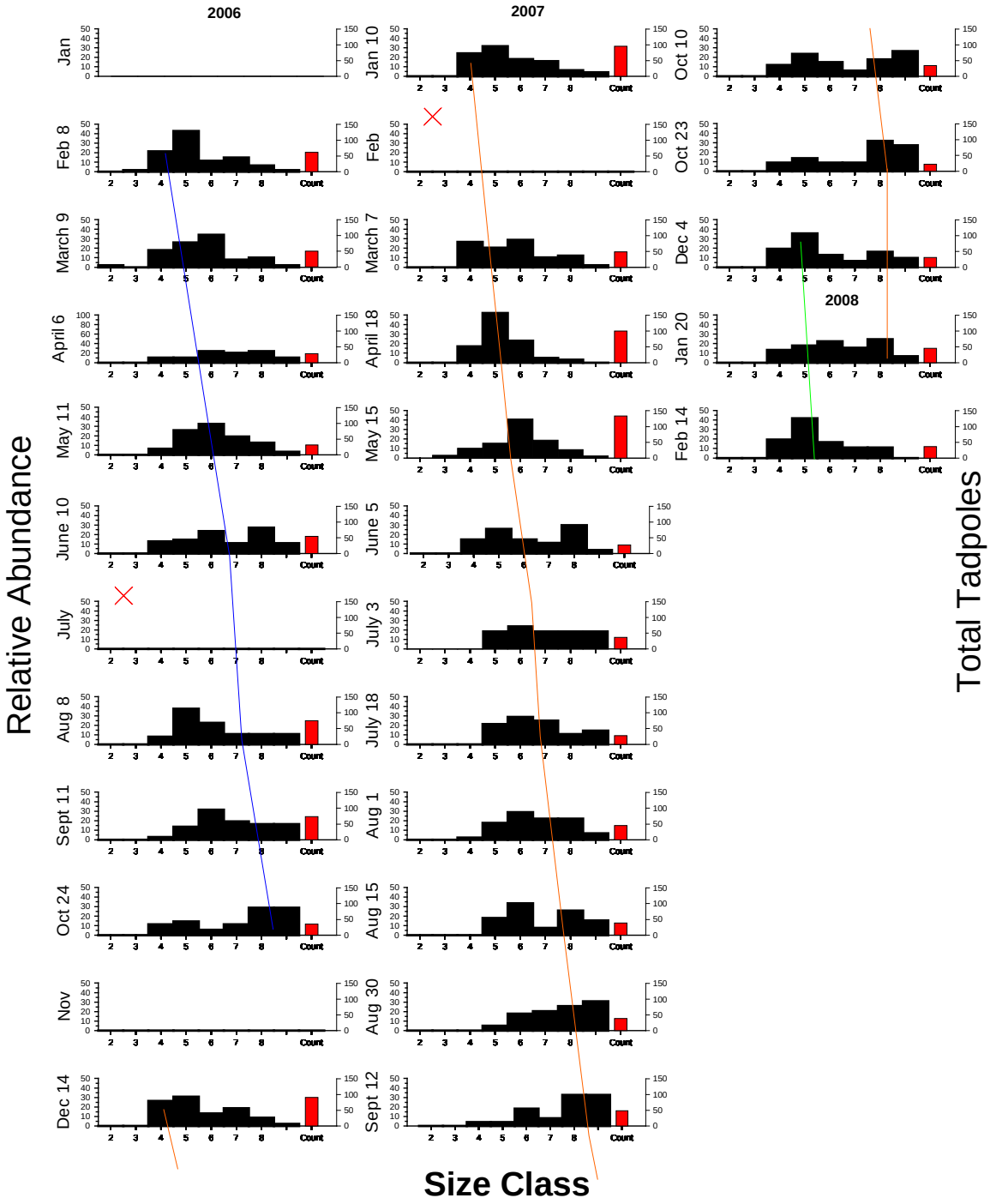


Figure 5.17. Black bars: Size-frequency distributions of *Litoria nannotis* tadpoles at Tully Gorge National Park. Red bars represent the total number of tadpoles captured. Black bars correspond to the left hand Y-axis. Red bars correspond to the right hand Y-axis. Lines represent the modal progression of each year's summer cohort.

Litoria rheocola

Tadpoles of *L. rheocola* were less abundant at all times over the 2 y sampling period than *L. nannotis.* As were *L. nannotis*, *L. rheocola* tadpoles were present in a range of size classes throughout the year; however, *L. rheocola* appeared to be more seasonal than *L. nannotis*. Few *L. rheocola* tadpoles were present in the creek between February and April in both years. In May-June, the abundance drastically increased with most captured tadpoles occurring in the small size-group (Fig.5.19). This peak recruitment of small *L. rheocola* tadpoles occurred 1-2 months following the end of peak *L. nannotis* recruitment (January-April; Figure 5.19). Only four metamorphosing *L. rheocola* tadpoles were captured over the 2 y study, however, as with *L. nannotis*, these metamorphs occurred exclusively in summer (between late October and February), coinciding with the period when tadpoles were largest (Fig. 5.19). This data, combined with the modal progression of size classes, suggests that *L. rheocola* tadpoles hatch in late autumn, grow over winter, and metamorphose the following spring-summer, taking between 5-9 months to metamorphose. Thus, like *L. nannotis* it appears that most growth and development of *L. rheocola* occurs over winter. *Litoria rheocola* tadpoles, however, achieve significantly smaller body sizes than *L. nannotis* (Fig. 5.18).

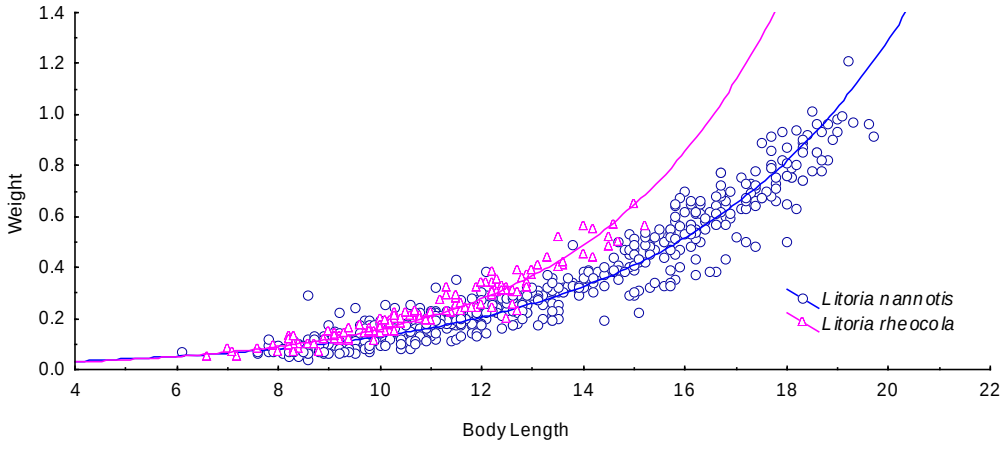


Figure 5.18. Length (mm) vs mass (g) scatterplots for *Litoria nannotis* and *Litoria rheocola*. Lines are to illustrate differences more clearly.

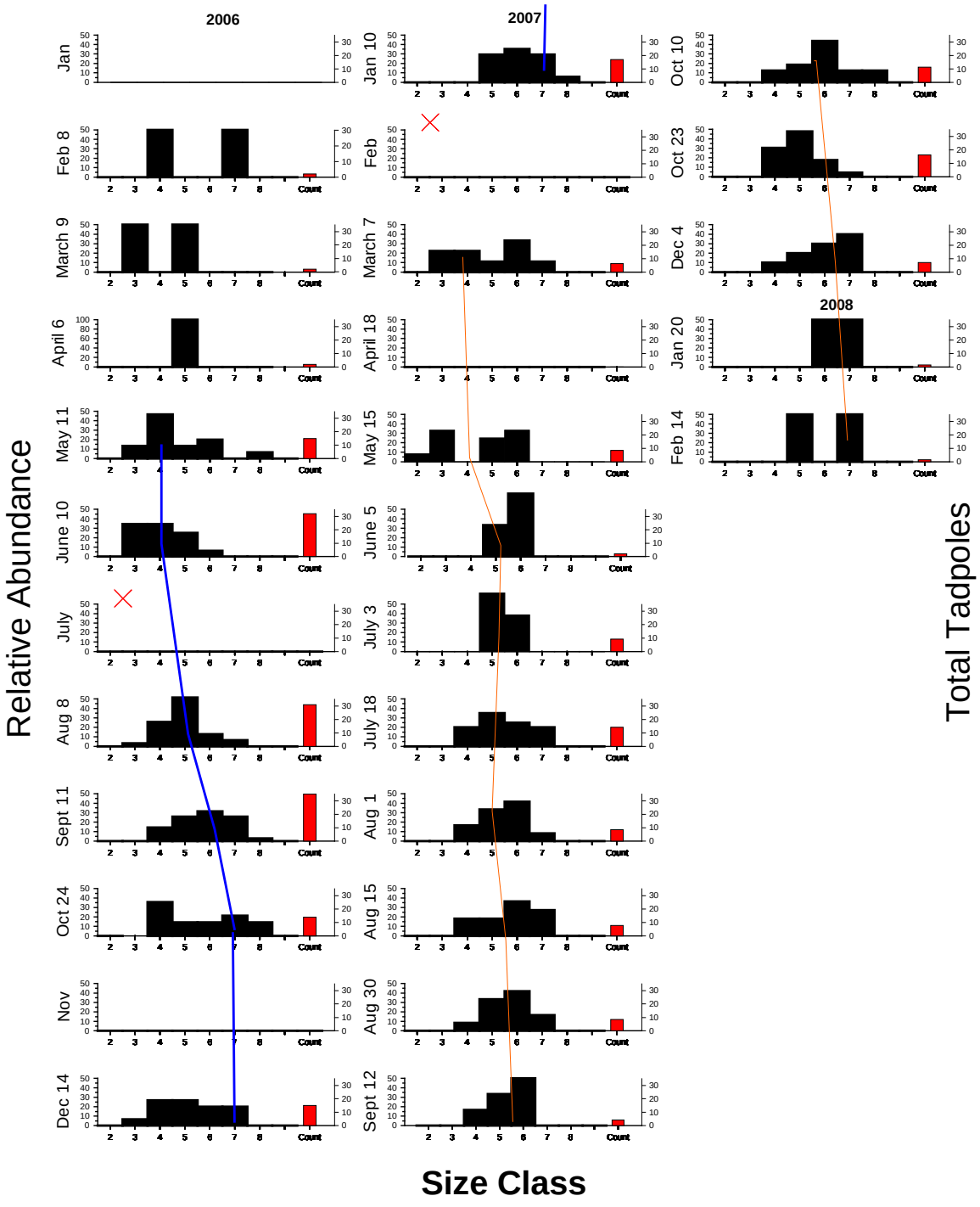


Figure 5.19. Black bars: Size-frequency distributions of *Litoria rheocola* tadpoles at Tully Gorge National Park. Red bars: Total number of tadpoles captured. Black bars correspond to the left Y-axis. Red bars correspond to the right Y-axis. Lines indicate modal progression of tadpoles from each late summer's cohort

Litoria xanthomera

Tadpoles of *L. xanthomera* were present throughout the year except for a 2-3 month period in winter when the isolated stream-side pools were dry (July-September). The modal progression shows very little temporal overlap of cohorts (Fig. 5.20). In total, four distinct size cohorts occurred over the course of the year (Fig. 5.20). It is not known if cohorts were from single or multiple egg clutches. Developmental time of *L. xanthomera* tadpoles appears to be approximately 8-12 wk.

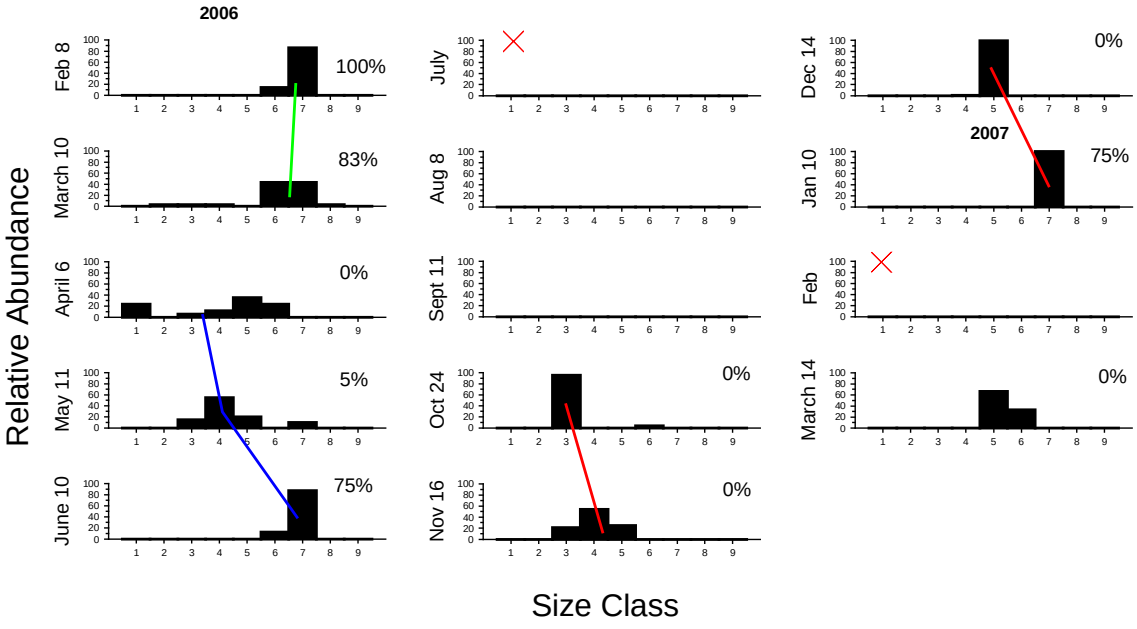


Figure 5.20. Size class distribution of *Litoria xanthomera* tadpoles at Tully Gorge National Park. Percentages in the upper right corner of each graph represent the percentage of tadpoles with emergent limbs. Colored lines indicate developmental cohorts. Red crosses represent sampling periods when data were not collected.

Litoria genimaculata

Tadpoles of *L. genimaculata* were present at intervals throughout the year, however, there were no clear patterns in seasonal size structure (Fig. 5.21 and 5.22).

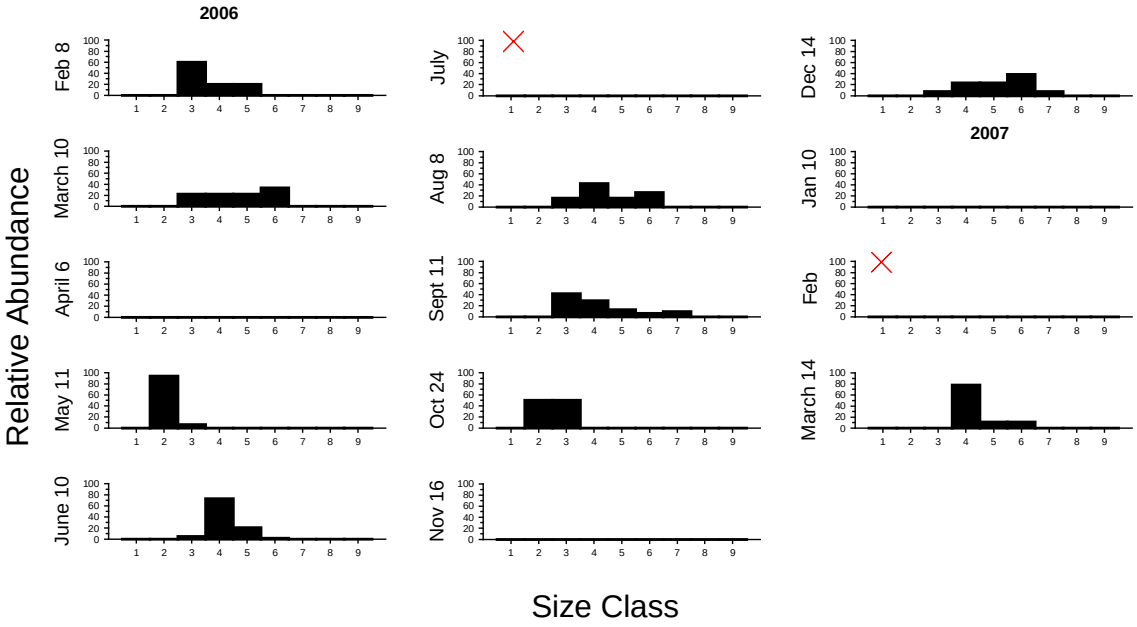


Figure 5.21. Size class distribution of *Litoria genimaculata* in Tully Gorge National Park. Red crosses represent sampling periods when data were not collected.

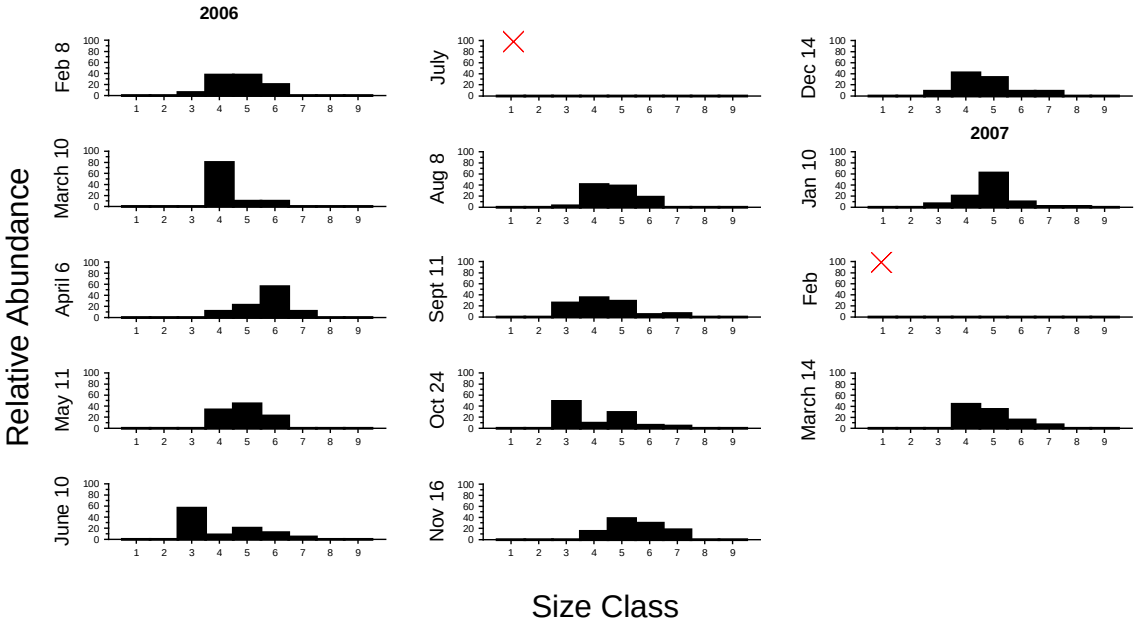


Figure 5.22. Size class distribution of *Litoria genimaculata* in Murray Upper National Park. Red crosses represent sampling periods when data were not collected.

Mark recapture

There was little change in the population sizes estimated using the removal census regression technique between May 15, 2007 (184 tadpoles) and August 16, 2007 (190 tadpoles; Fig. 5.23). The recapture rate of torrent tadpoles three months postrelease was 2.2% (4/180). In the samples taken 24 hr post-release, 7/32 tadpoles (21.9%) collected within the original sampling stretch (50-55 m) were marked. Only 1/26 (3.9%) tadpoles captured upstream (55-60 m) and 0/6 tadpoles captured downstream (45-50 m) were marked. The total numbers of tadpoles captured within the central 5 m and in the two surrounding 5 m sections of the stream are equal (32 in each sample). I calculated a crude estimate of the rate of emigration of tadpoles from that section over 24 h as the ratio of the number of recaptured animals that had emigrated from the central 5 m to the number that remained within the central 5 m (1/7 = 0.1429). Adjusting the available number of marked animals (130) downward by this fraction suggests that at the time of the 24 h postrelease sample, 111 marked individuals should have been available for recapture within the central 5 m. If this was the case, then a simple ratio-based mark recapture estimate of the number of *L. nannotis* tadpoles within the central 5 m at 24 h postmarking would suggest that there were approximately 507 (111*32/7). The fact that this number is roughly 2.5 times the removal estimate of the number available in that section suggests that the data underestimate the rate of emigration over 24 h. However, 3 wk postrelease, 3/105 tadpoles (2.9%) within 45-60 m were marked. This is very close to the proportion (3.9%) that would be predicted to remain if the emigration rate was 14.29%/day, as estimated above.

Overall, the results indicate that *L. nannotis* tadpoles are not highly sedentary, but disperse relatively rapidly on the small spatial scale sampled. They also indicate that, on average, individuals that leave an area are replaced by immigrants, maintaining a relatively constant density. The larger-scale spatial behaviour of *L. nannotis* tadpoles remains unresolved. The fact that the percentage of individuals that remained in the central 5 m after 3 wk agreed reasonably closely with the percentage that should have remained if all individuals had a constant probability of emigration per unit time

suggests either of two hypotheses regarding *L. nannotis* tadpoles. Either they are nomadic, lacking fixed areas of occupancy within the stream, and simply move with some combination of current, habitat availability, and resource availability, or they have very large areas of occupancy within which they move. The results of the sample taken three months after the initial sample suggest that the latter hypothesis may be correct; because four of the original 130 tadpoles were found in the original 5 m sampling area after 3 months, if survival over that period had been 100%, this would suggest that the original 130 tadpoles were drawn from a population of individuals inhabiting approximately 165 m of the creek, and had dispersed evenly over that distance after 3 months. Since survival was almost certainly less than 100%, the actual area occupied by tadpoles is likely to be less than this, on the order of 50 m of the creek. This is, of necessity, a very loose approximation, based on little data. It is also possible that individual tadpoles vary, with many being nomadic and a few highly sedentary.

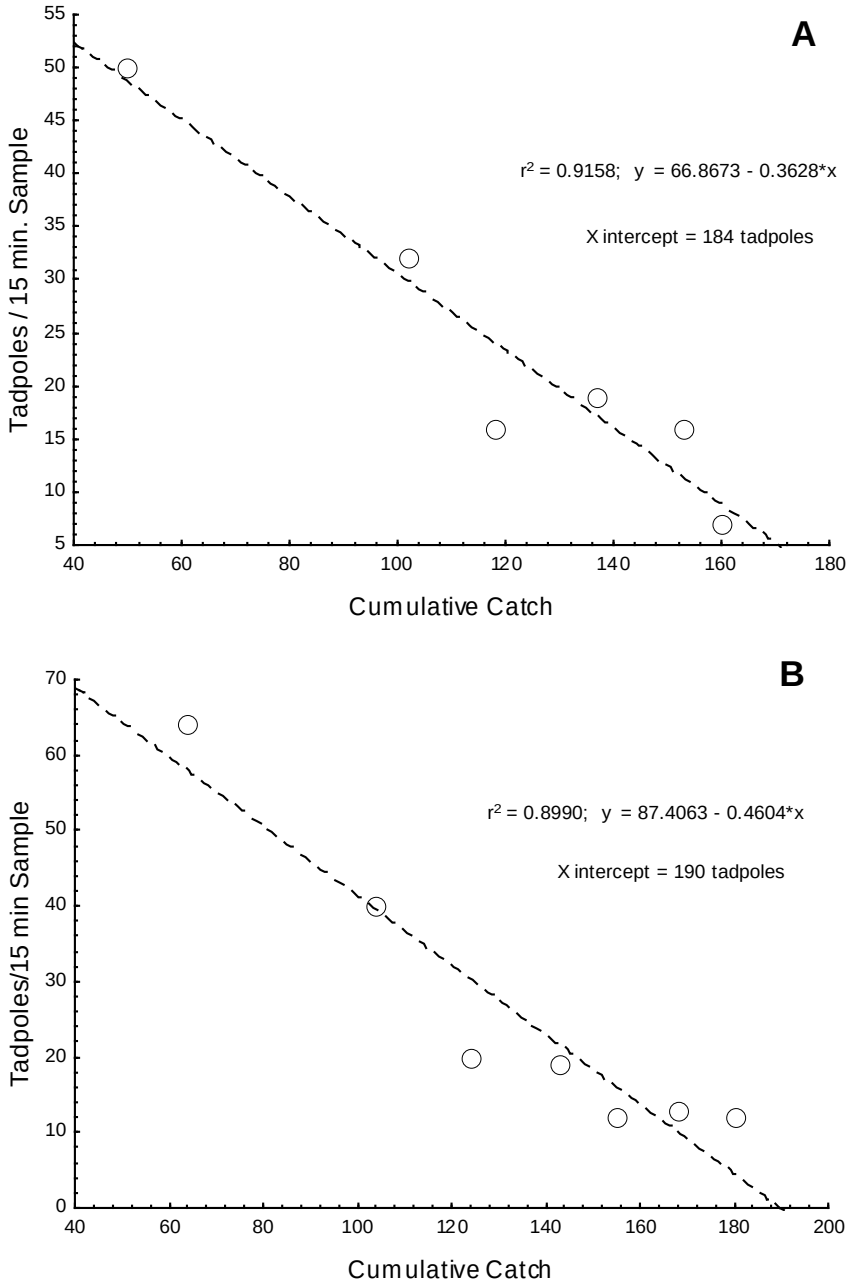


Figure 5.23. Population estimate of torrent tadpoles in a 5 meter stretch of stream at Tully Gorge NP before and after peak mouthpart loss. A) May 15, 2007. B) August 16, 2007

Discussion

The tadpoles within this rainforest stream assemblage varied significantly in their use of space by habitat type (isolated pools, connected pools and torrents) and within habitat type (torrents) by water velocity. Peaks in tadpole abundance within torrents also varied temporally as *L. rheocola* hatched approximately 2 months later than *L. nannotis*. Development in *L. nannotis* was seasonal, with a discernable cohort that hatched in summer/early fall and developed over the ensuing year, metamorphosing the following summer. Torrent tadpoles (*L. nannotis*, *L. rheocola* and presumably *N. dayi*) maintained close contact with the stream substrate almost continuously while *L. genimaculata* were more active in the water column. Dispersal rates of *L.nannotis* were high, indicating a population with a high level of mixing on at least an intermediate (ca. 100 m of stream) spatial scale.

The suctorial morphology allows tadpoles to exploit the available resources present in high energy streams. The mechanism of adhesion has not been studied in Australian species, however, it has been studied in the functionally similar tadpoles of the North American *Ascaphus truei*. In these tadpoles the water trapped between the oral disc and substrate is sucked through the buccal cavity and out the spiracle, creating a negative pressure that draws the oral disc tightly to the substrate surface (Gradwell, 1971, Cannatella, 1999). Tooth rows, particularly the two outermost rows, appear to aid in adhesion (Altig and Johnston, 1989). Once adhered to the rock, an oral valve seals the mouth from within, permitting respiration through the nostrils and movement ("mouth-hitching") across the substrate without compromising the partial vacuum (Gradwell, 1971). Mouth-hitching across rocks is accomplished by a reduction in suction by partially opening the oral valve, combined with a forward movement of the upper and lower labia (Cannatella, 1999, Gradwell, 1971). I have observed *L. nannotis* tadpoles advancing up rocks of a small waterfall under a thin film of water (Figure 5.7 A). To move longer distances tadpoles will release suction and enter the current before reattaching to substrate downstream or slide across the rock surface before reengaging. In slower flows they may use their strong tails to swim in short bursts (pers. obs.).

Hitching is also a primary method of feeding as the labial tooth rows and jaw sheath scrape against the substrate during movement. Following movement, the transfer of water from the oral disc and substrate interface, through the buccal cavity to reestablish the partial vacuum also acts to draw the newly dislodged material across the filter apparatus and then into the stomach for digestion (Viertel and Richter, 1999). I frequently observed *L. nannotis* and *L. rheocola* tadpoles hitching in place, repeatedly scraping a particular section of the substrate, apparently feeding. I also observed this behavior in the laboratory, while tadpoles grazed on glass feeding slides covered in algae.

Richards (2002) proposed that pool tadpoles were excluded from torrents by the inability to maintain position in turbulent or fast water. Tadpoles that can occur in fast water vary in their abilities. The three torrent-adapted tadpoles studied here occupied habitats with differing mean flow rates (Fig. 5.12). *Nyctimystes dayi* occupied the fastest flowing water, *L. rheocola* the slowest flowing riffles, and *L. nannotis* was intermediate. Morphological differences among these tadpoles suggest that the ability to maintain position is the factor limiting entry into higher velocity environments (Richards, 2002). *Litoria rheocola* have the least hydrodynamic, flattened body shape, the smallest oral disc and the least developed tail musculature. *Nyctimystes dayi* tadpoles have a more flattened body shape than *L. rheocola,* a very large oral disc that occupies up to 50% of their body (Trenerry, 1988), and the most developed tail musculature with the most shallow fins of the three species. *Litoria nannotis* also have a highly depressed body form, but an intermediate-sized oral disc and intermediately developed tail musculature. While the degree to which tadpoles exhibit these morphological characteristics may control their ability to occur in higher velocity water, the converse does not necessarily follow; it is not clear that being highly suctorial should exclude tadpoles from slow-flowing habitats; however, the more suctorial species *L. nannotis* and *N. dayi* generally avoid these habitats. One possible explanation is predation. The highly suctorially adapted tadpoles studied here give up the ability to swim quickly in still water in favor of the ability to remain attached to

substrates. Their characteristic anitpredator mechanism, which is highly effective in fast-flowing, turbulent habitats, is simply to release suction and allow the current to erratically and rapidly displace them from their positions. This would be ineffective in still water.

Although torrent tadpoles were usually found within a preferred velocity profile, they can be highly dispersive. The recapture rate of marked *L. nannotis* 24 h postrelease was high (~22%), but soon dropped below 3% 3 wk postrelease and did not markedly change after 3 months. During this time the size of the population did not decrease significantly, indicating widespread mortality did not occur.

Pulses of small tadpoles, indicating reproductive peaks, entered the stream in summer, initially in *L. nannotis* and approximately 2 months later in *L. rheocola*. Most tadpoles grew and developed over winter until metamorphosis, which was apparent by a spike and then drop in large tadpole abundance during the subsequent summer. Despite this seasonality, a wide range of size classes was observed throughout the year, indicating that some reproduction occurred at times outside the peak; however, individuals with emergent limb buds and metamorphs were not seen between May and September. This indicates that tadpoles that were not of an appropriate size to metamorphose during the spring/summer period overwintered and metamorphosed the following summer. Metamorphosis during the summer months ensures that froglets emerge in a moist terrestrial environment at a time when invertebrate prey are most abundant (Frith and Frith, 1985, Richards and Alford, 2005, Frith and Frith, 1990)

Litoria genimaculata tadpoles are members of the "clasping" ecomorphological guild (Altig and Johnston, 1989) and were found in connected pools and backwaters with accumulated detritus and slow water flow (<5 cm/s; Figure 5.5). In experimental flow tanks small *L. genimaculata* tadpoles could not physically maintain position in 25 cm/s flow and even large individuals could not withstand 50 cm/s flow or turbulence at any speed (Richards, 2002). *Litoria genimaculata* have ventrally positioned tooth rows that are partially concealed by the upper and lower labia. They have a rounded body form,

relatively weak tail muscle (compared with the torrent adapted tadpoles), and tail fins of medium depth (Figure 5.7). They feed by rasping and picking at the substrate, including leaf packs (Iwai et al., 2009) and probably by filtering particulates from the water column (Alford, 1999). During timed observations, *L. genimaculata* tadpoles frequently left the substrate to swim to nearby locations or to remain suspended in the water column, presumably filter feeding (Figure 5.3). Trenerry (1988) speculated that dispersal in *L. genimaculata* is low, with individuals remaining within a single pool until metamorphosis, death or possible flushing during heavy rains.

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

In summary, the tadpoles within these rainforest streams occupy a series of distinct microhabitats and display morphologies and behaviours suited to these habitats. The torrent-adapted tadpoles *L. rheocola* and *L. nannotis* displayed seasonality with obvious peaks in recruitment and metamorphosis. These microhabitat and behavioral differences among species, differences in rate of development, and seasonal variation in species abundance, are likely to affect the disease processes within this multi-host system. In subsequent chapters this data on the ecology of the tadpole assemblage will be used to interpret patterns of *Bd* prevalence, intensity and pathology.

Chapter 6 : Dynamics of *Batrachochytrium dendrobatidis* Infection in Rainforest Stream Tadpoles

Introduction

Many diseases of wildlife are highly seasonal; infection rates, prevalence, and intensity of infection change in predictable cycles (Hosseini et al., 2004). This seasonality can reflect a variety of changes in host-pathogen interactions, including changes in host behavior, 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). These processes govern both pathogen transmission to naïve individuals and the subsequent proliferation of infection on the host. The degree to which each occurs determines the severity and scope of disease within a population. It is therefore crucial to first understand the underlying processes that determine prevalence and intensity of infection before designing conservation strategies.

The recently emerged and highly virulent pathogen of amphibians *Batrachochytrium dendrobatidis* (*Bd*) poses a global threat to amphibian biodiversity (Beebee and Griffiths, 2005, Fisher et al., 2009, Lips et al., 2006). However, little is known of the factors driving infection and intensity, particularly in the tropics where declines have been most severe.

Batrachochytrium dendrobatidis is a parasitic fungus with flagellated aquatic zoospores that requires moisture, ceases growth in culture at 28º C, is not known to enter a resting stage and is readily transmitted via water (Piotrowski et al., 2004, Longcore et al., 1999, Berger et al., 1998, Rachowicz and Vredenburg, 2004, Berger et al., 2005a). These characteristics and physiological requirements of the pathogen likely explain why amphibians with a stronger association with water are at a greater risk of decline and disappearance (Lips et al., 2003, Hero et al., 2005, Mahony, 1996, Williams and Hero, 1998, Bielby et al., 2008, Brem and Lips, 2008, McDonald and Alford, 1999). In tropical rainforests, declines appear most severe in riparian species, however, pond and terrestrial species can be affected as well, indicating transmission can extend well

beyond the stream, particularly during epizootics (Brem and Lips, 2008, Lips et al., 2006, McDonald and Alford, 1999, Williams and Hero, 1998). As *Bd* becomes enzootic, transmission appears to contract closer to the water bodies, as suggested by higher prevalence in more aquatic frogs (Brem and Lips, 2008). This combined evidence suggests that water bodies, and streams in particular, form a "central artery" of *Bd* transmission in rainforests.

While streams may be an active zone of *Bd* transmission, with many opportunities for exposure of uninfected animals to infectious zoospores, theory indicates it is unlikely for species extinction to occur without the presence of a reservoir host or saprobic life stage to keep transmission rates high as species decline (Boots and Sasaki, 2002, de Castro and Bolker, 2005). To date, efforts to identify a non-amphibian reservoir have been unsuccessful (Rowley et al., 2007; Chapter 8, Rowley et al., 2006) and the physiology of *Bd* suggests that it may be amphibian-specific (Berger et al., 2005a). However, non-amphibian reservoirs may not be necessary to cause extinction, because although nearly all amphibians can become infected with *Bd*, not all are equally vulnerable, and some can carry sublethal infections (Daszak et al., 2004).

Stream tadpoles may be a highly effective reservoir, as they are susceptible to infection but do not usually die as a result, and negative environmental effects on *Bd* will typically be lower in the aquatic environment. Many terrestrial habitats are too warm or too dry for *Bd* survival (Ron, 2005, Puschendorf et al., 2009) and seasonal or even daily temperature fluctuations can affect prevalence and intensity of infection (Woodhams and Alford, 2005, Kriger and Hero, 2007) either by acting 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, Richmond et al., 2009). In contrast, tropical stream larvae reside within an environment that is typically cooler and more thermally consistent. Therefore, infection dynamics in these tadpoles should be less variable than in the terrestrial stage, which could allow *Bd* to persist or thrive, even when prevalence and intensity is decreasing in terrestrial adults. In many streams, tadpoles are present continuously throughout the year, as opposed to pool and pond systems where tadpoles

are typically not present year-round (Alford, 1999). This may allow stream tadpoles to be a more consistent pathogen reservoir and could help explain why non-terrestrial frogs in the Australian wet tropics 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). Similarly, the extended larval development times in two out of ten species in Peñalara National Park in Spain were offered to explain why these were the only two species to have suffered declines (Bosch et al., 2001, Bosch and Martinez-Solano, 2006). The number of tadpoles present at a site may also be an important factor in determining the probability of declines. Data-based models predict that increased numbers of larvae increase the likelihood of species extinction by increasing the rate of transmission (Mitchell et al., 2008, Briggs et al., 2005). Thus, the picture of *Bd* dynamics and transmission within an amphibian assemblage is incomplete without information on both the terrestrial and aquatic life stages. Infection in the larval stage, however, remains largely unstudied.

This chapter describes the epidemiology of enzootic chytridiomycosis in tadpoles. I monitored the dynamics of *Bd* infections and host populations in tadpoles of five species in two rainforest streams over two years. As an epidemiological study I draw together observations, patterns, statistical correlations, experimental results, and data from published studies to generate a conceptual model of the factors affecting health and illness, and prevalence and intensity of *Bd* in tadpoles.

Materials and Methods

Field Sampling and Measurements

See Chapter 5 Materials and Methods for description of field sampling and measurements.

Swabbing and Real Time Taqman Quantitative PCR

Infection of tadpoles occurs within the keratinized oral structures of the mouthparts and associated tissues. Infection can spread to the epidermis during metamorphosis as

keratinized cells form (Marantelli et al., 2004). Currently the only way to non-destructively sample tadpoles for *Bd* infection is by swabbing the mouthparts and using PCR to test for the presence of *Bd* (Retallick et al., 2006, Boyle et al., 2004). Following capture by dip-net, tadpoles were placed in a sorting tray and then quickly transferred to individual clickseal bags, avoiding any direct handling (Fig 5.8, photo of tadpole in bag). Each tadpole was handled with well-rinsed vinyl gloves to prevent mortality and gloves were changed between individuals to prevent disease transmission (Cashins et al., 2008a). For swabbing, 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; eight times horizontally across the upper and lower tooth rows and jaw sheath and eight times vertically across all rows for a total of 24 strokes. During 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 15 min to confirm their unaffected condition 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 *Bd* 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 min at 16.1 x 10^3 RCF. Extraction tubes were incubated at 100 °C for 10 min, cooled at room temperature (23 °C) for 2 min and then centrifuged at 16.1 x 10^3 RCF for 1 min 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). Standard dilutions were 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 (Hyatt et al., 2007).

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 $\log_{10}$ transformed prior to analysis as the data showed a highly skewed distribution ranging from 1 to 36,000. Uninfected tadpoles were excluded from analyses of intensity of infection. Statistical analyses and graphs were performed using Statistica 7 (StatSoft), SigmaPlot 10 (Systat) and SPSS (SPSS Inc.). Prevalence values were compared using chi square. Yates' continuity correction was used in 2x2 tables. Correlations between intensity and prevalence or density of infected tadpoles were calculated using time series cross correlation analyses. *Nyctimystes dayi* and *Litoria xanthomera* were both only found at Tully Gorge National Park, and therefore could not be included in comparisons between sites.

Logistic Regression Model (Infection status)

Following analysis of the intensity and prevalence of infection, 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 used to construct a binomial logistic regression model using SPSS (version 16, SPSS Inc.). The dichotomous dependent variable was infection status. Models were constructed as recommended 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 maximum temperature and 2. at minimum temperature). All continuous variable data (except flow rate) were averaged over the previous 7, 14, 21 and 28 d 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 collected on site during each sampling block. All 14 variables with 66 total versions (i.e. 7, 14, 21 and 28 d) 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 versions, the one with the lowest Wald statistic, or best predictive value, was selected. The following variables were selected: site, species, body size class, 7 d mean air temperature, 7 d mean water temperature, 28 d mean rainfall, and mean flow rate. All variables were examined for collinearity. 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 more accurately reflect stream-level conditions, compared to rainfall data which was interpolated from area weather stations. Rainfall was therefore eliminated. A model was fit to the remaining five variables. Any variable that did not significantly predict infection status was removed. Each pair-wise interaction was then added to the model individually and tested for significance ($p<0.05$). 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 using a model chi-square test. The Hosmer and Lemeshow goodness-of-fit test was used to determine whether the predicted data fit the observed values and Cox and Snell R^2 and Nagelkerke R^2 values 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*, a linear regression model of intensity of infection was only constructed for *L. nannotis*, the species with the largest available sample size. Only infected (≥ 2 PCR positive wells) tadpoles were included in the analysis. Variables were selected *a priori* 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 $\log_{10}$ transformed as the data were strongly positively skewed (4.65) and leptokurtotic (26.76). Following transformation, values of skewness and kurtosis were -0.326 and -0.784 respectively. The *a priori* selected independent variables included water temperature, air temperature, body length, water flow rate, solar radiation, rainfall, and site. Rainfall was positively skewed and was thus $\log_{10}$ transformed to achieve normality. A univariate regression analysis was performed on each version (i.e. 7,14, 21, and 28 d means) of all selected variables. The version with the highest R^2 value from each variable was included in the multiple regression analysis. In the univariate analyses, air temperature was not a significant predictor and was eliminated from further consideration. Mean 28 d rainfall (R^2: 7.9%), mean 28 d minimum water temperature (R^2: 5.2%), site (R^2: 5.0%), body length (R^2: 4.5%), mean 28 d solar radiation (R^2: 2.6%) and mean water flow rate (R^2: 1.1%) were all significant univariate predictors. These variables were included in an initial multiple regression model; in this model mean flow rate was no longer significant and was removed and the model was refitted. The final suite of independent variables was checked for bivariate correlations using Pearson product-moment correlation and for multicollinearity using the Tolerance statistic (T) and the variance inflation factor (VIF). Variables were considered correlated if $r > 0.7$ and to display multicollinearity if $T < 0.1$ and $VIF > 10$. A total of

806 cases were available for analysis. The overall predictive ability of the model is presented via the R^2 statistic. The contribution of each of the independent variables was assessed via the standardised beta coefficient values.

Results

Prevalence

A binary logistic generalized linear model with infection status as the dependent variable and site, species and the site x species interaction as model factors was created. There was a significant effect of species (Wald chi-square = 186.152, $p < 0.0005$, df = 4; Fig. 6.1) and site (Wald chi-square = 4.218, $p = 0.04$, df = 1; Fig. 6.2), but not the site x species interaction (Wald chi-square = 1.730, $p = 0.421$, df = 2) on infection status. A series of pair-wise chi-square tests revealed that torrent-adapted tadpoles had a higher prevalence of infection than pool-adapted tadpoles (Table 6-1). Within the torrent-adapted group, *N. dayi* had a lower prevalence than either *L. nannotis* or *L. rheocola*. Prevalence did not differ between sites in *L. genimaculata* or *L. rheocola*, however, *L. nannotis* had a higher prevalence at Murray Upper NP (Table 6-1, Fig. 6.2).

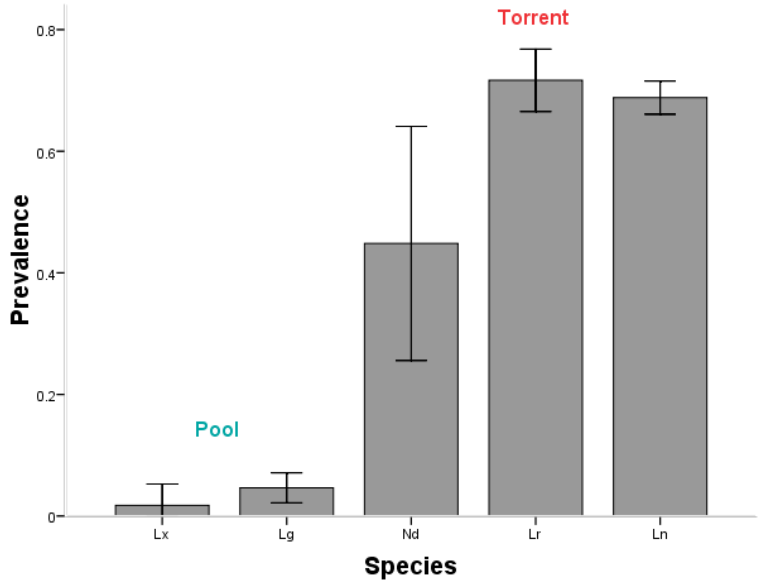


Figure 6.1. Mean prevalence of *B. dendrobatidis* +/- 95% CI in tadpoles of five species over two years (2006-2008), data for Murray Upper NP and Tully Gorge NP combined.

Table 6-1. Chi square comparisons of prevalence between tadpoles of all species and between sites within species.

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

* significant difference

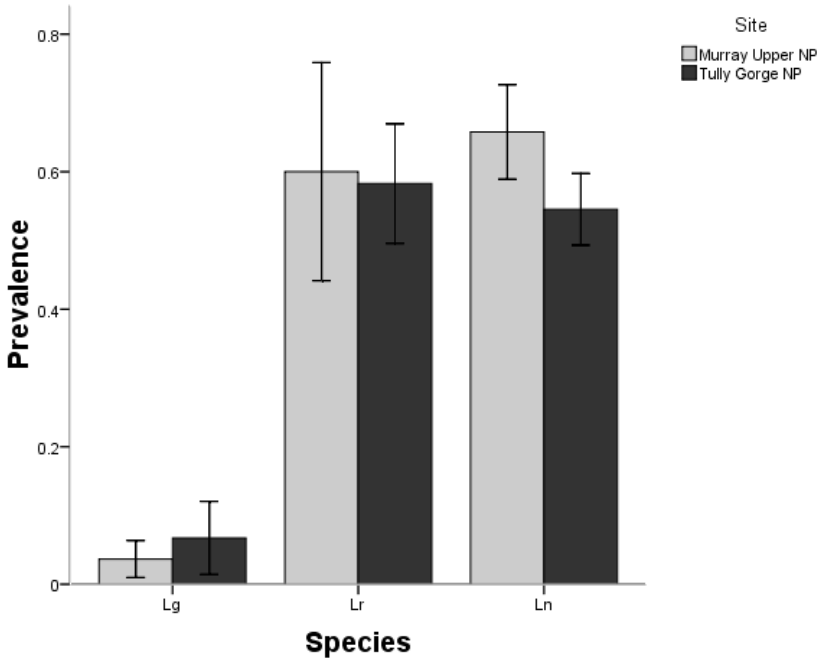


Figure 6.2. Mean prevalence +/- 95% CI of *B. dendrobatidis* infection in tadpoles by site (2006-2007).

Prevalence by Size Class

Prevalence in both *L. nannotis* and *L. rheocola* increased with tadpole size across seasons (Fig. 6.3). Prevalence was low in the smallest tadpoles, substantially higher in the mid-range size classes, and slightly higher again in the largest tadpoles. The decreasing rate of change in prevalence as tadpoles increased in size suggests that transmission occurs primarily in the smaller size classes, and that tadpoles are unlikely to lose infections. Prevalence in *L. genimaculata* is also higher in larger tadpoles (independent samples t-test: $t = -4.580$, $p < 0.0005$, $df = 278$; Fig. 6.5), however, prevalence only increased in the largest two size classes (Fig. 6.4). Prevalence in *L. xanthomera* was very low across all size classes.

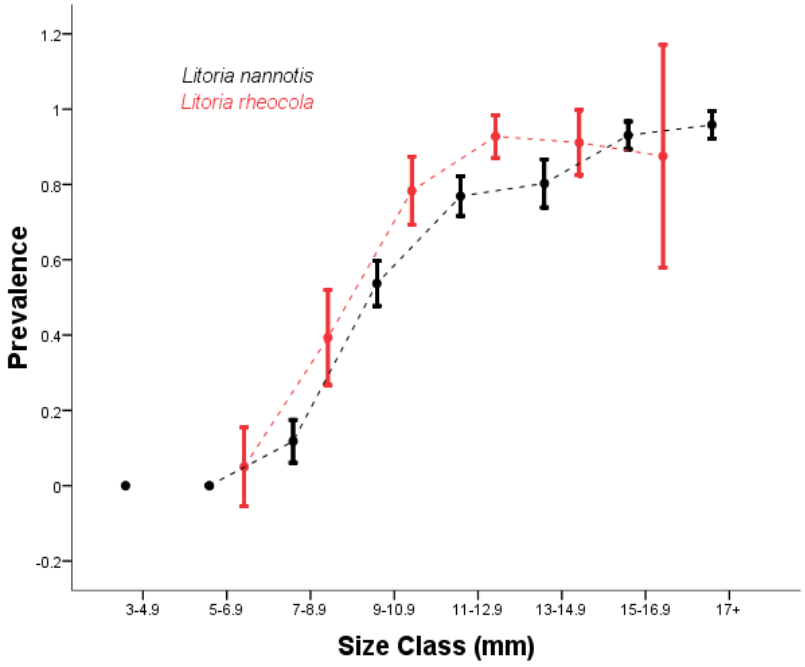


Figure 6.3. Mean prevalence +/- 95% CI of *B. dendrobatidis* in *L. nannotis* and *L. rheocola* by size class. Tully Gorge NP and Murray Upper NP (2006-2008). Connecting lines are for visualization.

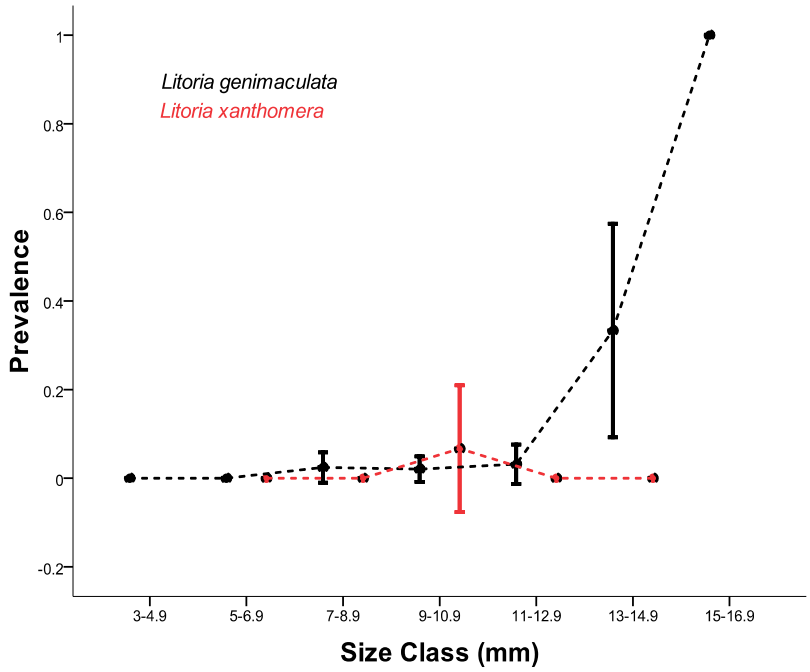


Figure 6.4. Mean prevalence +/- 95% CI of *B. dendrobatidis* in *L. genimaculata* and *L. xanthomera* by size class. Tully Gorge NP and Murray Upper NP (2006-2008). Connecting lines are for visualization.

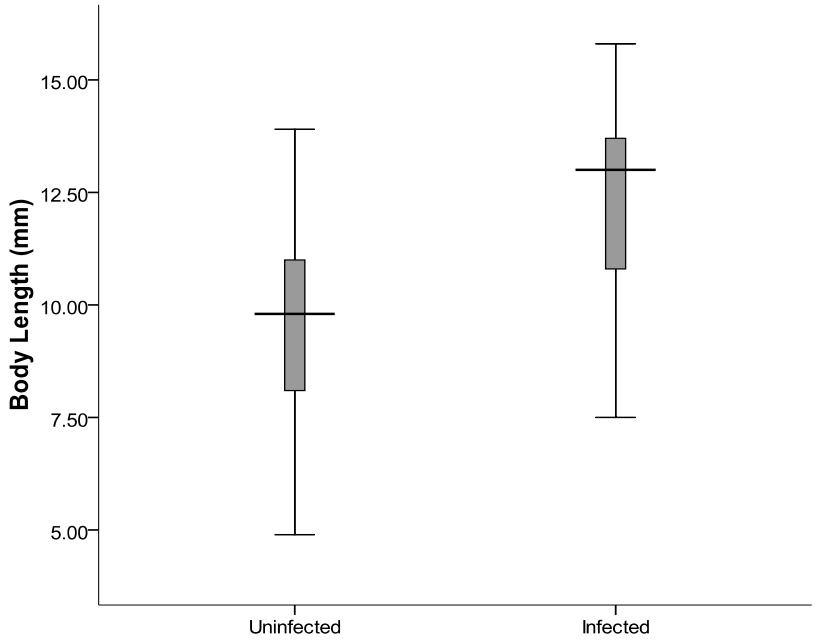


Figure 6.5. Mean body length of infected and uninfected *L. genimaculata* tadpoles. Independent samples t-test: t=-4.580, df=278, p<.0005

Prevalence by Water Temperature

Independent samples t-tests indicated 7-day mean water temperature was significantly cooler for infected tadpoles of *L. nannotis* (t = 11.076, p <0.0005, df = 988), *L. rheocola* (t = 4.467, p <0.0005, df = 286) and *N. dayi* (t = 3.219, p = 0.004; Fig. 6.7) compared with uninfected individuals. No significant difference in water temparature was detected between infected and uninfected *L. genimaculata* tadpoles (t = -0.586, p = 0.558, df= 222; Fig. 6.7)

Yearly water temperature extremes ranged between approximately 15 and 25 ºC (Figs. 6.8 – 6.9) with a mean of approximately 20 ºC. For this analysis, I considered "winter" the period when mean temperature was below 20 ºC (May1-September 30) and "summer" the period when mean water temperature was above 20 ºC (October 1-April 30). There were significant differences in prevalence of infection within size classes of *L. nannotis* between seasons. Prevalence was significantly higher in winter than in

summer in tadpoles larger than 9 mm, except size class 15.0-16.9 mm which was near significant (Fig. 6.6, Table 6-2). No tadpoles smaller than 7 mm were captured in winter and there was no difference in prevalence between winter and summer in tadpoles sized 7.0-8.9 mm (Fig. 6.6).

Table 6-2. Chi squared tests of prevalence of *Bd* infection in *L. nannotis* between seasons (as determined by water temperature) within size classes.

	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

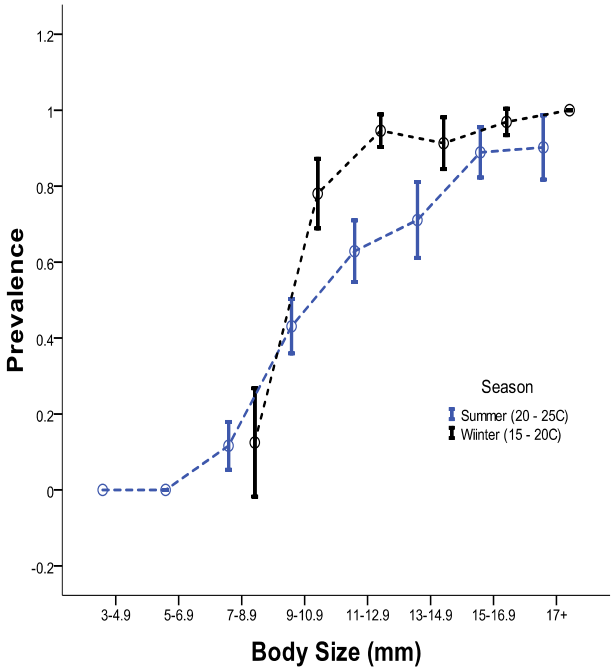


Figure 6.6. Mean prevalence +/- 95% CI of *B. dendrobatidis* in *L. nannotis* by size class and season (as defined by water temperature). Tully Gorge NP and Murray Upper NP (2006-2008). Connecting lines are for visualization.

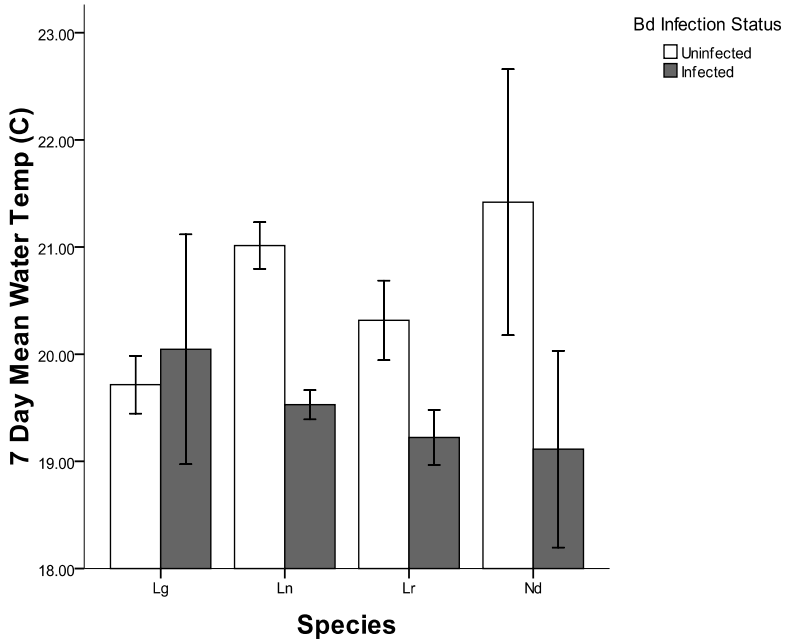


Figure 6.7. Bar graphs illustrating 7-day mean water temperature +/- 95% CI for *B. dendrobatidis* infected and uninfected tadpoles of *L. genimaculata, L. nannotis, L. rheocola, N. dayi*.

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 (Figs. 6.8-6.9). Periods of increased prevalence in pool tadpoles are associated with the presence of larger *L. genimaculata* tadpoles (Fig. 6.5). 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 predictable 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. 6.8 – 6.9). This seasonality, however, is primarily determined by changes in prevalence in the medium size-group tadpoles (Fig. 6.11). Prevalence in small tadpoles remains low throughout the year and prevalence in large tadpoles remains high throughout the year, with the notable exception of a decrease in summer between January and March of each year. It therefore appears that transmission occurs primarily in medium sized tadpoles, as suggested by figure 6.3. The seasonal decrease in prevalence occurs during the period when large tadpoles metamorphose and exit the population and new, small tadpoles hatch and enter the population (Chapter 5 and Fig. 6.10). The decrease in prevalence within the medium size-group is therefore caused by the largest, infected tadpoles exiting into the large size-group and new, uninfected tadpoles entering from the small size-group (Fig. 5.16 and Fig. 6.11).

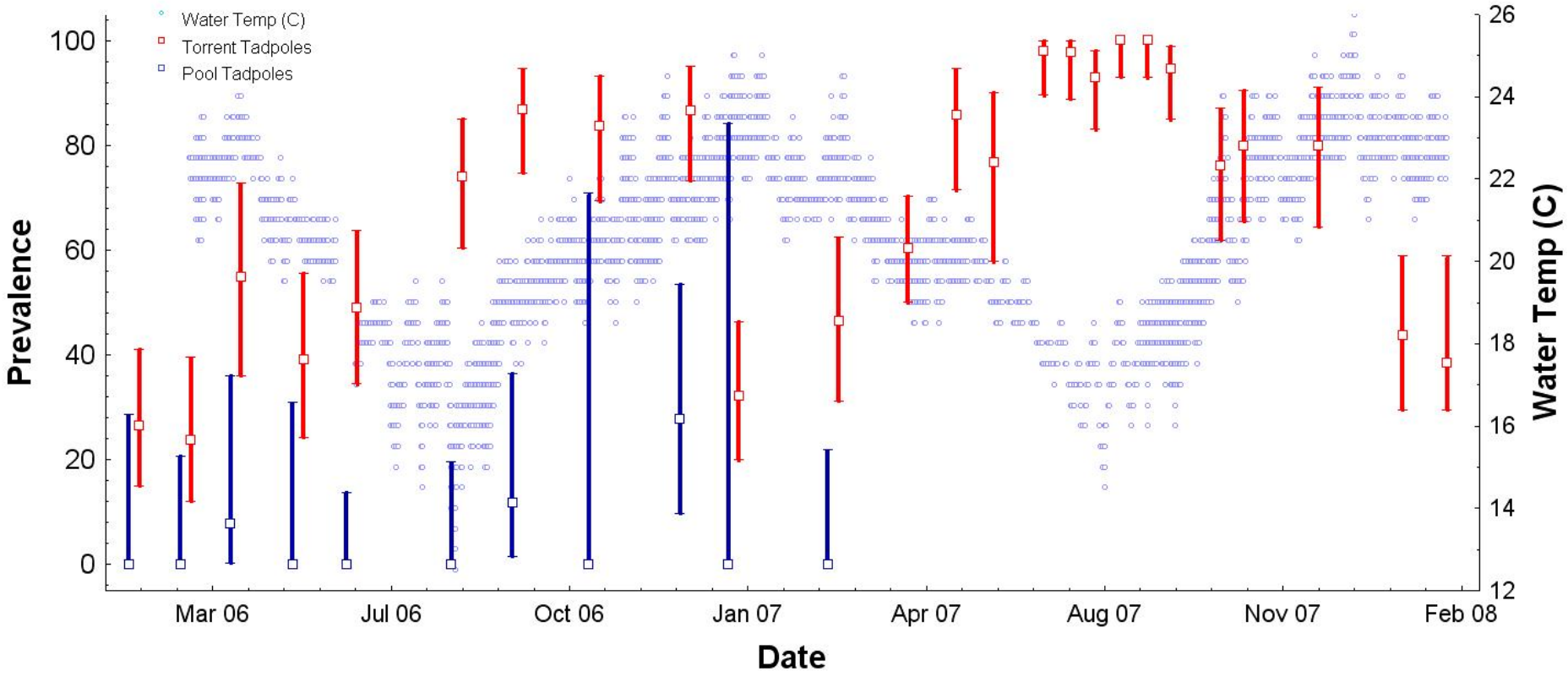


Figure 6.8. Seasonal prevalence +/- 95% CI of *B. dendrobatidis* in torrent-adapted (*L. nannotis*, *L. rheocola*, *N. dayi*) and pool-adapted (*L. genimaculata* and *L. xanthomera*) tadpoles at Tully Gorge National Park, 2006 – 2008. Bars are offset three days on either side of actual date to prevent overlap. Blue dots represent the range in water temperature.

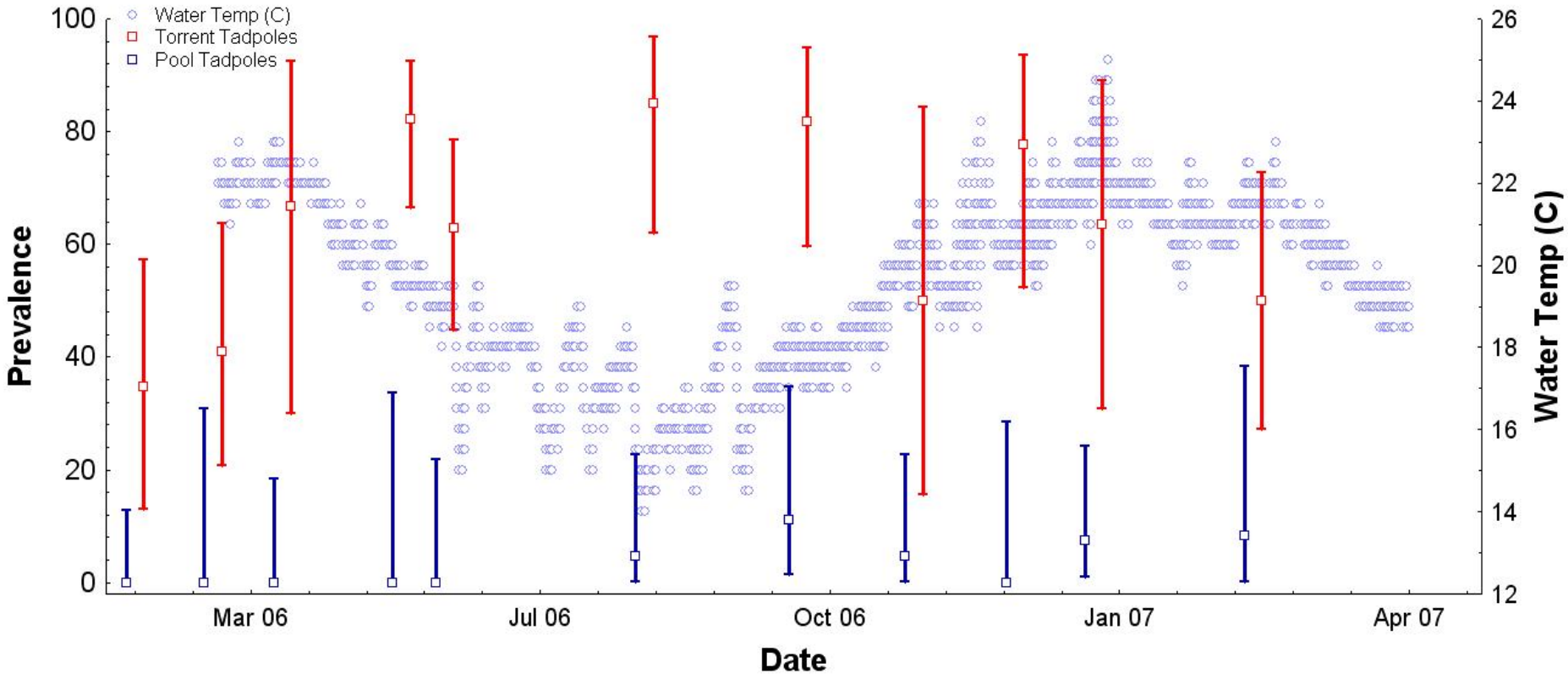


Figure 6.9. Seasonal prevalence of *B. dendrobatidis* +/- 95% CI in torrent-adapted (*L. nannotis*, *L. rheocola*) and pool-adapted (*L. genimaculata*) tadpoles at Murray Upper National Park, 2006 – 2007. Bars are offset three days on either side of actual date to prevent overlap. Blue dots represent the range in water temperature.

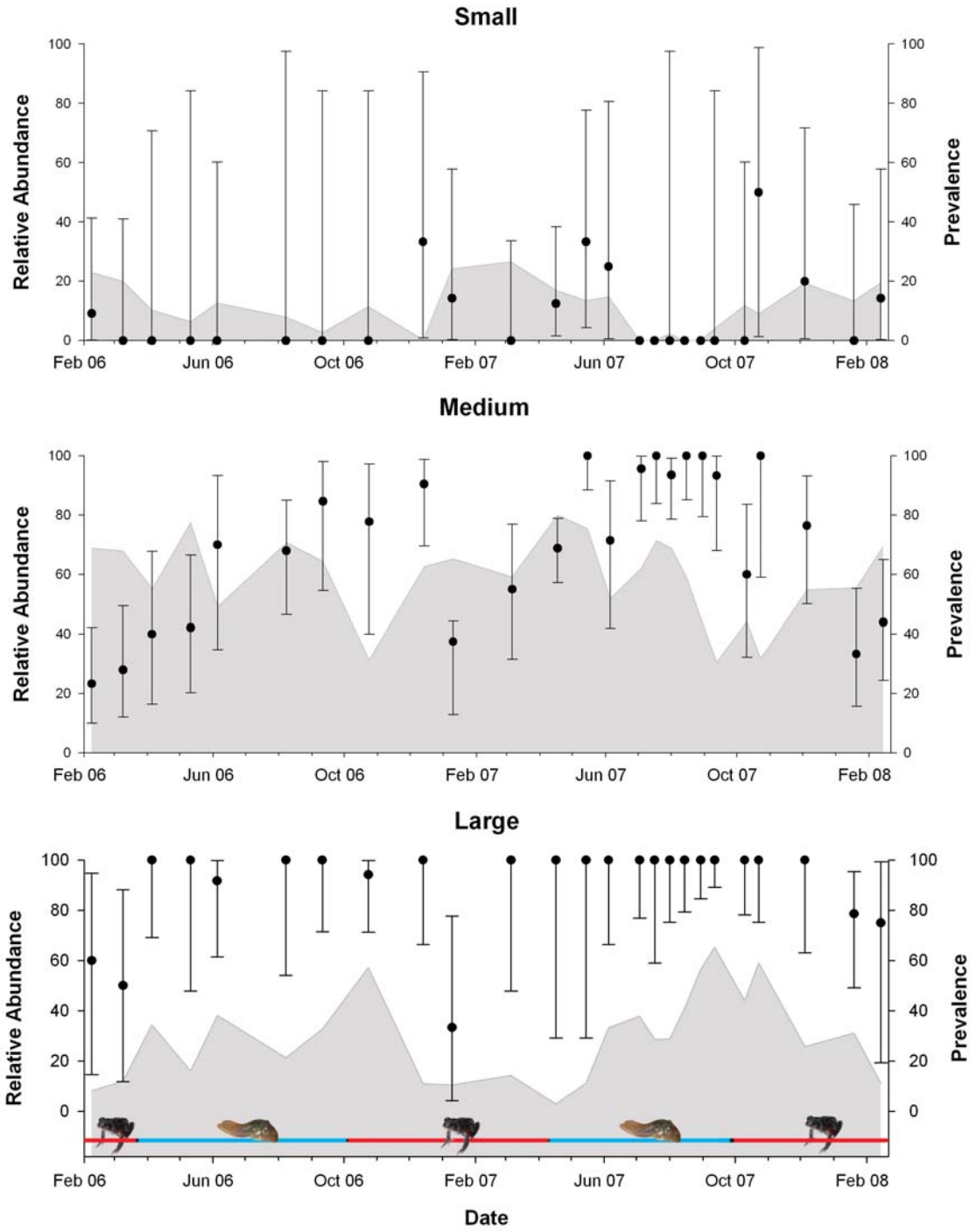


Figure 6.10. Prevalence +/- 95% CI of *B. dendrobatidis* in *L. nannotis* tadpoles at Tully Gorge National Park (2006-2008). Separate panels present data from small, medium and large size-group tadpoles. Shaded gray areas represent the relative abundance of each size-group. The bar at the bottom depicts periods during which large tadpoles are undergoing metamorphosis in red.

Binary Logistic Regression Model

The significant variables in the logistic regression model included 7 d mean water temperature, species, and size class (Table 7-3). 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 with species was the only 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 (Table 7-3). Size class was the best predictor of infection status. With every increase in size class tadpoles were more likely to be infected. The largest size class was 1098 times as likely to be infected as the smallest size class. The significant interaction indicates that torrent adapted tadpoles have an increased likelihood of infection when water flow rates are slower.

The 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, $x^2_8 = 10.359$, $p = 0.241$). The Nagelkerke R^2 indicated that 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.

Table 6-3 Binary logistic regression model examining the effect of independent variables on the probability of infection with B. dendrobatidis

Variable	*Coefficient*	*SE*	*Wald* [a]	*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

Intensity of infection differed significantly among species (ANOVA, $F_{4,1002} = 93.043$, $p<0.00005$; Fig. 6.11). Tukey's post-hoc comparisons showed that the only significant pairwise differences were between *Litoria nannotis, L. genimaculata,* and *L. rheocola;* the mean intensity of infection was significantly higher in *L. nannotis* than in the other two species. Intensity did not differ significantly between any other species pairs. Only two infected *L. xanthomera* were found, however, the intensity in one of them was very high (33,000 zoospore equivalents). Although clearly an outlier, this individual was included in analyses as there was no indication the value was false. Intensity differed

significantly among size classes in *L. nannotis* (ANOVA, $F_{5,758} = 7.9755$, $p<0.000005$; Fig. 6.12) and tended to increase with increasing size. Tukey's post hoc test indicates the smallest size class (7.0-8.9 mm) had a lower mean intensity than all of the larger size classes, and tadpoles sized 9.0-10.9 mm had a lower mean intensity than size classes 13.0-14.9 mm and above. Intensity tended to increase with size class in *L. rheocola*, but this was not significant (ANOVA, $F_{4,209} = 1.9931$, $p=0.0968$). There was no significant difference among size classes within *N. dayi* (ANOVA, $F_{3,9} = 2.1770$, $p=0.16051$) or *L. genimaculata* (ANOVA, $F_{4,8} = 1.2435$, $p=0.3663$; Fig.6.17), however, sample sizes were small. When all torrent tadpoles are combined, intensity tended to increase with size with a significant difference among classes (ANOVA, $F_{6,985} = 10.245$, $p<0.000005$; Fig. 6.13). Tadpoles in the 7.0-8.9 mm size class have lower mean zoospore equivalents compared with tadpoles larger than 11 mm, and tadpoles in the 9.0-10.9 mm size class have lower mean infection intensity than tadpoles larger than 13 mm.

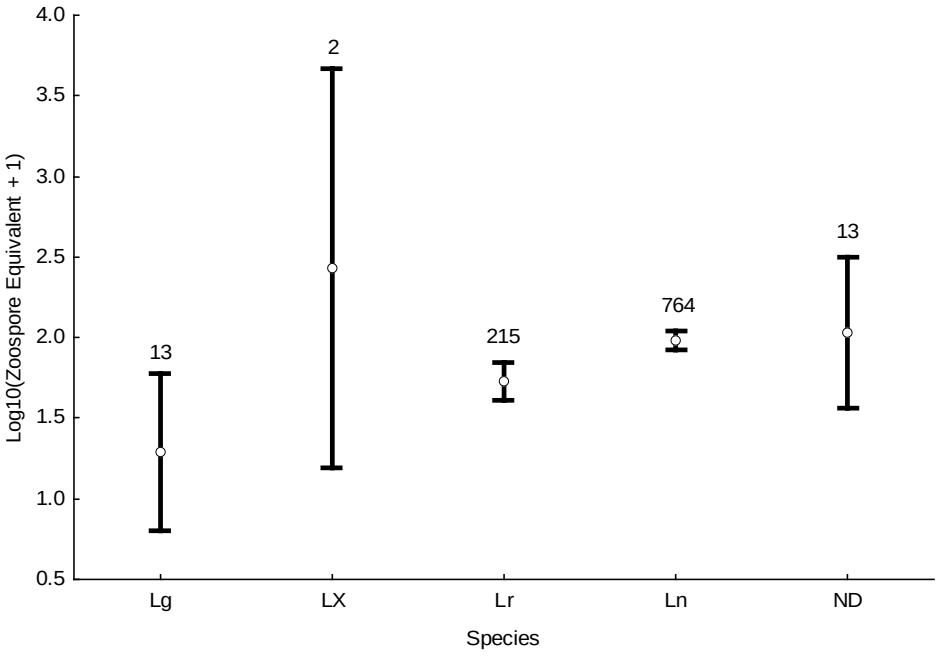


Figure 6.11. Mean intensity +/- 95% CI of *B. dendrobatidis* infection in infected tadpoles of each species. Tully Gorge NP and Murray Upper NP (2006-2008). Numbers above are sample sizes. ANOVA, $F_{4,1002} = 93.043$, $p<0.00005$

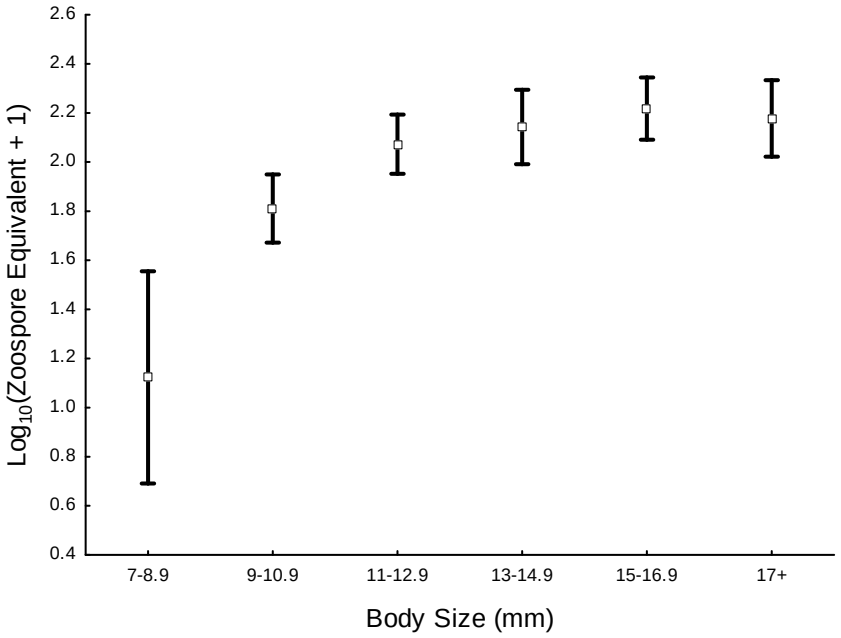


Figure 6.12. Mean intensity +/- 95% CI of *B. dendrobatidis* infection by size class in infected *L. nannotis*. Tully Gorge NP and Murray Upper NP (2006-2008). ANOVA, $F_{5,758} = 7.9755$, $p<0.000005$.

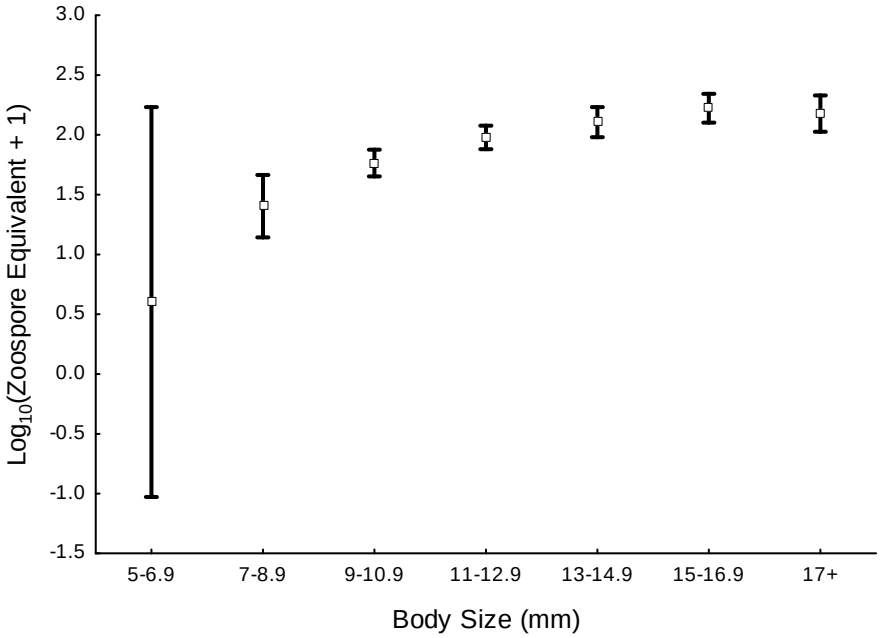


Figure 6.13. Mean intensity +/- 95% CI of *B. dendrobatidis* infection by size class in infected torrent adapted tadpoles. Tully Gorge NP and Murray Upper NP (2006-2008). ANOVA, $F_{6,985} = 10.245$, $p<0.000005$

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, $F_{2,455} = 0.1008$, p=0.9042; Fig. 6.14). 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 the effects of site and size class on intensity of infection (ANOVA, $F_{5,335} = 3.9968$, p=0.00155; Fig. 6.15). This difference coincided with higher intensity at Murray Upper in size class 9.0-10.9 mm (Tukeys HSD, p=0.0102). The remaining size classes did not differ significantly between sites. There was no significant effect of site on the intensity of infection among size classes in *L. rheocola* (ANOVA, $F_{4,90} = 1.9166$, p=0.1148; Fig. 6.16).

Sampling Period

Mean intensity in *L. nannotis* (ANOVA, $F_{10,325} = 8.017$, p<0.0001) and *L. rheocola* differed (ANOVA, $F_{9,139} = 6.5556$, $p < 0.00005$) between the two sites within sampling periods (Fig. 6.17 and 6.18), however, in both cases this was due largely 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,* $p < 0.0005$; Fig. 6.17 and *L. rheocola,* p=0.0003; Fig. 6.18). Intensity did not differ significantly within the remaining sampling periods. Overall, there was a high level of agreement between the two sites, across both size class and sampling period, in *L. nannotis,* and *L. rheocola*.

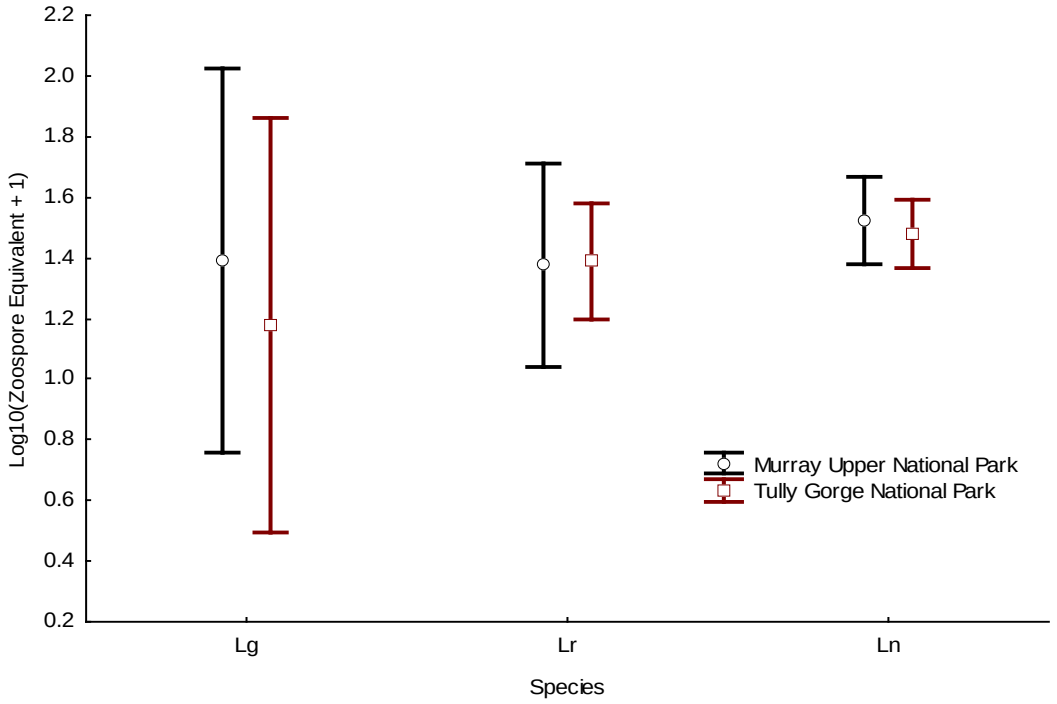


Figure 6.14. Mean intensity of *B. dendrobatidis* infection +/- 95% CI in tadpoles by species and site, 2006 – 2007.

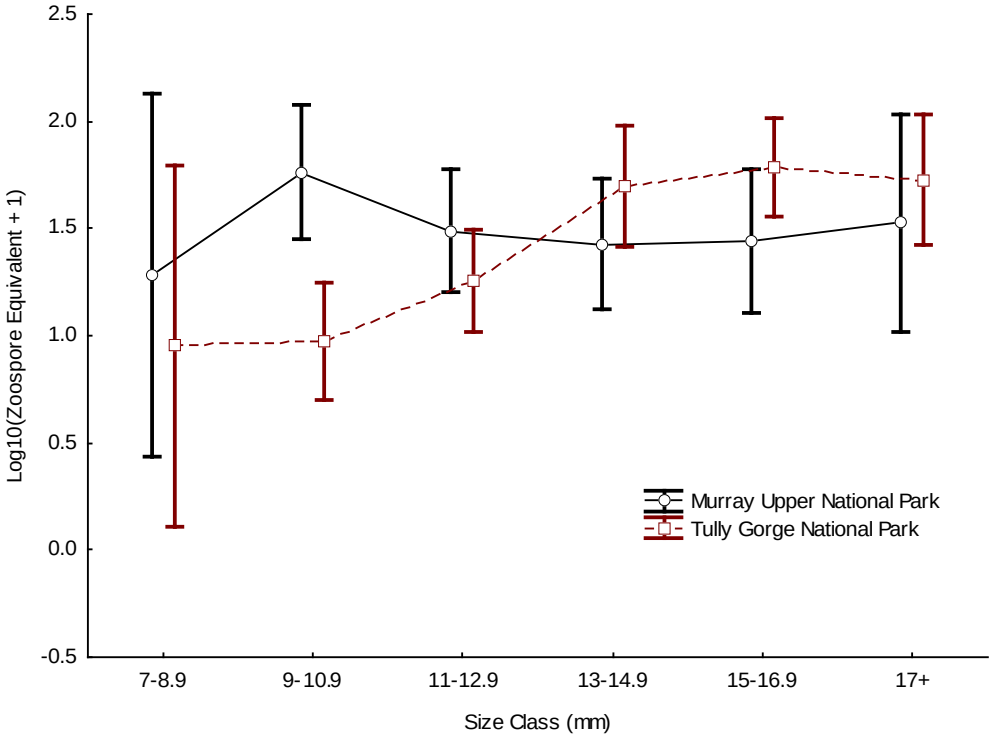


Figure 6.15. Mean intensity of *B. dendrobatidis* infection +/- 95% CI in *L. nannotis* tadpoles by size class and site. Connecting lines are a visual aid only, 2006 – 2007.

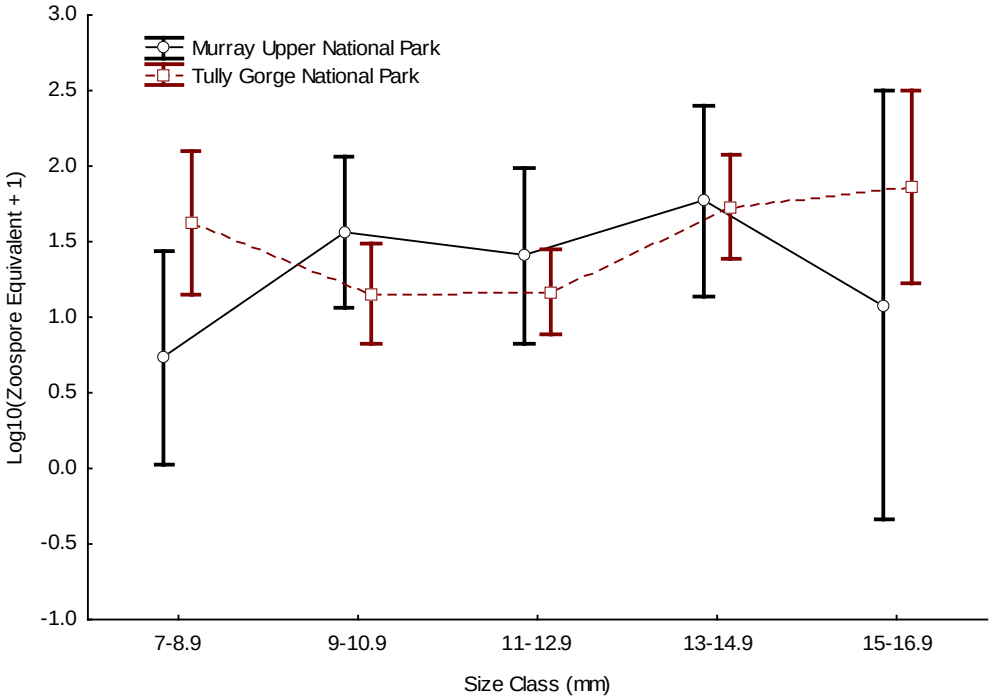


Figure 6.16. Mean intensity of *B. dendrobatidis* infection +/- 95% CI in *L. rheocola* tadpoles by size class and site. Connecting lines are a visual aid only, 2006 – 2007.

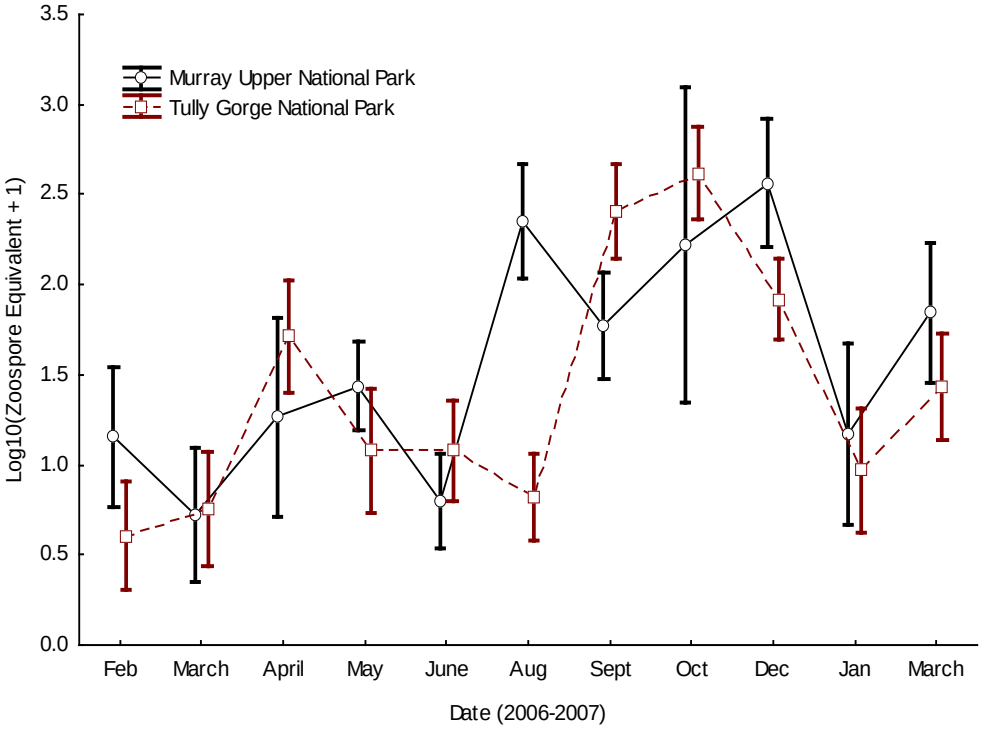


Figure 6.17. Mean intensity of *B. dendrobatidis* infection +/- 95% CI in *L. nannotis* tadpoles by sampling period and site. Connecting lines are a visual aid only, 2006 – 2007.

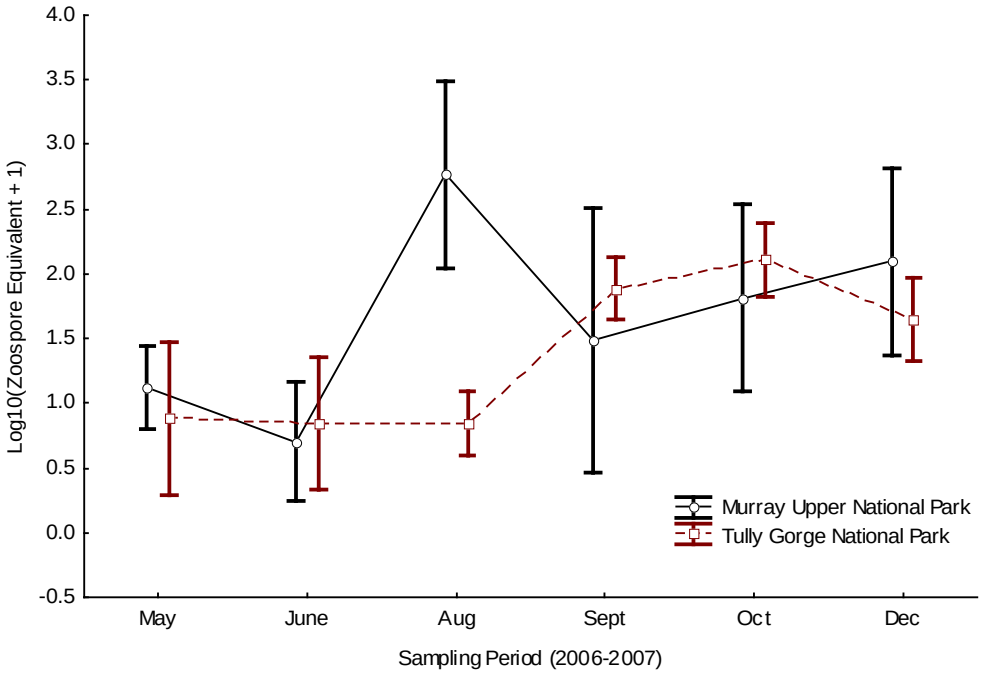


Figure 6.18. Mean intensity of *B. dendrobatidis* infection +/- 95% CI in *L. rheocola* tadpoles by sampling period and site. Connecting lines are a visual aid only, 2006 – 2007.

Seasonality

Intensity of infection in torrent tadpoles changed throughout the year (ANOVA, $F_{24,967}$ = 32.355, $p < 0.0005$; Fig. 6.20). Peak intensities occurred in spring of 2006 and winter of 2007. The mean intensity of infection in *L. rheocola* was strongly positively correlated with infection intensity in *L. nannotis* when they were paired by sampling period (Time series cross-correlation: r=0.865; Fig. 6.19) indicating that temporally varying factors that affect tadpoles of both species determine a large proportion of the variation in infection intensity, with species differences resulting in a consistently higher mean intensity in *L. nannotis*.

Prevalence and Intensity

Although intensity of infection is strongly correlated between *Litoria rheocola* and *L. nannotis*, *L. rheocola* was excluded from the following analysis because *L. rheocola* tadpoles were not captured during all sampling periods and differences between the species in overall mean intensity could therefore bias mean intensity. Mean intensity of infection in infected *L. nannotis* is significantly positively correlated with prevalence

over the two year sampling period (Time series cross-correlation: r=0.713; Fig. 6.20). The strength of the correlation, however, decreased 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=0.991; Fig. 6.20). From the first sign of mouthpart loss until the following February, when prevalence and intensity reach minima, the correlation between the two is far weaker (Time series cross correlation: r=0.471; Fig. 6.20). Following the first indication of mouthpart loss, intensity suddenly decreased, however, prevalence continued to increase, although at an apparently slower rate than before. When mouthpart loss was most severe, intensity was at its minimum (Fig. 6.20). Mouthparts then began to recover (Discussed in detail in Chapter 7). As they recovered, intensity increased until reaching a mean maximum of 630 zoospore equivalents. Even as intensity increased above or near pre-mouthpart loss levels, tadpoles maintained their full complement of teeth. Within 4-8 weeks tadpoles begin to show signs of metamorphosis and new recruits enter the population (Fig. 6.20). Mean prevalence begins to decrease.

Standard Multiple Regression Model (Intensity of Infection)

Variables that contributed significantly to the multiple regression model for *L. nannotis* infection intensity were, in descending importance, the preceding 28 day mean rainfall, body length, site and solar radiation (Table 6-4). The whole model explained 20.1% of total variance ($F_{4,801} = 50.347$, $p<0.0005$).

Table 6-4. Multiple regression model of intensity of *Bd* infection in *L. nannotis*

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

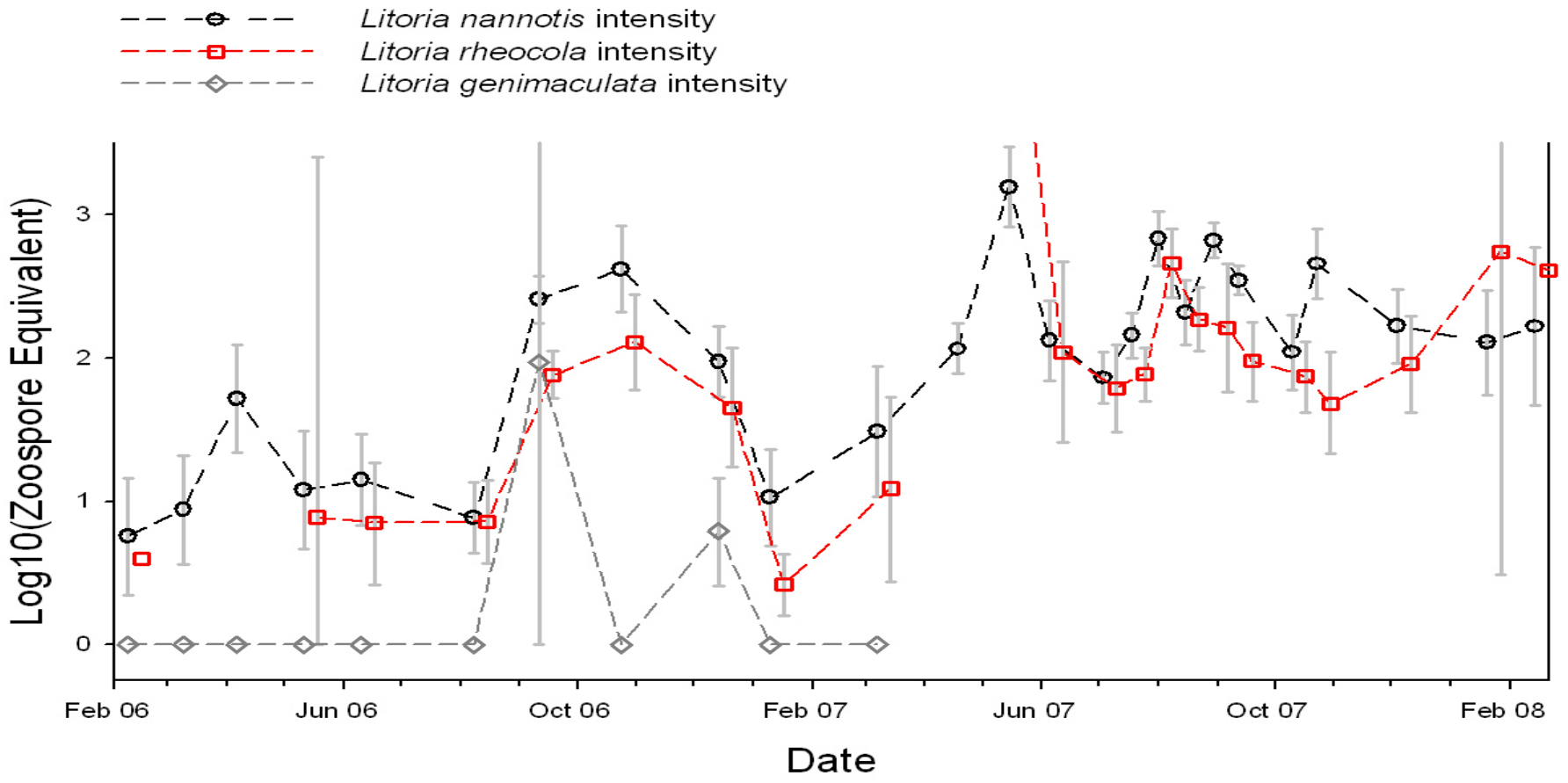


Figure 6.19. Intensity in infected *Litoria nannotis, L. rheocola* and *L. genimaculata* over time at Tully Gorge NP (2006-2008). Values of 0 were added to indicate tadpoles were captured but were uninfected. Missing values indicate tadpoles were not found.

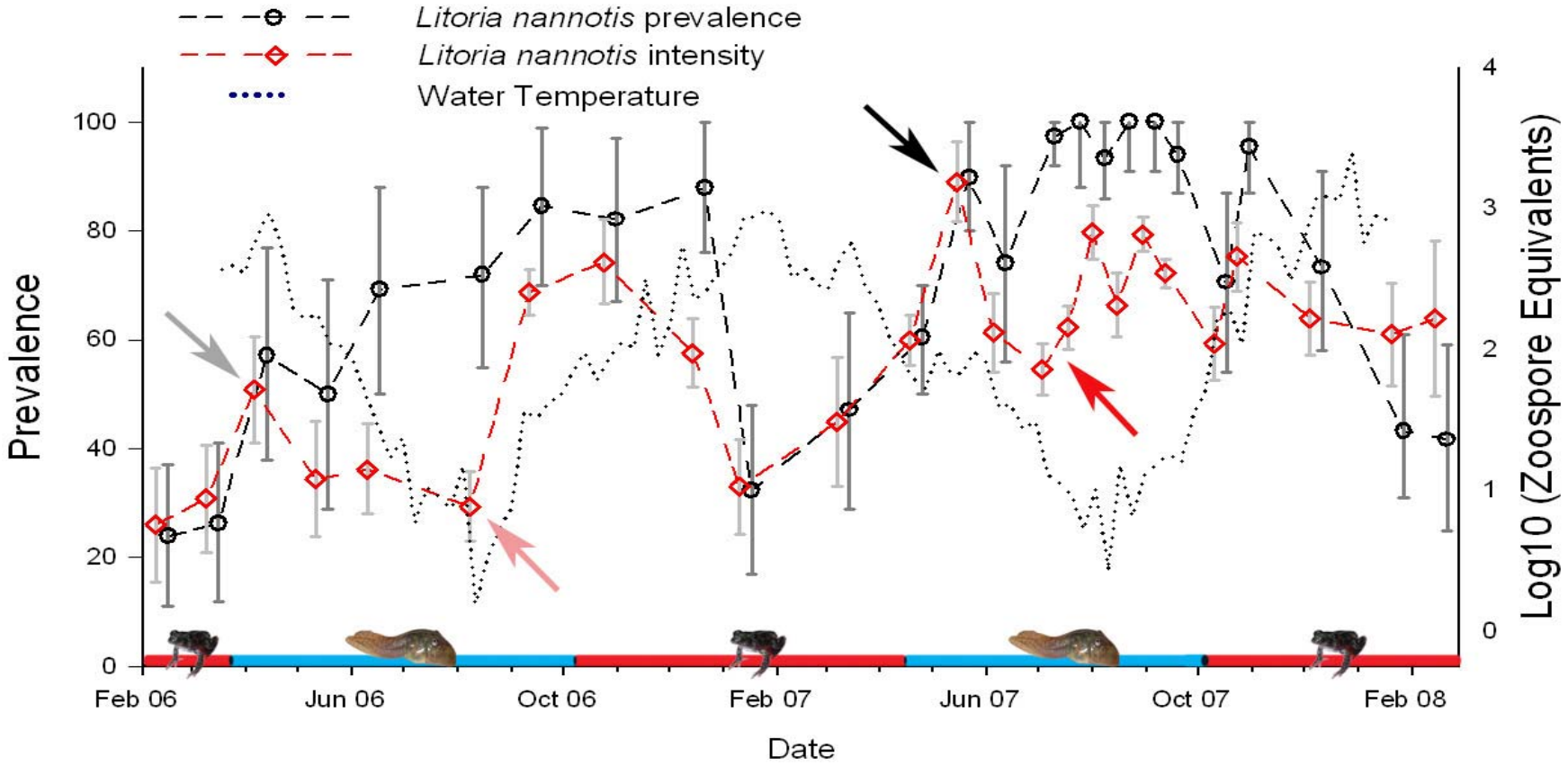


Figure 6.20. Prevalence and intensity of infection over time in *Litoria nannotis* and *L. rheocola* combined at Tully Gorge National Park (2006-2008). Blue dots represent weekly mean water temperature and are provided as an indication of season. The black arrow indicates the observed start of mouthpart loss. The red arrow indicates the observed peak of mouthpart loss. The lighter color arrows indicate the estimated timing of the mouthpart loss events based on field notes and intensity data. The blue and red bar illustrates the periods of growth and metamorphosis

Effects of Density

Transmission

In the first year, the tadpole sampling design allowed the total number of tadpoles captured to be used as an estimate of host relative abundance (discussed in Chapter 5 methods). If transmission is density dependent, prevalence of infection should increase with abundance of infected hosts. This analysis was restricted to *L. nannotis* within the medium size-group from Tully National Park. The two sites could not be combined because host abundance differed between sites and the medium size-group was selected because most new infections occurred within this group (Fig 6. 10). The relative abundance of infected hosts was estimated by multiplying the total number of captured torrent adapted tadpoles of each species (from all size-groups) by the prevalence of infection in the subset of tadpoles of each species that were sampled for infection status. The correlation between prevalence in medium sized *L. nannotis* and infected torrent tadpole abundance is significant (Spearmans rho, $r_{11} = 0.891$, $p < 0.005$; Fig.6.21) indicating transmission to uninfected animals increased with density of infected conspecific hosts.

Intensity

If intensity within individuals increases due to self-reinfection, intensity levels should vary primarily due to within-individual factors such as host size or exposure time. On the other extreme, if increases in intensity are primarily caused by external re-infection, intensity levels within an individual should vary in response to the intensity of infection of infected tadpoles at large (this assumes that more intense infections will release more zoospores) and the local density (which combined with intensity is a measure of the density of *Bd* within the stream). The timed sampling method employed in 2006-2007 allows the number of tadpoles collected to be used as an estimate of relative population density (discussed in Chapter 5). To control for the effect that size has on intensity (Fig. 6.13), 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

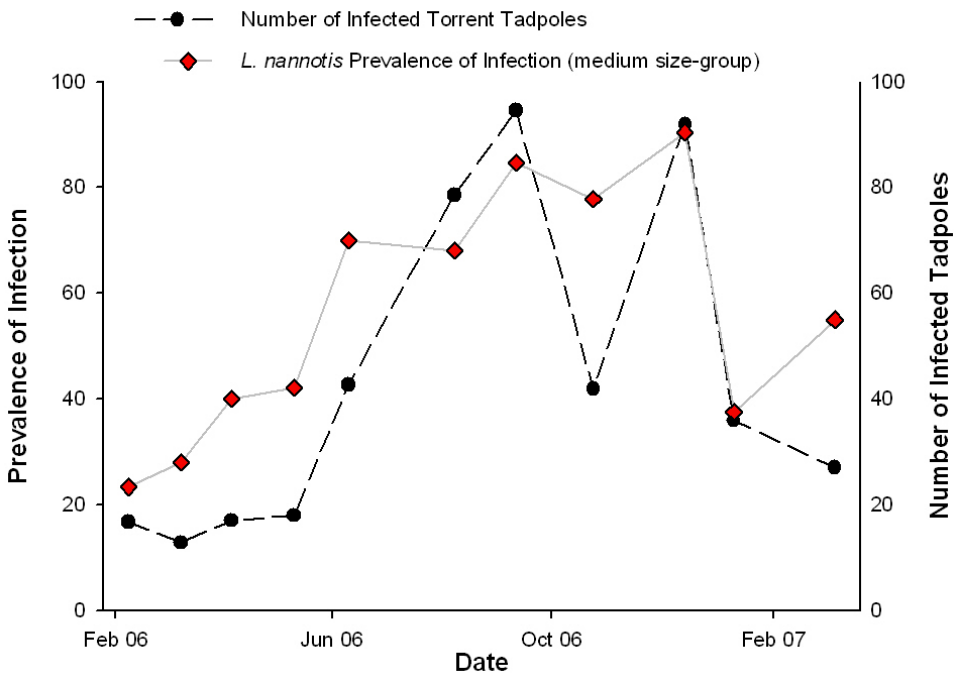


Figure 6.21. Infected host relative abundance and prevalence of infection in medium size-group *Litoria nannotis* tadpoles. Tully Gorge National Park, 2006-2007.

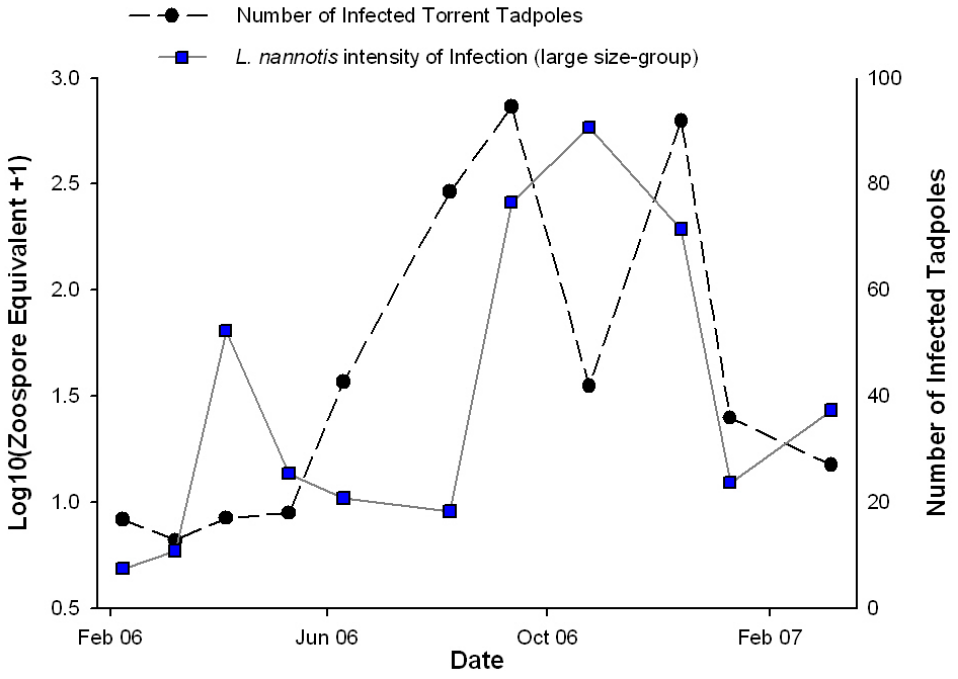


Figure 6.22. Intensity of *B. dendrobatidis* infection in large size-group *L. nannotis* tadpoles and relative density of infected torrent-adapted tadpoles, Tully Gorge NP, 2006-2007.

infected tadpoles. A time series cross-correlation (r_{11} = 0.563; Fig. 6.22) reveals that intensity of infection in large size-group *L. nannotis* was positively correlated with density of infected torrent tadpoles.

Discussion

I found that risk of infection varied 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. A model combining species, body size, temperature and water velocity correctly predicted the infection status of 85.8% of swabbed 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. The data suggest that *L. nannotis* tadpoles have a degree of acquired immunity or tolerance to infection following a period of severe mouthpart loss. This resistance or tolerance allows tadpoles to regrow mouthparts, acquire nutrients and metamorphose. Tadpoles in 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 6-1). The differences in prevalence among these species are best explained by an interaction of host-specific characteristics (species term in model), flow rate (flow rate x species term) and exposure time (size class term).

Host-specific characteristics

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 2 of

66 *L. xanthomera* found were infected, suggesting either that these tadpoles are more resistant to infection or, more likely, that *Bd* is not commonly present in this habitat. Prevalence and intensity of *Bd* infection in *L. genimaculata* tadpoles were not significantly different from those in *L. xanthomera* (Fig. 6.1 and 6.11). *Litoria genimaculata* tadpoles 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. Larger *L. genimaculata* tadpoles had a higher prevalence but not intensity of infection (Fig. 6.5), 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) and suggests the observed low prevalence is due to infrequent environmental transmission, but predicts prevalence may increase if the larval stage were longer, increasing exposure time.

Torrent-adapted tadpoles have significantly higher prevalences and intensities of infection than *L. xanthomera* and *L. genimaculata.* Torrent tadpoles are infected at smaller sizes in the wild (Fig. 6.3) compared to pool tadpoles, indicating a higher cumulative risk of infection, caused mainly by differences in growth rates but also likely due to differences in rate of transmission. Tadpoles harbour infection only within the mouthparts (discussed in Chapter 5), differences in the interaction of mouthparts with the environment between functional groups may affect transmission of *Bd*. Torrent tadpoles are attached to rocks in fast flowing water almost continuously (Chapter 5). In comparison, pool tadpoles are far less substrate adherent (Richards, 2002, Chapter 5). This suggests that prolonged substrate contact may increase risk of transmission (in Chapter 8 I sample the environment for *Bd* to address this hypothesis).

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

Zoospores entering through the nares from the water column and infecting tooth rows is possible, but is unlikely to be the primary avenue of transmission. In the North American torrent tadpole *Ascaphus truei*, the valves of the internal nares and the internal oral disc valve are both only open while the sucker is in the process of engaging. When the sucker is engaged or disengaged from the substrate, one or both of the valves is closed (Gradwell, 1971), preventing water that has entered through the nares from contacting tooth rows. Therefore, tooth rows will be separated from water entering through the nares most of the time. In addition, the filtration of respiratory currents from the nares is inconsequential for collecting particles from the environment (Altig and Brodie Jr, 1972). Instead, virtually all ingested organic material is collected by the scraping of substrate surfaces (Altig and Brodie Jr, 1972). Observation of feeding behavior in the field and lab suggested that torrent tadpoles of Australia function similarly, indicating that the bulk of mouthpart contact with organic matter will be via substrate surface scraping during feeding, further suggesting this is the primary avenue of 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 (Ryan et al., 2008, Rowley and Alford, 2007)

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 a complex biofilm forms, 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 thickness of the boundary layer, causing settlement rates to decrease as a result of a combination of both reductions in instantaneous settlement rates and increases in resuspension rates (Stevenson et al., 1996). Like any microorganism, *Bd* is expected to settle within the boundary layer on rocks in torrents, and its 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 6-3)

is inversely related to the velocity of their preferred microhabitat (Chapter 5). *Litoria rheocola* occupy lower velocity currents and have the highest risk of infection and *N. dayi* occupy the fastest currents and have lowest risk of infection. Within species, infection risk was significantly higher in *L. nannotis* and *L. rheocola* captured in slower flowing water (controlled for temperature; Table 6-3). Infection intensities were also higher following drier months when flow rates were low (Table 6-2 and 6-3).

Batrachochytrium dendrobatidis can survive up to 7 weeks in sterile pond water (Johnson and Speare, 2003). Therefore, zoospores settling out of the current may be able to survive within the boundary layer for an extended period of time if they are not consumed by predators or 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 in different stream microhabitats will be important for future modeling. I successfully detected low numbers of *Bd* from rock samples within torrents using filtration and PCR, indicating *Bd* is present but not ubiquitous (Chapter 8). Analogous to the properties of the community of microflora on amphibian skin (Harris et al., 2006), the composition of microorganisms within biofilms can differ among sites, seasons and even rock faces (Lear et al., 2008). Bacteria within biolfilms can 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 could affect zoospore settlement include negative phototaxis, chemotaxis, surface chemistry and topography (Patel et al., 2003).

If increased velocity deposits fewer zoospores, larger numbers of zoospores should accumulate 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). Therefore higher mortality rates in nutrient rich pools may reduce zoospore survival and transmission.

Seasonality

Prevalence

Batrachochytrium dendrobatidis is the only member of the Chytridiomycota known to invade vertebrate cells (Berger et al., 1998). Many other chytridiomycotes are parasites of plankton, and are often host specific (Bruning et al., 1992, Holfeld, 1998, Canter and Jaworski, 1982). Epidemics of these chytrids have been partly explained by factors including light, temperature, nutrients, pH, turbulence and zooplankton grazing (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 followed by a parasitic chytrid bloom. Prevalence of infection can be over 90% (Ibelings et al., 2004). Both taxa then rapidly decline (typically within weeks) following host death, and the cycle is repeated when host densities are again high enough (Ibelings et al., 2004).

Similarly to other chytrids, the prevalence of *Bd* in torrent tadpoles is seasonal and becomes very high before decreasing back to a low level; 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 caused by the very different amphibian host life cycle and the response of larvae to infection. Seasonality in prevalence was driven primarily by tadpole population dynamics (predictable seasonal breeding and development patterns; Chapter 5, Fig. 6.11), with a relatively small, but significant influence from higher water temperatures. Many tadpoles captured during winter had been in the stream and were exposed to *Bd* for a longer period of time than tadpoles of the same body size captured in summer. The low prevalence of *Bd* infection in small (7-8.9mm) tadpoles across seasons indicates that recently hatched tadpoles are unlikely to be infected at any time. However, the consistently lower prevalence in summer across all other size classes (including the largest tadpoles: Fig. 6.5, Table 6-2) suggests that higher temperatures exert a relatively small, but significant downward pressure on infection either through a decreased rate of transmission or via the clearing of existing

infections. The highest 7-day mean maximum water temperature over the two year study was 24.1 ºC, which is within the optimum temperature range for *Bd* growth in culture (Piotrowski et al., 2004). This indicates that increased temperature may have 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 to cause widespread clearing of infections. Consequently, decreases in the prevalence of *Bd* only occurred when infected tadpoles left the population via metamorphosis.

My results indicate that the dynamics of *Bd* infections in tadpoles operate independently of those in terrestrial stages. This suggests that the majority of infectious zoospores to which tadpoles are exposed originate from 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 torrent frogs are found on wet rock surfaces at night and in cracks or underwater in riffles during the day (Rowley and Alford, 2007). Therefore, they are often in direct contact with tadpole habitats.

Descriptive Prevalence Model

A pulse of egg hatching occurs during the summer of each year (Fig. 6.10). Smaller numbers of small, recently hatched tadpoles are found in other seasons as well, however, infection prevalence in this size-group remains low throughout the year, indicating that infections are acquired after hatching. After the pulse of recruitment in summer, most tadpoles grow during the cooler, drier winter. No metamorphosis takes place over this time, and fewer eggs hatch. As individual larvae graze rock surfaces in search of food, their risk of infection increases, even as water temperatures reach a minimum in midwinter and then begin to rise. The combination of reduced immigration and emigration and increasing infection prevalence in growing tadpoles causes the seasonal pattern of prevalence. Transmission appears to be density dependent as prevalence of infection in medium size class tadpoles is strongly correlated with density of infected tadpoles of all size classes. As early summer approaches, the proportion of large tadpoles in the population grows until approximately 60% of all tadpoles are of

adequate size to metamorphose. Prevalence in these large tadpoles is at or near 100%. Tadpoles 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.

The prevalence in large 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, leaving the uninfected tadpoles to remain in the population to continue development. Alternatively, this may indicate that some infected tadpoles have cleared their *Bd* infections.

Intensity

Variation in intensity of infection and density of hosts should indicate when *Bd* zoospore numbers are high and transmission is likely to be maximal. Understanding the causes of increased intensity may lead to direct management strategies such as removing tadpoles prior to peak infection intensity in order to lower zoospore numbers within the stream and reduce transmission to adults during periods of high risk. This form of management could prove useful in limited circumstances, such as the conservation of critically endangered species at selected sites or during the reintroduction of captive bred populations (Gascon et al., 2007, Australian Government 2006)

Four variables in a multiple regression model explained 20.1% of the variation in *Bd* intensity in *L. nannotis*: 1) *28-day mean rainfall* (inversely related to intensity), 2) *body length* (positively related to intensity) 3) *28-day mean solar radiation* (positively related to intensity) and, 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 is a major predictor of infection status (Table 6-2 and discussion above).
2. Greater body lengths indicate both a larger mouthpart surface area and a longer exposure time that plausibly leads to increased infection intensity. *L. rheocola* tadpoles are smaller than *L. nannotis* tadpoles. This size difference may explain why intensity in *L. rheocola*, although strongly correlated with intensity in *L. nannotis* was consistently lower than *L. nannotis* (Fig. 6.19). However, intensity did not vary 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 or survival time of zoospores. Alternatively, solar radiation may simply be a proxy for something else, such as season, which correlates with seasonal intensity patterns.

In theory, individual infection intensity should be determined by the difference between the rate of re-infection (self-reinfection or external-reinfection) and the rate at which individual *Bd* thalli form mature zoosporangia, shed zoospores, and die. If self-reinfection rates are low, the intensity of individual infections should be largely determined by environmental concentrations of zoospores, and should respond to the prevalence, intensity of infection, and density of the tadpole population as a whole. This would lead to high correlations of infection intensity among individuals. If self-reinfection rates are relatively high, the intensity of infection should vary independently among individuals. This could lead to lower correlations of intensity among individuals, however, because the demography of tadpoles is seasonal, intensity could be correlated even if it develops independently within individuals. Over a two year period, mean intensity was highly correlated between *L. nannotis* and *L. rheocola* (Fig. 6.19) despite differences in the timing of their development (Chapter 5), and there was a moderate correlation of intensity in large tadpoles with the overall density of infected tadpoles (Fig. 6.22) suggesting that the intensity of individual infections may be

strongly affected by rates of external-reinfection. Briggs et al. (2010) report a similar finding in endemically infected populations of *Rana muscosa* in California. Their 'best fit' model suggests that individual infection status and intensity of adult frogs are determined primarily by external re-infection of aquatic zoospores, likely produced by long-lived infected tadpoles (Briggs et al., 2010). Their model predicted that external reinfection rates would be primarily affected by density of infected hosts and water flow rates, further supporting my findings here.

Disease can be a powerful selective force (Altizer et al., 2003), and in response, hosts can evolve both resistance (the ability to reduce the success of infection or increase the rate of clearance) and tolerance (the ability to limit the severity of disease caused by a pathogen; Raberg et al., 2007, Restif and Koella, 2004). These two defenses are believed to be independent but not necessarily mutually exclusive, and can 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 (Mauricio, 2000, Mauricio et al., 1997, Tiffin et al., 1999) which can display both at the same time (Mauricio, 2000). The relative contribution of each may respond to disease parameters such as virulence and transmission rate (Roy and Kirchner, 2000), and defences may coevolve with the parasite. For example, hosts exposed to parasites with high transmission rates and low virulence are predicted to evolve greater tolerance, whereas hosts exposed to a highly virulent pathogen should be more likely to evolve resistance (Roy and Kirchner, 2000, Woolhouse et al., 2001). This can affect competitive interactions among hosts as increased tolerance may serve as a "biological weapon" (Restif and Koella, 2004, Roy and Kirchner, 2000) by allowing virulence against more susceptible competitors to increase (Restif and Koella, 2003). Ironically, tolerance to infection in tadpoles could help maintain virulence against adult life stages.

I will next consider a potential framework for factors affecting intensity, including the significant variables from the multiple regression model, the theoretical relationship between re-infection vs. shedding, the apparent 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 each other (Fig. 6.19), indicating a period of unimpeded pathogen replication and transmission. This exponential growth phase, however, appeared to be disrupted by the onset of mouthpart loss, likely initially resulting from the decrease in available substrate for infection (discussed in Chapter 7). As mouthpart condition worsened, intensity declined (Chapter 7), however, prevalence continued to increase up to 100% (in 2007; Fig. 6.20). Mouthpart loss eventually reached a peak, after which the oral structures recovered and intensity began to rapidly increase again. Mouthparts, however, continued to recover to near normal despite increasing intensity. Water temperatures decreased as mouthparts recovered (Fig. 6.20), eliminating the possibility that mouthpart recovery is influenced by warmer temperatures as seen in some temperate species (Rachowicz, 2002). The recovery of tooth functionality while infected strongly indicates a tolerance to infection has developed. In addition, it appears intensity may also be moderated by something other than mouthpart loss. Prior to the onset of mouthpart loss, intensity increased exponentially. As mouthparts recovered, intensity fluctuated below a maxima of approximately 630 zoospore equivalents despite abundant available substrate (keratinized mouthparts). The absence of a continued exponential increase in intensity suggests tadpoles may be capable of limiting intensity, suggesting possible resistance.

Considering that: 1) torrent tadpole survival depends on functional teeth to extract nutrients from boulder surfaces 2) infection with *Bd* causes a loss of mouthparts and 3) prevalence of *Bd* in torrent tadpoles is at or near 100%, it follows that there has been strong natural selection on torrent tadpoles to evolve strategies to cope with infection and preserve the ability to gather nutrients in order to survive and metamorphose. In non-torrent tadpoles, which can continue feeding despite a loss in mouthparts, this selective pressure should be lower. Apart from Symonds et al. (2007), who looked at *Mixophyes* larvae that inhabit pools in streams, all detailed information on tadpole

mouthpart loss are from temperate pond tadpoles. None of these studies suggest that infected tadpoles may regenerate mouthparts; however, the studies were not designed to determine this (Vredenburg and Summers, 2001, Fellers et al., 2001, Obendorf, 2005, Smith et al., 2007).

Because tadpoles can be a significant contributor of zoospores within a water body, selection on tadpoles for tolerance and/or resistance to infection could have substantial effects on both the number of *Bd* zoospores in water bodies and even its virulence. For instance, highly effective larval tolerance could produce an increase in pathogen virulence (as experienced by the terrestrial stage) as there would be less pressure on *Bd* to limit host damage, leading to more aggressive strains (Restif and Koella, 2003). Virulence in terrestrial stages that spend substantial periods in contact with water could also increase through increased rates of external reinfection. Conversely, an increase in larval resistance could result in a decrease of aquatic zoospore density. Metamorphosis is a period of immune system reorganization in which 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 caused by *Bd*. However, studies indicate that some lymphocytes generated in the tadpole life stage persist through metamorphosis (Rollins-Smith, 1998). This suggests that if tadpoles mount a successful immune response it could help prepare them for challenges from the same pathogen as adults and metamorphs. These interactions are likely to be 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). Also, resources partitioned to prevent or reduce the impacts of *Bd* infection as larvae may leave individuals more susceptible to mortality as adults (Garner et al., 2009). Clearly, the presence of tolerance and resistance in the tadpole stage may have significant effects on the evolution of both the host and the pathogen.

Descriptive Intensity Model

At the beginning of the year in summer (January – February) the mean intensities of infections were low (Fig. 6.20). There had just been recruitment of uninfected tadpoles into the population and many of the infected tadpoles were recent recruits that had not been in the stream long and thus had low intensities (Fig. 5.16, 6.12, 6.13). The water then became cooler and the last of the metamorphs exited the stream, leaving the remaining tadpoles to grow and develop over winter (Fig. 5.16). From this time, the intensity of infection increased at an increasing rate, in lockstep with prevalence (Fig. 6.19). These increases ceased around May as tadpoles began to lose mouthparts, presumably as a reaction to high intensity infections (Chapter 7). It is unclear why the levels of intensity reached before mouthpart loss commenced differed between years (Fig. 6.20). This difference suggests that intensity alone may not trigger mouthpart loss. As the keratinized teeth and jaw sheaths were shed due to infection, infection intensity decreased (Fig. 6.20, Chapter 7). Prevalence, however, continued to increase despite the decrease in intensity, although at an apparently slower rate. Mouthpart condition and intensity of infection declined for about two months before reaching minima around July (Fig. 6.20). Mouthparts then began to regrow and infection intensity increased as water temperatures continued to become cooler. Despite increasing intensity, tadpole mouthparts remained in good condition, suggesting increased tolerance. Following mouthpart recovery, intensity did not increase as rapidly as it did prior to mouthpart loss, but remained below 630 mean zoospore equivalents, suggesting that in addition to increased tolerance, there may be resistance. This intensity is lower than the maxima achieved prior to mouthpart loss in 2007, but not in 2006 (Fig. 6.20).

This pattern suggests that: 1) tadpoles develop some degree of tolerance that allows mouthparts to regrow despite relatively high intensities of infection and 2) tadpoles may have some mechanism of resistance, apart from the shedding of teeth, that limits infection intensity despite abundant substrate (keratinized teeth and jaw) for infection to grow.

Mean intensity tends to decrease during the period when tadpoles exit the stream during metamorphosis. This drop in mean intensity is caused by a combination of large, heavily infected tadpoles leaving the population and decreases in mean intensity within all size-groups (Fig. 6.23). This drop in intensity within size-groups may be caused in part by a decrease in external reinfection. The intensity of infection in large *L. nannotis* was moderately correlated (r=0.563; Fig.6.22) with the density of infected torrent tadpoles. This relationship suggests that external re-infection from environmental zoospores may contribute to intensity. Self re-infection may be lower in torrent tadpoles than in frogs because as they feed they continuously pass water over their mouthparts, through the spiracle and back out into the current behind them. Therefore, zoospores exiting zoosporangia may be actively flushed away before being able to re-re-encyst on on the mouthparts.

Conclusions

Tadpoles, particularly the torrent tadpoles of *L. nannotis*, *L. rheocola*, and *N. dayi*, are effective reservoir hosts. These tadpoles are present in the stream throughout the year and can harbour infections at up to 100% prevalence. They are distributed widely throughout torrent habitats and disperse readily (Chapter 5) ensuring that *Bd* is present throughout the system independent of adult density, adult infection status, or terrestrial environmental conditions. Seasonal changes in prevalence of infection in torrent tadpoles are driven primarily by length of time in water and seasonal demographics indicating that risk of infection is cumulative and not associated with a single time of year or event.

Pathogens can exert powerful selective forces on wildlife (Altizer et al., 2003) and some amphibian populations that have survived initial *Bd* epidemics are now persisting despite infection with the pathogen, indicating that a degree of resistance has evolved (Retallick et al., 2004, McDonald et al., 2005). Significant mortality due to disease, however, may still be experienced (Murray et al., 2009). In Chapter 7 I show that infections can cause torrent tadpoles to lose mouthparts and the ability to feed. This strong negative effect of infection means that the presence of, or selection for, tolerance

or resistance in torrent tadpoles may be necessary for terrestrial recruitment and species survival. The patterns of prevalence, intensity and mouthpart loss support the suggestion that torrent tadpoles can mitigate the negative effects of infection and may be able to limit infection intensity as well. These results highlight the important role of tadpoles in *Bd* dynamics and in species recovery following an epidemic and illustrate that 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 how this affects susceptibility to chytridiomycosis 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 may be important in understanding this process.

Research on the interaction of *Bd* with stream microbiota should 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 determining intensity of infection in tadpoles will also significantly help in understanding how *Bd* levels in the stream vary. If the source of reinfections leading to increases in intensity is primarily external, 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 and reinfection rates could increase the odds of survival and provide more time for adults to mount an acquired immune response (Richmond et al., 2009). This form of active management may only be feasible in

habitats with low amphibian diversity or during a reintroduction program when densities are low

Chapter 7 : Effects of Infection with Batrachochytrium dendrobatidis on Tadpoles

Introduction

While metamorphosis may appear to represent a clean break from the larval stage, there is a growing body of evidence demonstrating that conditions experienced by larvae can have latent or carry-over effects on the adult stage and on population dynamics. These 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 metamorphosis, 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, respond to negative conditions with reduced recruitment below the maximum achievable under optimal conditions (Metcalfe and Monaghan, 2001, Werner, 1986). For instance, phenotypic plasticity allows multiple phenotypes from a single genotype in response to environmental conditions. In a resource-poor year, individual fitness may be maximized by reducing fecundity (West-Eberhard, 1989). Therefore, an introduced pathogen that causes yearly, widespread ontogenetic disruption can generate the equivalent of an "off" year in perpetuity and could cause cohort effects and alter population dynamics in both the short and long term.

In addition to adaptive phenotypic plasticity, life history traits can be shaped 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 by *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 (Davidson et al., 2003, Berger et al., 2005c). 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 that mortality occurs when *Bd* infection creates an electrolyte imbalance that leads to cardiac standstill (Voyles et al., 2007, Voyles et al., 2009a). In most 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). Infection is not known to cause death in tadpoles (although see Blaustein et al., 2005), however, 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" (Obendorf and Dalton, 2006, Padgett-Flohr and Goble, 2007, Felger et al., 2007, Smith and Weldon, 2007, Rachowicz and Vredenburg, 2004). As Altig (2007a) 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, since histopathology has confirmed that the individual teeth and cells within the jaw sheath matrix are physically absent after infection and not simply lacking pigment. I suggest the description "mouthpart loss" be used in place of "depigmentation".

Tadpole mouthparts are composed of two jaw sheaths 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 that are contained within the series (Altig and McDiarmid, 1999). When a tooth falls out because of 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, 2007a). As some cells fail to regenerate teeth, the overall appearance of the tooth row becomes discolored or gray in appearance (pers. obs.). If enough adjacent basal cells are disrupted, a gap appears in the tooth row, and if all cells are disrupted, no teeth are present. The same should 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 and Cornelius, 2004, Parris, 2004, Parris and Baud, 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, direct effects on survival of infected tadpoles have not been found (Parris, 2004, Parris and Cornelius, 2004), however, mortality caused by chytridiomycosis can be extreme soon after metamorphosis when keratinizing cells first develop on the skin of juveniles (Banks and McCracken, 2002, Rachowicz et al., 2006, Marantelli et al., 2004).

Based on available evidence, tadpoles are considered to not be substantially affected by *Bd* 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, and Chapter 6). While this effect is probably 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 affected by a range of factors including host environment and behavior (Richards, 2008, Berger et al., 2004, Rowley and Alford, 2007, Woodhams et al., 2003). Tadpoles, like frogs, are morphologically and ecologically diverse. 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 may affect the interaction of the host with the pathogen in both time and space and lead to very different disease interactions and outcomes within a single environment. However, detailed information on the effects of *Bd* infection on tadpoles in the wild are lacking, particularly in tropical streams where declines affecting the greatest number of species occur (Stuart et al., 2004, Bielby et al., 2008).

In this chapter I investigate the relationships between *Bd* prevalence, intensity of infection, host species and mouthpart loss, the pattern of tooth loss over time, and the effects 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

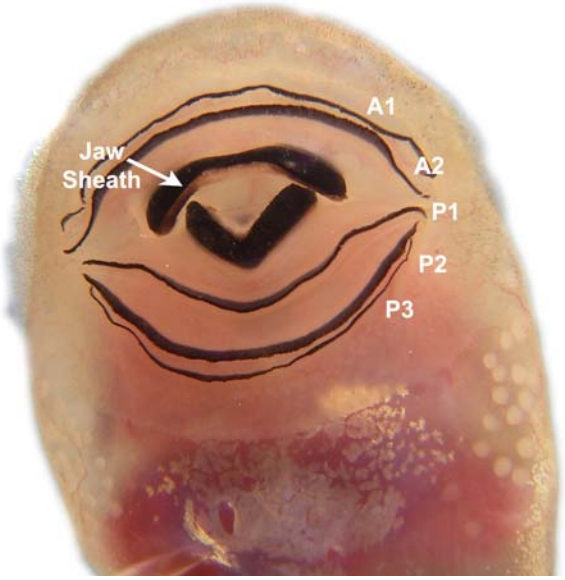


Figure 7.1. *Litoria nannotis* mouthparts with labeled tooth rows (A1-P3) and jaw sheath.

Sampling

Tadpole sampling was conducted at Tully Gorge National Park as described in Chapter 5. Following capture, tadpoles were placed into small, individually labeled click-seal bags. Torrent-adapted tadpoles adhered to the inside of the bag as in Fig 5.8, and their mouthpart loss was observed through the bag and described as detailed below. All tadpoles were then transferred to a well-rinsed gloved hand (Chapter 2). Body length was measured to 0.01 mm (Chapter 5). The mouthparts of pool-adapted tadpoles were then examined (with a 10X hand lens if necessary), mouthparts were swabbed and the tadpole was weighed using a portable electronic balance to 0.05 g.

During the first year, mouthpart loss was recorded simply as present or absent. Beginning in the second year, mouthpart loss was scored as 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.

In Year 2, Tooth loss was noted for each of 5 individual tooth rows. Tadpoles of *L. nannotis* and *L.* rheocola have two anterior tooth (A1 and A2) rows and three posterior (P1 – P3) rows (Fig. 7.1). 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 using a combination of the scores for each tooth row and an average of the percentages of tooth loss estimated for each row. Individual tooth row scores of 1 and 2 were combined into a single "discolored" category. Overall tooth loss scores 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 in total, 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 7-3 and Fig. 6.1). Due to the very small effect of *Bd* on these tadpoles, the data were not analyzed further. In contrast, tadpoles of *L. nannotis*, *L. rheocola* and *Nyctimystes dayi* had high prevalences of mouthpart loss and *Bd* infection during year 1 (Fig. 6.1). Accordingly, mouthpart loss in tadpoles of these species was examined in more detail in year 2. *Nictimystes dayi* was not included in analyses because of small sample size.

Prevalence of Tooth Loss

The proportions 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* (Pearson chi-square = 150.2, $p < 0.0005$, df = 5, n = 664; Table 7-1, Fig. 7.2) and *L. rheocola* (Pearson chi-square = 57.13, $p < 0.0005$, df = 4, n = 152; Table 7-1, Fig. 7.2). *Litoria nannotis* tadpoles with no loss (Pearson chi-square = 146.8, $p < 0.0005$, df = 1, n = 664) and with discolored mouthparts (Pearson chi square = 13.94, p = 0.001, df = 1, n = 319) had lower prevalences of *Bd* than tadpoles with greater levels of tooth loss. The prevalence of infection in *L. nannotis* did not significantly differ among tooth loss levels greater than discoloration (Pearson chi-square = 4.4, p = 0.224, df = 3, n = 170). *Litoria rheocola* with normal mouthparts had lower prevalences of *Bd* than tadpoles displaying any level of tooth loss, including discoloration (Pearson chi-square = 53.3, $p < 0.0005$, df = 1, n = 152). In contrast to *L. nannotis*, prevalence in *L. rheocola* with discolored teeth did not significantly differ from *L. rheocola* tadpoles with more advanced loss (Pearson chi-square = 0.354, p = 0.552, df = 1, n = 106). The sample size for *L. rheocola* with >75% loss was small (n = 2) and was removed for analyses but included in Fig. 7.2.

Prevalence of Jaw Sheath Loss

The proportion of tadpoles with no loss, thinning, <10%, 10-50%, 50-90% and >90% jaw sheath loss differed significantly between infected and uninfected tadpoles of both *L. nannotis* (Pearson chi-square = 75.2, $p < 0.0005$, df = 5, n = 616; Table 7-1, Fig. 7.3) and *L. rheocola* (Pearson chi-square = 30.9, $p < 0.0005$, df = 4, n = 151; Table 7-1, Fig. 7.3); however, over 60% of tadpoles of both species with no loss were infected. Prevalence was 100% in tadpoles of both species presenting any level of jaw sheath loss (except for one *L. rheocola* with 50-90% jaw loss that was uninfected as determined by PCR).

Table 7-1. Numbers of *B. dendrobatidis*-infected and uninfected *L. nannotis* and *L. rheocola* tadpoles by degree of tooth loss and jaw loss. Tully Gorge NP, 2007- 2008.

	Litoria nannotis			*Litoria rheocola*		
	Uninfected	**Infected**	**Prevalence**	**Uninfected**	**Infected**	**Prevalence**
Tooth loss Score						
No loss	159	186	53.9%	25	21	45.7%
Discoloration	14	134	90.5%	2	34	94.4%
<25%	0	52	100.0%	1	21	95.5%
25-50%	0	65	100.0%	0	39	100.0%
50-75%	1	31	96.9%	0	9	100.0%
>75%	0	22	100.0%	1	1	50.0%
Jaw Loss Score						
No loss	156	297	65.6%	28	48	63.2%
Thinning	0	81	100.0%	0	29	100.0%
<10%	0	44	100.0%	0	15	100.0%
10-50%	0	18	100.0%	0	11	100.0%
50-90%	0	19	100.0%	1	19	95.0%
>90%	0	1	100.0%			

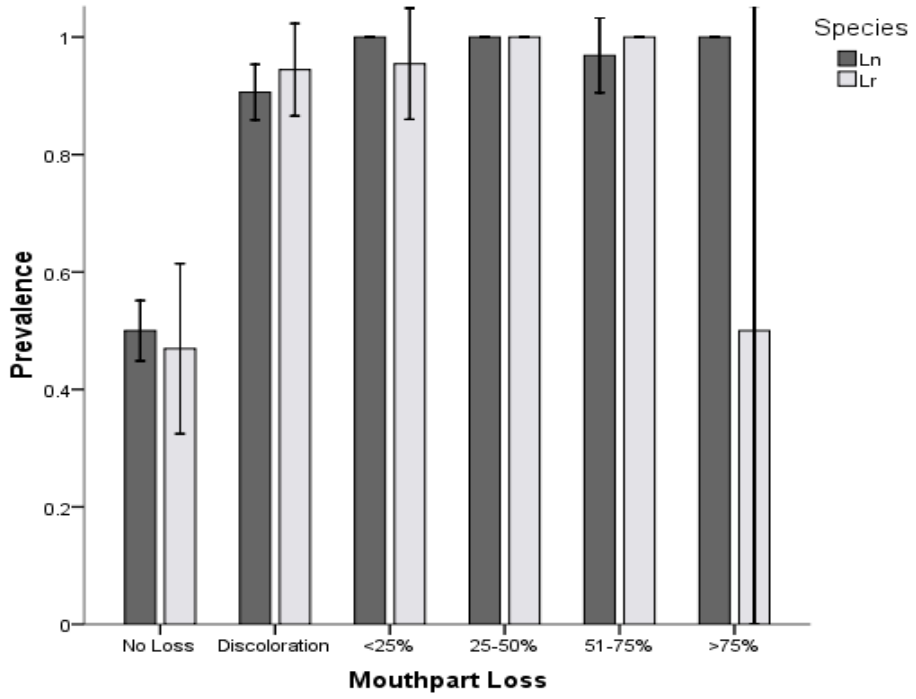


Figure 7.2. Mean prevalence +/- 95% CI of *B. dendrobatidis* infection in *L. nannotis* and *L. rheocola* by degree of overall mouthpart loss. Tully Gorge NP, 2007-2008.

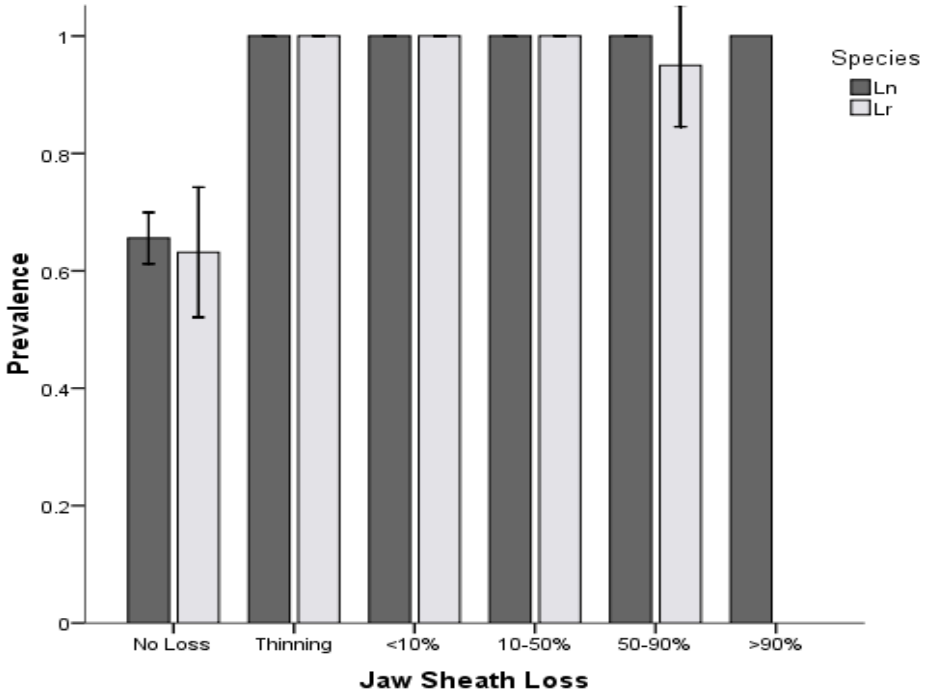


Figure 7.3. Mean prevalence +/- 95% CI of *B. dendrobatidis* infection in *L. nannotis* and *L. rheocola* by degree of jaw sheath loss. Tully Gorge NP, 2007-2008.

Binomial Logistic Regression

Binomial logistic regression was employed to determine which of mouthpart loss or jaw sheath loss was the better predictor of *Bd* infection status and if there were any differences in this predictive ability between *L. nannotis* and *L. rheocola*. 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 a significant indicator of infection status while species and jaw sheath loss were not (Table 7.2). 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 significantly improve the ability to predict infection status (Table 7.2).

Table 7-2. Binomial logistic regression examining the predictive abilities of species, jaw sheath loss, and tooth loss for 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			

Sensitivity and Specificity

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 7-4). 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 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 7-4). Visual assessment of

jaw sheath loss as an indicator of infection status produced similar results. Specificity was very high, however, sensitivity was low.

Due to the low prevalences of both 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 7-3). In the case of *L. xanthomera*, the sensitivity was 0% simply because no mouthpart loss was observed.

Table 7-3. Evaluation of overall tooth loss as an indirect test for 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.

			PCR Result			Percent		
Species	Mouthpart	Visual Assessment	Bd-	Bd+	Prevalence	Accurate	Sensitivity	Specificity
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%

Species	Mouthpart	Visual Assessment	PCR Result Bd-	PCR Result 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%

Table 7-4. Evaluation of overall tooth loss as an indirect test for infection with *B. dendrobatidis* in *L. 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.

Intensity

Mouthpart loss is a more precise indicator of infection status than jaw loss, therefore, the following analyses focused on mouthpart loss data. *Litoria rheocola* with >75% loss were excluded from the analyses because of small sample size (n=2).

Intensity of infection differed significantly among tooth loss categories in both *L. nannotis* (ANOVA, $F_{5, 484} = 12.56$, $p<0.0005$, Fig. 7.4) *and L. rheocola* (ANOVA, $F_{4, 119} = 12.72$, $p<0.0005$, Fig. 7.4). 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. Results of Tukey's HSD tests indicate that mean intensity in *L. nannotis* with no tooth loss was significantly lower than in tadpoles with discoloration, <25% and 25-50% loss, but did not differ significantly from tadpoles with 50-75% or >75% loss. Intensity did not differ significantly among groups displaying discoloration or tooth loss.

Infection intensity was generally lower in *L. rheocola* than *L. nannotis*, however, the pattern of intensity in relation to tooth loss was very similar. Mean intensity in *L. rheocola* tadpoles with no tooth loss was significantly lower than in tadpoles with any level of tooth loss, including discoloration. Mean pathogen load peaked in tadpoles with <25% tooth loss and then decreased. Tukey's HSD tests indicated that tadpoles with normal mouthparts had a significantly lower mean intensity than tadpoles in any of the tooth loss categories. However, the difference between the no loss and 50-75% loss group was near non-significant (0.044). There were no significant differences among groups showing tooth loss.

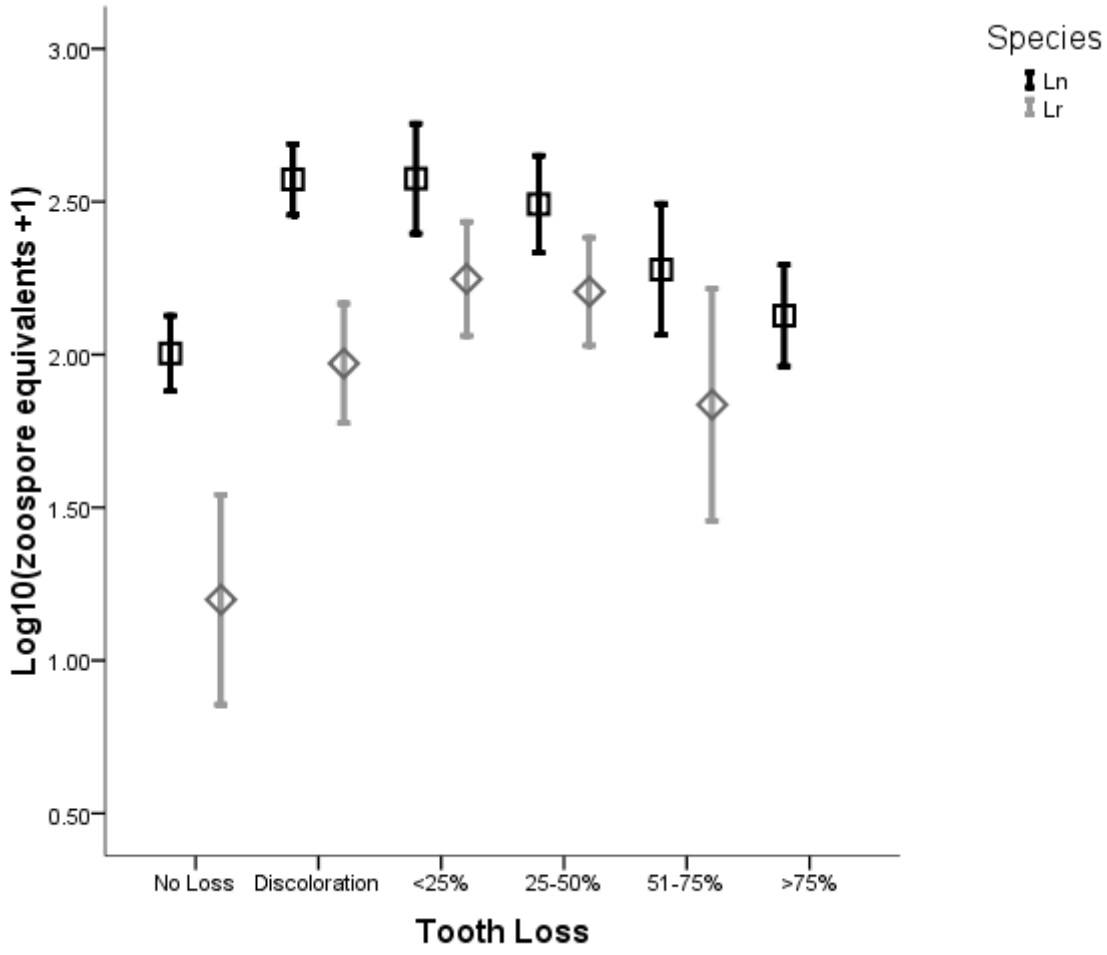


Figure 7.4. Mean intensity +/- 95% CI of *B. dendrobatidis* infection in *L. nannotis* and *L. rheocola* by degree of overall tooth loss. Tully Gorge NP 2007-2008.

Body Size Effects

Because *L. nannotis* and *L. rheocola* are 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. 7.9). Relatively few large tadpoles with normal teeth were uninfected, however, the only large tadpoles that were uninfected had normal teeth and, in one case, discoloration (Fig. 7.9). Although large tadpoles had very high prevalences and intensities of infection (Chapter 6) they did not have high levels of mouthpart loss. The proportion of tadpoles in the large size-group decreased with increasing mouthpart loss (Fig. 7.9), and no large tadpoles were

captured with >75% tooth loss. Tadpoles suffering advanced stages of tooth loss (>50% loss) were primarily medium sized tadpoles. Nearly all small tadpoles had no tooth loss, with only one small tadpole captured displaying a level of loss greater than discoloration (Fig. 7.9).

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. 6.3 and 7.9). Therefore, uninfected tadpoles were primarily present only when small, new tadpoles entered the population (Fig. 5.16, Fig. 7.10). 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 7.11). Leading up to this time, the prevalence of infection and mean intensity in infected individuals in the population increased (Fig. 6.20, Fig. 7.12). From May 15, tooth loss began to increase while mean infection intensity decreased. When tooth loss severity peaked around July 18, all tadpoles were infected and missing over 25% of their teeth, but mean intensity was ten times lower, and only medium sized tadpoles were missing over 75%. Over the following two months, until September 21, this pattern was reversed. Overall mouthpart condition improved, the number of large tadpoles increased as tadpoles developed towards metamorphosis, and mean infection intensity began to trend upwards again until September 12, around the time that metamorphosis began. From here, the number of large tadpoles decreased and the numbers of small to medium sized tadpoles increased, with the majority of small tadpoles uninfected and with no mouthpart loss. Intensity in infected tadpoles trended downward after December 23 until sampling stopped at the culmination of the year-long cycle on February 14, 2008.

Although detailed tooth loss data was not recorded during the first year of the study in 2006-07, I can compare patterns in the intensity of infection data (Fig. 6.20) with patterns seen in 2007-2008. While no definitive conclusions regarding patterns of tooth loss in the first year can be drawn, the same general pattern of infection intensity (which may indicate tooth loss patterns) was seen in 2006-07 as well as 2007-08; infection intensity of infected individuals was low in summer, increased over autumn, then decreased temporarily in winter (as tooth loss severity increased). Intensities then increased again (as mouthparts recovered) before peaking in spring and then declining back into summer as frogs metamorphose and new tadpoles enter the population.

Progression of Infection

The progression of mouthpart loss can be used as a measure of the progression of infection. Understanding the foci of infection is important to understand where and how transmission occurs. I monitored the progression of infection by recording the condition of each tooth row.

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. 7.1) suffered significantly more loss than the three interior rows at all stages of overall tooth loss, except when overall loss exceeded 75% (Fig. 7.6). Row P3 had significantly greater mean individual tooth loss than row A1, except when overall tooth loss was greater than 50%.

Prior to the period of peak overall tooth loss, increasing loss is associated with increasing prevalence and intensity of infection. During the period of peak mouthpart loss, the interior rows suffered tooth loss equivalent to the exterior rows (Fig. 7.8). Following the peak loss period, as teeth recovered, the pattern was reversed. 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.

Jaw Sheath

As overall tooth loss intensified, so did loss of the keratinized jaw sheath (Fig. 7.5). 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 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 7.3, which shows that 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.

Habitat

Over the period of peak mouthpart loss, when mean overall loss was greater than 25% (July 4 – Aug 1 2007, Fig. 7.7), tadpoles with increasingly severe mouthpart loss tended to occupy habitats with lower mean flow rates. This restricted time period was chosen to minimize the effects of seasonal differences on flow rates. Mean flow rate differed significantly among *L. nannotis* individuals with different categories of overall tooth loss score (ANOVA, $F_{3, 94} = 3.055$, $p = 0.032$); differences were near significant for *L. rheocola* (ANOVA, $F_{3, 37} = 2.613$, $p = 0.066$). Tukey's HSD tests indicated, for *L. nannnotis*, that tadpoles with greater than 75% loss occupied habitats with lower mean water flow rates than tadpoles with <25% loss. There were no other statistically significant comparisons.

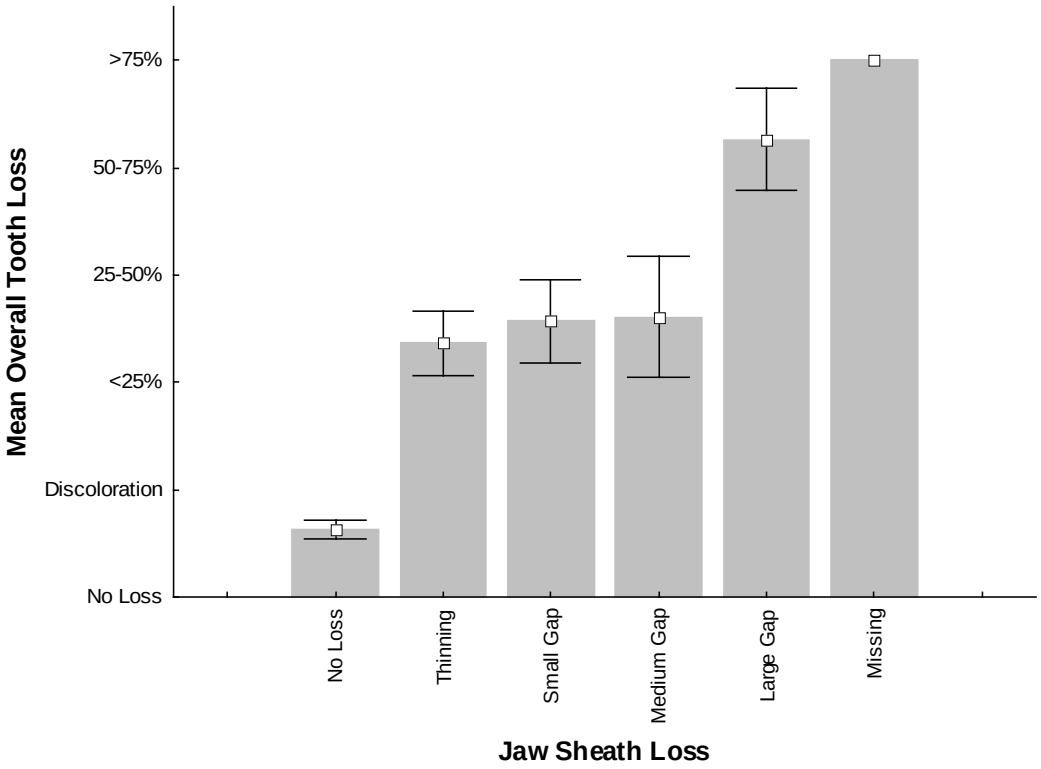


Figure 7.5. Mean overall tooth loss score +/- 95% CI of L. nannotis and L. rheocola tadpoles with varying degrees of jaw sheath loss. Tully Gorge NP 2007- 2008.

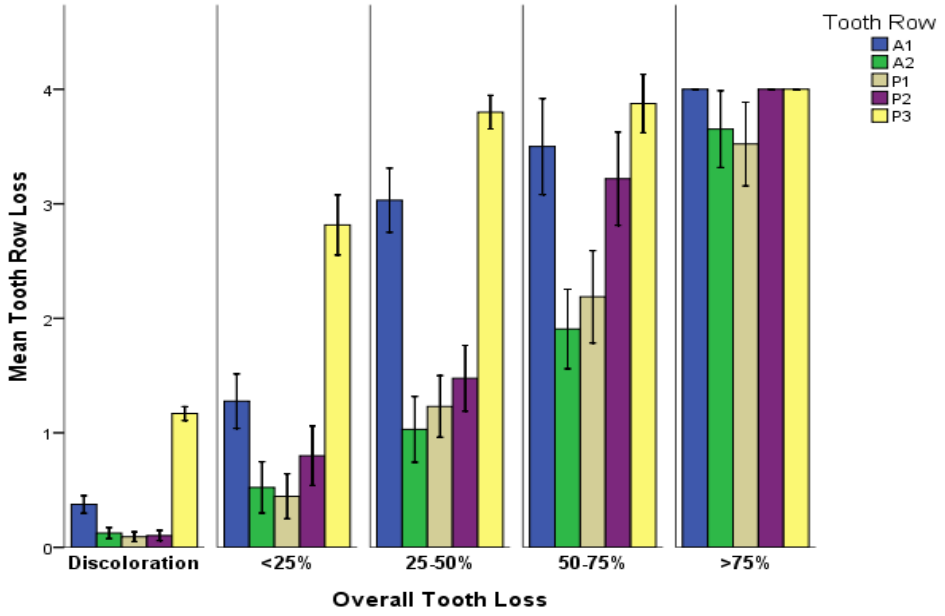


Figure 7.6. Mean individual tooth row loss +/- 95% CI with varying degrees of overall tooth loss in *Litoria nannotis* and *L. rheocola*. Tully Gorge NP, 2007 - 2008.

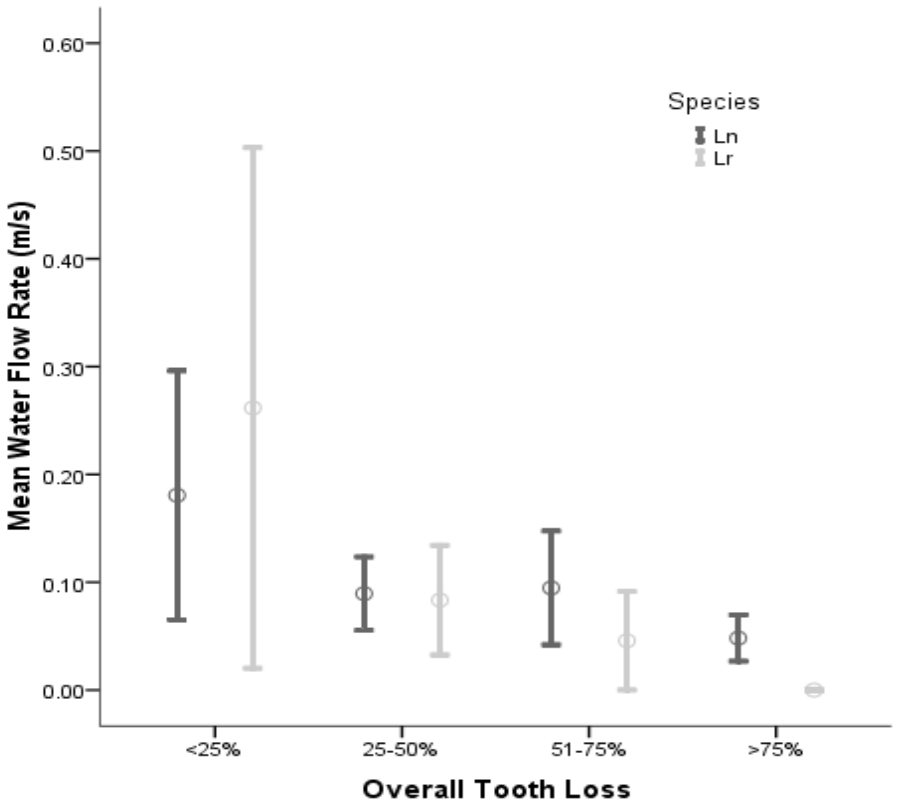


Figure 7.7. Mean water flow rate +/- 95% CI where *Litoria nannotis* tadpoles with varying degrees of overall tooth loss were captured. Tully Gorge NP, July 4 – August 1, 2007.

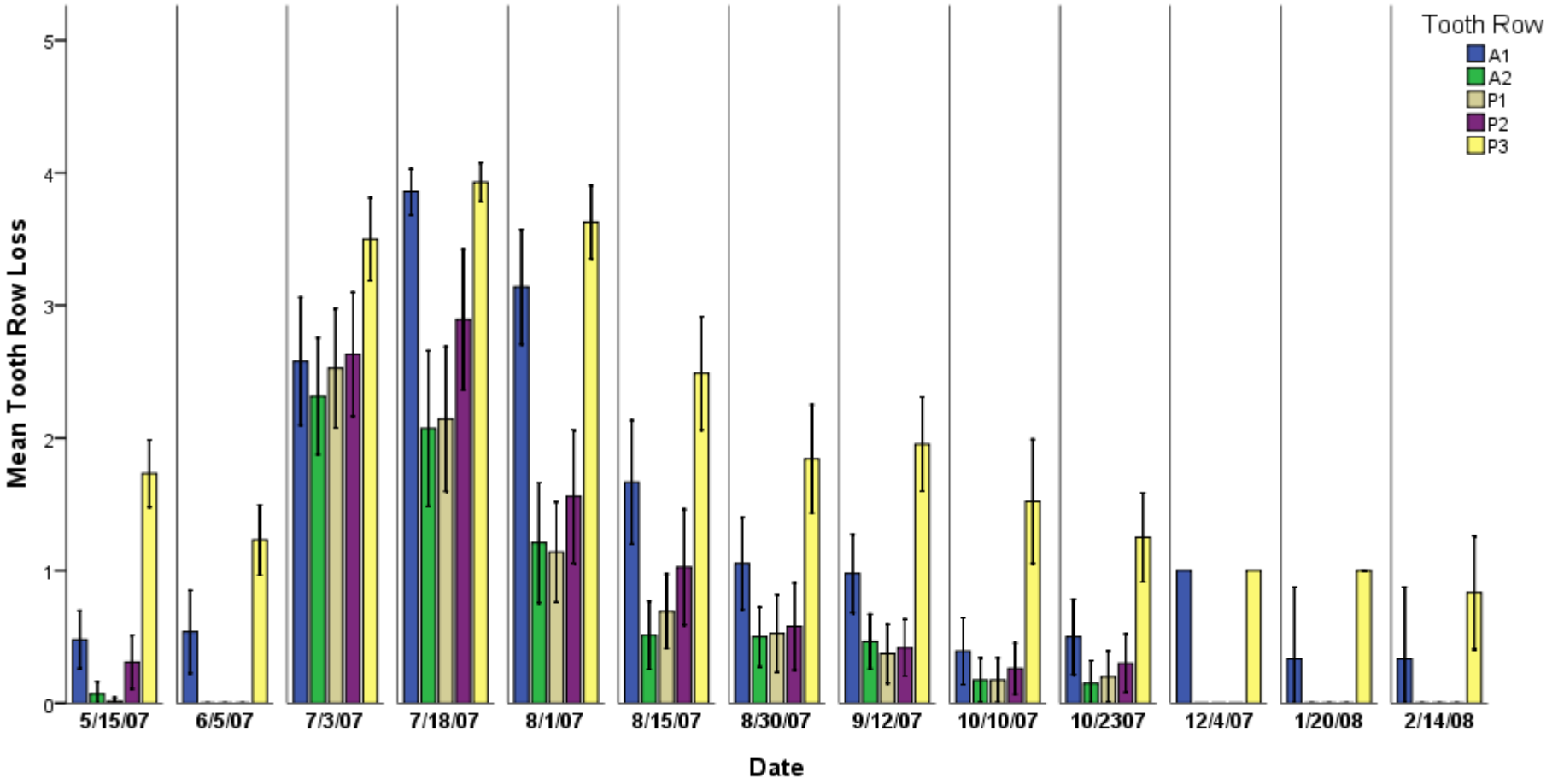


Figure 7.8. Mean individual tooth loss +/- 95% CI in *L. nannotis* over time at Tully Gorge NP. Graph begins when tooth loss was first observed

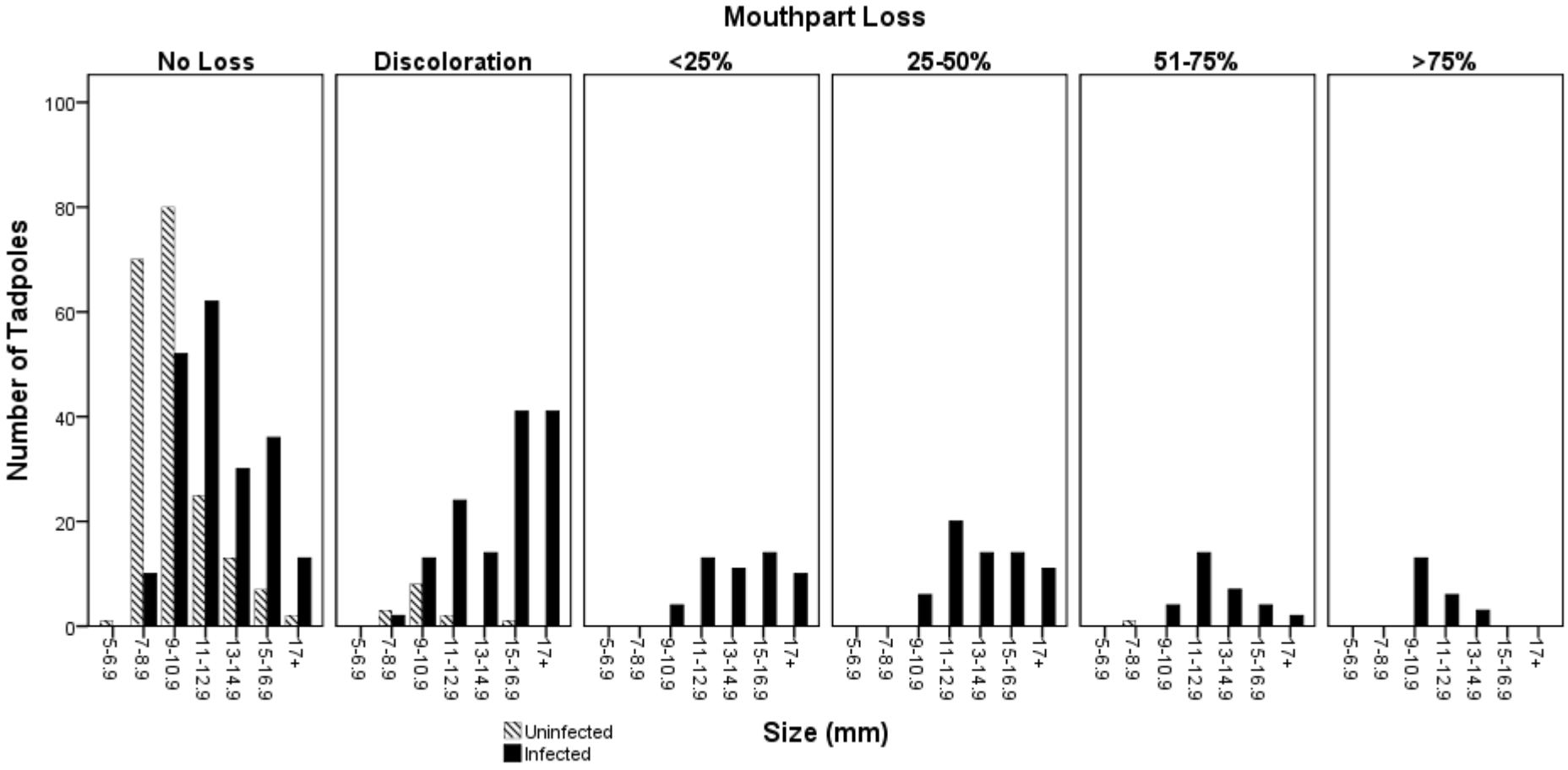


Figure 7.9. Number of infected and uninfected *Litoria nannotis* tadpoles by overall tooth loss and size class. Tully Gorge NP, 2007-2008.

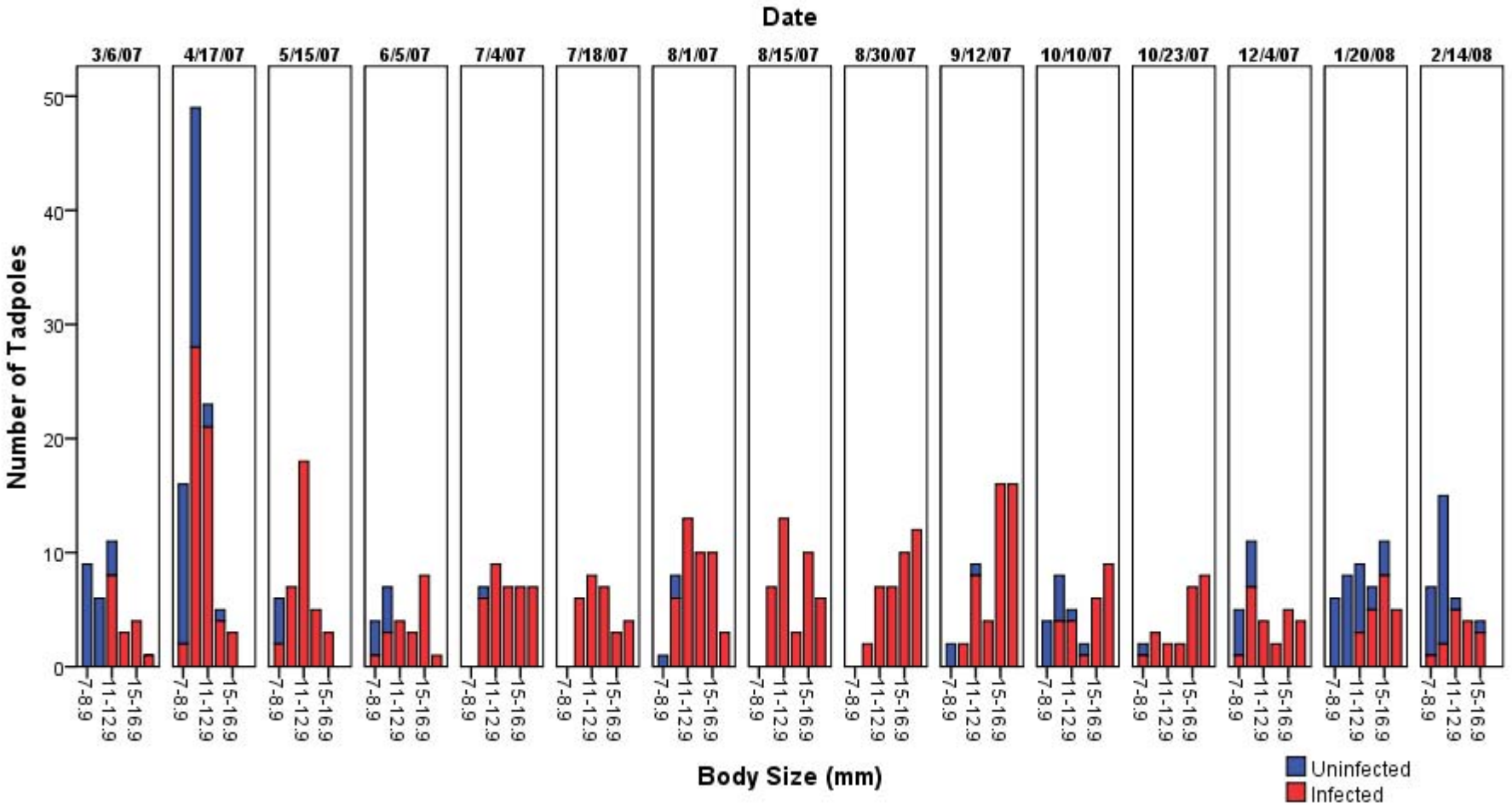


Figure 7.10. Number of infected and uninfected *Litoria nannotis* tadpoles in Tully Gorge NP by size class over time (2007 – 2008).

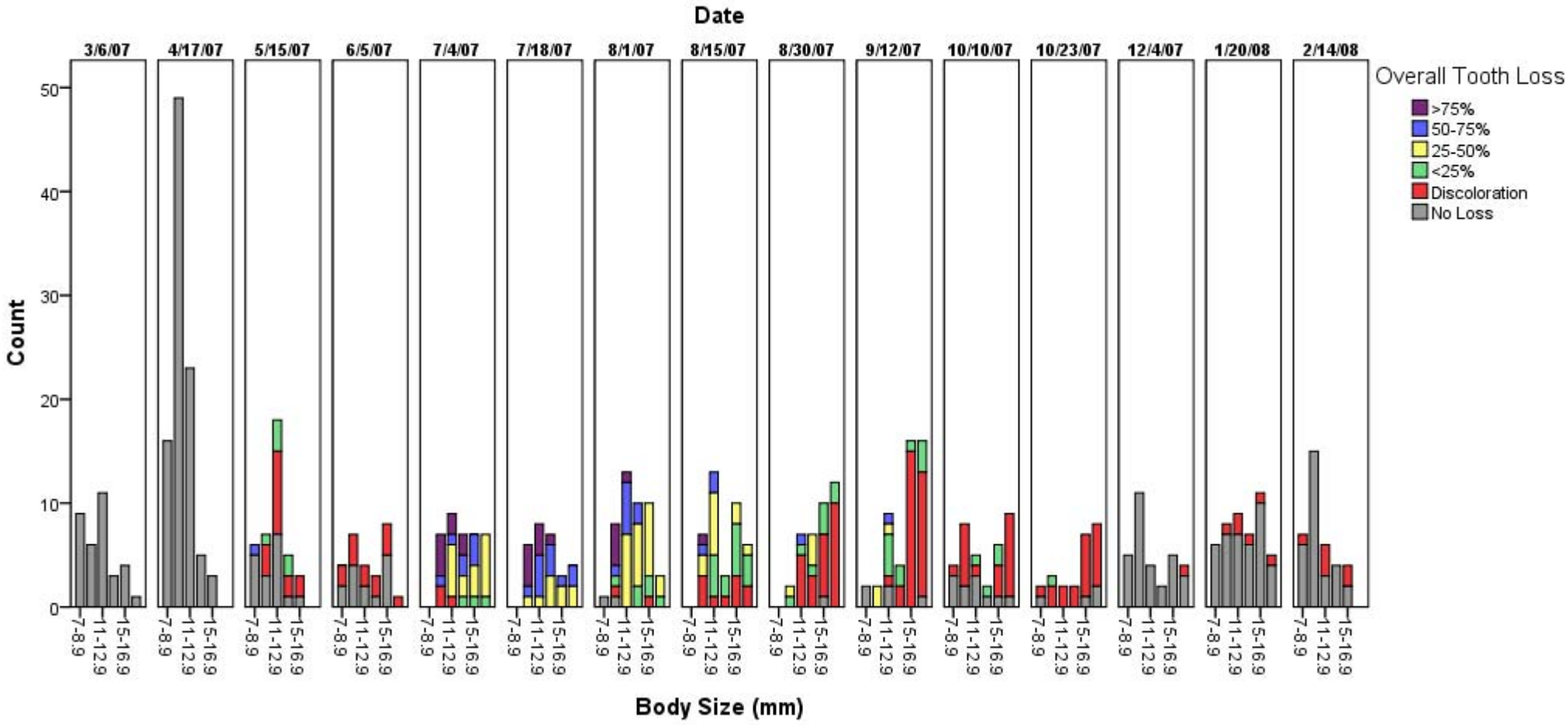


Figure 7.11. Overall tooth loss in *L. nannotis* in Tully Gorge NP by size class over time (2007- 2008).

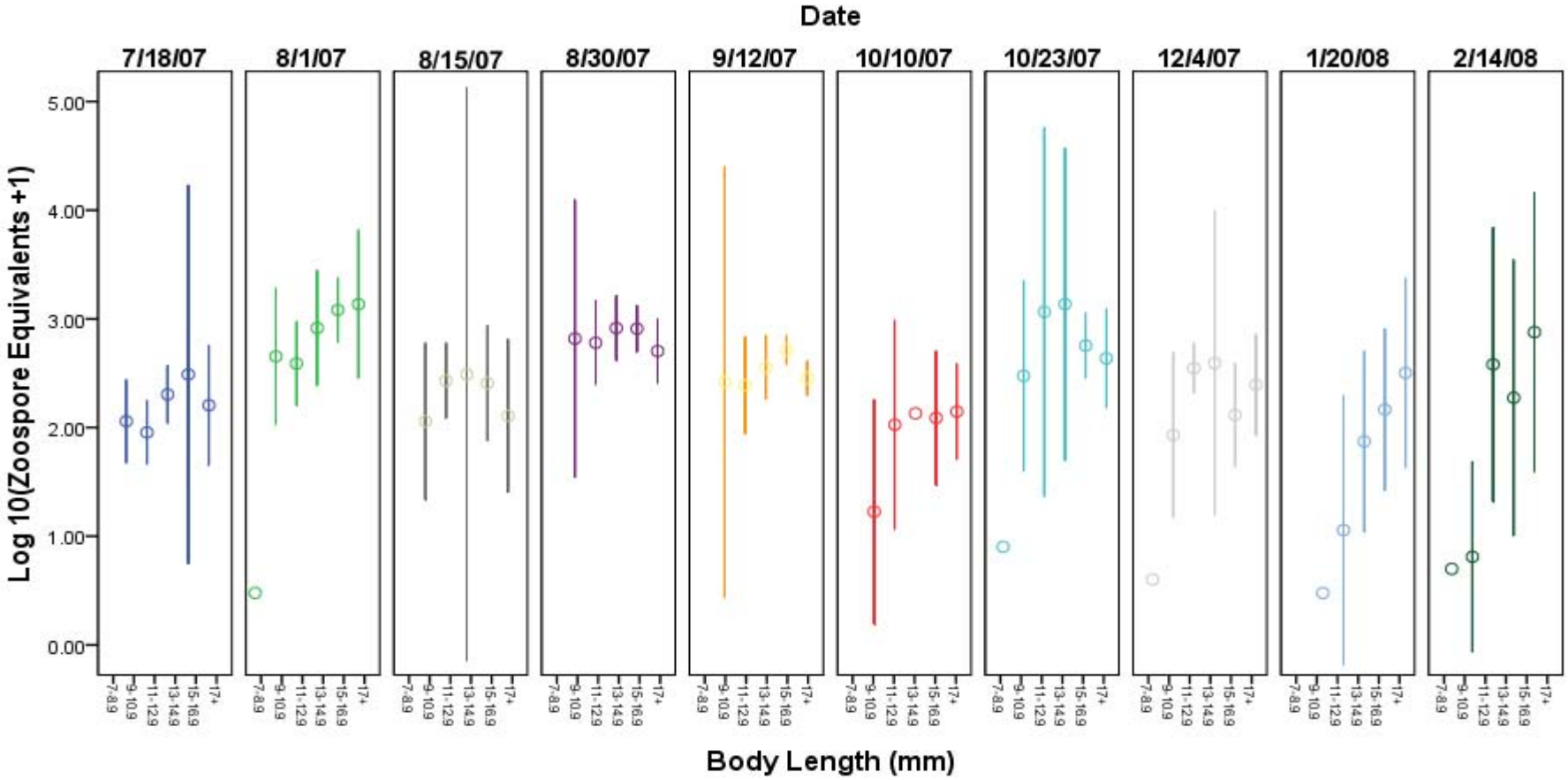


Figure 7.12. Intensity of *Bd* infection +/- 95% CI in *Litoria nannotis* tadpoles by size class over time. Tully Gorge NP, 2007- 2008.

Body Condition

Tadpole body condition was estimated by first regressing $\log_{10}$ body length (mm) against $\log_{10}$ mass (g) using ordinary least squares (Fig 7.13, *L. nannotis*: $R^2 = 90.9\%$, *L. rheocola* $R^2 = 90.4\%$). From the regression line, the mass residual for each tadpole was determined. These residuals represent the difference in mass from the expected for a given 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* (Fig. 7.14). 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 the mass residuals changed significantly over time (ANOVA, *L. nannotis*, $F_{13, 579} = 56.414$, $p < 0.0005$; *L. rheocola*, $F_{12, 121} = 4.782$, $p < 0.000005$, Fig 7.13). From April 17, 2007 until the observed onset of metamorphosis on October 10, 2007, tadpole body condition (mass residuals) was negatively correlated with mean mouthpart loss (Time series cross-correlation: *L. nannotis*; $r = -.719$, *L. rheocola*; $r = -.655$; Fig. 7.13). As the severity of mouthpart loss increased, body condition decreased. As mouthparts recovered, mean body condition improved. Then, as the levels of mouthpart loss returned to zero, and during the period of metamorphosis and recruitment, the mean value of the mass residuals significantly decreased (Fig. 7.14).

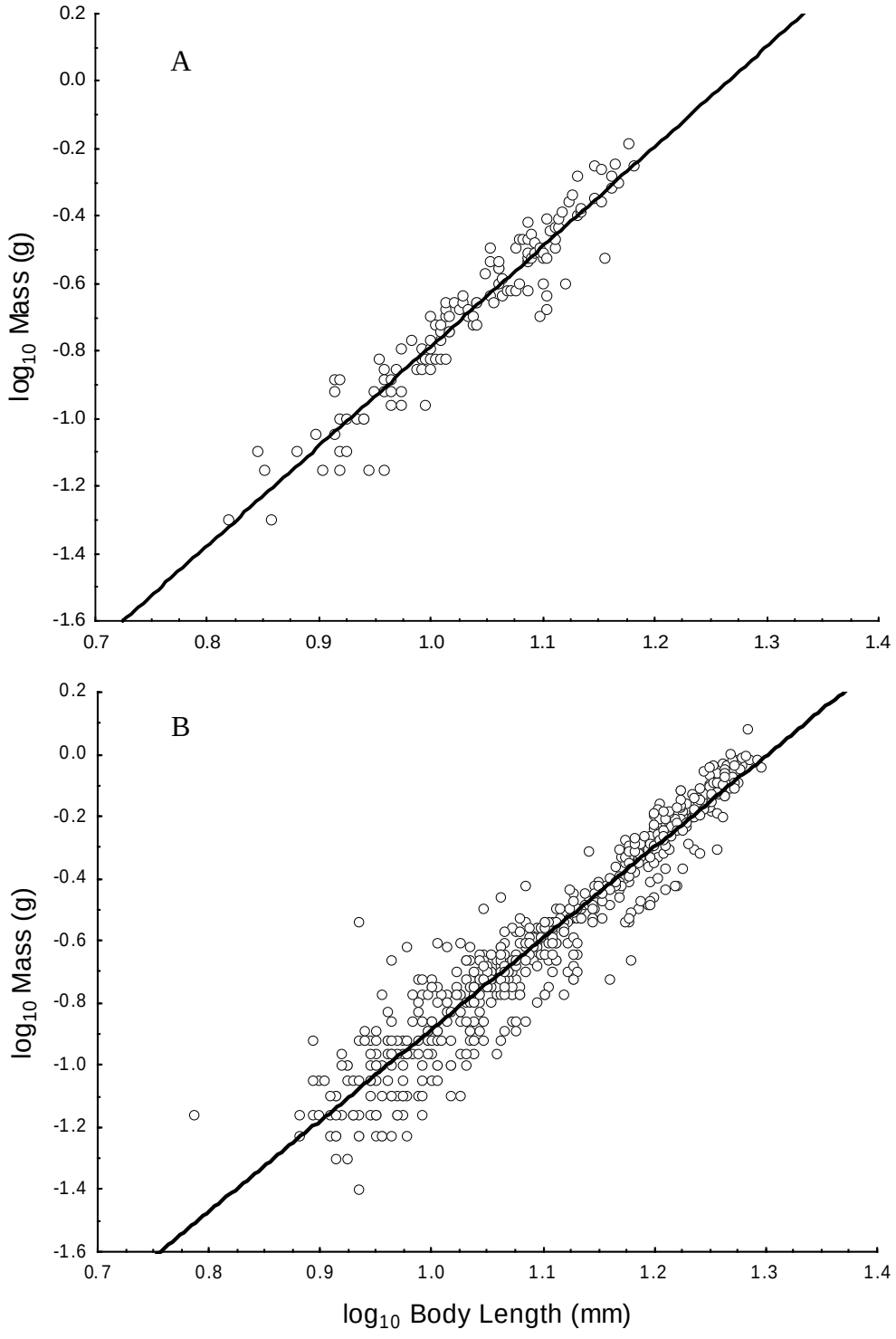


Figure 7.13. Scatter plot and linear regression of log_{10} mass and log_{10} body length of (A) *L. rheocola* (log_{10} Mass = -3.8138+2.9299x, r^2= 90.4%, N=134) and (B) *L. nannotis* (log_{10} Mass = -3.7444 + 2.959x, r^2= 90.9%, N=593). Tully Gorge National Park 2007 – 2008.

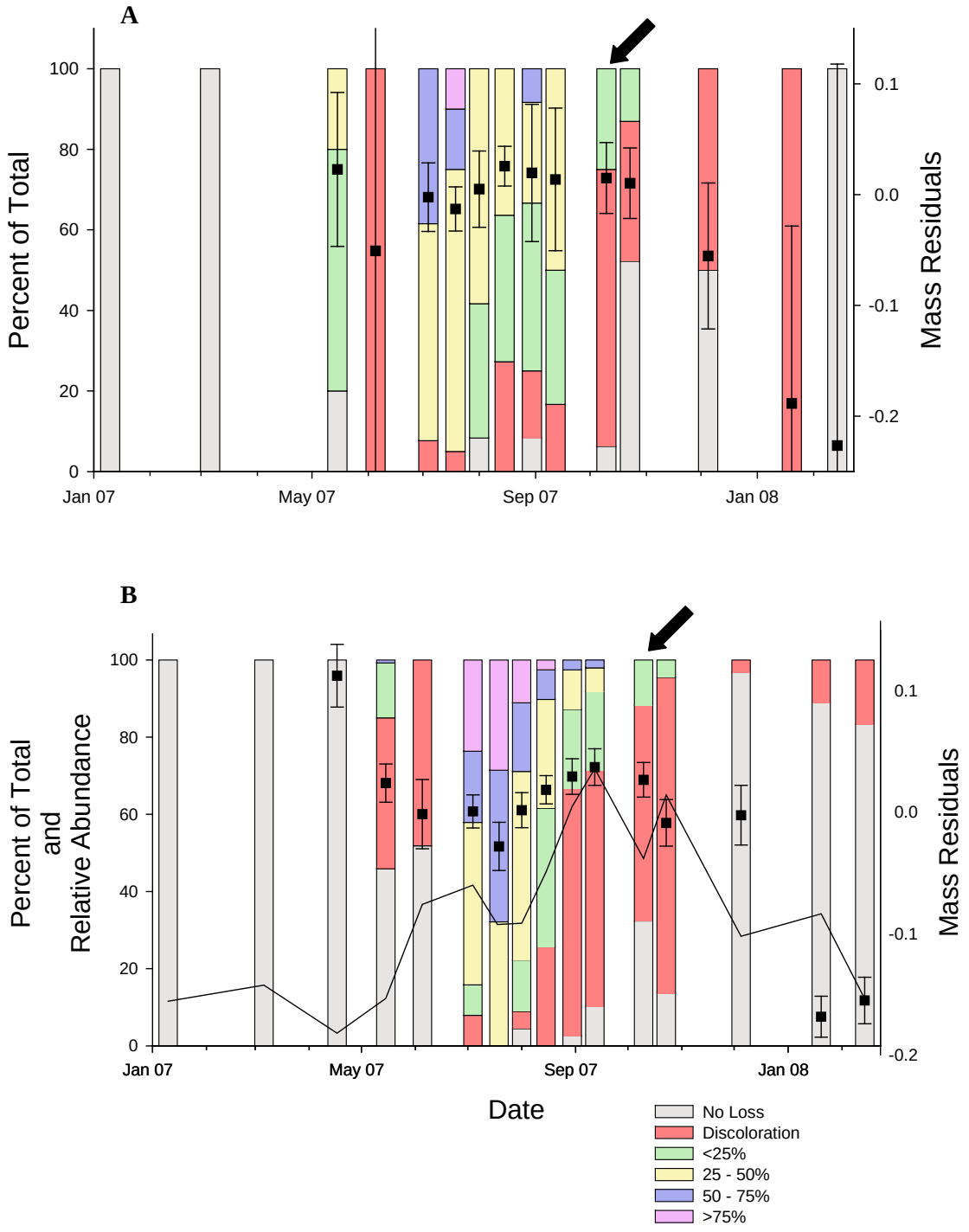


Figure 7.14. Mean mass residuals +/- 95% confidence limits overlaid on stacked bar graphs representing degrees of tooth loss over time. A) *L. rheocola*, B) *L. nannotis*. Tully Gorge NP (2007-2008). The solid black line in B is the relative abundance of large size-group tadpoles. Black arrow indicates earliest occurrence of metamorphosis.

Observations

Over the course of the two-week period of peak overall loss, many of the tadpoles captured with >50% loss showed a substantial decrease in body condition (Fig. 7.15). 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 few 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.

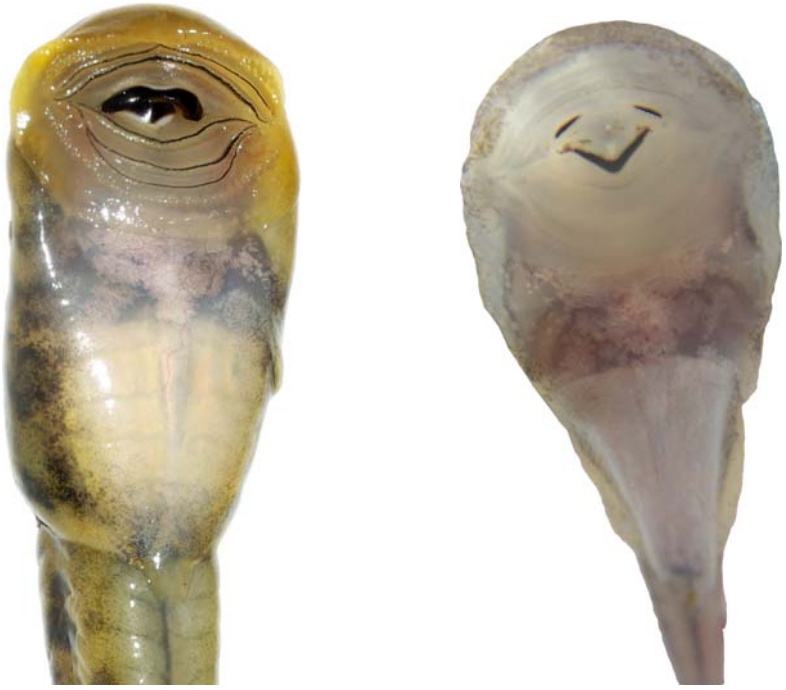


Figure 7.15. Left: A healthy *L. nannotis* with normal tooth rows, full gut and well-developed tail musculature. Right: An unhealthy *L. nannotis* with >75% overall tooth loss and 50-90% missing jaw sheath, empty gut, and poorly developed tail musculature.

On at least two occasions, segments of tooth rows dislodged from the mouthparts of severely weakened *L. nannotis* during handling and swabbing. Dislodged segments of teeth were either found on the glove following handling or 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. This suggests that poor body condition was a true indicator of very poor health.

Effects on Population Size and Development

To determine whether tadpole population size changed significantly following the period of peak body condition loss, population estimates were conducted on a 5 m section of stream at Tully Gorge. *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 min blocks, separated by 5 min breaks. As the stream is an open system it is not feasible to truly exhaust the population. The X-intercept of a linear regression of the number of tadpoles captured per sampling block against 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 that the population density remained relatively constant (Fig. 7.17).

In an initial effort to determine whether tadpoles metamorphose at a smaller size as a result of mouthpart loss, a comparison was made with a site (McLeod Creek) on the Carbine Tablelands where mouthpart loss in torrent tadpoles caused by *Bd* infection does not appear to be as severe (pers. obs.). For this analysis, only tadpoles within the largest size class (>17 mm) from the period just prior to metamorphosis (8/30/07 and 9/12/07) were included from Tully Gorge NP in order to select the largest tadpoles captured. In comparison, tadpoles from McLeod Creek were captured on 7/28/08, the middle of winter, and approximately 8 weeks away from the metamorphic period, suggesting McLeod tadpoles had not yet reached maximum size. Body sizes were compared using an independent samples t-test.

Tadpoles were significantly larger at McLeod Creek (N= 28, mean = 21.2 mm, SD = 2.36) than at Tully Gorge NP (mean = 18.1 mm, SD = 0.63, N=28; $t_{54} = -6.74$, $p <$

0.0005; Fig. 7.16). Many factors can affect size at metamorphosis (Alford, 1999), however, these results provide tentative support for the hypothesis that mouthpart loss may affect size at metamamorphosis in *L. nannotis*.

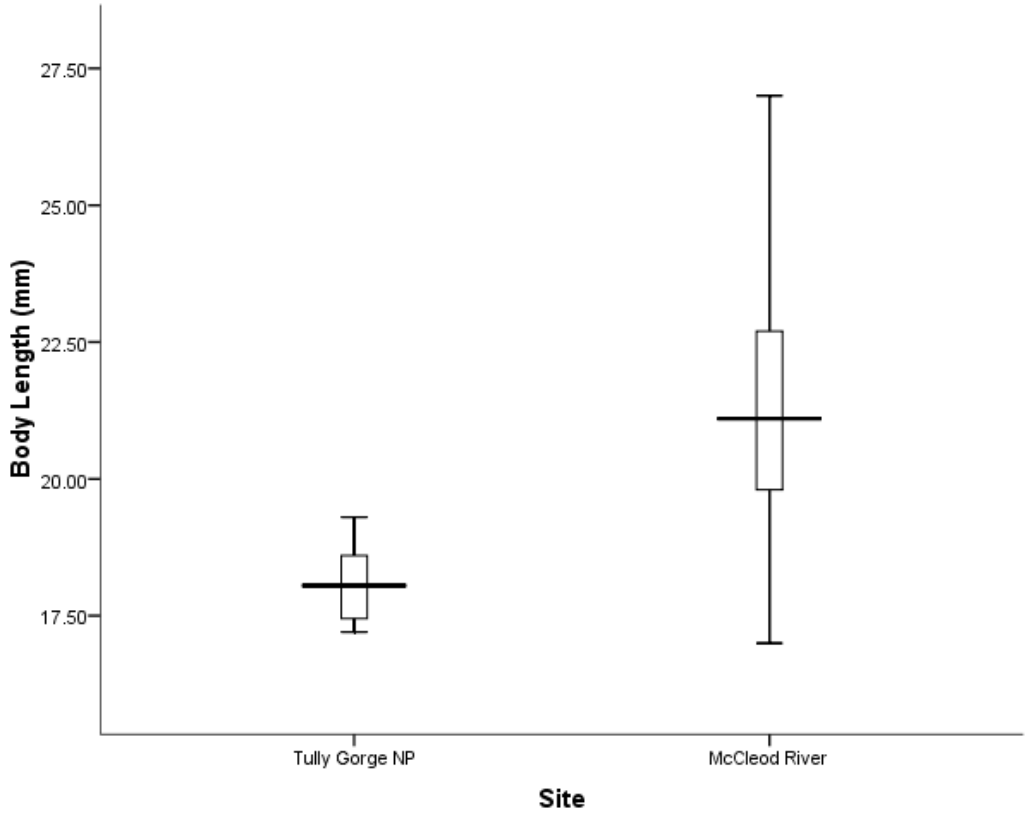


Figure 7.16. Box plot of body lengths of *Litoria nannotis* tadpoles within the >17 mm size class at Tully NP and McLeod Creek. Tully NP data are from the 4 weeks prior to the onset of metamorphosis.

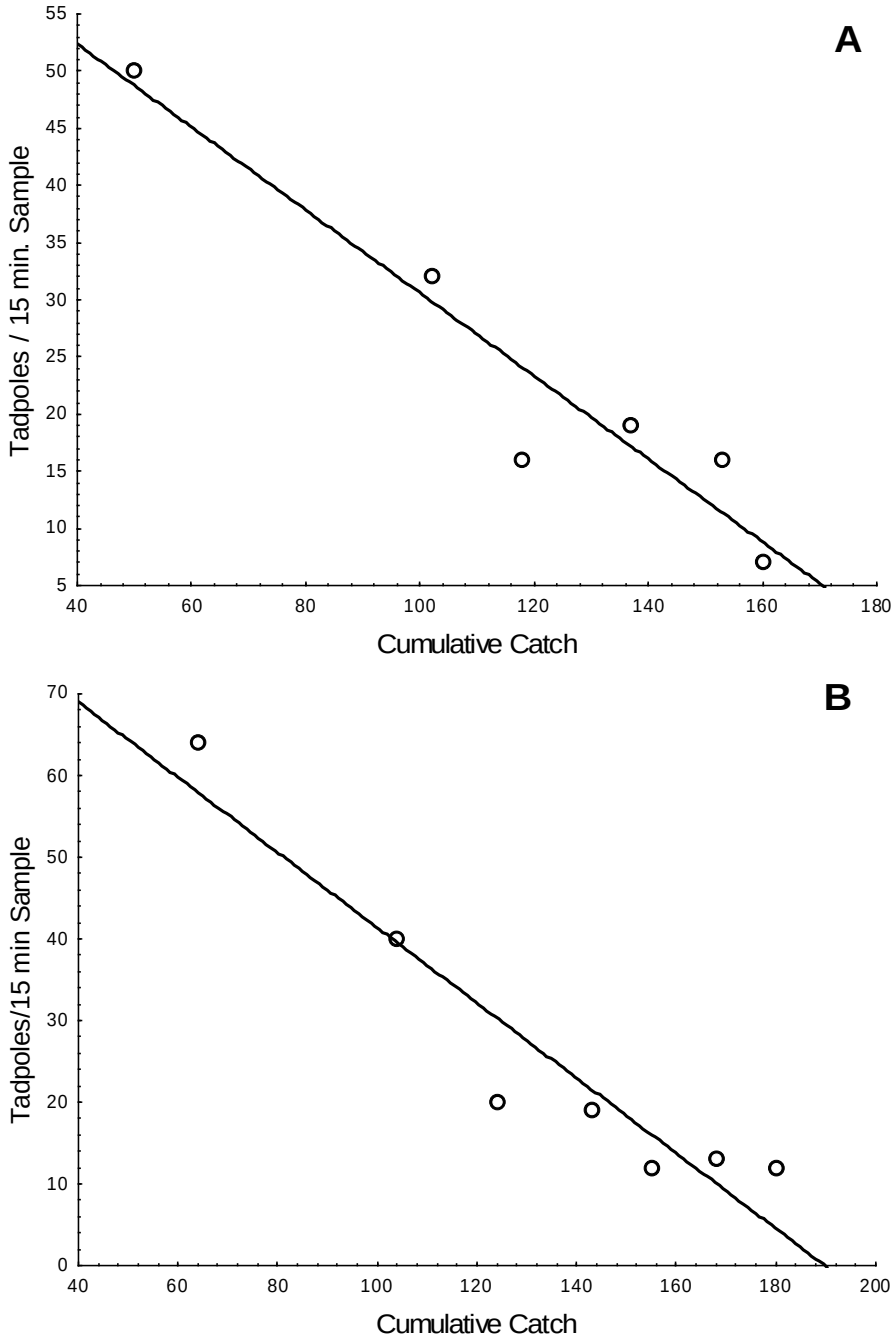


Figure 7.17. Population estimates of number of torrent tadpoles in a 5 meter stretch of stream at Tully Gorge NP before and after the time of peak mouthpart loss. A) May 15, 2007 (r^2=0.9158, y=66.8673- 0.3628x, X-intercept = 184 tadpoles). B) August 16, 2007 (r^2=0.8990, y=87.4063-0.4604x, X-intercept = 190 tadpoles).

Discussion

Oral deformities are associated with *Bd* infection in larvae of a number of species (Lips et al., 2004, Symonds et al., 2007, Vredenburg and Summers, 2001, Fellers et al., 2001, Marantelli et al., 2004). Knapp and Morgan (2006) found that the presence of oral deformities was a good predictor of *Bd* infection at both the individual and population levels (86% and 92% correctly predicted respectively), however, mouthpart deformities can have a number of causes apart from disease, including temperature, contaminants, physical damage and phylogeny (Rachowicz, 2002, Drake et al., 2007, Padgett-Flohr and Goble, 2007). It also appears that larvae of some species may exhibit no mouthpart deformities when infected with *Bd* (Knapp and Morgan, 2006). To date, most detailed data on mouthpart deformities in *Bd*-infected populations have come from pond-dwelling tadpoles in temperate regions (the sole exception is Symonds et al. 2007). I present the first information on infection and deformities in torrent-adapted tadpoles and on 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.

This study indicates that *Bd* can interact with tadpoles in a variety of ways, probably as a result of differences in host morphology, behaviour, and microhabitat. It produced 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 mouthpart loss associated with *Bd*.

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 emaciated and physically weak. The data strongly suggest that mouthparts were subsequently regenerated by most individuals, despite persistent infection by *Bd*, allowing feeding to resume, body condition to improve, and preventing widespread mortality. The recovery of mouthparts is evidenced by the fact that all tadpoles experienced at least >25% loss

during mid-winter (Fig. 7.8, 7.14), however, before metamorphosis and the large-scale recruitment of new tadpoles into the population, the condition of mouthparts improved across all size classes (Fig. 7.11). Further, the pattern of loss within individual tooth rows indicates that infection progressed from the outer tooth rows in and then recovered from the innermost tooth rows outward (Fig. 7.8).

The pattern of loss and regrowth of tooth rows may shed light on the mechanism by which the intensity of *Bd* infection decreased following the period of peak intensity in winter. If intensity decreased because of shedding of teeth and tooth rows, one would expect tooth rows to recover in the order in which they were damaged, since the tooth progenitor tissue in shed areas would be free of infection while that in less damaged tooth rows would not. If some other aspect of immune function slowly gains competency as tooth row damage accumulates, leading to an overall decrease in intensity regardless of shedding, then one would expect less damaged tooth rows to regrow first. The latter pattern was clearly shown in the field, suggesting that the decrease in intensity is caused by increase in some aspect of immune function, rather than simple mechanical loss of infected tissue.

Mouthpart Loss and Prevalence of Bd

Tadpoles in rainforest streams are likely to have fewer non-disease induced mouthpart deformities than are temperate pond species. Tropical streams are often permanent water bodies with a small minimum to maximum temperature range. Therefore, the stresses of seasonally cold temperature or evaporating habitat leading to overcrowding, which occur in some temperate species (Rachowicz, 2002), are unlikely to occur in rainforest streams. The sites visited for this study were within and downstream from protected, undisturbed areas, thereby limiting the exposure to pesticides or contaminants that may cause deformities.

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 were strongly

associated with *Bd* infection (Table 7-2, 7-4). Mouthpart deformities were very rare in *L. genimaculata* and were not strongly associated with infection (Table 7-3). Deformities were never observed in *L. xanthomera*.

Mouthpart Damage as an 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 7.3). This indicates that a longer duration of infection is required before loss of jaw sheath cells is noticeable (Fig. 7.5). 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), and only a limited number of PCR tests can be performed, using the jaw sheath loss score to select individuals for subsequent direct testing (PCR or histology) would be preferred as the 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, less ambiguous, and equally effective method for identifying infected individuals (Table 7-4). 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. 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* (Obendorf, 2005, Knapp and Morgan, 2006). If uninfected torrent tadpoles are desired, overall tooth loss score is the appropriate indirect screening tool because of a lower rate of false negatives (higher sensitivity).

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

Mouthpart Loss and Intensity of Bd

The intensity of infection in infected tadpoles was lowest in tadpoles with no mouthpart loss, and increased to a peak at loss less than 25%, before decreasing as loss increased above 25%. In *L. nannotis* intensity did not differ significantly between tadpoles with no loss and tadpoles with >50% loss. The initial increase of intensity of infection with increasing tooth loss is consistent with an infection spreading across the mouthparts and disrupting tooth production, leading to more advanced loss. Decreases in intensity at mouthpart losses above 25% may have occurred as a result of a decrease in the quantity of substrate available for infection as keratinized teeth are shed. Infections may therefore "burn themselves out" to some extent, although infections are rarely cured completely (Chapter 6). When few keratinized teeth remained (and the tadpoles were in poor condition), intensity dropped, at which point mouthparts were able to recover and intensity began to increase again). However, in order to achieve the near full recovery of mouthparts population-wide that was observed (Fig. 7.13), even as infection persisted at an elevated level, some form of resistance or tolerance is required (Chapter 6). Histological examination of preserved tadpoles is needed to investigate possible mechanisms.

Intensity of infection over two years was best explained by a multiple regression model including body length, rainfall, site and solar radiation (Chapter 6). Mouthpart loss was not included in this analysis because it was considered a sign of and not a cause of infection intensity, although it clearly has an effect, as would the presence of resistance/tolerance.

Figure 7.12 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 the highest intensity infections; intensity was lower in smaller and larger tadpoles. As large size-group tadpoles left the population and small tadpoles hatched (Fig. 7.11, December 4, 2007) the original pattern of increasing intensity with body size returned (Fig. 7.12). This shifting pattern of intensity of infection suggests a combination of increasing resistance and cessation of feeding due to mouthpart loss in heavily infected individuals. It appears that at the time of peak mouthpart loss, few tadpoles were able to grow into the larger size classes. Figure 7.13 indicates that *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.

Progression of Infection

Tooth Row Loss

The outermost tooth rows (A1 and P3) were the first to show damage and the last to recover (Fig. 7.8). This 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 if torrent tadpoles acquire their infections 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. As mentioned earlier, this pattern suggests that recovery occurs because resistance to infection has increased, rather than simply because heavily infected parts have been shed; if the latter were the case, the outer rows should recover first, since they are shed first.

Given that tadpoles themselves 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 from the external environment will drive both prevalence and 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 intensity of infection, then the progression of clinical signs and intensity of *Bd* within an individual should proceed independent of external factors. Removing infected tadpoles from the stream would eliminate the zoospores shed by those tadpoles and could decrease rates of transmission but should have no effect on intensity of infection in other tadpoles. However, if external re-infection is important, removing infected tadpoles should decrease both the rate of transmission and the mean intensity of *Bd* infections in tadpoles across the population. This could multiply the effect of removing infected animals and might mean that reducing density below a critical threshold would greatly reduce the impact of *Bd.* 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.

Jaw Sheath Loss

I observed jaw sheath loss only in tadpoles with missing tooth rows, indicating that jaw sheath loss occurs after tooth loss commences. 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 occurs but tooth row loss does not (Rachowicz and Briggs, 2007, Nieto et al., 2007). This may indicate a difference in how these tadpoles become infected with *Bd*. For instance, the jaws of the ranids may have closer contact with *Bd* in the environment compared to those of the torrent tadpoles. Alternatively, it may indicate variation in jaw sheath susceptibility to infection among species.

Habitat

Over the period of peak mouthpart loss (July 4 – August 1, 2007), *Litoria nannotis* tadpoles with greater than 75% loss were found in significantly slower-flowing water than tadpoles with less than 25% loss. Tadpoles with extensive loss were often emaciated and frail. They were weak and had difficulty swimming even within sorting trays. These tadpoles are likely to have had difficulty navigating swift flow rates without being swept downstream. This suggests that tadpoles with heavy mouthpart loss may select habitat with slower water flows to conserve dwindling energy reserves or may be unable to maintain their positions in fast flowing water. Richards (2002) found that tadpoles with smaller, less effective oral disc adhesion were excluded from swifter moving water. If a substantial proportion of reinfection occurs from the external environment, the relegation of heavily infected individuals to slower-flowing areas may lead to positive feedback in infection intensity, since zoospores should accumulate more readily in areas with reduced flow rates.

Body Condition

The severe loss of teeth due to infection very likely restricted the ability of torrent tadpoles to extract nutrients from the surfaces of rocks (Chapter 6). This restriction in feeding ability apparently caused a significant decrease in body condition prior to metamorphosis. This decrease was severe in some individuals, and probably caused some mortality. This widespread period of starvation during development appears to have affected growth and time to metamorphosis and could have negative carryover effects on adult fitness traits and population dynamics.

The general goal of tadpoles is to maximize size at, and minimize 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 (Wilbur, 1980, Pfennig, 1992). Growth and development rates, and thus the timing of metamorphosis in anurans, are plastic (Wilbur and Collins, 1973) in response to a number of factors, including but not limited to tadpole density (Gromko et al., 1973, Scott, 1994), predation (Nicieza et al., 2006, Lardner, 2000, Benard, 2004), temperature (Smith-Gill and Berven, 1979, Blouin and Brown, 2000), photoperiod

(Wright et al., 1990) water level (Denver et al., 1998) and food availability (Morey and Reznick, 2000, Alford and Harris, 1988). The often complex interaction of these factors reflects tradeoffs between the risk of remaining in the larval environment and the risk of transitioning to the unknown terrestrial environment (Werner and Gilliam 1984).

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 metamorphosis occurs when a minimum body size is obtained. The minimum size is likely established evolutionarily by decreased survival in metamorphs below the limit. If nutritional conditions improve and growth rate increases, time to metamorphosis (developmental rate) can increase 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 widespread starvation in the middle of growth and development could significantly affect not only larval survival, but also developmental rate, 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 mouthpart loss in *L. nannotis* (Fig. 7.11) the maximum period of starvation, or severely reduced feeding efficiency, as a result of >75% tooth loss, for any individual tadpole was 4-8 weeks. For many individuals, the time spent at >75% loss appears to be less than 4-8 weeks based on the recovery rate of tooth loss in the population. Regardless, many *L. nannotis* tadpoles probably can survive starvation periods of this duration.

Although many captured tadpoles were visibly emaciated, in poor condition and occasionally near death, population density estimates obtained by exhaustive sampling before and after peak overall loss do not indicate that widespread severe tooth loss caused significant mortality (Fig. 7.16), however, under different circumstances it might. Emaciated tadpoles may have a decreased ability to avoid predators or maintain position against the water current (Fig. 7.15). If predator densities were higher, any decrease in ability to avoid predation would lead to increased mortality. Unseasonal heavy rains, which occur in some years, could wash many individuals downstream. Any increase in the length of the period of maximum tooth loss, caused for example by lower temperatures or higher environmental densities of *Bd* zoospores, could lead to increased death through starvation.

No tadpoles in the large size-group were observed with >75% overall loss. In conjunction with the lack of evidence for decreases in tadpole density, 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 graphs of overall tooth loss (Fig. 7.11) and relative abundance of large tadpoles (Fig. 7.13) reveal 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 thus suggest that tadpoles recovered mouthparts, resumed growth, added body

mass and quickly entered the large size-group with primarily discolored mouthparts. Metamorphosis was observed after September 12 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.

Conceptual model

As mouthparts first began to disappear, growth rate began to decrease. According to the Wilbur-Collins model, if tadpoles at that time were of adequate size to metamorphose, they 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, so some metamorphosis may have occurred. Any tadpoles too small to metamorphose at this time would need to remain in the stream and continue to grow. Increased mouthpart loss due to *Bd* infection then caused the remaining tadpoles to lose body mass. Once mouthpart loss increased above 25%, the intensity of infection dropped, because there are fewer teeth available to support the infection, because resistance has increased, or a combination of both factors. This allowed teeth an opportunity to regenerate. As teeth were partially regenerated, feeding ability increased and body mass began to recover allowing development towards metamorphosis to resume; however, because of the slow overall growth rate imposed by mouthpart loss (analogous to crowding in Wilbur-Collins) and the likely continued suboptimal growth rate due to persistent infection (as observed in other species; Parris and Cornelius, 2004) most tadpoles metamorphose near the minimum threshold size in advance of winter (when terrestrial conditions for emerging froglets are suboptimal and metamorphosis was not observed; Fig. 6.10).

This proposed model leads to the prediction that tadpoles that do not suffer severe mouthpart loss should metamorphose at larger sizes. 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 an opportunity for a preliminary test of this prediction. Drier sites may serve as a refugia from *Bd*-associated rainforest declines because the environment is less conducive to *Bd* (Puschendorf et al., 2009). 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, it was at levels below those seen during the winter peak at Tully Gorge NP. The tadpoles at this site were, as predicted, significantly larger than those at Tully Gorge NP. Increased size at metamorphosis in populations such as this may assist in maintaining a healthier adult population.

Although many traits of larvae and adults are decoupled by metamorphosis, some traits are linked, and adult fitness can be affected by growth and development in the larval stage (Nicieza et al., 2006). Negative effects on larvae during development can carry over into later life stages in many organisms with complex life cycles (Pechenik et al., 1998). Altwegg and Ryer (2003) tracked *Rana lessonae* and *R. esculenta* over 3 years and found that 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 may have had lower survival. Twenty-one percent 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 that 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, Smith, 1987, Scott et al., 2007).

Smaller clutch size and longer time to first reproduction negatively affect fitness and population growth rate (Scott, 1994). These 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 declines caused by epizootics. 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 unaffected by *Bd* would be predicted to have much lower effects on recruitment and thus should recover more rapidly following declines of adults caused by epizootics. Unhindered recruitment may help explain why *L. genimaculata* was particularly resilient following decline and appeared to return to pre-decline levels more quickly than species having torrent-adapted tadpoles (McDonald et al., 2005). 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), behaviour and microhabitat use (Rowley and Alford, 2007) and *Bd* strain differences (Berger et al., 2005b, Retallick and Miera, 2007) are important factors affecting adult susceptibility to chytridiomycosis and are also expected to govern rates of population recovery.

The most dramatic effect of chytridiomycosis is certainly the mortality of terrestrial frogs. It is this mortality that can quickly reduce many species in an amphibian assemblage from abundant to endangered. As a result, the study of the impact of *Bd* infection in tadpoles has been comparatively neglected, however, the terrestrial and aquatic life stages are inextricably linked. Insults experienced as larvae can affect performance as adults. This study revealed that *Bd* infection of tadpoles not only affects rates of growth and development but can also cause mortality through starvation from

the loss of keratinized mouthparts. The carryover effects of a period of seasonal starvation are unknown; however, they could affect long term population health and recovery. The fact that tadpoles are not susceptible to mortality from *Bd* infection in the same way as many adult frogs may make them a good life stage to investigate the presence of an acquired immune response to *Bd*, as prolonged sublethal exposure to the pathogen could provide the time needed for an immune response to develop. The relatively long lifespan of the torrent tadpoles studied here combined with the recording of toothloss and infection intensity over time revealed an apparent increase in tolerance and resistance to infection over the course of development. This response to infection may be very important to species survival and recovery because without the ability to regrow mouthparts, torrent tadpoles would be unable to feed and complete development, and immunity developed as larvae may carryover through metamorphosis. This result highlights the potential importance of the amphibian acquired immune response in both adults and larvae for survival following *Bd* emergence.

Chapter 8 : Environmental Sampling for *Batrachochytrium dendrobatidis*

Introduction

Although many free-living animal populations are regulated, in part, by disease (Dobson and Foufopoulos, 2001, Daszak et al., 2000, McCallum and Dobson, 1995, Hudson et al., 1998), few cases are known of pathogens, acting alone, driving species to extinction. Less than 4% of all known plant and animal extinctions since 1500 have been 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, the Australian thylacine and the Christmas Island rat (Daszak and Cunningham, 1999, Guiler, 1961, Van Riper et al., 1986, 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 (Bielby et al., 2008, Stuart et al., 2004), with strong supportive evidence for the role of *Bd* in a few well-studied representative cases (Lips et al., 2006, Schloegel et al., 2006, Berger et al., 1998). This arguably makes *Bd* the infectious disease with the greatest negative effects on biodiversity ever recorded (Mitchell et al., 2008, Skerratt et al., 2007), and raises the question of what separates this disease from others.

Theoretically, a pathogen is most likely to drive species to 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 and Sasaki, 2002, de Castro and Bolker, 2005). Small populations are more vulnerable to stochastic events, and this vulnerability could increase 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 and 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*.

Aspects of *Bd*'s reproductive physiology support the hypothesis that it is an amphibian-specific pathogen (Berger et al., 2005a), however, its ability to reproduce on a broad range of nutrient sources *in vitro* (Piotrowski et al., 2004, James, 2007) suggests that it may be able to reproduce or survive for extended periods of time in the environment. Di Rosa et al. (2007) believed that they had discovered a potential resting stage of *Bd* based on the discovery of an organism that was neither a zoospore nor a sporangium but that was stained by immuno-histology using a polyclonal antibody for *Bd* and that was associated with skin samples positive by PCR for *Bd*; however, these antibodies are not specific and cross-react with other organisms (Berger et al. 2002) and the preliminary study did not determine the organism's identity, life stage (e.g., resting stage, saprobic stage) or its ability to infect amphibians. Regardless, the possibility remains that *Bd* may persist in the environment for longer periods of time while not on 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 increase as the length of time *Bd* can survive in the environment increases (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 and Speare, 2005). The survival time of *Bd* in nature is probably shorter and is likely to be influenced by abiotic factors such as temperature and pH (Piotrowski et al., 2004) and by biotic factors such as predation (e.g., zooplankton; Kagami et al., 2004) and interactions with other microbiota (Chapter 7; Harris et al., 2006). Seasonal or spatial variation in survival rates of *Bd* 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 incorporated these results into the development of a sampling device that is portable, can be operated in remote and wet conditions and can filter reasonably large (>1L) volumes of stream water to increase the chances of detecting low zoospore densities in the environment. I then used this method to test water and rock surfaces at Tully Gorge NP (2006 – 2007), at the same time and on the same 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 the number of *Bd* zoospores 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.

Previous work has indicated that dirt and detritus in samples can lead to inhibition of the PCR reaction by preventing target DNA replication (Hyatt et al., 2007). This could obviously occur in environmental samples. 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 four TGhL agar plates at 23 ºC for 8 d. 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 min to harvest zoospores. The four 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. Ten microlitres 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 -30 ºC 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 that might naturally be present in the water.

Two replicates from each of the above treatments were then extracted with each 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) because of 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, five valves were fit so that the connections were 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 as tadpole sampling. During each visit water was filtered from 16 different stream locations, with four replicates within each of 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 was sampled from the water column by gently dipping a 5 L bucket into the surface of the water and filling it with approximately 4 L of water. Water was then filtered directly from the bucket. For rock surface sampling, enough rocks (each less than 15 cm long) to displace 1 L of water were placed into a bucket containing 4 L of stream water collected as described above. Rock surfaces were comprehensively scoured with a stiff brush to remove as much material from them as possible. The water within the bucket was then filtered. Before each sample, 5 L 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 1 hr and thoroughly rinsed to eliminate possible cross contamination of samples (Cashins et al., 2008b).

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 and 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.

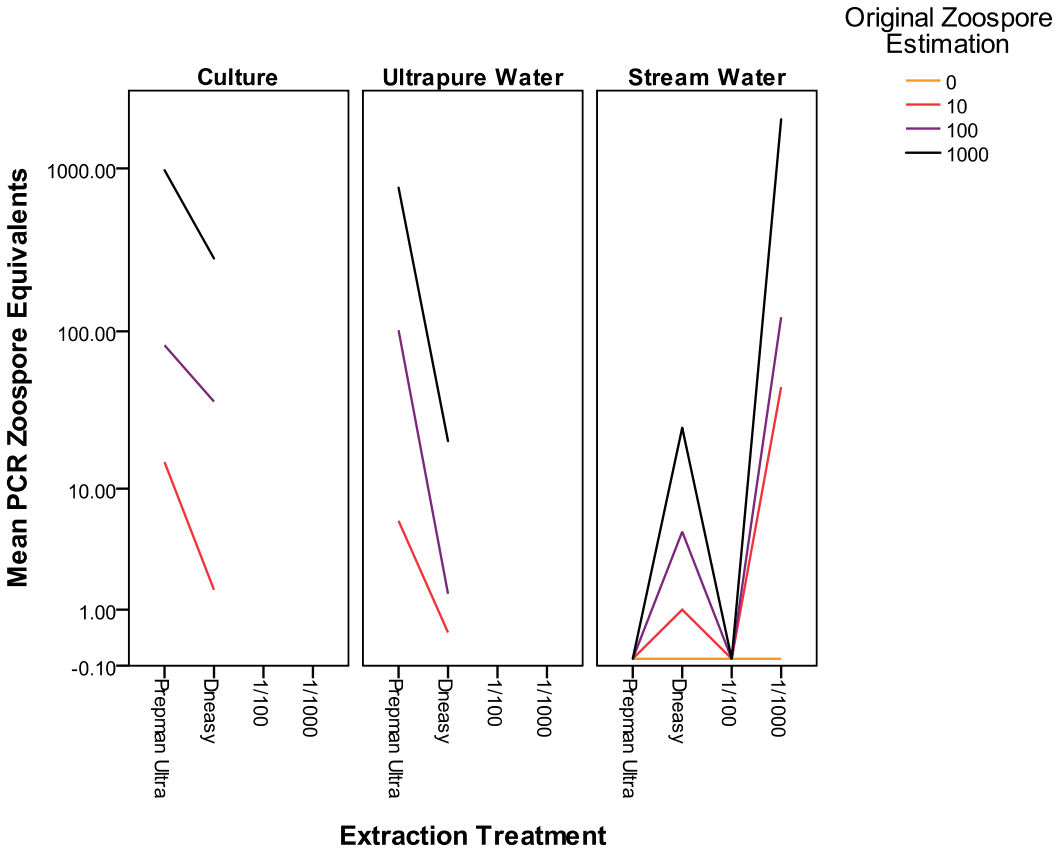


Figure 8.1. Comparison of mean *Bd* zoospore equivalents as determined by qPCR for each treatment. Each box is a separate treatment (pure culture control, 2 µm membranes that filtered ultrapure water and 2 µm membranes that filtered stream water. X axis represents the various extraction protocols trialed (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.

Mean zoospore equivalents of the 1/1000 dilution treatments were greater than those estimated from the Dneasy treatments, however, the high dilution factor reduced the expected accuracy of zoospore equivalent estimates.

Field Sampling

To determine whether inhibition affected field samples in the manner suggested by the preliminary experiment, I initially extracted two replicate filters per sample from 53 samples (taken throughout the year) using Prepman Ultra, and diluted 1/100 prior to PCR. Results from internal positive controls indicated that 23 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 with 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 for each habitat and add to 100% 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

Because the preliminary result indicated that 1/1000 dilution was effective at reducing inhibition (Fig. 8.1), additional replicate filters from many of the inhibited samples were extracted and diluted 1/1000 to generate interpretable results. Filters from all remaining

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.

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

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 in the number of *Bd* zoospores in the environment, however, the result is not significant (Runs test, P = 0.124).

Discussion

In Chapter 3, I demonstrated 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 demonstrated 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* within a stream. 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, but 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 the elevated number of *Bd* zoospores in the environment were the result of, and not the cause of, high prevalence and heavy infections in 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 (Fig. 5.6) 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 within the entire stream 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 per unit water filtered from rock surface samples is more than double that of the water column samples.

The presence of *Bd* on rock surfaces may represent an important avenue 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 of a pathogen with propagules constantly present at relatively low densities. 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 of infection (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 in this species as discussed in Chapter 6.

The detection of *Bd* zoospores in the environment occurred only at the end of a steady increase in prevalence that reached 100% in torrent tadpoles (Chapter 6) suggesting that the environmental zoospores detected were shed from infected amphibian hosts. This, combined with the evidence that seasonal patterns in torrent tadpole prevalence are best explained by duration of exposure combined with metamorphosis and recruitment (Chapter 6) strongly suggests that 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 the stream, however, the low number of *Bd* zoospores 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 the 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 (Walker et al., 2007, Kirshtein 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 suggested to have further increased density of *Bd* (Walker et al., 2007). Assuming that 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.

If *Bd* could survive in the environment for long periods of time or can grow saprobically, the risk of species extinction would be increased since a positive force of infection could be maintained as amphibian hosts declined (Mitchell et al., 2008). My study, however, provided no evidence to support the possibility of either a saprobic life stage or a life stage with a long lifespan in the environment. Although transmission among tadpoles is likely due to zoospores released on the surfaces of shared substrates, it appears that the lifespan of these zoospores is short. Factors affecting survival of *Bd* in the absence of amphibian hosts likely play an important role in transmission. Additional study of these factors will be needed for a fuller understanding of the dynamics of transmission and for the possible development of management actions to reduce pathogen spread.

Chapter 9 : General Discussion

Introduction

Amphibians were the first vertebrates to leave water over 340 million years ago. The species present today reflect the successes of ancient lineages that have survived four mass extinctions, two major ice ages and undoubtedly numerous unknown biological assaults. Over this time amphibians have evolved to survive the conditions posed by a wide variety of habitats, from desert to rainforest. The biphasic life cycle has been an important part of this success, allowing larvae to exploit unpredictable, ephemeral (thus largely predator-free) environments as well as permanent waters with abundant resources (Wassersug, 1975, Wilbur, 1980). In recent times, however, landscapes have been increasingly dominated by humans, and amphibians face a rapidly changing and unfamiliar suite of threats, including sudden, complete habitat destruction, novel man-made chemical pollutants, over-exploitation in terms of human use of wild amphibians for food and pets, introduced competitor and predator species, climate change and human-mediated disease outbreaks (Collins and Storfer, 2003, Sodhi et al., 2008, Stuart et al., 2004, Alford and Richards, 1999, Berger et al., 1998). Many species are ill-equipped to deal with these new and potential threats in isolation, or in concert. As a result, amphibians have been declining across the globe at an alarming rate. Over one-third of the 6,300 species are at risk of extinction (Wake and Vredenburg, 2008). In recent years the amphibian extinction rate has been 211 times as great as estimates of the long-term historical average, and if all species currently in imminent danger of extinction go extinct, this multiple will rise to 25,039 – 45,474 times the long-term average rate (McCallum, 2007).

In many cases, the complex life cycle may now be more a detriment than an asset to survival, as species with larvae, particularly in streams, are at greater risk of decline (Bielby et al., 2008, Becker et al., 2007, Todd, 2007). A primary reason for this is the recent, global emergence of the amphibian pathogen *Batrachochytium dendrobatidis* (*Bd*). This virulent, microscopic, fungus with aquatic propagules infects the skin of

adult amphibians and the mouthparts of tadpoles and its spread has decimated amphibian populations around the world, causing the extinction of dozens of species (Skerratt et al., 2007, La Marca et al., 2005, Schloegel et al., 2006).

The host-pathogen relationship in adult frogs has received significant and well-deserved research attention. Numerous aspects of this relationship are now better understood compared to when *Bd* was first discovered in 1998 (Berger et al., 1998, Fisher et al., 2009). In contrast, little is known of the host-pathogen relationship in tadpoles. In chapter 6 and 7 I argued that rainforest stream tadpoles reside within the "central artery" of *Bd* transmission, are a major *Bd* reservoir host, can suffer pathophysiological changes as a result of infection (mouthpart loss), and are an essential developmental stage in the amphibian life cycle. Certainly, tadpole infection could play an important role in disease dynamics and health at the population and even assemblage levels.

Similar to adult frogs, the large diversity of tadpole morphology, physiology, behavior and developmental strategies is likely to result in interspecific variation of the host-pathogen relationship. Because tadpoles spend their lives in aquatic environments, knowledge of their susceptibility to infection, and of temporal patterns in prevalence and intensity, provide a better indication of how and where *Bd* persists in aquatic environments than is possible to obtain by studying only adults.

Aims and Approach

The general aim of this thesis was to improve our understanding of the epidemiology of *Bd* infection on larvae in rainforest streams and to develop a greater understanding of the contribution of larval infections to total population and assemblage dynamics, including species declines and extinctions. To achieve this I studied an assemblage of tadpoles (5 species) at two sites (Murray Upper National Park for one year and Tully Gorge National Park for two years). I examined the habitat preferences, behaviour, dispersal, development patterns, prevalence of infection, intensity of infection and effects of infection on mouthparts, body condition and survival. My specific aims were to:

- Develop techniques to study the epidemiology of *Bd* in tadpoles in rainforest streams, such as being able to filter *Bd* in the environment for PCR detection
- Describe how tadpoles vary among species in behaviour, space, time, resource use and development
- Determine how tadpole behaviour, space, time, resource use and development affect risk of infection
- Determine additional environmental and biological factors that drive prevalence and intensity of *Bd* infection in susceptible tadpoles
- Describe the pathophysiological effects of and responses to infection in susceptible tadpoles
- Describe seasonal variation in the number of *Bd* zoospores in the stream environment

Development of Techniques

In order to accomplish these aims I first developed a series of methods. During a preliminary trial designed, in part, to assess the ability to detect *Bd* infection in tadpole mouthparts via swabbing, I discovered that disposable gloves worn to prevent transmission of disease were lethal to the tadpoles of interest (Cashins et al., 2008a, Greer et al., 2009; Chapter 4). Following a series of experiments, I found that latex, vinyl and nitrile gloves can be toxic to tadpoles. I also discovered that well-rinsed vinyl gloves did not cause harm. I therefore, incorporated the careful rinsing of vinyl gloves before handling each tadpole into my standard sampling protocol.

The diagnostic PCR test used to detect *Bd* is extremely sensitive because it amplifies mitochondrial DNA. This test can consistently detect as little as $1/10^{th}$ and often $1/100^{th}$ of a single zoospore (Boyle et al., 2004). This analytical sensitivity presents a problem when reusing equipment and tools in the field. Even though *Bd* may be dead, the presence of its DNA can lead to cross-contamination of samples and the generation of false positives. In particular, I wanted to develop a filtration method to sample water bodies and rock surfaces for the presence of *Bd*. In order to reuse equipment and not cross-contaminate, all *Bd* DNA had to be rendered unrecognizable by PCR. Sodium

hypochlorite (NaOCl) has been used effectively to denature proteins as well as DNA for other applications. I tested three concentrations of NaOCl (1, 6, and 12%) over four time periods (1, 6, 15, and 24 h) and found that 12% NaOCl at any exposure period was the only 100% effective treatment (Cashins et al., 2008b; Chapter 2). I therefore washed reusable equipment that had the potential to cross-contaminate samples in a 12% NaOCl solution.

As part of the development of an environmental *Bd* detection method I tested the effects of swab samples, filter membrane types, and pore sizes on the sensitivity of the PCR assay to determine the best option for filtration of water and rock surface scrapings. I found there was little effect of the filter types tested on the sensitivity of detection and selected the Millipore Isopore 2.0 µm membrane due to its ease of use in the field and lab (Chapter 3). To determine how to remove inhibition from detritus that was blocking 49% of filter samples I examined the effect of dilution of the standard extraction product with the effect of using a commercially available DNA soil extraction kit (Dneasy™). I found that both methods were effective at removing inhibition (Chapter 8). I opted for dilution of the standard extraction product because it is less expensive and time intensive and was at least equally effective at reducing the effects of inhibition.

Variation in Space and Resource Use

Competition for resources to maximize size at, and minimize time to, metamorphosis is a significant factor driving the wide diversity of tadpole body forms and their abundance in time and space (Altig and Johnston, 1989). In the Wet Tropics of Australia, stream tadpoles occupy a range of habitat types: 1) Isolated pools; *L. xanthomera*. 2) Connected pools; *L. genimaculata*. 3) Torrents; *L. nannotis*, *L. rheocola*, *N. dayi*. Within torrents, tadpoles of each species occupy distinct water velocity profiles. *Nyctimystes dayi* were found within the fastest flowing water and *L. rheocola* were typically found in the slowest flowing water (Chapter 5). Peak breeding of *Litoria nannotis* and *L. rheocola* was asynchronous, with *L. rheocola* showing a pulse of egg hatching 1-2 months after *L. nannotis*. Development time of *L. nannotis* was 9-12 months and *L. rheocola* developed in 5-9 months (Chapter 5). In conjunction

with the extreme differences in water velocity among habitat types, the tadpoles that occupied each displayed specialized behaviours. *Litoria nannotis* and *L. rheocola* establish a partial vacuum with the rock surface and rarely break contact as they scrape the substrate for food. *Litoria genimaculata* spend more time in the water column and tend to pick and rasp at food items on leaf litter and detritus. *Litoria xanthomera* are similar to *L. genimaculata* but also showed cannibalistic behaviour. A mark-recapture study of *L. nannotis* showed they are not highly sedentary and disperse rapidly on a small (15 m of creek) spatial scale. They may, however, exhibit philopatry on the scale of 50-100 m stretches of creeks.

Prevalence and Risk of Infection

Tadpoles within the stream assemblage had significantly different infection risks. This differential susceptibility was probably due to a combination of factors, including variation in habitat affecting zoospore settlement and survival in the environment, variation in behaviour affecting contact rate with zoospores, and variation in the length of larval duration affecting cumulative risk of *Bd* exposure. Torrent tadpoles were infected at up to 100% prevalence, while prevalence in pool tadpoles was generally very low (Chapter 6). This may be in part because zoospores settle into the boundary layer on rocks in torrents, and the mouthparts of torrent tadpoles are in frequent close contacts with those substrates. It is also likely that the longer larval periods of torrent tadpoles contribute to higher prevalences, because they experience greater cumulative risk of exposure to infective zoospores. The lower prevalences in the pool dwelling tadpoles of *L. genimaculata* may have been caused by some combination of decreased rates of zoospore survival due to competition with or predation by other microbes in pools, lower rates of mouthpart contact with substrate because of their different feeding behaviour, and a lower cumulative exposure because of their shorter larval duration (Chapter 6). *L. xanthomera* are likely to have a low risk of infection for these reasons and also because their habitat is typically isolated from the stream, decreasing the likelihood of *Bd* entering the habitat.

Risk of infection was negatively correlated, among the three torrent tadpoles, with mean water velocity (Chapter 6). *Litoria rheocola* occupy the slowest torrents and had the highest risk of infection, while *N. dayi* occupied the fastest water flows and had the lowest risk of infection. Similarly, risk of infection increased within species (*L. nannotis* and *L. rheocola*) when flow rate was slower suggesting faster water flows may decrease zoospore settlement or reinfection rate.

Drivers of Seasonal Bd Prevalence and Intensity in Tadpoles

Prevalence and intensity of *Bd* in torrent tadpoles (*L. nannotis* and *L .rheocola*) was highly seasonal (Chapter 6). Over a 12 month cycle, mean prevalence increased with exposure risk (indicated by body size) from a low in summer (February) to a high in winter and spring. This was followed by sharp decrease in summer as a result of seasonal demographic changes; specifically the concurrent metamorphosis of large infected tadpoles and recruitment of small uninfected tadpoles. Elevated water temperatures in summer (> 25° C) appear to assist in clearing infections in a small percentage of tadpoles (Chapter 6).

A multiple regression model found that 20.1% of variation in intensity of infection in *L. nannotis* was explained by a combination of 28-day mean rainfall, body length, 28-day mean solar radiation, and site. Host responses post-infection were also found to affect intensity, including mouthpart loss and the apparent presence of, and possible adaptive increases in, host immunity. A seasonal period of severe mouthpart loss following infection caused a decrease in intensity possibly because the most heavily infected areas of tissue were shed. Despite continued infection, the mouthparts were able to regrow, and less infected areas that had shed less tissue regrew first, suggesting that tadpoles had developed a measure of tolerance or immunity to infection (Chapter 6). Following mouthpart recovery the mean intensity of infection was significantly lower than mean intensity just prior to the onset of tooth loss and fluctuated below 630 zoospore equivalents despite fully intact mouthparts and cooler water temperatures, suggesting host immunity was limiting infection. Density of infected hosts may also affect intensity, as the density of infected torrent tadpoles was positively correlated with

intensity of infection in the large tadpoles. *Batrachochyrium dendrobatidis* was only detected in the water column and on rock surfaces during the period when prevalence in torrent tadpoles was at its highest.

These results suggest that tadpoles are the principal drivers of infection dynamics in tadpoles. There was no evidence for an external factor impacting tadpole infection dynamics, such as a bloom of saprobic growth in the environment or infection of the adult life stage.

Effects of Infection on Tadpoles

Litoria genimaculata and *L. xanthomera* were infected at very low prevalences; therefore I was unable to draw conclusions on any possible effects of infection apart from the observation that, when infected, *L. genimaculata* can lose keratinized tooth rows and their jaw sheath. Torrent-adapted tadpoles, however, are at a high risk of infection, and also suffer mouthpart loss when infected. The use of mouthpart loss as an indicator of *Bd* infection in *L. nannotis* and *L. rheocola* is a highly specific, but not very sensitive, test and therefore has a very low rate of false positives, but is prone to false negatives (Chapter 7).

Tooth loss in *L. nannotis* is generally first apparent on the outermost tooth rows and then moves inward. During mouthpart recovery, this pattern is reversed and the outermost rows are the last to recover. The initial signs of jaw sheath loss are typically only seen once overall tooth loss increases above 25%. This pattern of progression and recession of tooth and jaw loss suggests the outermost rows may be the initial point of *Bd* transmission with infection progressing inward. The outermost rows will make first contact, and are likely to be in closer contact with the substrate during grazing. Therefore, these rows may be in closer and more frequent contact with zoospores present on the substrate surface. It also suggests that regrowth is facilitated by an increase in immune function, rather than simply by regeneration of mouthparts following shedding, since regrowth is typically initiated first in the inner rows, which typically suffer the least loss.

It is clear that the ability of torrent tadpoles to acquire nutrients suffers significantly as a result of tooth loss. Body condition (calculated as the mass residual of body length vs. mass) decreased with increasing severity of mouthpart loss. Then, as mouthparts recovered, body condition increased (Chapter 7). During the period of peak mouthpart loss, dramatically less food was in the gut, tadpoles appeared emaciated, had less developed tail musculature, and were weaker swimmers. Tadpoles with severe mouthpart loss were found in slower flowing water, suggesting they had difficulty maintaining position within the higher velocity currents. Some emaciated tadpoles were extremely weak and died during handling indicating that mortality of tadpoles due to *Bd* could be significant. Based on population estimates, however, most tadpoles survived this period and were able to regrow mouthparts, resume normal feeding, increase in size and metamorphose. Similar to other factors that limit food during tadpole development, it is predicted that torrent tadpoles suffering from *Bd* infection and severe mouthpart loss will metamorphose at a smaller size due to a slower and effectively shorter developmental period prior to the onset of winter when metamorphosis does not occur. A comparison with a site where tadpoles appear to suffer less tooth loss showed that at that site *L. nannotis* tadpoles grew to a significantly larger size prior to metamorphosis, providing tentative support for this hypothesis.

Implications

An annual, population-wide loss of mouthparts, and a consequent decrease in feeding ability and physical condition in *L. nannotis* and *L. rheocola*, may not only exert a direct effect on their survival, but may also cause individuals to metamorphose at a less than optimal size. Metamorphosis at a less than optimal size can have significant latent, or carry-over effects in many species with complex life cycles. In amphibians, this can impair immune defenses (Gervasi and Foufopoulos, 2008), increase susceptibility to parasites (Dare et al., 2006), decrease survival (Scott et al., 2007, Chelgren et al., 2006), and increase time to first reproduction and lead to smaller clutch sizes following metamorphosis (Altwegg and Reyer, 2003, Scott, 1994). All of these effects contribute to a decrease in lifetime fecundity and could negatively effect rates of population

growth following an epidemic of chytridiomycosis. Such effects may be one cause of the patterns of species recovery observed within the assemblage I studied. The species with more *Bd*-susceptible torrent tadpoles were slower to recover following *Bd* emergence, whereas *L. genimaculata* (stream connected pool tadpoles) recovered much faster and *L. xanthomera* (isolated pools) did not appear to decline at all (McDonald and Alford, 1999). Because of the small sample of species, these patterns may be caused by other factors such as host defenses and adult behaviour patterns (Woodhams et al., 2006, Rowley and Alford, 2007), however, this hypothesis merits further examination in other systems.

Mortality of larvae or recent metamorphs, and reduced fecundity of adults due to infection and loss of mouthparts in the larval stage, should strongly favour tadpoles that can tolerate and actively resist infection. The endemic host-pathogen relationship observed in some susceptible adult populations following initial dramatic declines indicates that resistance to *Bd* may evolve (McDonald et al., 2005, Richmond et al., 2009, Retallick et al., 2004). Unlike adult frogs that die within days or hours of the first clinical signs of chytridiomycosis, the signs and progression of infection in some tadpoles, such as *L. nannotis* and *L. rheocola* can be observed with the naked eye via the loss of keratinized mouthparts. Observing the degree of tooth loss, combined with a sensitive quantitative PCR assay to directly estimate pathogen load, provided the opportunity to study the effect of and host response to infection over time. This was particularly useful in *L. nannotis* and *L. rheocola*, which appeared to develop both tolerance and immunity to infection. These responses appeared to both limit replication of the pathogen and reduce the degree of pathological changes produced by the disease. Additional work to determine the mechanisms behind these processes may lead to a greater understanding of how all amphibian hosts can limit the effects of *Bd* infection and disease.

Tadpoles are a significant producer of zoospores within a stream. All evidence indicates that infection dynamics in the tadpoles studied here were driven by tadpoles; not by adults and not by an independent saprobic life stage. Increased larval resistance

or tolerance could therefore affect pathogen virulence (Woolhouse et al., 2001), environmental zoospore density, transmission to other life stages and hosts, and may contribute to adult resistance if an effective immune response in larvae is retained through metamorphosis. The prevalence of infection in torrent tadpoles was elevated throughout the year, even when adult prevalence was very low. Therefore, as suggested by others, tadpoles can act as an effective intraspecific reservoir host and maintain *Bd* in the environment when adult host densities are low and when terrestrial conditions limit or eliminate infections in adults, such as during warm summers.

The results of this thesis indicate stream tadpoles may play a larger role in many aspects of chytridiomycosis and associated amphibian declines than previously appreciated and suggest that the continued study of both aquatic and terrestrial life stages are needed to understand the epidemiology of chytridiomycosis and to direct conservation and management strategies of endangered amphibians

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