Novel approaches for elucidating drivers of *Batrachochytrium dendrobatidis* epidemiology in amphibians

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Summary

Batrachochytrium dendrobatidis (Bd) is the skin-invasive fungus that causes chytridiomycosis, a disease implicated as a proximate cause of global amphibian declines. While this pathogenic system is well-researched, our understanding of how environmental drivers regulate chytridiomycosis is incomplete. I aimed to develop and evaluate approaches to address how interactions between co-infecting Bd strains and host, pathogen and temperature affect Bd epidemiology. I also aimed to develop and evaluate methods to reduce the cost of qPCR-based detection in amphibians.

Two methods for reducing the cost of qPCR detection were identified: a reduced volume (10 µL) SYBR green qPCR on DNA extracted using either a CTAB extraction protocol or the standard extraction reagent, PrepMan Ultra. Reducing the volume of the SYBR green assay from 20 µL to 10 µL resulted in a slight reduction of assay sensitivity, from one zoospore in a sample to ten. However, the 10 µL assay performed well in validation against the standard Taqman qPCR assay, agreeing on positive or negative detection of Bd in 84.6% (CTAB) and 92.3% (PrepMan) of the samples tested. Therefore both of these protocols are suitable for use for detecting Bd in experimentally infected tadpoles; however the PrepMan extraction performed better than CTAB. The consumable costs of these protocols ranged from 38% (CTAB) to 42% (PrepMan) of the cost of the standard assay.

Hosts are commonly infected with multiple strains of a pathogen, and interactions between co-infecting strains can influence several aspects of disease epidemiology and evolution. Distinct strains of Bd have been identified but at present nothing is known about how they might interact. In Chapter 3 of this thesis I aimed to trial fluorescent probes for labelling Bd cells to distinguish between strains and track their fate. Two BODIPY and two CellTracker dyes (Molecular Probes, Invitrogen) were selected for trial. Both BODIPY dyes (558/568 and FL) and CellTracker orange CMTMR produced fluorescent Bd cells, but CellTracker green CMFDA did not. BODIPY 558/568 and FL were the most suitable for long-term tracking, at a concentration of 10 µM. At this concentration, BODIPY-labelled cells were brightly fluorescent for 12-16 days, distinguishable after being mixed together, and Bd growth was not inhibited.

In Chapter 4, I aimed to determine whether thermal patterns in Bd infection are explained by Bd thermal responses, or alternatively, by interacting host and pathogen thermal responses. Growth of Bd in culture was measured at the thermal optimum for Bd, 23°C,
at two sub-optimal temperatures, 15°C and 27°C. *Litoria raniformis* tadpoles were experimentally exposed to Bd at the three temperatures. The response of tadpole size, weight and developmental stage was measured and Bd infection was detected with qPCR. The growth response of Bd to temperature did not correlate with infection prevalence or abundance. Bd grew more rapidly at 23°C than at 15°C and 27°C. However Bd prevalence in tadpoles decreased linearly with temperature and was 0% at 27°C. There was no significant difference in mean Bd abundance at 15°C and 23°C. Neither did infection correlate with tadpole responses; tadpoles reached higher developmental stages and sizes at 23°C and 27°C than at 15°C. However, infected tadpoles were heavier and larger than uninfected conspecifics. Therefore it is likely that thermal patterns in Bd infection were determined by the interaction between tadpole and Bd responses to temperature.
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List of Abbreviations

AAHL  Australian Animal Health Laboratory
AIC   Akaike Information Criterion
Bd    *Batrachochytrium dendrobatidis*
BODIPY 558/568  4,4-difluoro-5-(2-thienyl)-4-bora-3a,4a-diaza-s-indacene-3-dodecanoic acid
BODIPY FL  4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-s-indacene-3-dodecanoic acid
bp     Base pairs
CMFDA  5-chloromethylfluorescein diacetate
CMTMR  5-(and-6)-(((4-chloromethyl)benzoyl) amino) tetramethylrhodamine
C_p    Crossing point
C_t    Threshold value
CTAB   Cetyltrimethyl ammonium bromide
DEH    Department of Environment and Heritage (Australia)
DNA    Deoxyribonucleic acid
EDTA   Ethylenediaminetetraacetic acid
GLM    Generalised linear model
GLMM   Generalised linear mixed model
MilliQ 0.22 μm Millipore filter purified water
MS-222 Tricaine methane sulfonate
NZD    New Zealand Dollars
OD     Optical density
qPCR   Quantitative real-time polymerase chain reaction
SDS    Sodium dodecyl sulphate
SE     Standard error
SVL    Snout-vent length
T-broth 1% tryptone broth
TE buffer Tris-EDTA buffer
T_m    Melting (dissociation) temperature
T-plate 1% tryptone, 1% agar plate
UDG    Uracil DNA glycoslyase
USD    United States Dollars
ZE     Zoospore genomic equivalents
CHAPTER 1: General Introduction
Batrachochytrium dendrobatidis (Bd) is a skin-invasive pathogen of amphibians. The spread of this Chytridiomycete fungus has been linked to the decline or extinction of around 200 amphibian species since 1980 (Skerratt et al. 2007), including populations in pristine areas of Panama, Australia (Berger et al. 1998), New Zealand (Bell et al. 2004) and Spain (Bosch et al. 2001). Bd has now been reported in wild amphibian populations on every continent inhabited by amphibians (Berger et al. 1998, Ron and Merino 2000, Waldman et al. 2001, Bradley et al. 2002, Hopkins and Channing 2002, Garner et al. 2005, Kusrini et al. 2008, Une et al. 2008). Investigations of the ecology and epidemiology of this fungus are viewed as a priority for amphibian conservation (Young et al. 2001, DEH 2005, Gascon et al. 2007).

While much is known about the life history and ecology of this pathogen, there still remain gaps in our knowledge of why the observed patterns in Bd epidemiology exist, and why the consequences of infection for the host are so inconsistent. The first section of this chapter aims to place the role of Bd amongst the suite of factors implicated in global amphibian declines. This is followed by a review of the current knowledge about the life history and ecology of Bd and the factors that may drive its epidemiology. Possible roles of one known and one potential driver of Bd epidemiology, temperature and co-infection, are discussed and, lastly, the aims and objectives of this thesis are described.

1.1 Global amphibian declines and the role of infectious diseases

The global amphibian assessment conducted between 2001 and 2004 found that amphibian species have undergone alarmingly rapid global declines over the past four decades, with a third of the approximately 6500 extant amphibian species now threatened with extinction (Stuart et al. 2004, Stuart et al. 2008). Some factors driving these declines are fairly visible agents such as overexploitation, habitat loss and invasive species. However, a significant proportion of these declines are enigmatic, where populations living in pristine habitats have declined without an easily identifiable driver. These declines have been
attributed to factors as diverse as climate change, infectious disease, UV radiation, environmental acidity, toxicants or interactions between these factors (reviewed in Alford and Richards 1999, Blaustein and Kiesecker 2002, Semlitsch 2003, Stuart et al. 2004, Stuart et al. 2008).

Emerging infectious diseases are often suggested as drivers of enigmatic amphibian declines (Daszak et al. 1999, Daszak et al. 2003, Rachowicz et al. 2006, Skerratt et al. 2007). Emerging infectious diseases are defined as a pathogen that has undergone a change in an aspect of its ecology, most commonly driven by an anthropogenic influence (Daszak et al. 2004b). These alterations in ecology can include changes in pathogenicity, incidence, severity, impact, geography or host range. Alternatively, the pathogen may have recently evolved or been discovered (Daszak et al. 2004b).

Amphibians host a wide diversity of macro- and micro-parasites (e.g. Green et al. 2002, Burrowes et al. 2004, Nieto et al. 2007), however micro-parasites are usually implicated in amphibian declines. Particular suspects for global declines include a skin-invasive chytrid fungus *Batrachocheitrium dendrobatidis*, and iridoviruses of the genus *Ranavirus*, since Koch’s postulates¹ have been fulfilled for both of these pathogens (Carey et al. 2003). Other parasites that have been implicated in population declines include the oomycete spawn and tadpole parasite *Saprolegnia ferax* (Blaustein et al. 1994, Kiesecker and Blaustein 1995, Romansic et al. 2008) and digenetic trematodes of the *Ribeiroia* genus that cause limb deformities (Rohr et al. 2008b). However, it is becoming increasingly apparent that, of these suspects, *Batrachocheitrium dendrobatidis* may pose the most significant disease threat to amphibian diversity (Daszak et al. 2003, Skerratt et al. 2007).

1.2 Biology of *Batrachocheitrium dendrobatidis*: what do we know? And what don’t we know?

**Discovery and classification.** *Batrachocheitrium dendrobatidis* (Bd) was first identified as a disease-causing agent of amphibians in 1998 (Berger et al. 1998, Nichols et al. 1998). Surveys of montane rainforest frogs during mass mortality events in Panama and Australia revealed the presence of a novel chytridiomycete fungus infecting the epidermis of sick and dead frogs (Berger et al. 1998). The same fungus was reported in sick captive frogs

¹ Koch’s postulates are a research methodology for identification of the specific organism that causes a disease. In order to fulfill Koch’s postulates, one must isolate a disease-causing organism from a sick individual, purify it, and infect more individuals with it. These newly infected individuals must show the same clinical signs as the original sick individual, and furthermore, the researcher must isolate the same pathogen from these infected individuals.
held at the National Zoological Park in Washington, USA (Nichols et al. 1998). Due to the unique ultrastructure of the spores, this fungus was placed in a new genus, *Batrachochytrium* (Longcore et al. 1999).

**The zoosporic true fungi.** The phylum Chytridiomycota traditionally represented an early-diverging group containing all true fungi that produce motile, flagellated spores, termed zoospores (Alexopoulos et al. 1996). However, recent taxonomic revision of the Chytridiomycota has resulted in the zoosporic fungi being split into three phyla; the Blastocladiomycota, Neocallimastigomycota and the somewhat reduced Chytridiomycota (James et al. 2006a, James et al. 2006b, Hibbett et al. 2007). There also remains another group of unrelated fungi of the *Olpidium* and *Rozella* genera which have not yet been resolved into phyla (James et al. 2006b). Bd, however, remains within the Chytridiomycota. While Bd was originally placed in the *Rhizophydium* clade of the order Chytridiales (Longcore et al. 1999, James et al. 2006b), this group has been split from the Chytridiales and now forms its own order, the Rhizophydiales (Letcher et al. 2006, Hibbett et al. 2007). Members of the Chytridiomycota, also known as chytrids, display a variety of life histories, although all chytrids have flagellated, motile zoospores as the dispersive stage and thalli and zoosporangia as encysted, sessile stages. They are ecologically diverse, and may be free-living, saprobic inhabitants of water or soil, or parasitic on plants, invertebrates or other fungi (Alexopoulos et al. 1996, James et al. 2006b). Bd is a rather unusual chytrid, given that it is the only known member to parasitise a vertebrate (Longcore et al. 1999).

**Life cycle.** The life cycle of Bd is well-described in Longcore et al. (1999) and Berger et al. (2005a), and is summarised in Figure 1.1. In brief, infection is disseminated via the movement of flagellated zoospores, which encyst within 24 hours under optimal conditions (Longcore et al. 1999, Berger et al. 2005a). There is some evidence of chemotaxis of zoospores towards nutrient sources (Moss et al. 2008). A thallus will either develop into a single zoosporangium (monocentric development) or into two zoosporangia divided by a septum (colonial development) (Longcore et al. 1999). Thalli typically produce rhizoids in culture medium, but not as frequently in amphibian skin (Longcore et al. 1999). Zoospores are produced asexually by mitotic division of the cytoplasm and released from the zoosporangium via an operculum-capped discharge tubule (Longcore et al. 1999, Berger et al. 2005a). The entire lifecycle takes four to five days to complete (Longcore et al. 1999, Berger et al. 2005a).
Figure 1.1 Life cycle of *Batrachochytrium dendrobatidis* (Bd), adapted from lifecycle diagram and light and electron micrographs by Berger et al. (2005a). A: The dispersive stage of Bd, the zoospore, as it appears under light microscopy. The zoospore is propelled by a posterior whiplash flagellum (F) and the black dots within the cytoplasm represent what are presumably lipid globules. B: The encysted zoospore or germling. The flagellum is reabsorbed into the body of the germling (Bi) and fine, branching rhizoids (R) may appear (Bii). Rhizoids presumably anchor the germling on the substrate and assist with nutrient uptake. C: The germling becomes a multinucleate thallus by mitotic division. The thallus may either develop (Ci) monocentrically or (Cii) colonially, with a thin septum (S) dividing the cytoplasm. One (Ci) or more (Cii) discharge papillae (P) may begin to develop. D: The cytoplasm of the thallus cleaves, giving rise to multiple uninucleate zoospores (Z) within (Di) the monocentric parent cell or (Dii) the colonial parent cells, which are now considered to be zoosporangia. E: The operculum capping the discharge papillae dissolves, releasing the zoospores into the environment, generally an aqueous medium. (Ei) is monocentric and (Eii) is colonial. Zoospores are often elongate and amoeboid upon emergence from zoosporangia, but subsequently become spherical and begin swimming with the use of the flagellum (Pessier et al. 1999). The empty zoosporangia die.  
**Figure notes:** A: zoospore, B: germling, C: thallus, D: zoosporangium, E: zoosporangium releasing zoospores, F: flagellum, M: mitochondria, N: nucleus, P: discharge papilla, R: rhizoids, S: septum, V: vacuole, Z: zoospore.
Reproduction. Chytrids are either solely diploid, or predominantly haploid with diploidy only seen in zygotes (Doggett and Porter 1996, Morehouse et al. 2003, Idnurm et al. 2007). Bd is diploid and predominately asexual (Morehouse et al. 2003, James et al. 2009). Some studies of local population genetics suggest sex could also occur (Morgan et al. 2007, Fisher et al. 2009). However, further research into the genotype of globally disparate strains of Bd has suggested that the loss of heterozygosity that occurs during asexual disparate reproduction would be sufficient for explaining the observed genotypic diversity (James et al. 2009). While evidence leans strongly towards the absence of sex in Bd, this has not yet been conclusively demonstrated. Sexual processes occur in other (former and current) Chytridiales as plasmogamy, the fusion of cell cytoplasm, and can occur by fusion of zoospores, differentiated gametes, or somatic structures (Idnurm et al. 2007). In the Chytridiales, plasmogamy is always followed by the formation of a resting spore (Idnurm et al. 2007). However, the Rhizophydiales appear to be asexual, although some have resting spores (Letcher et al. 2006). An account of a resting stage of Bd has been published, however this is not supported by strong evidence (DiRosa et al. 2007). Mathematical modelling has demonstrated that the presence of a resting stage would allow Bd to drive a host population to extinction without need of an external factor, given that a resting stage would release Bd from obligate dependence upon the host (Mitchell et al. 2008). Therefore the question of whether Bd is capable of sexual processes is of great interest but has yet to be answered.

Pathology. Good descriptions of the pathology of Bd infection can be found in Berger et al. (1998, 2005a, 2005c), Nichols et al. (2001) and Pessier et al. (1999). To date, Bd infections have been confirmed in anurans and salamanders; but it has not yet been found in caecilians (Berger et al. 1998, Davidson et al. 2003, Gower and Wilkinson 2005). Bd infects both adult and larval amphibians, encysting within the cytoplasm of epidermal keratinocytes (Berger et al. 1998, Pessier et al. 1999, Nichols et al. 2001). Bd zoospores presumably encyst in the deeper epidermal layer, the stratum granulosum, since immature thalli tend to be found here (Berger et al. 2005a). The reproductive zoosporangia are found in the sloughing outer skin layers, the stratum corneum (Berger et al. 2005a). The exact mechanism by which Bd penetrates host skin is currently unknown, however other chytrids tend to attach to the host prior to cell invasion and enter host cells through enzymatic degradation of the host cellular membrane (Garner et al. 2009). In susceptible adults, chytridiomycosis may cause clinical signs such as: anorexia, increased skin sloughing, electrolyte loss, epidermal lesions,

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2 Keratinocyte: cell that makes keratin. Keratinocytes represent about 95% of the cells present in the epidermis.
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Erythemia and thickening of the epidermis and behavioural changes such as inappetence, loss of righting reflex, seizures and lethargy (Pessier et al. 1999, Nichols et al. 2001, Voyles et al. 2007, Andre et al. 2008). Ventral areas of the skin tend to become the most heavily infected and have the most marked pathological changes (Berger et al. 2005c). Infection usually leads to morbidity and death in susceptible adults (Pessier et al. 1999, Nichols et al. 2001), although adults of some species do not succumb to infection (Daszak et al. 2004a, Gibble et al. 2007). Clinical signs do not always manifest and may vary between conspecifics or species (Pessier et al. 1999); therefore it is difficult to diagnose infection based upon clinical signs. The mechanism causing death is not fully understood. Hypotheses include 1) the release of a currently unknown toxin, which may explain neurological effects such as seizures, or 2) epidermal damage and/or thickening disrupting the respiratory or osmotic function of the skin (Berger et al. 1998, Pessier et al. 1999, Nichols et al. 2001, Voyles et al. 2007).

While chytridiomycosis is highly pathogenic to adults of several species, Bd infection is not lethal to tadpoles of most species (Berger et al. 1998, Blaustein et al. 2005). This may be because Bd infections are restricted to the keratinised jaw sheaths and tooth rows in tadpoles, along with the feet in older tadpoles (Berger et al. 1998, Marantelli et al. 2004). Bd rapidly colonises newly keratinised areas during tadpole development and metamorphosis (Marantelli et al. 2004). Infected tadpoles may die upon the onset, or soon after metamorphosis, possibly due to the rapid expansion of Bd across the skin surface (Lamirande and Nichols 2002, Marantelli et al. 2004). Chytridiomycosis can however cause minor pathology in tadpoles, such as mouthpart abnormalities (Fellers et al. 2001, Obendorf 2005, Knapp and Morgan 2006, Parris et al. 2006), reduced mass at metamorphosis, increased larval period and increased hind limb asymmetry (Parris and Beaudoin 2004, Parris and Cornelius 2004, Garner et al. 2009). In addition, Bd infection can cause behavioural effects such as reduced activity and changes in anti-predator response (Parris et al. 2006).

Transmission. Transmission of Bd infection has been demonstrated between tadpoles, between tadpoles and adults, and via water (Nichols et al. 2001, Davidson et al. 2003, Rachowicz and Vredenburg 2004, Carey et al. 2006). There is a potential role for environmental transmission, since Bd can remain viable for three to six weeks in sterile lake water and actively grow for at least twelve weeks in sterile wet river sand (Johnson and Speare 2003, 2005). These observations, in addition to the ability of Bd to grow on culture

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1 Erythemia: reddening of the skin (West 1982).
2 Both as abnormal proliferation of tissue (hyperplasia) and production of an excess of keratin, resulting in thickening and hardening of the skin (hyperkeratosis).
3 Hyperkeratosis and segmented or total loss of pigment.
substrates and on the skin of dead frogs suggests that Bd may be capable of a saprobic lifestyle (Longcore et al. 1999). At present it remains unclear whether Bd is an obligate or facultative pathogen, but as mentioned previously, the likelihood of Bd driving an amphibian population to extinction is tied to its ability to persist outside of the host (Mitchell et al. 2008). There is no strong evidence for a non-amphibian animal carrier (Rowley et al. 2006, Rowley et al. 2007b), although Bd can persist on bird feathers for one to three hours (Johnson and Speare 2005).

**Global dissemination.** Competing hypotheses exist for the global emergence of Bd. The first of these is that Bd was globally distributed and a recent change in the environment facilitated its emergence and the second is that Bd has been rapidly spread around the globe from a previously restricted origin (reviewed in Rachowicz et al. 2005). The second hypothesis is better supported by existing evidence, as globally disparate isolates of Bd have extremely low genetic polymorphism, suggesting rapid dissemination from one, or very few, sources (Morehouse et al. 2003, James et al. 2009). If it is thus accepted that Bd spread rapidly from a single origin, then how did it become globally distributed, and where did it come from? Again, two competing hypotheses exist; the first being that Bd originated from Africa and was spread via the global trade of African clawed frogs *Xenopus laevis* for laboratory use in pregnancy tests (Weldon et al. 2004), and the other is that global trade of the American bullfrog *Rana catesbeiana* has spread Bd to new areas (Fisher and Garner 2007). Relative to the global mean, Bd strains carried by bullfrogs display higher heterozygosity, whereas those carried by *X. laevis* have lower heterozygosities (James et al. 2009). Bullfrogs appear to have played an important role in disseminating diverse strains from North America, but this observation does not rule out *X. laevis* as the carrier of Bd “out of Africa” (Weldon et al. 2004, James et al. 2009). Naturalised populations of both these species exist outside of their original home range, and Bd has been found in several invasive bullfrog populations (Garner et al. 2006, AmphibiaWeb 2009). However, the oldest record of Bd infection comes from museum specimens of South African *X. laevis* that date back to 1938 (Weldon et al. 2004). Bd has also been identified in other amphibian species involved in anthropogenic movement over global and local scales, such as in the food and pet trades, in captive laboratory and zoo populations and in amphibians released as biocontrol agents (Daszak et al. 2003) and also in invasive amphibians such as *Eleutherodactylus coqui* and the Cane Toad *Bufo marinus* (Berger et al. 1998, Beard and O'Neill 2005). Bd has probably been introduced into the wild from these sources via accidental or intentional release of infected amphibians, and spread by subsequent contact between carriers and indigenous amphibians or through co-inhabited water (Lips et al. 2006). Therefore anthropogenic spread, or “pathogen pollution”
is the most likely candidate for the emergence of Bd (Daszak et al. 2003), and there are probably many agents of spread acting concurrently. Lips et al. (2008) found evidence for multiple introductions of Bd to South America, strengthening the evidence for human-mediated long-distance spread. Variable, but rapid, rates of local spread have been estimated, ranging from between 25 and 282 km/year in South and Central America (Lips et al. 2008). Analysis of historical population data and diagnosis of museum samples suggests that Bd had spread to Australia by 1978 (Speare and Berger 2005), North America by 1961 (Ouellet et al. 2005) and South America by the late 1970’s to early 1980’s (Ron and Merino 2000, Lips et al. 2008).

**Bd in New Zealand.** Bd was first detected in New Zealand in 1999 in a population of Southern Bell frogs *Litoria raniformis* at Godley Head near Christchurch (Waldman et al. 2001). Bd has since become dispersed across the North and South Island (Šadić and Waldman 2004, Potter and Norman 2006) and has been linked to population crashes of the critically endangered Archey’s frog *Leiopelma archeyi* in the Coromandel region (Bell et al. 2004). Very little is currently known about the biology of Bd in New Zealand frog populations.

### 1.3 Factors influencing Bd epidemiology

Parasites and pathogens are generally defined as symbionts that cause harm to their host; however the actual degree of harm done in a host-parasite interaction may vary considerably between cases. Organisms usually considered parasites may become relatively benign or even confer benefits to the host in some situations (Thomas et al. 2000, Thompson and Cunningham 2002, Leung and Poulin 2008a). Conversely, usually benign symbionts may become virulent (Rosenberg et al. 2007, Leung and Poulin 2008a). In less extreme cases, circumstances may induce a reduction or an increase in virulence of a parasite (Brown et al. 2000, Massey et al. 2004, Blaser and Schmid-Hempel 2005, Cable and van Oosterhout 2007). The virulence expressed during parasitism often results from interactions between host and pathogen genotypes (or phenotypes) and environmental factors (Thomas and Blanford 2003, Mitchell et al. 2005, Wolinska and King 2009).

Could the outcome of Bd infection be explained by the interaction between pathogen, host and environmental characteristics? The virulence of Bd in amphibians appears to be highly plastic, despite the global near-homogeneity of isolate genotypes (Morehouse et al. 2003, James et al. 2009). However, isolates can be distinguished by genotype and differ in virulence, proteomics and phenotype (Berger et al. 2005b, Retallick and Miera 2007, Fisher et
Bd appears to be a true generalist, with no link between strain genotype and host species, or strain virulence and host resistance, suggesting that Bd has not yet coevolved with any known host (James et al. 2009).

Different host species, and conspecific individuals, may respond differently to Bd infection; they may succumb rapidly, eliminate Bd infection, or carry an infection without any resulting pathology (Nichols et al. 2001, Daszak et al. 2004a, Blaustein et al. 2005, Kriger and Hero 2006b, Bishop et al. 2009). Much research has focussed on linking differences in innate immune skin peptides to a species’ vulnerability to Bd (reviewed in Rollins-Smith and Conlon 2005, Woodhams et al. 2006, Woodhams et al. 2007), however adaptive immunity may also play an important role (Richmond et al. 2009). Conspecific host genotypes may differ in their resistance to Bd, as major histocompatibility complex (MHC) genotype of X. laevis tadpoles has been linked to survival when challenged with the opportunistic bacterium Aeromonas hydrophila (Barribeau et al. 2008). Within a species, larger adult individuals seem to be less likely to succumb to Bd infection (Bell et al. 2004, Carey et al. 2006, Kriger et al. 2006b, Garner et al. 2009). Conversely, likelihood of infection has been noted to be higher in larger tadpoles (Smith et al. 2005). There also appears to be a link between host niche and likelihood of Bd-associated decline, with montane and/or stream breeding amphibians being the worst affected (Daszak et al. 1999, Skerratt et al. 2007). Host behaviour may also affect the transmission or virulence of Bd (Woodhams et al. 2003, Rowley and Alford 2007).

Abiotic environmental factors appear to have strong impacts upon Bd infection, with temperature and humidity being most strongly correlated with geospatial patterns in Bd infection (Ron 2005, Puschendorf et al. 2009). Environmental stressors such as UV-B radiation, climatic change and ecotoxins are postulated to increase virulence of Bd and other amphibian pathogens via causation of host stress and/or immunosuppression (Kiesecker and Blaustein 1995, Carey et al. 1999, Blaustein et al. 2003, Rohr et al. 2008b). Environmental stress can also result in increased virulence of pathogens, for example by increasing pathogen abundance (Johnson et al. 2007, Rohr et al. 2008b) or by altering expression of virulence factors (Rosenberg et al. 2007). Bd has been shown to have an interactive effect with copper exposure on larval period length, but not on survival (Parris and Baud 2004). Bd interacts strongly with temperature, but this will be covered in the following section. Biotic factors known or postulated to affect Bd infection include microbial co-inhabitants of amphibian skin, presence of non-susceptible reservoir species or tadpoles and the density of host organisms (Rachowicz and Vredenburg 2004, Rachowicz and Briggs 2007, Harris et al. 2008). The co-occurrence of certain skin bacteria in salamanders appears to have a probiotic effect, inhibiting Bd growth in culture and reducing pathogenicity of chytridiomycosis when
applied to the skin of infected salamanders (Harris et al. 2006, Harris et al. 2008). The consequences of Bd infection may also depend on the presence of predators or competitors (Parris and Beaudoin 2004, Parris and Cornelius 2004, Parris et al. 2006). Two environmental mediators of chytridiomycosis that form the focus of this thesis are discussed in the following sections.

1.4 Temperature as a mediator of chytridiomycosis

**Effects of temperature on Bd and chytridiomycosis.** Environmental temperature appears to play a strong role in determining Bd epidemiology. Bd growth in vitro is sensitive to changes in temperature, with a known tolerance range of 4 – 28°C and an optimal range of 17 – 25°C (Piotrowski et al. 2004). It cannot withstand prolonged temperatures above 29°C (Johnson et al. 2003, Piotrowski et al. 2004). Bd growth is reduced by low temperatures, but greater numbers of zoospores are produced per zoosporangia at 7 – 10°C (Woodhams et al. 2008). This may be a means for Bd to compensate for reduced growth at low temperatures, and suggests that Bd could become more infective at low temperatures (Piotrowski et al. 2004, Woodhams et al. 2008). Virulence expressed within adult amphibian hosts is altered by temperature; for example captive adult *Mixophyes fasciolatus* died from Bd infection at 17°C and 23°C, but half of the frogs held at 27°C survived infection (Berger et al. 2004). Similarly, in Bd-infected *Litoria chloris*, disease development was slowed at 8°C and when temperature fluctuated between 13.5 – 23.2°C, whereas infected frogs held at 37°C eliminated the disease (Woodhams et al. 2003). However *L. chloris* frogs rapidly succumbed to infection at 20°C (Woodhams et al. 2003). Conversely, no difference in survival time was found in experimentally infected juvenile *Bufo boreas* held at 12°C and 23°C (Carey et al. 2006). This suggests that thermal effects on infection outcome may differ between host species. Results of surveys in some wild amphibian populations over seasonal, temporal and geographical scales show that Bd tends to be more virulent at lower temperatures and limited by temperatures above 29°C (Bradley et al. 2002, Berger et al. 2004, Woodhams and Alford 2005, Kriger and Hero 2006a, Kriger et al. 2007b). Given this breadth of empirical and observational evidence, it is reasonable to assume that temperature plays a major role in determining infection patterns of Bd.

**Is there a link to climate change?** It has been recently hypothesized that a synergistic relationship between climate change and disease may be driving amphibian declines, known as the “climate-linked epidemic hypothesis” (Harvell et al. 2002, Pounds et
Pounds et al. (2006) examined climatic data from neotropical mountain ranges and proposed that climatic warming has caused elevation of the cloud layer surrounding mountain ranges. This lifting effect, Pounds and colleagues hypothesised, was causing the temperatures in the increasing area underneath cloud to become more uniformly distributed around the thermal optimum for Bd. This “chytrid-thermal-optimum hypothesis” suggests that montane amphibian extinctions are caused by a synergism between Bd and climate change, and appears to explain the positive correlation between altitude and last year observed for now-extinct *Atelopus* populations (Pounds et al. 2006) and similar patterns of decline in montane-dwelling *Alytes* toads in Spain (Bosch et al. 2007). However, this hypothesis has recently received substantial criticism (Lips et al. 2008, Rohr et al. 2008a). In relation to climatic warming, a “drought-linked chytridiomycosis hypothesis” (Kriger 2009) exists, where periods of reduced rainfall are thought to increase the severity or incidence of chytridiomycosis (Lampo et al. 2006). Possible mechanisms include: increased host stress reducing immune function; increased likelihood of host desiccation as Bd infection often causes skin irregularities in the pelvic patch, an epidermal site of water uptake in amphibians; or by causing increased aggregation of hosts or concentration of parasites in decreasing water bodies or hibernacula, thus increased disease transmission (Burrowes et al. 2004, Lampo et al. 2006, P. Bishop, pers. comm., however see Kriger 2009 for a refute of this hypothesis).

**A role for host thermal response?** Most of the literature concerning the thermal epidemiology of chytridiomycosis uses the thermal biology of Bd to explain patterns, whereas little consideration has been given to how the host’s thermal biology may affect chytridiomycosis. This is surprising considering that amphibian physiology, including immunology, is strongly influenced by temperature (Duellman and Trueb 1986, Carey et al. 1999, Raffel et al. 2006). Rödder et al. (2008) recently sought to resolve this by modeling the climate envelope of both Bd and Stony creek frogs, *Litoria lesueuri*, finding that climate suitability for the host followed a similar pattern to Bd infection intensity in these frogs. Experimental infection of tadpoles, which were then allowed to metamorphose at two temperatures considered optimal for Bd growth, 17°C and 22°C, showed that while only 5% of infected metamorphs held at 17°C survived, 50% survived infection at 22°C (Andre et al. 2008). The results of both Rödder et al. (2008) and Andre et al. (2008) suggest that host thermal biology has a role in determining Bd epidemiology. It is possible that the varied susceptibility of amphibian host species to chytridiomycosis may be explained by the interactions between host and pathogen thermal responses, as has been documented in host-pathogen interactions in insects (Thomas and Blanford 2003) and between the sea fan *Gorgonia ventalina* and *Aspergillus* fungi (Ward et al. 2007).
Could tadpoles be a good host for modelling thermal effects? Tadpoles may be an excellent model for testing the interactions between host and pathogen thermal responses, given that the effect of temperature on their development is highly visible and has important fitness consequences (Semlitsch et al. 1988). Amphibian larvae develop and grow faster with increasing temperature, until the upper bound of thermal tolerance is approached (Harkey and Semlitsch 1988). Generally, large size is beneficial, as larger tadpoles are more tolerant to stressors such as low pH and because larger and faster growing tadpoles tend to achieve large size at metamorphosis (Berven 1982, Rosenberg and Pierce 1995). However, Travis (1980) found the opposite; slower developing *Hyla* tadpoles achieved larger size at metamorphosis, possibly conferring an advantage in spite of prolonged development. This observation appears to conform with the majority of thermal studies on ectotherms that report a negative association between body size and temperature (Atkinson 1994). In amphibians, larval development has consequences for post-metamorphic fitness, because smaller metamorphs suffer from higher mortality in addition to lower reproductive success due to smaller size at maturity (Berven 1981, Berven and Gill 1983, Smith 1987, Semlitsch et al. 1988). The study of Bd in larval amphibians is important, as tadpoles may be an important reservoir for Bd, given that they usually carry subclinical infections (Blaustein et al. 2005) and because long-term persistence of Bd may occur in overwintering tadpoles (Rachowicz and Briggs 2007).

1.5 Co-infection as a potential influence on chytridiomycosis

It is becoming apparent that Bd isolates do form distinct strains that differ in genotype, phenotype, virulence and protein expression (Berger et al. 2005b, Fisher et al. 2009). However, nothing is currently known about how strains may interact. It is likely that amphibians could become infected with multiple strains of Bd, due to the rapid rate of its global dissemination (Morehouse et al. 2003, James et al. 2009) and evidence of multiple introduction sites in South America (Lips et al. 2008). Infection with multiple strains is common in other host-pathogen interactions (Read and Taylor 2001). The way in which strains interact can have strong consequences for the epidemiology and evolution of the pathogen, pathology for the host and the evolution of host immune systems (Read and Taylor 2001). Strains may compete against each other (de Roode et al. 2005a, Gower and Webster 2005), which often leads to selection for the most virulent strains, as these have a competitive advantage (de Roode et al. 2005b, Bell et al. 2006). Because competition selects for more virulent genotypes, multiple infection can increase disease severity for the host (Hodgson et
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Interference competition, resource competition and immune-mediated apparent competition may occur between co-infecting strains (Massey et al. 2004, Råberg et al. 2006). However, strains may also co-operate, particularly if they are closely related (Buckling and Brockhurst 2008). Identifying if and what type of interactions occur between Bd strains in amphibian hosts would therefore be a valuable topic to address. It may be that strains are indeed too genetically similar to compete, however if the opposite is the case then this would lend support for increasing efforts to limit the further spread of Bd.

1.6 Thesis objectives

Bd-amphibian pathogenic interactions present an intriguing system for disease research. Although the system is becoming well-characterised, there still remain many knowledge gaps, notably centred on the problem of why the expression of virulence is so plastic. Several aspects of host and pathogen biology, plus environmental factors, have been shown to change virulence or infectivity of Bd. However, very little is known about how these factors interact to produce the observed infection patterns. This thesis sets out to develop and evaluate approaches to enable researchers to look at two current knowledge gaps in what affects the course of chytridiomycosis: how might multiple strains of Bd interact, and how do interactions between amphibian, Bd and temperature affect chytridiomycosis.

However, accurate detection and quantification of Bd infection in tadpoles was required for this work. The recommended method detecting Bd in amphibians is a Taqman-based quantitative polymerase chain reaction (Taqman qPCR; Boyle et al. 2004), but this technique is relatively expensive (Speare et al. 2005, Kriger et al. 2006a). It was reasoned that other research groups may have similar problems with the assay costs for qPCR. Therefore, in Chapter 2, alternative techniques for qPCR were examined and evaluated against the standard method to determine whether the cost of qPCR detection of Bd could be reduced while maintaining assay quality.

It is becoming apparent that Bd isolates differ in phenotype, virulence, protein expression and genetic make-up (Berger et al. 2005b, Fisher et al. 2009), however to date nothing is known about how strains may interact. To examine multi-strain infections, researchers need to be able to discriminate strains after they are mixed, and at present the only reliable methods include proteomics and genotyping (Fisher et al. 2009). Two types of fluorescent probe have proven useful for discriminating metazoan parasites (Kurtz et al. 2002,
Keeney et al. 2008), and their potential for discriminating groups of Bd is trialled and evaluated in Chapter 3 of this thesis.

For the most part, the thermal biology of Bd is used to explain patterns in chytridiomycosis in response to temperature variation. In contrast, little is known about how amphibian thermal biology might contribute to chytridiomycosis epidemiology. However, studies of the effect of temperature on other host-parasite systems suggest that the interaction between temperature, host and parasite is important for determining infection epidemiology. Therefore, the aim of Chapter 4 is to evaluate whether Bd thermal responses can adequately explain infection patterns at different temperatures, or alternatively whether infection patterns are better explained by a temperature × host × pathogen framework. Tadpoles of the New Zealand-naturalised frog *Litoria raniformis* were used as model hosts in this study.

The specific objectives of this thesis were as follows:

- Chapter 2: To reduce the material costs of qPCR diagnosis while maintaining assay sensitivity, accuracy, specificity and reproducibility.
- Chapter 3: To evaluate whether fluorescent labelling of Bd cells could be a feasible system for distinguishing strains in mixed-strain experiments.
- Chapter 4: To examine the effects of temperature on Bd growth, tadpole phenotype and Bd infection of *Litoria raniformis* tadpoles and determine whether Bd thermal growth response can adequately explain the observed infection response.

Each of these objectives is dealt with in separate chapters, with each being written in the style of an independent paper. Therefore, some overlap may exist between common sections, particularly basic introductory information about Bd. Different aspects of the same data set are presented in both Chapters 2 and 4. The final chapter discusses the overall results of this thesis and their wider implications for Bd research and amphibian conservation. There is one reference section covering all chapters at the end of this thesis.
CHAPTER 2: Reducing reagent costs associated with real-time qPCR detection of the amphibian pathogen, *Batrachochytrium dendrobatidis*
CHAPTER 2

Reducing reagent costs associated with real-time qPCR detection of the amphibian pathogen, *Batrachochytrium dendrobatidis*

2.1 Introduction

The emergence of the pathogen *Batrachochytrium dendrobatidis* (Bd) has been implicated in the decline or extinction of around 200 amphibian species since 1980 (Skerratt et al. 2007). This skin-invasive Chytridiomycete fungus has now been reported on every continent inhabited by amphibians, and investigations of its epidemiology are viewed as a priority for amphibian conservation (Berger et al. 1998, Ron and Merino 2000, Waldman et al. 2001, Young et al. 2001, Bradley et al. 2002, Hopkins and Channing 2002, DEH 2005, Garner et al. 2005, Gascon et al. 2007, Kusrini et al. 2008, Une et al. 2008).

Disease surveys of wild and captive amphibians is an important and widely-used technique for obtaining information on the ecology and spread of Bd (Skerratt et al. 2008). However, infected individuals can be difficult to identify since some amphibians carry Bd without clinical signs (Parker et al. 2002, Daszak et al. 2004a, Obendorf 2005, Kriger and Hero 2006b, Padgett-Flohr and Goble 2007). Therefore laboratory, rather than clinical, diagnosis is the only way to confirm Bd presence or absence in an individual. Detection of Bd DNA in amphibian skin using Taqman real-time quantitative PCR (qPCR) (Boyle et al. 2004) is currently being developed as the Office Internationale Epizootes (OIE) standard (Hyatt et al. 2007). This technique is well-validated, specific to Bd and extremely sensitive, being capable of detecting the genomic equivalent of one zoospore in a sample (Boyle et al. 2004, Hyatt et al. 2007). Some authors have even gone as far as to recommend Taqman qPCR as the “gold standard” assay, to the exclusion of other detection techniques such as histology (Kriger et al. 2006a, Kriger et al. 2006b, Kriger et al. 2007a). However, Taqman qPCR is comparatively expensive; with approximate prices to analyze each sample ranging from $5.34-$40 USD ($8.35-$62.52 NZD, as at June 2009) per sample depending on methodology and whether researchers perform their own analysis or outsource samples to a commercial diagnostic laboratory (Speare et al. 2005, Kriger et al. 2006a). Given that chytridiomycosis may occur at prevalences as low as 2% in wild populations, it is recommended that 149 samples be taken per population to be 95% confident of detecting Bd (Speare et al. 2005,
Reducing qPCR costs for detecting Bd (Skerratt et al. 2008). Surveys for Bd often cover multiple populations, meta-populations, species or regions, therefore sample sizes generally range between 100 and >10,000 host individuals (e.g. Berger et al. 2004, Garner et al. 2005, Speare et al. 2005, Obendorf and Dalton 2006, Puschendorf et al. 2006, Kriger et al. 2007b, Rowley et al. 2007a, Alemu I et al. 2008, Frias-Alvarez et al. 2008, Ruiz and Rueda-Almonacid 2008). Thus, while efforts have been made to reduce the cost of Taqman qPCR (Kriger et al. 2006a, Hyatt et al. 2007), the cost of monitoring Bd in wild amphibian populations may still be prohibitively expensive for some research groups. Therefore, further work on reducing the cost of qPCR-based diagnosis as well as validation of alternative diagnostic assays against Taqman qPCR is likely to be valuable for Bd research.

Two other PCR assays for Bd are currently in existence, a SYBR® green I-based qPCR for detecting Bd in soil and water (Kirshtein et al. 2007) and an endpoint PCR assay (Annis et al. 2004). While the endpoint assay is capable of determining the presence of absence of Bd, it cannot quantify the amount of Bd and it appears to be less sensitive than qPCR, reliably detecting the genomic equivalent of 10 zoospores in a sample (Annis et al. 2004, Kriger et al. 2006a). SYBR green qPCR appears to have comparable sensitivity to Taqman qPCR (Kirshtein et al. 2007) but may be less specific. However, the endpoint, SYBR green and Taqman PCR assays have not yet been directly compared against each other.

The aims of this chapter are to trial methods to reduce the cost of qPCR detection of Bd in amphibians. Approaches were developed and validated using mouthpart tissue of experimentally infected tadpoles, since accurate detection of Bd in these samples was of immediate concern for Chapter 4 of this thesis. To achieve this, a standard cetyltrimethyl ammonium bromide (CTAB) DNA extraction protocol (Ausubel et al. 2002, page 2-11) and a SYBR green qPCR protocol for detection of Bd in soil and water (Kirshtein et al. 2007) were optimized and trialled for detection and quantification of Bd DNA in experimentally infected tadpoles. These were reasoned to be potential cost-saving measures because extraction kits (the standard Taqman assay uses PrepMan Ultra extraction reagent) are often comparatively expensive and SYBR® green qPCR reagents are often cheaper than their Taqman counterparts.
2 Reducing qPCR costs for detecting Bd

2.2 Methods

2.2.1 Pilot trial: Extraction and amplification of Bd zoospores from pure culture

The CTAB extraction was initially trialled on serial dilutions of Bd strain JEL197 consisting of $10^7$, $10^6$, $10^5$, $10^4$, $10^3$, $10^2$ and $10^1$ Bd zoospores in 1 mL 1% tryptone broth (T-broth). Zoospores were harvested from agar plate culture and counted, then diluted to the desired concentration in T-broth. See section A1.1 in Appendix 1 for further information on Bd culture methods. Prior to extraction, 1 mL of each serial dilution of Bd was placed into 1.5 mL tubes and heat-killed by immersion in a 65°C water bath for > 5 minutes (Johnson et al. 2003) to prevent contamination of the clean area designated for DNA work.

**DNA extraction.** Zoospores were pelleted by centrifugation at 664 × g for 15 minutes. The top 900 μL of broth was aspirated and discarded. Three hundred and sixteen microlitres of TE buffer (10 mM Tris, 1 mM EDTA, pH 7.5), 30 μL 10% sodium dodecyl sulphate (SDS) and 3 μL Proteinase K (20 mg/mL) were added to each tube, which was then mixed and incubated at 37°C for one hour. After incubation, 100 μL 5 M NaCl and 80 μL of CTAB/NaCl solution (10% CTAB, 4.1% NaCl (w/v); Ausubel et al. 2002) was added to the preparations, which were then mixed and incubated for ten minutes at 65°C. Six hundred and eighty-one microlitres of 24:1 chloroform/isoamyl alcohol (v/v) solution was added to the tubes, which were then inverted vigorously to mix the resulting layers and centrifuged at 14,000 × g for 5 minutes at room temperature. The top 500 μL of the resulting aqueous phase was transferred to a new tube. Five hundred microlitres of 25:24:1 phenol/chloroform/isoamyl alcohol (v/v/v) was added to the phase in the new tube. The preparations were mixed, and centrifuged again. Four hundred and fifty microlitres of the aqueous phase was transferred to a new tube and 18 μL of 5 M NaCl, 100 μL glycogen solution (Oyster type II, 1 mg/mL in TE, pH 8.0) and 378 μL ice-cold isopropanol were added (Fermentas 2007). The tubes were gently inverted and incubated overnight at -20°C. The next day, the tubes were centrifuged at 14,000 × g for 20 minutes at 4°C to pellet the glycogen and DNA. All supernatant was removed, replaced with 500 μL 70% ethanol and the tubes were centrifuged again at 14,000 × g for five minutes at room temperature. After all ethanol was removed, the pellet was suspended in 20 μL TE buffer (pH 7.5) and stored at -20°C.

**Initial qPCR.** This test uses the ITS1-3 Chytr and 5.8S Chytr primers designed by Boyle et al. (2004) which target a 146 base pair (bp) segment of the multicopy region of the Bd ribosomal operon (Kirshtein et al. 2007). Samples were amplified in triplicate 20 μL
Reducing qPCR costs for detecting Bd

reactions consisting of 10 μL Platinum® SYBR® green qPCR SuperMix - UDG (Invitrogen, Carlsbad, California), 1.25 μM of each primer (Sigma-Genosys, St. Louis, Missouri), 4 μL sterilized 0.22 μm- Millipore filter-purified water (MilliQ water) and either 4 μL template DNA or 4 μL MilliQ for no-template negative controls. PCR was run on 96-well PCR plates sealed with optically clear cap strips (ABgene Ltd., Epsom, UK) in a Stratagene MX3000P quantitative thermocycler. Cycling conditions were: one initial cycle of 2 minutes uracil DNA glycosylase (UDG) incubation at 50°C, followed by 2 minutes Taq activation/UDG inactivation at 95°C, then 40 cycles of 95°C for 30 seconds, 57°C for 30 seconds and 72°C for 30 seconds. A dissociation curve was run at the completion of the amplification cycles to identify qPCR products. Carry-over contamination of amplified DNA is controlled for by the Invitrogen SYBR green mix. Because uracil is incorporated into amplified DNA in place of thymine, any contaminating amplified DNA is denatured during the initial UDG incubation step (Longo et al. 1990).

**Modifications to qPCR.** Other published primer concentrations were compared to that of the initial reaction (1250 nM): 900 nM (Boyle et al., 2004) and 1200 nM (Kirshtein et al., 2007). 1200 nM of each primer produced slightly lower C_i values than the other two primer concentrations; thus was used for all subsequent qPCRs. The thermal profile used in Kirshtein et al. (2007) was also trialled, but this resulted in sub-optimal (< 90%, Bustin 2004) DNA amplification efficiency.

### 2.2.2 Validation of standard curves

Eight 1.5 mL tubes containing $10^6$ zoospores in 1 mL T-broth were pelleted by centrifugation and all supernatant was removed. DNA was extracted by either the CTAB methodology or by the standard PrepMan Ultra protocol for DNA standards in Boyle et al. (2004). Each extraction protocol was performed on four replicates. Extraction product was suspended in 1 mL TE buffer (pH 7.5) and stored at -20°C. Immediately prior to qPCR, a sub-sample of each extraction was diluted in MilliQ to $2.5 \times 10^5$ zoospore equivalents mL$^{-1}$, corresponding to $10^3$ zoospore genomic equivalents (ZE) per reaction. One replicate of each extraction was further diluted to create a standard curve consisting of $10^5$, $10^4$, $10^3$, $10^0$ and $10^{-1}$ ZE per reaction. In addition, a Bd DNA standard ($2 \times 10^5$ ZE mL$^{-1}$) extracted with PrepMan Ultra was obtained from the Australian Animal Health Laboratory (AAHL), which is the global reference laboratory for qPCR diagnosis of Bd. This standard was diluted in MilliQ to create $10^2$, $10^1$, $10^0$ and $10^{-1}$ ZE per reaction. The initial concentration of AAHL
DNA was too low to make a $10^3$ standard for the required 4 μL volume. These ZE values represent the lower range of whole-sample zoospore equivalents detected by Taqman qPCR in amphibian tissue (Hyatt et al. 2007). These three standard curves (CTAB, PrepMan and AAHL), the remaining CTAB and PrepMan Ultra extractions (diluted to $10^3$ ZE/reaction), the undiluted negative extraction controls and a no-template control were amplified in triplicate 20 μL SYBR reactions (1200 nM primer concentration). The resulting threshold cycle ($C_T$) values were compared against each other and against typical standard $C_T$ values for the Kirschtein et al. (2007) SYBR green qPCR (J. Kirschtein, USGS, Reston, Virginia, pers. comm., 2008).

### 2.2.3 qPCR volume reduction and subsequent lower detection limits

Reduction of the qPCR volume to 10 μL was trialled as a potential means to reduce assay cost. The 10 μL reaction volume was produced by halving the volume of each component of the 20 μL reaction. The primer concentration for both reactions was 1200 nM. To test the sensitivity of these reactions, DNA standards consisting of $10^2$, $10^1$, $10^0$, $10^{-1}$ and $10^{-2}$ ZE per reaction of CTAB-extracted DNA, $10^{-2}$ ZE of PrepMan Ultra-extracted DNA and a no-template negative control (4 or 2 μL MilliQ instead of DNA) were amplified in triplicate 10 and 20 μL reactions. Zoospore equivalent values were log-transformed and differences between the standard curves were tested using ANCOVA in R 2.8.0 (R Development Core Team 2008).

### 2.2.4 Detection of Bd in tadpole mouthparts

**DNA extraction.** Mouthpart samples were sourced from 47 experimentally infected *Litoria raniformis* tadpoles from the experimental exposure of Chapter 4 (see Sections 4.2.1 and 4.2.3). Samples were stored at 4°C in 70% ethanol. The whole mouthpart was excised from the tadpoles and sectioned down the vertical midline, as Bd infection in tadpoles below Gosner stage 39 is limited to the mouthparts (Marantelli et al. 2004). One half-mouthpart was placed back in storage for later validation (Section 2.2.5). The other half was blotted dry with a new piece of paper towel and re-hydrated in 316 μL TE (pH 7.5) buffer on ice for 30 minutes. New equipment and gloves were used for each tadpole to prevent cross-contamination. The CTAB extraction protocol was essentially the same as for Bd cells in culture, with modifications as follows. After addition of SDS and Proteinase K, the
mouthparts were ground against the side of the tube with a glass pestle until fragments were < 0.25 mm. To denature nucleic acids, proteins and enzymes prior to use, glass pestles were soaked in 4.8% (w/v) sodium hypochlorite (domestic bleach; Janola or Dynawhite) for > 4 hours, rinsed with distilled water, wrapped in aluminium foil and baked at 250°C for 4 hours (Walker et al. 2007). The volume of 24:1 chloroform / isoamyl alcohol was reduced to 529 μL, due to the smaller starting volume of the sample. In addition to the samples, a negative extraction control was made by extracting DNA from the ethanol-stored half-mouthpart of a L. raniformis tadpole that had never been exposed to Bd. A positive extraction control was created by spiking the remaining half of the negative control mouthpart with an unknown large quantity of Bd prior to extraction.

**qPCR.** Tadpole samples were run in triplicate SYBR green 10 μL reactions. Standard curves consisted of 100, 10, 1 and 0.1 ZE of CTAB-extracted DNA. The positive and negative extraction controls, along with a no-template negative control were included in each qPCR plate. A second, ‘quantitative’ positive control was included to test for PCR inhibition. This was made by adding 1 μL each of the negative extraction control and the 100 ZE standard to triplicate wells (resulting in 50 ZE per reaction). The amount of Bd in each positive sample was estimated in ZE via comparison of the average Ct value of the triplicate against the standard curve Ct values. Interpretation of qPCR results followed Hyatt et al. (2007), with tadpoles considered “positive” if all three triplicate wells amplified Bd-specific DNA. Tadpoles were considered “equivocal” if one or two wells amplified Bd DNA and “negative” if all three wells tested negative.

**Overcoming PCR inhibition.** Diluting the DNA by 1:2, 1:5 and 1:10 was trialled to counter inhibition. Dilution was trialled on both types of positive control: the ‘extraction positive’ and the ‘quantitative positive’. The extraction negative control was also trialled at 1:10 dilution to ensure the prior negative PCR results were not an artefact of inhibition. Based on the results of the dilution trial (see Section 2.3.4 and Table 2.2), DNA from the 47 tadpoles was diluted 1:10 in MilliQ and assayed again. Extraction and no-template negative controls were assayed in each PCR plate in addition to the quantitative positive control. The 2 μL of extract used in each qPCR reaction represented 1% of the total extract since a 1:10 dilution of the initial 20 μL product was used. Therefore, the total amount of zoospores per sample was calculated by multiplying the average ZE by 100.

**Assay specificity.** SYBR green qPCR is potentially less specific than Taqman qPCR because SYBR green I dye fluoresces in the presence of any double-stranded DNA, rather than in the presence of the target sequence only as in Taqman qPCR (Bustin 2004). To overcome this problem, dissociation curves were performed at the end of every reaction to
discriminate target and non-target amplicons on the basis of their unique dissociation temperature (Ririe et al. 1997). The accuracy of the dissociation curve was confirmed by running qPCR products on a 2% agarose gel. Presence of a band at 146 bp signified presence of the target amplicon.

2.2.5 Validation against the standard Taqman assay

**CTAB and SYBR green assay vs. standard Taqman assay.** The detection and quantification capacities of the CTAB extraction and 10 μl SYBR green qPCR were directly compared against a version of the standard Taqman assay currently run by M. Butler and R. Poulter at the Biochemistry Department, University of Otago. This Taqman protocol performed well (88% positive/negative agreement and 100% sensitivity) in previous validation against the standard Taqman qPCR assay run at AAHL (Bishop et al. 2009). The complementary half-mouthpart of all 11 Bd- “positive” and “equivocal” tadpoles plus 15 randomly selected “negative” tadpoles were given to M. Butler for blind diagnosis. The half-mouthparts were re-hydrated in 400 μL TE (pH 7.5) at 4°C overnight. Methodology for extraction and Taqman qPCR is essentially the same as for Boyle et al. (2004), with modifications as described in Bishop et al. (2009). The full protocol for this trial can be viewed in Appendix 1 (Section A1.2). Four of the positive samples were tested twice to ensure the quantified values were replicable. Total zoospore equivalents in each half-mouthpart were calculated by multiplying the average ZE per triplicate by 100, since the 5 μL DNA added to each reaction represented 1% of the total 1:10 diluted extraction product.

**Direct comparison of SYBR green and Taqman qPCR.** The PrepMan Ultra-extracted DNA from the Taqman validation was used to directly compare the Taqman and SYBR green qPCRs. This DNA was diluted 1:10 and amplified in triplicate 10 μL SYBR green reactions. The extraction-negative control was not used, since it had already been confirmed negative twice by the Taqman qPCR, but ‘quantitative’-type positive and no-template negative reactions were included to control for PCR errors. A standard curve of 100, 10, 1 and 0.1 ZE was constructed from AAHL-extracted DNA for use in this assay. Upon analysis of Bd quantification of the three assays, it was shown that use of a CTAB standard curve had underestimated the amount of Bd in samples. All CTAB-extracted positive samples were subsequently re-quantified against the AAHL standard curve using triplicate 10 μL SYBR green reactions.
Statistical analysis. Statistics were performed in SPSS (version 16 for Windows, SPSS Inc., Chicago, Illinois). Differences between the CTAB-SYBR, PrepMan-SYBR and PrepMan-Taqman assays were analysed using a Chi squared test for estimated prevalence and a Friedman test to compare the quantification of Bd. A series of Wilcoxon signed rank tests were performed to test for differences in quantification between each pair of assays. A Bonferroni adjustment was applied to the alpha value (adjusted $\alpha = 0.017$) for this test. The zoospore equivalent values for the CTAB-SYBR assay were taken from the re-quantification assay (Test 2, Table 2.3). Only those tadpoles that had either a positive or equivocal result in both the initial and re-quantification tests (Tests 1 and 2, Table 2.3) were scored as ‘Bd positive’ by the CTAB-SYBR assay.

2.2.6 Calculation of assay costs

Prices for the triplicate reagent and consumable plasticware costs were calculated in New Zealand dollars for the Boyle et al. (2004) Taqman qPCR; the reduced-volume Taqman qPCR proposed by Kriger et al. (2006a); and the assays trialled in this study. Chemicals needed for the CTAB extraction were priced either according to their supplier or via New Zealand prices on the Sigma-Aldrich website (www.sigma-aldrich.com). Prices are current for 2008. Complete lists of the calculations used to estimate consumable costs of each protocol, including suppliers and product details, are provided in Appendix 2.
2.3 Results

2.3.1 Pilot trial: Extraction and amplification of Bd zoospores from pure culture

DNA was successfully extracted and amplified from $10^5$, $10^4$, $10^3$, $10^2$ and $10^1$ zoospores in 1 mL broth culture ($2 \times 10^4$, $2 \times 10^3$, $2 \times 10^2$, $2 \times 10^1$ and 2 ZE per reaction) using the CTAB extraction and 20 μL SYBR green qPCR. An acceptable standard curve was produced from the dilutions ($C_t = -3.39 \times \log(ZE) + 33.82$, $R^2 = 0.981$, PCR efficiency = 97.2%). The no-template negative controls did not amplify. The dissociation curve showed that all amplification had a dissociation temperature ($T_m$ product) between 71.65°C and 72.28°C (Fig. 2.1).

![Figure 2.1](image-url)  
**Figure 2.1** Dissociation curve for SYBR green qPCR for Bd detection. Amplified material was CTAB-extracted DNA from dilutions of Bd zoospores in T-broth. The temperature corresponding with maximum loss of fluorescence (i.e. the peak in each curve) is considered the dissociation temperature of the double-stranded DNA fragment ($T_m$ product) and is used to identify the target amplicon.


2.3.2 Validation of standard curves

The 20 μL SYBR green assay detected CTAB- and PrepMan Ultra-extracted DNA in all triplicate reactions spanning the ten-fold dilution series of 0.1-1000 ZE. AAHL DNA was also detected in all triplicate reactions for 0.1-100 ZE. Bd standards created by diluting DNA extracted from $10^6$ zoospores had higher $R^2$ values (all $R^2 > 0.998$, $n = 7$) than those made from DNA extracted from dilutions of zoospores in T-broth ($R^2 = 0.981$, section 2.3.1). $C_t$ values from CTAB, PrepMan and AAHL extractions are shown alongside $C_t$ values typically obtained from a 1000-1 ZE standard curve using the Kirshtein et al. (2007) SYBR green qPCR in Table 2.1. The AAHL standards were the most similar to Kirshtein and colleagues’ standard $C_t$ values, differing by -0.44 to 0.43 $C_t$. CTAB standards had consistently lower $C_t$ values than PrepMan standards, even though both were extracted from the same stock of zoospore broth. $C_t$ values of $10^3$ ZE of PrepMan extract ranged between 18.09 and 18.7 ($n = 4$) and CTAB extract ranged between 15.64 and 16.21 ($n = 4$).

Table 2.1: $C_t$ values from triplicate 20 μL SYBR green qPCR of DNA standard curves produced from CTAB and PrepMan Ultra extractions plus Bd standards from the global reference laboratory, AAHL. These are compared against typical standard $C_t$s produced by the Kirshtein et al. (2007) SYBR green qPCR (J. Kirshtein, pers. comm.). All values are average $C_t$ ± 1 standard error calculated from the triplicates for each ZE/reaction value.

<table>
<thead>
<tr>
<th>Ze/reaction</th>
<th>CTAB</th>
<th>PrepMan Ultra</th>
<th>AAHL</th>
<th>Kirshtein et al. (2007)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1000</td>
<td>16.21 ± 0.08</td>
<td>18.70 ± 0.06</td>
<td>-</td>
<td>20.6</td>
</tr>
<tr>
<td>100</td>
<td>19.57 ± 0.23</td>
<td>22.19 ± 0.12</td>
<td>23.36 ± 0.18</td>
<td>23.8</td>
</tr>
<tr>
<td>10</td>
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<td>25.16 ± 0.29</td>
<td>27.06 ± 0.09</td>
<td>27</td>
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<tr>
<td>1</td>
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<td>29.29 ± 0.08</td>
<td>30.63 ± 0.11</td>
<td>30.2</td>
</tr>
<tr>
<td>0.1</td>
<td>30.08 ± 0.32</td>
<td>33.11 ± 0.92</td>
<td>34.03 ± 0.87</td>
<td>-</td>
</tr>
</tbody>
</table>

2.3.3 qPCR volume reduction and subsequent lower detection limits

There was no significant difference in standard curves between the 20 μL and 10 μL reactions (ANCOVA, PCR volume: $F_{1,27} = 3.76$, $p = 0.063$) despite the fluorescence output of the 10 μL reactions being lower (Fig. 2.2). The $R^2$ value of the 10 μL curve was slightly lower ($R^2 = 0.9943$) than that of the 20 μL curve ($R^2 = 0.9985$). Standard errors of each triplicate were slightly higher for 10 μL (average SE = 0.09 $C_t$, range 0.02-0.14 $C_t$) than for 20 μL (average SE = 0.06 $C_t$, range 0.03-0.12 $C_t$). 0.01 ZE of CTAB-extracted DNA was detected in
all triplicates at 20 and 10 μL. The 20 μL assay detected 0.01 ZE of PrepMan Ultra-extracted DNA in one well, but the 10 μL assay did not detect this amount.

**Figure 2.2** (Top) Standard curves produced by triplicate 10 μL qPCR reactions and 20 μL qPCR reactions of CTAB-extracted DNA and corresponding amplification plots for 20 μL (bottom left) and 10 μL (bottom right) standards in 10⁻¹ serial dilution, from 100 to 0.01 zoospore equivalents.
2.3.4 Detection of Bd in tadpole mouthparts

**DNA extraction.** The first extraction from three of the samples (T7, T8 and T28) failed and was performed again on the complementary half-mouthpart. While the initial failure of T7 and T8 was a result of human error, the failure of T28 was due to inversion of the layers after addition of the phenol:chloroform:isoamyl alcohol solution, presumably caused by high salt concentration (Ausubel et al. 2002). High salt concentration could be caused by improper separation of the aqueous layer after addition of chloroform:isoamyl alcohol.

**qPCR.** Bd DNA was detected in five out of the 47 tadpoles when undiluted CTAB extract was used. Of these, tadpoles T1 (8 ZE), T9 (4 ZE) and T10 (6 ZE) were considered positive for Bd infection (Bd DNA detected in all triplicates). Two tadpoles, T8 (4 ZE, two positive wells) and T35 (0.28 ZE, one positive well), were considered equivocal. Both the extraction and quantitative positive extraction controls tested Bd-negative in addition to the extraction and no-template negative controls.

**Overcoming PCR inhibition.** Inhibition was overcome by diluting the DNA in TE buffer (pH 7.5). A 1:10 dilution was the most effective, resulting in 43.4 out of 50 (86.8%) zoospores being detected (Table 2.2). Bd DNA was amplified in one of the 1:2 extraction control triplicates, but the averaged amplification curve did not cross above the threshold line. Neither the extraction nor the no-template negative control amplified at the 1:10 dilution. Diluting the CTAB extract from tadpole samples by 1:10 produced better results (Test 1, Table 2.3). Eleven tadpoles tested positive and two were equivocal. Zoospore equivalents were quantified by CTAB standards in the first 1:10 trial, yielding 0.02-22 ZE per sample for equivocal results and 6-527 ZE per sample for positive results. However, 61-63% of the 50 ZE in the quantitative positive controls were detected. Both the extraction and no-template negative controls tested negative.
Table 2.2: Average number of zoospore equivalents (ZE) per reaction volume detected by 1:2, 1:5 and 1:10 dilutions of CTAB extract. The positive extraction control consists of an unknown large amount of Bd added to a mouthpart prior to extraction. The positive quantitative control consisted of 50 zoospore equivalents plus 1 μL Bd-negative tadpole mouthpart extract per PCR well, thus allowing for the quantification of inhibition.

<table>
<thead>
<tr>
<th>Dilution</th>
<th>Positive extraction control</th>
<th>Positive quantification control</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ze\textsubscript{av}/reaction</td>
<td>Number of +ve replicates</td>
</tr>
<tr>
<td>1:2</td>
<td>No Ct</td>
<td>1</td>
</tr>
<tr>
<td>1:5</td>
<td>14650</td>
<td>3</td>
</tr>
<tr>
<td>1:10</td>
<td>10370</td>
<td>3</td>
</tr>
</tbody>
</table>

**Assay specificity:** The SYBR green qPCR commonly amplified non-target DNA but the dissociation curve could distinguish between target and non-target amplification. All Bd standards produced a T\textsubscript{m} product between 71°C and 73°C (e.g. Fig 2.1). All tadpole samples that had a T\textsubscript{m} product between 71°C and 73°C produced a band approximately half-way between 100 and 200 base pairs (bp) on agarose gel, corresponding to both the target amplicon length of 146 bp and the band produced by the Bd standards (Fig. 2.3). All tadpole samples that had a T\textsubscript{m} product outside of the 71-73°C range did not produce a band at 146 bp, but often produced a faint band of differing size. These non-specific bands can be seen in Figure 2.3 between 400 and 800 bp in the NC tadpole, T2 and T6 samples, and as a thick band centred on 100 bp for the T10 sample with a T\textsubscript{m} product of 81.1. An additional faint band was also sometimes seen in samples with specific amplification (T8 in Fig. 2.3). The bands smaller than 100 bp on the gel demonstrate primer-dimer bonding (Fig. 2.3).
Figure 2.3 Photograph of 2% agarose gel of selected post-qPCR products from triplicate tadpole CTAB-SYBR test 1. The ladder is on the far left of each row, ranging from 100 to 10,000 base pairs (bp). Bd-specific bands can be seen halfway between the 100 and 200 bp ladder bands. Bands are labelled with their sample type. “Standard” is taken from a 100 ze quantitation standard, “NTC” is a no-template negative control, “NC tadpole” denotes product from a negative control tadpole, “PC tadpole” denotes product from tadpole DNA spiked with 50 Bd zoospore equivalents and tadpole samples are labelled T1 to T10. Tm product for each sample is noted next to the sample name in brackets.
2.3.5 Validation against the standard Taqman assay

The number of tadpoles that Bd was detected in (positive or equivocal) did not differ significantly between the three assays (Chi squared test: $\chi^2 = 0.110$, d.f. = 2, $p = 0.947$). However, there was a slight disagreement between assays about which samples were positive. The CTAB-SYBR and PrepMan-Taqman tests agreed on positive/negative result in 22 out of the 26 samples (84.6%). Twenty-four (92.3%) diagnoses agreed between PrepMan-Taqman and PrepMan-SYBR. Twenty-two diagnoses (84.6%) agreed between CTAB-SYBR and PrepMan-SYBR (Table 2.3). The CTAB-SYBR and PrepMan-SYBR assays detected Bd in 80% and 90% of the samples determined positive by the Taqman assay, respectively. Detection of Bd in samples with lower chytrid loads was generally more equivocal between tests, whereas all tests agreed on samples that scored more than 1670 ZE in the Taqman assay (Table 2.3). The amount of zoospores amplified by the quantitative positive controls was similar for both CTAB and PrepMan extractions. On average, 66.46% ($n = 3$) of the 50 zoospores added to PrepMan tadpole extract amplified and 61.7-73.4% ($n = 3$) of those added to CTAB extract.

There was a large discrepancy in quantification between the initial CTAB-SYBR assay and the PrepMan-Taqman and PrepMan-SYBR tests (Tests 1, 3 and 4, Table 2.3). Re-quantification of all positive and equivocal samples with the AAHL standards resulted in better agreement on quantification and confirmed ten positive results (Test 2, Table 2.3). Only two wells of the 0.1 ZE AAHL standard amplified in this test, but all other AAHL standards amplified in three wells.

Where all three assays (Tests 2, 3 and 4) detected a positive sample, there was a significant difference in the amount of Bd detected (Tests 2, 3 and 4, Table 2.3; Friedman test: $\chi^2 = 10.75$, d.f. = 2, $p = 0.002$). The (re-quantified) CTAB-SYBR test returned the lowest zoospore counts (1272±444 ZE), followed by the PrepMan-SYBR (4573±2172 ZE) then PrepMan-Taqman (6654±2721). However, only the PrepMan-SYBR and PrepMan-Taqman were significantly different ($Z = -2.521$, $p = 0.008$, all other $Z \geq -2.240$ and $p \geq 0.023$, Bonferroni adjusted $\alpha = 0.017$).
Table 2.3 Results of assay validation tests on tadpole mouthparts. Threshold (C\textsubscript{t} or C\textsubscript{p}) values and number of zoospore equivalents (ZE) in the sample half-mouthpart (= average ZE \times 100) are displayed. Test 1 consisted of DNA extracted with the CTAB protocol, diluted 1:10 and amplified with SYBR green qPCR against CTAB-extracted standards. Test 2 re-quantified samples found positive or equivocal by tests 1, 3 or 4 using SYBR green qPCR and AAHL standards. For test 3, DNA was extracted with PrepMan Ultra and amplified by Taqman qPCR by Margi Butler, Department of Biochemistry, University of Otago. For test 4, DNA from test 3 was amplified with SYBR green qPCR and quantified with AAHL standards. Tadpoles T7 and T8 were not included in the validation, but are shown here for completeness.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Test 1</th>
<th>Test 2</th>
<th>Test 3</th>
<th>Test 4</th>
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<tbody>
<tr>
<td></td>
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<td>(re-quantified)</td>
<td>PrepMan-Taqman</td>
<td>PrepMan-SYBR</td>
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<tr>
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<td>ZE / sample</td>
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<td>-</td>
<td>0</td>
</tr>
<tr>
<td>T27</td>
<td>-</td>
<td>0</td>
<td>-</td>
<td>0</td>
</tr>
<tr>
<td>T31</td>
<td>25.17</td>
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</tr>
<tr>
<td>T32</td>
<td>24.39</td>
<td>216</td>
<td>25.51</td>
<td>2035</td>
</tr>
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<td>T35</td>
<td>25.64</td>
<td>90</td>
<td>26.95</td>
<td>849</td>
</tr>
<tr>
<td>T36</td>
<td>-</td>
<td>0</td>
<td>-</td>
<td>0</td>
</tr>
<tr>
<td>T37</td>
<td>28.61</td>
<td>11</td>
<td>30.09</td>
<td>126</td>
</tr>
<tr>
<td>T38</td>
<td>31.09</td>
<td>2</td>
<td>31.97</td>
<td>40</td>
</tr>
<tr>
<td>T39</td>
<td>-</td>
<td>0</td>
<td>-</td>
<td>0</td>
</tr>
<tr>
<td>T40</td>
<td>-</td>
<td>0</td>
<td>-</td>
<td>0</td>
</tr>
<tr>
<td>T41</td>
<td>37.60\textsuperscript{a}</td>
<td>0.02\textsuperscript{b}</td>
<td>-</td>
<td>0</td>
</tr>
<tr>
<td>T42</td>
<td>-</td>
<td>0</td>
<td>-</td>
<td>0</td>
</tr>
<tr>
<td>T45</td>
<td>-</td>
<td>0</td>
<td>-</td>
<td>0</td>
</tr>
<tr>
<td>T46</td>
<td>23.11</td>
<td>527</td>
<td>24.51</td>
<td>3729</td>
</tr>
<tr>
<td>T47</td>
<td>24.56</td>
<td>191</td>
<td>25.71</td>
<td>1804</td>
</tr>
</tbody>
</table>

Notes:
\textsuperscript{a} sample negative for Bd, i.e. no C\textsubscript{t} or C\textsubscript{p} value
\textsuperscript{b} Bd detected in 1/3 wells, an equivocal result
\textsuperscript{av} the value displayed is an average of two qPCR tests
C\textsubscript{p} the threshold cycle out of 46 PCR cycles used in the Taqman test
C\textsubscript{t} the threshold cycle out of 40 cycles used for the SYBR green tests
n/a not tested
2.3.6 Assay costs

For consumable costs, the most cost-effective assay was a combination of CTAB extraction and 10 μL SYBR green reaction, which reduced the consumable cost per tadpole sample to 38% of the price of the standard Taqman assay (Table 2.4). Combination of PrepMan Ultra extraction and the 10 μL SYBR assay costs 42.3% of the standard assay (Table 2.4). Reducing the SYBR green reaction volume to 10 μL accounted for most of the reduction in consumable cost. In terms of time costs, PrepMan Ultra extraction was more efficient, taking just over an hour to complete, whereas the CTAB extractions took between six and nine working hours plus an overnight incubation to complete. Thus, when labour costs are considered (estimated at $20 NZD per hour), the PrepMan Ultra and SYBR green protocol would be the least expensive ($12.11 for CTAB-SYBR versus $8.23 for PrepMan-SYBR per tadpole sample; Appendix 2). Thus, taking time and consumables into account, the PrepMan and 10μL SYBR green assay was the most cost-efficient of the three assays validated in this study.

Table 2.4 Comparison of consumable (reagents and disposable plastics) costs of different triplicate qPCR assay combinations: CTAB extraction and SYBR green qPCR with 20 μL or 10 μL reaction volumes, PrepMan Ultra extraction and Taqman qPCR with 25 μL (Boyle et al. 2004, Hyatt et al. 2007) or 20 μL (Kriger et al. 2006a) reaction volumes, and PrepMan Ultra extraction with 20 μL or 10 μL SYBR green qPCR. PrepMan Ultra extraction is calculated to use 100 μL of PrepMan reagent per tadpole, following the methods described in section 2.2.6. All prices are quoted in NZD.

<table>
<thead>
<tr>
<th>Extraction</th>
<th>qPCR</th>
<th>Total price / sample</th>
<th>Price / 100 samples</th>
</tr>
</thead>
<tbody>
<tr>
<td>CTAB: $1.08</td>
<td>SYBR 20 μL: $6.11</td>
<td>$7.19</td>
<td>$719</td>
</tr>
<tr>
<td></td>
<td>SYBR 10 μL: $3.43</td>
<td>$4.51</td>
<td>$451</td>
</tr>
<tr>
<td>PrepMan Ultra: $1.60 (100 μL)</td>
<td>Taqman 25 μL: $10.29</td>
<td>$11.89</td>
<td>$1189</td>
</tr>
<tr>
<td></td>
<td>Taqman 20 μL: $8.38</td>
<td>$9.98</td>
<td>$998</td>
</tr>
<tr>
<td></td>
<td>SYBR 20 μL: $6.11</td>
<td>$7.71</td>
<td>$771</td>
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<tr>
<td></td>
<td>SYBR 10 μL: $3.43</td>
<td>$5.03</td>
<td>$503</td>
</tr>
</tbody>
</table>

2.4 Discussion

Two potential options for reducing the cost of qPCR detection of Bd in experimentally infected tadpoles were explored in this chapter. The first was a modified CTAB-based extraction (Hengen 1996, Ausubel et al. 2002, Fermentas 2007) and the second was the use and reduction of the reaction volume of an existing SYBR green qPCR protocol for detection of Bd in water and sediment (Kirshtein et al. 2007). Two suitable protocols were identified,
Reducing qPCR costs for detecting Bd

the first combining CTAB extraction with a 10 μL triplicate SYBR green qPCR, and the second combining PrepMan Ultra extraction (Boyle et al., 2004) with the 10 μL assay. Of these two protocols, the PrepMan-SYBR performed better in validation and was more efficient. However, the consumable costs of the CTAB-SYBR protocol were slightly cheaper. The recommended protocols for these assays based upon the findings of this study are summarized in Figure 2.4.

PrepMan Ultra extraction (modified by M. Butler from Boyle et al. (2004))

Modifications as follows:
1. Mouthpart re-hydrated from 70% ethanol in 400 μL TE buffer (pH 7.5).
2. 100 μL volume of PrepMan Ultra in Roche ‘green beads’ tubes used with a MagNa Lyser for DNA extraction.
3. 50 μL supernatant removed and diluted 1:10 for qPCR.

The full protocol can be viewed in Appendix 1 of this thesis.

CTAB extraction (modified from Hengen (1996), Ausubel et al. (2002) and Fermentas (2007))

Summary protocol:

Re-hydrate mouthpart in 316 μL TE buffer (pH 7.5).
Add 30 μL 10% SDS and 3 μL Proteinase K (20mg/mL). Grind, then incubate 1 hr at 37°C.
Add 100 μL 5M NaCl and 80 μL CTAB/NaCl solution. Incubate 10 min at 65°C.
Add 529 μL 24:1 chloroform:isoamyl alcohol, mix and centrifuge.
Remove 500 μL supernatant to a new tube, add 500 μL 25:24:1 phenol:chloroform:isoamyl alcohol, mix and centrifuge.
Remove 450 μL supernatant to a new tube and add 18 μL 5M NaCl and 100 μL glycogen solution (1 mg/ml) and 378 μL isopropanol. Incubate overnight at -20°C.
Wash with 500 μL 70% ethanol and suspend pellet in 20 μL TE buffer.
Dilute 1:10 for PCR.

SYBR green qPCR (for PrepMan and CTAB extracts)

10 μL triplicate reactions: 5 μL SYBR green SuperMix with UDG (Invitrogen), 1200 nM ITS1-3 Chytr and 5.8S Chytr primers, 2 μL DNA, made up to 10 μL with MilliQ water.

Thermocycler conditions: Stratagene MX3000P instrument, 2 min 50°C, 2 min 95°C then 40 cycles of 95°C 30 seconds, 57°C 30 seconds, 72°C 30 seconds. Melting curve used machine default settings.

Standards: 100, 10, 1, 0.1 zoospore genomic equivalents, extracted using PrepMan Ultra following Boyle et al. (2004).

Figure 2.4 Summary of the recommended protocols for SYBR green qPCR detection of Bd in experimentally infected tadpoles
Different assay sensitivity for different sample types? The 20 µL SYBR green qPCR had the same theoretical sensitivity as the standard Taqman assay, 0.01 AAHL ZE per reaction or one zoospore per sample (Boyle et al. 2004). The sensitivity of the 10 µL assay was slightly lower, detecting 0.1 ZE or 10 zoospores per sample, comparable to that of the Annis et al. (2004) endpoint PCR assay. For the purpose of experimentally infected tadpoles, the likelihood of missing an infection is low since Bd is restricted to the mouthparts (Berger et al. 1998, Marantelli et al. 2004) and DNA extraction can be performed on the entire mouthpart. It has been argued that detection of one zoospore is important for correct diagnosis of amphibian skin swab samples (Kriger et al. 2006a, Kriger et al. 2006b, Kriger et al. 2007a), however one zoospore detected in an amphibian does not necessarily constitute an infection (Smith 2007). It must be taken into consideration that while qPCR is extremely sensitive, it does not actually confirm infection, merely the presence of pathogen DNA in, or on, the sample (Smith 2007, Burreson 2008). Experimental amphibians are typically exposed to large amounts of Bd (e.g. Carey et al. 2006, Andre et al. 2008), increasing the probability of false positive samples, therefore use of a slightly less sensitive assay may be preferable. However, the probability of missing Bd infection in wild amphibians is considerably higher since non-lethal sampling for qPCR is vastly preferable (Retallick et al. 2006, Hyatt et al. 2007). Non-lethal methods can only sample a fraction of the entire keratinised epidermal areas available for infection, with the repercussion that the likelihood of detecting low-level infections is somewhat reduced (Retallick et al. 2006). Therefore, while the theoretical lower detection limit of the 10 µL assay should be adequate for the detection of Bd in experimentally infected tadpoles, the 20 µL assay should be developed for use with samples from wild amphibians.

Assay validation: differences in detection of Bd. Greater than or equal to 84.6% can be regarded as good positive/negative agreement between assays, since this figure is close to that of the agreement between the Taqman assay run by M. Butler and the standard Taqman assay run at AAHL (88%, Bishop et al. 2009). The CTAB-SYBR and PrepMan-SYBR assays detected Bd in 80% and 90% of the samples determined positive by the Taqman assay, respectively. This suggests these two assays may be slightly less sensitive than the Taqman assay. However, the CTAB-SYBR assay detected Bd in two samples where Bd was not detected by Taqman qPCR and the PrepMan-SYBR assay detected Bd in one sample not determined positive by the other assays. Why were these differences observed? The CTAB-SYBR had a lower percentage agreement with Taqman than PrepMan-SYBR, which could indicate either a difference between assays or asymmetry in the aggregation of Bd cells between the mouthpart halves. Berger (2001) found that Bd may be clustered around a single focus in light infections of adult frogs, whereas Bd was usually widespread throughout the
epidermis in heavily infected individuals. In Bd-infected tadpoles, a continuum from localized to complete depigmentation of keratinized mouthpart structures has been observed (Fellers et al. 2001, Obendorf 2005, Knapp and Morgan 2006), suggesting that different levels of aggregation can occur in tadpole mouthparts. The qPCR results appear to support this; tadpoles whose diagnoses conflicted between mouthpart halves (T9, T10, T36, T41, Table 2.3) had lower estimated chytrid loads, suggesting low level infection could be aggregated asymmetrically in the mouthpart. Therefore it is recommended that the whole mouthpart be used for further assays.

Assay validation: differences in quantification of Bd. The PrepMan-SYBR green assay detected significantly lower zoospore equivalent values than the Taqman assay, signifying that the 10 µL SYBR green qPCR is less sensitive than Taqman qPCR. However, this loss of sensitivity did not significantly affect the detection capability of the SYBR green assay. The 20 µL SYBR green qPCR assay had a similar theoretical sensitivity to Taqman qPCR, thus it would be interesting to trial this method against Taqman qPCR to determine if the quantification of Bd in samples would differ. The CTAB-SYBR green assay returned zoospore equivalent values ranging from 64.6× lower to 3.3× higher than the Taqman assay, but the difference was not significant. This can probably be attributed to differences in the amount of Bd infecting each mouthpart half. The Wilcoxon analyses would have had more power to detect a difference between the PrepMan-SYBR and Taqman assays than between the Taqman and CTAB-SYBR assays. This is because the PrepMan-SYBR and Taqman assays were performed on the same mouthpart half. However, on average, the CTAB-SYBR method detected lower zoospore equivalent values than the Taqman and PrepMan-SYBR assays, suggesting that CTAB extraction may recover lower amounts of Bd DNA from tadpole samples than PrepMan Ultra. This was unexpected because CTAB extraction consistently produced lower C_t values (corresponding to higher zoospore equivalents) than PrepMan extraction from zoospores in broth culture. The Ausubel et al. (2002) extraction was originally designed for extraction of genomic DNA from large amounts of bacteria in culture, which may explain why it was more effective at recovering DNA from large amounts of Bd in culture. However the discrepancy in the efficiency of CTAB extraction from culture and samples meant that CTAB-extracted standards underestimated Bd load in samples. Standards extracted with PrepMan Ultra provided more realistic C_t values and should therefore be used for future CTAB-SYBR assays.

Assay costs. Both CTAB- and PrepMan-SYBR 10 µL assays were comparable in price, reducing consumable reagent costs to 37.9-42.3% of the standard Boyle et al. (2004) assay. Though more expensive, the 20 µL assay still reduced assay consumable costs
Reducing qPCR costs for detecting Bd considerably, ranging between 60.5-64.8% of the cost of the standard assay. Overall, PrepMan Ultra extraction was less time-consuming and involved fewer steps, appeared to be more efficient at extracting from mouthparts and created more realistic standards. Among the benefits of the PrepMan Ultra protocol is that it has been evaluated for a wide range of sample types (Boyle et al. 2004, Retallick et al. 2006, Hyatt et al. 2007), and it is considerably less time-consuming than CTAB extraction. Additionally, the risk of cross-contamination of samples is lower in the PrepMan Ultra extraction because sample manipulation is kept to a minimum. In addition, the cost of PrepMan extraction could be further reduced if a smaller volume of PrepMan Ultra was used for extraction. Therefore the PrepMan Ultra and 10 μL SYBR assay was the most effective of the combinations trialled.

In conclusion, both the PrepMan-SYBR and CTAB-SYBR techniques appear to be suitable for detecting and quantifying Bd in experimentally infected tadpoles, as needed for Chapter 4 of this thesis. Combining PrepMan Ultra extraction with the 10 μL SYBR green qPCR was the most effective and efficient of the assays compared in this study. However, material cost of the CTAB-SYBR is slightly cheaper. Both assays appear to be inexpensive, sensitive, specific and replicable; demonstrating that assay cost could be reduced without necessarily compromising assay quality. Detection of large amounts of zoospores within some of the *L. raniformis* tadpoles suggests they can be experimentally infected and are a suitable model species for Chapter 4. The 20 μL assay may prove useful for reducing the costs of detecting Bd in wild amphibian populations, however further work needs to be done to fully evaluate the suitability of these assays for wild amphibians.
CHAPTER 3: Fluorescent probes as a tool for marking and tracking

*Batrachochytrium dendrobatidis*
CHAPTER 3

Fluorescent probes as a tool for marking and tracking Batrachochytrium dendrobatidis

3.1 Introduction

Host organisms are commonly infected with multiple strains of a parasite species (Read and Taylor 2001, Lagrue et al. 2007, Leung et al. 2009). Co-infecting strains often compete for host resources (Read and Taylor 2001, de Roode et al. 2005a, Gower and Webster 2005, Bell et al. 2006), although cooperation between strains has been observed in some parasites (Buckling and Brockhurst 2008). Interactions between co-infecting strains can shape the epidemiology of the parasite, pathology for the host and the evolution of parasite phenotypes such as virulence or drug resistance (Read and Taylor 2001, Hodgson et al. 2004, Massey et al. 2004, de Roode et al. 2005b, Alizon et al. 2009). Therefore, identifying and understanding multi-strain infections is a critical area of study for infectious disease research.

Batrachochytrium dendrobatidis (Bd) is a unicellular chytrid fungus that parasitises keratinised amphibian skin (Berger et al. 1998, Longcore et al. 1999). Bd is highly virulent in several amphibian species, and has been implicated in more than 200 amphibian declines or extinctions (Skerratt et al. 2007). While geographically disparate strains of Bd display very little genetic variation (Morehouse et al. 2003, James et al. 2009), recent papers have found that different strains differ in morphology, growth and virulence (Berger et al. 2005b, Fisher et al. 2009). It is hypothesised that Bd has been rapidly spread around the globe (Morehouse et al. 2003, James et al. 2009) and Lips et al. (2008) found evidence for multiple introductions of Bd into South America. Therefore while not yet documented, it is possible that amphibians could become infected with more than one strain of Bd.

While it is becoming apparent that distinct strains of Bd exist, nothing is known about how these strains may interact. However to address this knowledge gap, methods must be available to enable the researcher to distinguish between strains. Bd strains can be distinguished by genotype, proteomics and zoosporangia morphology (Morgan et al. 2007, Fisher et al. 2009).
Labelling the cells of known Bd strains could provide an additional, rapid and simple method to distinguish and track the fate of strains in multi-strain experiments.

Fluorescent probes have been successful for tracking and distinguishing conspecific metazoan parasites (Kurtz et al. 2002, Keeney et al. 2008). In addition, they have been found to be helpful for examining Bd growth, physiology and anatomy (Weldon 2005); but they have not yet been used for tracking multiple strains. Two types of fluorescent probe that have been previously used for tracking metazoan parasites (Kurtz et al. 2002, Keeney et al. 2008) were trialled in this study: CellTracker™ probes and BODIPY® fatty acid analogues (Molecular Probes, Invitrogen, Carlsbad, California). CellTracker reagents diffuse through cellular membranes and become fixed in the cytoplasm via conjugation with intracellular glutathione (Haugland 2005). Once fixed, fluorescence is inherited by daughter cells through several cell divisions (Haugland 2005). BODIPY fatty acids mimic the properties of naturally occurring lipids and are incorporated into the cell in via the same pathways (Haugland 2005). Fluorescence of both these dyes is excited by a specific wavelength of light and emitted as a specific wavelength, which is visualised as the colour of the dye. The specific excitation and emission characteristics of each dye allow specifically dyed cells or structures to be identified using techniques such as epifluorescent microscopy, laser scanning confocal microscopy or flow cytometry.

The aim of this chapter is to evaluate whether these dyes would be suitable for labelling and distinguishing different strains of Bd in mixed-strain situations. Specific objectives include: 1) to determine whether CellTracker and BODIPY dyes can label Bd cells; 2) to determine how long fluorescent labelling lasts; 3) to determine whether differently labelled Bd cells can be distinguished from each other, and from un-dyed cells; and 4) to assess whether the dyes affect Bd growth.
3.2 Methods

3.2.1 Staining and autofluorescence

Sourcing, culture and manipulation of Bd is described in Appendix 1 of this thesis. To determine which dyes would be most appropriate, un-dyed Bd cells (type isolate JEL197, Longcore et al. 1999) were examined for autofluorescence under a range of epifluorescent Olympus BX51 microscope filter sets: WIB (excitation wavelength (ex): 460-490 nm, emission wavelength (em): > 515 nm), WIG (ex: 500-520 nm, em: > 580 nm), W1Y (ex: 545-580 nm, em: > 610 nm) and WU (ex: 380-385 nm, em: > 420 nm). Based on the observed autofluorescence, the following probes were selected for trial: CellTracker orange CMTMR and green CMFDA and BODIPY FL C_{12} and 558/568 C_{12}, respectively (Table 3.1).

Table 3.1 Spectral characteristics of dyes trialled in this study.

<table>
<thead>
<tr>
<th>Dye</th>
<th>Appearance</th>
<th>Abs (nm)</th>
<th>Em (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CellTracker™ CMFDA (5-chloromethylfluorescein diacetate)</td>
<td>Green*</td>
<td>492</td>
<td>517</td>
</tr>
<tr>
<td>CellTracker™ CMTMR (5-(and-6)-(((4-chloromethyl)benzoyl)</td>
<td>Orange</td>
<td>541</td>
<td>565</td>
</tr>
<tr>
<td>amino) tetramethylrhodamine)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BODIPY® FL C_{12} (4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-s-</td>
<td>Green</td>
<td>505</td>
<td>511</td>
</tr>
<tr>
<td>indacene-3-dodecanoic acid)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BODIPY® 558/568 C_{12} (4,4-difluoro-5-(2-thienyl)-4-bora-3a,4a-</td>
<td>Red</td>
<td>559</td>
<td>568</td>
</tr>
<tr>
<td>diaza-s-indacene-3-dodecanoic acid)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Notes:
Abs: Absorption maxima, Em: Fluorescence emission maxima. Table adapted from (Molecular Probes 2003, 2008).
* CMFDA is colourless and non-fluorescent until it undergoes both conjugation with intracellular thiols and the acetate groups are removed by esterases (Haugland 2005).

Dyeing protocols were adapted from the manufacturer’s instructions for CellTracker probes (Molecular Probes 2008). Upon receipt, the dyes were dissolved in DMSO to a concentration of 100 µM and stored in the dark at -20°C. Zoospores were harvested from 1% tryptone-agar plates (T-plates) and counted using the protocols described in Appendix 1 of this thesis. The resulting 1% tryptone broth (T-broth) and zoospore mixture was placed into 1.5 mL tubes as 1 mL aliquots. The tubes were centrifuged for 15 minutes at 664 × g to pellet cells and then the top 950 µL of supernatant was removed. The dyes were diluted from the 100 µM stock
solution to make double the desired concentration in T-broth. Fifty microlitres of this 2× concentrated dye mixture were added to the remaining 50 µL of Bd broth culture in each tube, resulting in the zoospores being suspended in the desired concentration of dye. The cultures were protected from the light and incubated at 20 – 23°C for 1.5 hours. After incubation, the cells were washed by adding 950 µL of T-broth, centrifuging the cells again, removing the top 950 µL of broth and replacing with 1 mL of fresh T-broth. This procedure yielded on average a 71.11 ± 6.19% (n = 4 post-dye cultures) pure culture of zoospores in 1.05 mL broth at around half of the originally harvested zoospore concentration. The remaining 28.89% of cells in the dyed cultures consisted of zoosporangia and thalli.

Five suggested concentrations of the BODIPY dyes (Keeney et al. 2008) were trialled: 0.1 µM, 0.2 µM, 2 µM, 10 µM and 20 µM, and six concentrations of the CellTracker dyes (Molecular Probes 2008; A. McLellan pers. comm.): CMTMR: 3 µM, 5 µM, 10 µM, 15 µM, 20 µM and 25 µM; CMFDA: 1 µM, 2 µM, 3 µM, 5 µM, 10 µM, 20 µM. Un-dyed control cultures were made for comparison of fluorescence; these received 50 µL of T-broth instead of a dilution of dye.

3.2.2 Longevity of fluorescence

Cultures were checked 1-2 hours after dyeing and for up to 16 days after dyeing (Table 3.2). At each check, cultures were examined and photographed under an epifluorescent compound microscope at 200-400× magnification (Optronics SN GH043774-H camera and MagnaFire 2.1 software). Paired photographs of Bd cells under bright field and fluorescence filters were taken to determine which parts of the cell were fluorescent. Fluorescence was checked under specific and non-specific filter sets to examine the degree of cross-over fluorescence. At least three cultures per concentration of each dye were examined for longevity. Additionally, differently dyed Bd cells were mixed together on a slide and viewed under the appropriate filter sets to determine if they could be discriminated from each other.

3.2.3 Growth inhibition

Standard procedures (Rollins-Smith et al. 2002), with modifications, were used to test whether any dye concentration inhibited Bd growth. Bd zoospores were stained with each of the
3 Fluorescent probes for tracking Bd

dye concentrations used in Section 3.2.1 of this chapter. Un-dyed control zoospore cultures were made using the same methodology as for dyed cells, except 50 µL of T-broth was added in place of dye at the dyeing step. Half of these un-dyed cultures were retained as positive controls. The other half were made into negative (zero-growth) controls immediately prior to experimentation by killing the cells via incubation in a water bath at 65°C for > 15 minutes (Johnson et al. 2003). One hundred microlitre aliquots of zoospores from each treatment (five to six dye concentrations, plus negative and positive controls) were placed into 10 wells of a 96-well flat-bottomed, optically clear, microtiter plate (Costar, Corning, Lowell, Massachusetts (CMTMR) or Greiner Bio-One, Frickenhausen, Germany (CMFDA, BODIPY 558/568 and FL)). Each 100 µL aliquot consisted of 5 × 10⁵ zoospores in the CMTMR trial, 2 × 10⁵ zoospores in the CMFDA trial and 5 × 10⁴ zoospores in the BODIPY 558/568 and FL trials. Each trial aimed to use the previous standard of 5 × 10⁵ zoospores in each well (Rollins-Smith et al. 2002), however this number of cells was not always achievable due to cells being lost in the dyeing process. Each plate also included blank wells consisting of 100 µL T-broth. Two microplates were made for the CMTMR and the BODIPY dyes. The CMFDA trial consisted of one microplate, as this trial was discontinued after discovering that CMFDA did not stain Bd.

Plates were covered with a lid and wrapped in plastic clingfilm to prevent loss of moisture. The optical density (OD, measured by absorbance) of each replicate was measured at a 490 nm wavelength using a plate reader (EL340, BioTek Instruments Inc., Winooski, Vermont or FLUOstar Omega, BMG LABTECH, Offenburg, Germany). Plates were read immediately after set up, and again after eight days incubation at 23°C. Plates were shaken briefly before each reading to resuspend cells. To check that the growth measured was from Bd and not from contamination of the microwells, 20 µL of each microwell was plated onto T-plates at the end of the experiment and grown at 23°C for seven days. A blank plate was read under both of the plate readers used to determine whether the readers differed in their measurement of OD.

3.2.4 Statistical analyses

Statistics were performed in SPSS version 16 for Windows (SPSS Inc., Chicago, Illinois). The observed growth for each culture (ΔOD) was calculated as OD on day 8 minus initial OD on day 0. Normality and equality of variance of the data sets were evaluated using Shapiro-Wilk and Levene’s tests, respectively. The effect of BODIPY FL, BODIPY 558/568 and CMFDA on Bd
growth was evaluated with one-way ANOVA. Tamhane’s T2 post-hoc tests were used for the BODIPY dyes, since the error variances were unequal. The CMFDA data only deviated slightly from homoscedasticity; therefore a Dunnett post-hoc test was used, with the positive control as the control variable. CMTMR data was compared using a Kruskal-Wallis test, as the data deviated strongly from normality even after transformation. One outlier (ΔOD = 0.2455) was excluded from the CMTMR 25 µM data, because it was twice as high as all other values in this group. Post-hoc comparisons were made between the positive control and all other treatments using a series of Mann-Whitney tests with Bonferroni-adjusted alpha values of 0.0071. Differences in the OD readings given by the two plate readers used were assessed by a Wilcoxon signed ranks test, as the data failed to normalise after transformation.

3.3 Results

3.3.1 Autofluorescence and dye fluorescence

Autofluorescence of Bd was negligible under the WIY and WIG filter sets, and increased as the wavelength of emission/excitation became shorter. Very weak fluorescence was apparent under the WIB filter and there was slightly stronger fluorescence under the WU filter.

All dyes except CMFDA successfully dyed Bd cells. Fluorescence became apparent in stained cultures two days after dyeing, presumably because cells were larger and more easily seen under the 200-400× magnification. Dyed thalli and zoosporangia were brightly fluorescent. Zoospores were sometimes visible around the edge of clusters of encysted cells when the fluorescence produced was very bright, for example at days two (BODIPY FL) and 16 (BODIPY 558/568). The CMTMR dyes stained the whole cytoplasm and also produced pink rhizoids that were visible under the brightfield lens (Fig 3.1). BODIPY dyes produced a granular appearance of fluorescence in the cytoplasm (Fig 3.1).

Higher concentrations of dye produced the brightest and longest fluorescence (Table 3.2). Fluorescence from both BODIPY dyes lasted longer than that of CMTMR (Table 3.2). Bd cells dyed with 10 µM BODIPY FL and 558/568 were still brightly fluorescent at days 12 and 16, respectively (Table 3.2). Fluorescence of Bd dyed with 20-25 µM CMTMR had dimmed considerably by day 12 (Table 3.2).
While fluorescence was brightest under the specific filter set, there was some cross-over in fluorescence. Orange CMTMR fluorescence was sometimes visible under the non-specific filters WIY and WIB. BODIPY 558/568 produced weaker fluorescence under the WIG filter and yellow-orange fluorescence under the WIB filter. BODIPY FL produced very weak to weak red fluorescence under the WIY filter and green-yellow fluorescence under the WIG filter.

Bd cells dyed with BODIPY FL and BODIPY 558/568 or BODIPY FL and CMTMR could be distinguished from each other by their fluorescence characteristics (Fig 3.2). CMTMR labelling could be distinguished from BODIPY FL by the presence of bright pink rhizoids, fairly uniform orange-red fluorescence of the cytoplasm, cell wall and rhizoids under the WIG filter and by weak or nil fluorescence under the WIB filter (Fig 3.2 B, C). BODIPY FL labelling was characterized by green grainy fluorescence within the cytoplasm under both WIG and WIB filters, and either negligible or reduced fluorescence under the WIY filter (Fig 3.2 B, C, F). BODIPY 558/568 labelled cells could be distinguished from BODIPY FL- labelled cells by the presence of bright red fluorescence under the WIY filter and yellow-orange fluorescence under WIB (Fig 3.2E, F). However, the camera used was unable to accurately depict the orange-yellow colour produced by BODIPY 558/568. Bd cells dyed with this dye appear more green-yellow in photographs, and the contrast with BODIPY FL appears much more subtle (Fig. 3.2 E). BODIPY 558/568 and CMTMR – labelled cells were indistinguishable. When Bd cells were difficult to locate on the slide due to low concentration, scanning the slide under the fluorescent filters proved to be a useful mechanism for locating both cell clusters and individual thalli or zoosporangia.
Figure 3.1 Appearance of dyed cultures under bright field and fluorescence filter sets. CMTMR: 25 µM concentration, day 6, 400× magnification, fluorescence settings: WIG filter set, 200 ms exposure. BODIPY FL: 10 µM, day 2, 200× magnification, fluorescence settings: filter in one stop, WIB filter set, 200 ms exposure. BODIPY 558/568: 10 µM, day 2, 200× magnification, fluorescence settings: filter in one stop, WIY filter set, 100 ms exposure.
**Table 3.2** Summary of fluorescence checks for dyed Bd cultures. Symbols are as follows: - = checked, but no Bd seen; x = Bd seen, but no fluorescence; blank = not checked, F = fluorescent Bd seen (F_4 = very bright fluorescence, F_3 = bright fluorescence, F_2 = dim fluorescence, but clearly visible, F_1 = very dim, almost negligible fluorescence). “Day” refers to the number of days after dyeing, with day 0 being immediately after dyeing.

<table>
<thead>
<tr>
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<tr>
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<tr>
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<td>-</td>
</tr>
<tr>
<td>558/568</td>
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<td>-</td>
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<td></td>
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</table>
Figure 3.2 Appearance of mixed BODIPY FL- and CMTMR-dyed Bd cells under (A) bright field, (B) WIG filter and (C) WIB filter (200× magnification, 200 ms exposure, microscope filters out) and fluorescence of mixed BODIPY FL- and BODIPY 558/568-dyed Bd cells under (C) bright field, (D) WIB and (E) WIIY filters (200×, 200 ms, filters out). The colour of the red cluster under the WIB filter set (figure E) was not accurately captured by the camera. Here it appears yellow-green, whereas to the eye it appears orange-yellow. A green arrow denotes the cluster is dyed with BODIPY, an orange arrow CMTMR and a red arrow BODIPY 558/568.
3 Fluorescent probes for tracking Bd

3.3.2 Inhibition

There was a significant difference between treatments for each of the dyes (CMTMR: Kruskal-Wallis $\chi^2 = 86.93$, df = 7, $p < 0.001$; BODIPY 558/568: $F_{6,133} = 106.91$, $p < 0.001$; BODIPY FL: $F_{6,133} = 42.54$, $p < 0.001$; CMFDA: $F_{7,71} = 205.03$, $p < 0.001$). Dyeing Bd with 20 µM, 15 µM and 10 µM CMTMR significantly inhibited growth relative to the positive control (20 µM: $U = 291.5$, $Z = -3.207$, $p = 0.001$; 15 µM: $U = 75.0$, $Z = -3.382$, $p = 0.001$; 10 µM: $U = 100.0$, $Z = -2.706$, $p = 0.007$) (Fig. 3.3). Bd dyed with 20 µM BODIPY 558/568 and FL also had significantly inhibited growth (Tamhane T2, 558/568: $p < 0.0005$; FL: $p = 0.003$). Growth of all other CMTMR and BODIPY treatments were not inhibited relative to their respective positive controls (25 µM, 5 µM, 3 µM CMTMR: Mann-Whitney $p > 0.011$, 10 µM, 2 µM, 0.2 µM, 0.1 µM, BODIPY 558/568 and FL: Tamhane T2, $p > 0.050$) (Figs. 3.3, 3.4, 3.5). Average growth of cultures dyed with concentrations of CMFDA ranged between 0.115 ± 0.003 and 0.130 ± 0.003 OD and were not inhibited relative to the positive control, 0.122 ± 0.002 OD (Dunnett post-hoc test: all $p \geq 0.255$). Average ΔOD of the negative control in this trial was 0.003 ± 0.0004 OD. While 20 µM 558/568 completely inhibited Bd growth, 20 µM FL did not (Tamhane T2, 20 µM compared with negative control, BODIPY 558/568: $p = 0.799$; BODIPY FL: $p = 0.012$) (Figs. 3.4, 3.5).

There was a significant difference between OD readings of plates by both plate readers used (Wilcoxon signed ranks test, $Z = -2.057$, $p = 0.040$, $n = 96$ wells). However, the difference between mean OD reading for each well was very small, indicating that the two plate readers returned similar measurements (EL340 mean OD: 0.0383, FLUOstar mean OD: 0.0391).
Figure 3.3 Growth of Bd zoospores dyed with selected concentrations of orange Celltracker CMTMR dye over eight days (OD_{day8} – OD_{day0}). All $n = 20$, except 25 µM, where $n = 19$. Error bars are ± 1 standard error. Asterisks denote that growth of a particular group was significantly different from the positive control ($p < 0.0071$). An † denotes borderline significant difference from the positive control ($p = 0.007$).

Figure 3.4 Growth (OD_{day8} – OD_{day0}) of Bd zoospores dyed with selected concentrations of red BODIPY 558/568 dye. All $n = 20$. Error bars are ± 1 standard error. An asterisk above a point denotes significant difference from the positive control ($p > 0.05$).
Fluorescent probes for tracking Bd

3.4 Discussion

CellTracker CMTMR and BODIPY 558/568 and FL successfully labelled live Bd cells. Bright fluorescence was apparent in CMTMR labelled cells for up to 10 days at 23°C and in BODIPY labelled cells for at least 12-16 days. Fluorescence performed best at concentrations of 20-25 µM for CMTMR and at 10 µM for the BODIPY dyes. 25 µM CMTMR and 10 µM BODIPY 558/568 did not inhibit Bd growth relative to un-dyed Bd. Bd cells dyed with green-fluorescent BODIPY FL and orange-fluorescent CMTMR or BODIPY FL and red-fluorescent BODIPY 558/568 could be discriminated from each other. However, the results indicate that the best combination for tracking two Bd strains would be to use 10 µM of both BODIPY 558/568 and FL. This dyeing system could also be used in combination with a third, un-dyed strain of Bd.

The dyes appear to be heritable by daughter cells, since the fluorescence lasted for at least two lifecycles, which take four to five days to complete at 23°C (Berger et al. 2005a). Additionally, several fluorescent zoospores were observed at the edges of zoosporangia clusters two and 16 days after dyeing. It is extremely unlikely that these zoospores were those that were originally dyed, since Bd zoospores typically encyst within 24 hours (Piotrowski et
Fluorescent probes for tracking Bd al. 2004, Berger et al. 2005a). Presumably zoospores inherit labelled cytosol and lipids from parent cells during the mitotic division of the parent thallus (Berger et al. 2005a). The fairly uniform fluorescence observed with the CMTMR dye suggests that thiol-containing biomolecules are ubiquitous throughout the cytoplasm of the thallus and rhizoids (Haugland 2005). The grainy appearance of the BODIPY fluorescence in thalli and zoosporangia suggests that these probes are being incorporated into the numerous lipid globules at the periphery of the core of aggregated ribosomes in zoospores and thalli (Berger et al. 2005a). It is surprising that the CellTracker CMFDA did not produce fluorescence, because several of the concentrations trialled were the same as for CMTMR, and dyeing with CMFDA did not kill Bd or inhibit growth. In addition, CMFDA has successfully produced fluorescence in Ascomycete and Oomycete fungi (Stewart and Deacon 1995, Balajee et al. 2005). The optimal staining times found by Stewart and Deacon (1995) suggest that the staining time could be increased; however Balajee et al. (2005) were able to stain Aspergillus species using conditions similar to those trialled in this study. Unlike CMTMR, CMFDA is non-fluorescent until the acetate groups are cleaved by cytosolic esterases (Molecular Probes 2008). Enzymatic profiling of Bd showed a range of isolates to have mild to moderate esterase (C4) activity and mild or no esterase lipase (C8) activity (Symonds et al. 2008). Therefore it is possible that esterase activity was not strong enough to produce CMFDA fluorescence in the incubation time used.

The BODIPY dyes appear to be potentially more suitable than CellTracker for long-term tracking of Bd strains, as bright fluorescence could be achieved for at least 12 to 16 days. Bd infection appears to take seven days to develop in experimentally infected frogs, and is detectable via histology at 14 days post-infection (Hyatt et al. 2007, Shaw et al. in review). This suggests that the longevity of these dyes should be suitable for distinguishing different strains in experimentally infected hosts. However it still needs to be determined whether BODIPY dyes can be used in conjunction with epifluorescent microscopy for identification of Bd in frog skin. Possible procedures could include epifluorescent microscopy of wet mounted epidermal samples (taken by gently scraping the surface of a frog’s skin), histologically sectioned amphibian skin or zoospores shed from the frog into a water bath (Berger et al. 1999, Briggs and Burgin 2003, Hyatt et al. 2007).

CellTracker dyes may be useful for short-term studies of reproduction in Bd. Currently, it is thought that global populations of Bd represent a single, diploid, asexual clone (Morehouse et al. 2003). However, the observed rates of genetic recombination and local adaptation of Bd populations in wild frogs have created uncertainty about the mode of reproduction (Morgan et al. 2007, Fisher et al. 2009). Sexual reproduction has been
documented in other members of the Chytridiomycota and could be indicated by plasmogamy, the fusion of cytoplasm (Whistler et al. 1975, Alexopoulos et al. 1996, Doggett and Porter 1996). This study demonstrated that CMTMR stained the cytoplasm of Bd and that this staining was heritable for one generation. It has been previously demonstrated that cellular fusion can be detected using CMFDA and CMTMR probes (Jaroszeski et al. 1994). Identification of hybrid Bd cells with dual-fluorescent cytoplasm, as was seen for hybrid cells by Jaroszeski and colleagues (1994) may be a fairly robust indicator of plasmogamy, which would indicate whether sexual reproduction is possible. If another blue or green CellTracker probe could stain Bd, then these dyes could be used to indicate potential for a sexual mode of reproduction.

In summary, this study indicated that the best combination for labelling and tracking two Bd strains would be to use 10 μM of BODIPY FL and 558/568 dyes. These concentrations did not affect Bd growth, suggesting they could be used in combination with un-dyed Bd. Therefore, up to three distinctly labelled strains could potentially be tracked. The longevity displayed by the BODIPY dyes suggests that they might be suited to long-term tracking of Bd strains in experimentally infected hosts. It is recommended that further work trial experimental infections of frogs with BODIPY-dyed Bd to determine if this would be a suitable technique for tracking mixed strains within a host. CellTracker dyes may be useful for shorter-term studies of plasmogamy and reproduction in Bd. However, a suitable green-blue CellTracker alternative to CMFDA needs to be identified before this work can proceed.
CHAPTER 4: Thermal ecology of *Batrachochytrium dendrobatidis* in tadpoles
CHAPTER 4

Thermal ecology of *Batrachochytrium dendrobatidis* in tadpoles

4.1 Introduction

Understanding and predicting the dynamics of diseases is a difficult task, given the wide range of factors and synergisms that can impact upon host–parasite relationships. Several studies have demonstrated that the interactions between host and parasite genotypes and/or phenotypes with each other and with the environment, can affect parasite virulence, host resistance or tolerance and host-parasite coevolution (Brown et al. 2000, Thomas and Blanford 2003, Mitchell et al. 2005, Cable and van Oosterhout 2007, Seppälä et al. 2008, Wolinska and King 2009). The ‘environment’ encompasses abiotic and biotic factors, and may refer to either external conditions or the immediate environment within the host (Wolinska and King 2009). Examining how parasites and hosts react to environmental factors, and whether these responses interact, may provide a useful framework for predicting disease dynamics under a range of conditions.

Our understanding of the emerging infectious disease amphibian chytridiomycosis, caused by the chytrid fungus *Batrachochytrium dendrobatidis* (Bd), could benefit from the application of this framework. This pathogen has received a large amount of attention since its discovery a decade ago (Berger et al. 1998, Nichols et al. 1998), particularly because its rapid global spread has been implicated in the declines or extinctions of an estimated 200 amphibian species (Morehouse et al. 2003, Skerratt et al. 2007, James et al. 2009). Bd has a simple lifecycle with two stages; motile, infective zoospores and reproductive zoosporangia (Longcore et al. 1999, Berger et al. 2005a) and it is thought to be transmitted directly between amphibians or indirectly via environmental water or soil (Nichols et al. 2001, Rachowicz and Vredenburg 2004, Carey et al. 2006). The zoosporangia reproduce in keratinized areas of amphibian epidermis; in tadpoles this is restricted to the mouthparts (Berger et al. 1998, Marantelli et al. 2004).

Temperature has a pronounced effect on the ecology of Bd, and affects its pathogenicity (Berger et al. 2004), growth (Piotrowski et al. 2004), production of infective cells (Woodhams et al. 2008) and epidemiology in wild amphibian populations (Bradley et al. 2002, Woodhams and Alford 2005, Kriger and Hero 2006a, Kriger et al. 2007b). Bd increases production of the infective zoospores at cool temperatures (7-10°C, Woodhams et al. 2008)
and outbreaks have been observed during cool temperatures (Bradley et al. 2002, Berger et al. 2004, Woodhams and Alford 2005, Kriger and Hero 2006a, Kriger et al. 2007b). For example, all chytridiomycosis-related mortalities in Arizona of Rana and Hyla species observed by Bradley et al. (2002) occurred during the cooler months of October and February, with average air and water temperatures of 16.9°C and 12.9°C, respectively. However, climatic warming has been linked to Bd-related amphibian declines, presumably because increased cloudiness in montane areas is condensing thermal fluctuation around the optimum for Bd (Pounds et al. 2006, Bosch et al. 2007).

To date, most of the literature dealing with the effect of temperature on the epidemiology of Bd has focused on the thermal biology of Bd. Given that temperature strongly affects amphibian physiology (Duellman and Trueb 1986), including the immune system (Carey et al. 1999, Raffel et al. 2006), it is surprising that little consideration has been given to the role that host thermal biology plays in determining patterns in chytridiomycosis (although see Andre et al. 2008, Rödder et al. 2008). This study aims to simultaneously examine the effect of temperature on Bd and its host to determine whether the interaction between host, parasite and temperature would be a more suitable model for explaining observed infection patterns (prevalence, abundance and intensity).


This study aims to examine the effects of temperature on Bd infection of tadpole hosts. I hypothesize that observed infection patterns will be the product of an interaction between host and Bd thermal responses. This hypothesis predicts that 1) temperature affects Bd growth, 2) temperature affects Bd infection prevalence, intensity and abundance in tadpoles and 3) temperature affects tadpole phenotype. It is also possible that a correlation exists between tadpole phenotype and Bd infection characteristics. Prediction 1 is addressed by observing Bd growth at three selected temperatures, and predictions 2 and 3 by experimental exposure of tadpoles to Bd at the three temperatures.
4.2 Methods

4.2.1 Model organisms and temperatures

The model organisms for these experiments were Southern Bell frog, *Litoria raniformis*, tadpoles and Bd type isolate JEL197 (Longcore et al. 1999). *L. raniformis* (Hylidae) is a pond-breeding frog with large omnivorous tadpoles (Anstis 2002). It is native to South-Eastern Australia, but was introduced to New Zealand in the late 1800’s and is now ubiquitous throughout the North and South Islands (Bishop 2008). Tadpoles were collected in November 2007 from the Sweet Koura Inc. farm in Alexandra, New Zealand at Gosner developmental stages 20-25 (Gosner 1960). No evidence of Bd infection has been found in this area (P. & F. Diver, Sweet Koura Inc., pers. comm.; S. Melzer, Otago University, pers. comm.). Tadpoles were held in 4 L aquaria in aged filtered water at 18°C until they had grown to at least stage 25. Tadpoles were fed a variety of foods *ad libitum*, including commercial fish pellets (Tetrafin), spirulina powder (Lifestream) and pre-frozen lettuce leaves. Bd type isolate JEL197 was sourced from a cryoarchive (Boyle et al. 2003) held by M. Butler and R. Poulter in the Biochemistry Department, University of Otago, and was originally imported into New Zealand from a chytrid culture collection held by J. Longcore at the University of Maine, USA. Husbandry of Bd followed the standard protocols described in Appendix 1 of this thesis. Experimental infections of tadpoles and Bd growth were compared at one temperature optimal for Bd growth, 23°C, and two temperatures sub-optimal for Bd, 15°C and 27°C (Piotrowski et al. 2004).

4.2.2 Prediction 1: Bd growth at different temperatures

Zoospores for the growth trials were flushed from 10 seven-day old T-plates (1% tryptone and 1% agar in distilled water) with 6 mL 1% tryptone (T-) broth, pooled together in a tube, and counted with a haemocytometer. Twelve replicate cultures were created by adding 1 mL of this inoculum containing 2.6 ×10⁶ zoospores to 19 mL T-broth in a 50 mL falcon tube (BD, Franklin Lakes, New Jersey). Four replicate cultures were randomly allocated to incubators set at each temperature; 15°C (mean = 14.1°C, range = 13.5 – 17.0°C), 23°C (mean = 23.4°C, range = 21.5 – 24.5°C), or 27°C (mean = 26.3°C, range = 24.0 – 29.0°C). Bd growth was quantified by taking a 1 mL sample from each tube and measuring the optical density at 490 nm (OD) three times with a spectrophotometer (UV mini 1240, Shimadzu,
Kyoto). An initial measurement was taken immediately prior to placing the cultures into treatments and successive measurements were taken every two days for a period of two weeks. Cultures were also checked for contamination every second day by plating out 1 mL of each culture on to T-agar plates and growing these for four days at 23°C.

4.2.3 Predictions 2 and 3: experimental exposure of tadpoles to Bd at different temperatures

Tadpoles at stages 25-27 were randomly separated into aquaria holding 3 L of aged filtered tap water, with 10 tadpoles per aquaria. Aquaria were randomly allocated to incubators set at 15°C (14.5°C – 16.5°C), 23°C (20°C – 25.5°C) and 27°C (25°C – 28.5°C). Each incubator received two aquaria. Tadpoles were allowed to acclimatize to the incubators and containers for six days prior to infection. On day five of acclimatisation, the snout-vent length (SVL) of each tadpole was measured three times with callipers and their developmental stage was visually determined with the aid of a 15× hand lens or a stereomicroscope.

Tadpoles were infected on day six of the experiment. Bd for the experiment was harvested from ten inoculated T-agar plates kept for five days at 23°C. Immediately prior to infection, the water in each aquarium was reduced to 500 mL. 5 × 10⁷ zoospores in 8 mL T-broth were added to each aquarium, resulting in 1 × 10⁴ zoospores/tadpole/mL. After 24 hours had elapsed, during which time most zoospores should have encysted or infected tadpoles (Piotrowski et al. 2004, Berger et al. 2005a), the water was topped up to 3 L with water that had been adjusted to the appropriate temperature.

Tadpoles were kept in the experimental set-up for 41 days. During this time they were fed ad libitum with fish flakes (Tetrafin) and the water was changed every second day with aged filtered tap water of the appropriate temperature. All waste water from the tanks was decontaminated prior to disposal by adding > 0.1 mL/L TriGene disinfectant (Webb et al. 2007).

On day 41, tadpoles were euthanized in 1% tricaine methanesulfonate (MS-222) and their SVL, stage and weight were measured immediately. Fresh equipment was used for each tadpole or decontaminated with isopropanol between tadpoles to ensure no cross-contamination with Bd. Tadpoles were placed in 70% ethanol. Bd was detected and quantified in tadpole half-mouthparts using the CTAB extraction and 10 µL SYBR green qPCR protocols described in Sections 2.2.4 and 2.2.5 of Chapter 2. Negative (reagents only) extraction controls were included in each batch of tadpoles extracted. The complementary
Thermal ecology of Bd in tadpoles

Half-mouthparts were used for validation of the CTAB and SYBR green detection techniques. Infection intensity was estimated in zoospore equivalents (ZE) from the results of the re-quantification test (Test 2, Table 2.3, Chapter 2), and tadpoles were considered positive for Bd if three PCR wells were positive.

4.2.4 Statistical analyses

All statistical tests were performed in SPSS (version 16 for Windows, SPSS Inc., Chicago, Illinois) or R (version 2.8.0, R Development Core Team 2008). Methods used to select the best combination of explanatory factors for modelling the experimental exposure data followed Crawley(2007), and used either penalised log-likelihood values (Akaike’s Information Criterion, AIC) or Chi-squared factor deletion tests as criteria for model selection. The ‘best’ model (termed the minimal adequate model) was the one that had the simplest combination of factors, but did not have a significantly worse fit to the data than the initial model. Because tadpoles were grouped in aquaria within each temperature treatment, the data was spatially pseudo-replicated. To account for the potential effect that aquaria may have on tadpoles, the factor ‘aquarium’ was nested within ‘temperature’ and added to the models as a random factor. Variance components for the random factors were calculated to examine how much of an effect grouping the tadpoles had on the observations. T-values were considered significant if $t > 2$ (Crawley 2007) and $p$-values were considered significant at the 0.05 level. All tests were two-tailed.

**Prediction 1: Bd growth response to temperature**

Equations for observed growth at each temperature were fitted using a curve estimation function in SPSS, selecting the lowest power equation for which the $R^2$ value was greater than 0.97. Differences in growth between temperatures were evaluated using repeated-measures ANOVA in SPSS. A non-parametric Freidman test was performed on the data to validate the use of repeated-measures ANOVA, because the sample sizes were too small for robust normality and homogeneity tests to be performed.

**Prediction 2: Infection response to temperature**

Infection patterns were described as prevalence, abundance and intensity. Prevalence was calculated as the proportion of tadpoles exposed to Bd that became infected. Infection
intensity (the amount of Bd infecting each tadpole) was estimated from qPCR diagnosis as the average zoospore genomic equivalents (ZE) per triplicate. Mean Bd abundance was calculated as the back-transformed (geometric) mean of Ln(ZE +1) in all exposed tadpoles. Mean intensity of Bd infection was calculated as the geometric mean of Ln(ZE) in infected tadpoles only.

A tadpole’s size or development might affect Bd infection along with temperature. Therefore tadpole weight, SVL and developmental stage was summarised into a single composite term that could be added as a covariate to the analyses of temperature on infection. This composite term (hereafter referred to as “composite SD”) was derived from principal components analysis in R of tadpole SVL, weight and developmental stage. The first principal component axis (PC1) explained 93.3% of the variation in tadpole size and development while the second axis explained only 5.68% of the variation. SVL, weight and stage contributed similarly to PC1, with eigenvalues ranging between 0.587 (SVL) and 0.562 (stage). Composite SD was calculated as the weighted sum of SVL, stage and weight, with each variable weighted by their corresponding PC1 eigenvalue.

Generalised linear mixed-effects models (GLMMs) were performed in the lme4 library in R (Bates et al. 2008). The effect of temperature and composite SD on infection prevalence was analysed as a binomial GLMM with a logit link. The factor temperature was initially trialled as a quadratic term to test for non-linearity, because curvature was observed in the response of prevalence to temperature. The effect of temperature and composite SD on the number of zoospore equivalents in each tadpole (Bd abundance) was analysed as a GLMM with a Poisson distribution and log link. This analysis included only those tadpoles held at 15°C and 23°C, the only groups in which tadpoles became infected. The number of zoospore equivalents in each tadpole was ranked (Conover and Iman 1981) prior to analysis to give a better fit to a count distribution. The factor ‘aquarium nested within temperature’ was included as a random factor in both analyses. Chi-squared deletion tests were used for model selection. The minimal adequate model for abundance was overdispersed; therefore the model was re-fitted using quasi-likelihood to specify the variance function.

**Prediction 3: Tadpole responses to temperature**

The effects of temperature and infection (infection present or absent) on tadpole weight, SVL and developmental stage were of interest. Pre-existing differences in tadpoles’ initial SVL (natural logarithm transformed) and developmental stage between temperature groups were analysed with a one-way ANOVA and a Kruskal-Wallis test, respectively. A
natural logarithm transformation was applied to tadpole weight and SVL prior to analysis. The random factor ‘aquarium nested within temperature’ did not explain any of the variation in the tadpole measures, therefore it was excluded and each tadpole was assumed to be an independent sample. Two-factor ANOVAs were subsequently used for SVL and weight, and a Poisson generalised linear model (GLM) with log link was used for developmental stage. The minimal adequate model was determined for each measure using the automated step function in R, which evaluates models by their AIC. The resulting simplified model for stage was underdispersed, therefore was re-fitted using quasi-likelihood. Tukey HSD tests were used for post-hoc comparisons of weight and SVL between temperatures. Orthogonal treatment contrasts were used to compare differences in developmental stage between temperatures. Survival to the end of the experiment was analysed by linear regression of the proportion of surviving tadpoles in each aquarium.
4.3 Results

4.3.1 Prediction 1: Bd growth

Two cultures held at 15°C were excluded from the analysis due to contamination. Temperature had a strongly significant effect on Bd growth (repeated measures ANOVA, $F_{2,7} = 58.597$, $p < 0.001$, effect size (partial ETA squared) = 0.944). Growth at 23°C was significantly higher than at 15°C and 27°C (Tukey HSD, both $p < 0.001$) (Fig. 4.1). There was no significant difference in growth between 15°C and 27°C (Tukey HSD, $p = 0.139$) (Fig. 4.1). Fitted equations for growth at each of the temperatures are provided in Appendix 3 (Fig. A3.1). By examining derivatives of these equations, it was observed that while Bd grew the fastest at 23°C between 15.29 and 218.05 hours, cultures held at 27°C and 15°C grew faster than cultures at 23°C after 218.05 and 225.96 hours, respectively (Appendix 3, Fig. A3.1).

![Figure 4.1](image-url)

**Figure 4.1** Bd growth in broth culture at 27°C ($n = 4$), 23°C ($n = 4$) and 15°C ($n = 2$), measured as optical density (OD). Lines labelled with different letters were significantly different ($p < 0.05$).
4 Thermal ecology of Bd in tadpoles

4.3.2 Prediction 2: Bd infection at 15°C, 23°C and 27°C

*Prevalence.* Infection prevalence decreased in a linear fashion as the temperature increased (Table 4.1). Mean prevalence was 46.7% at 15°C, 31.3% at 23°C and 0% at 27°C. The most parsimonious model for prevalence included both temperature and composite SD, with no interaction (Table 4.1; Table A3.1 in Appendix 3). Infected tadpoles had a significantly higher composite SD than uninfected tadpoles (Table 4.1).

**Table 4.1** Results of the minimum adequate GLMM for infection prevalence. Significant p-values are flagged by asterisks, * for 0.05 < p < 0.01 and ** for 0.01 < p < 0.001.

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<td>Temperature</td>
<td>-0.481</td>
<td>0.179</td>
<td>-2.691</td>
<td>0.007**</td>
</tr>
<tr>
<td>Composite SD†</td>
<td>0.010</td>
<td>0.004</td>
<td>2.446</td>
<td>0.014*</td>
</tr>
</tbody>
</table>

**Notes:**
Model AIC: 43.63, deviance: 33.63 on 41 residual df, dispersion: 0.82.
See table A3.1, Appendix 3 for model selection statistics (AICs) and goodness of fit deletion tests.
†Composite SD is each individual’s weight, SVL and stage weighted by their corresponding PC1 eigenvalues derived from principal components analysis of these three measures (see section 4.2.5, prediction 2).
Variance components: aquarium(temperature) = 0.00%, temperature ≈ 0.00%.

*Mean abundance and intensity.* Mean abundance of Bd was not significantly different between 15°C and 23°C (Table 4.2), despite the mean abundance being slightly higher at 15°C (geometric mean = 13.5 zoospore genomic equivalents (ZE), mean rank = 16.47) than at 23°C (geometric mean = 8.5 ZE, mean rank = 15.56). However, the amount of Bd carried by tadpoles increased with the increasing magnitude of the composite SD (Table 4.2). The interaction between temperature and composite size-development was not significant; however its inclusion was important for the fit of the model (Table 4.2). While the abundance at 15°C was 1.5 times greater that at 23°C, the geometric mean intensity was 4.38 times higher at 23°C (1336.4 ZE) than at 15°C (305.4 ZE).
4 Thermal ecology of Bd in tadpoles

Table 4.2 Minimum adequate GLMM for mean Bd abundance (ranked amount of Bd per exposed tadpole in ZE). Significant t values are > 2 (Crawley 2007) and are marked with an asterisk.

<table>
<thead>
<tr>
<th>Factor [level]</th>
<th>Estimate</th>
<th>SE of estimate</th>
<th>t value</th>
</tr>
</thead>
<tbody>
<tr>
<td>(Intercept)</td>
<td>11.778</td>
<td>2.748</td>
<td>4.285*</td>
</tr>
<tr>
<td>Temperature [23]</td>
<td>-4.356</td>
<td>4.49</td>
<td>-0.970</td>
</tr>
<tr>
<td>Composite SD†</td>
<td>0.029</td>
<td>0.011</td>
<td>2.617*</td>
</tr>
<tr>
<td>Temperature [23] × composite</td>
<td>-0.006</td>
<td>0.014</td>
<td>-0.466</td>
</tr>
</tbody>
</table>

Notes:
†Composite SD is a weighted combination of tadpole SVL, weight and stage.
Result of model simplification: simplified model (no interaction term): AIC = 1305; comparison with maximal model: $\chi^2 = 8.97$, $df = 1$, $p = 0.003$. Thus, maximal model is the minimum adequate model.
Variance components: aquarium(temperature) = 7.44%, temperature = 0.00%, residual = 92.56%.
Intercept = Temperature [15]

Relationships between tadpole size-development and infection. Generally, the largest and most developed tadpoles available in each aquarium were more likely to be infected than smaller individuals (Fig. 4.3), as shown by the positive effect of the composite-size development term on infection presence (Table 4.1). Similarly, the largest and most developed tadpoles tended to be infected with more Bd (Fig. 4.3; Table 4.2). These patterns appeared to be similar to those displayed if SVL, weight and stage were examined separately (see Figures A3.2, A3.3 and A3.4 in Appendix 3).
4.3.3 Prediction 3: Tadpole responses to temperature

**Survival.** Of the 20 tadpoles included in each temperature treatment, 15 survived to the end of the experiment in the 15°C group, and 16 in each of the 23°C and 27°C groups. There was no relationship between temperature and the proportion of surviving tadpoles per aquaria (linear regression: $F_{1,4} = 0.082, p = 0.789, R^2 = 0.020$).

**Initial measures.** There were differences between temperature groups in SVL (ANOVA: $F_{2,56} = 4.468, p = 0.016$, means: 15°C: $7.26 \pm 0.35$ mm, 23°C: $8.40 \pm 0.43$ mm, 27°C: $7.04 \pm 0.20$ mm) and stage (Kruskal-Wallis test: $\chi^2 = 6.535$, df = 2, $p = 0.038$, means: 15°C: $25.00 \pm 0.00$, 23°C: $25.32 \pm 0.13$, 27°C: $25.10 \pm 0.069$) at the initial measurement on
day 5 of the acclimatisation period. Tadpoles held at 23°C had a significantly larger mean SVL than tadpoles at 27°C at the start of the experiment (Tukey HSD: \( p = 0.024 \)).

**Temperature effects.** Comparison of AIC values showed that both temperature and the infection status of a tadpole were important explanatory variables for weight and SVL (Appendix 3, Table A3.2). However, only temperature was important for model parsimony for developmental stage (Table A3.2). Temperature had a significant effect on tadpole weight (two-factor ANOVA, \( F_{2,43} = 10.845, p < 0.001 \)), SVL (two-factor ANOVA, \( F_{2,43} = 10.579, p < 0.001 \)) and development (Table 4.3). By the end of the experiment, tadpoles held at 23°C and 27°C had attained larger weights and SVLs, and had reached higher developmental stages, than tadpoles held at 15°C (Tables 4.3 and 4.4, Figs. 4.3A, 4.4A and 4.4C). Developmental stages of tadpoles approached a significant difference between 23°C and 27°C (Table 4.4). Tadpoles held at 15°C generally grew little and developed little during the experiment.

**Infection presence/absence.** Tadpoles that became infected with Bd had significantly higher weights and SVLs than tadpoles that did not become infected (weight: two-factor ANOVA, \( \ln(\text{estimate}) \) for infection presence = 0.696 ± 0.224, \( t = 3.114, p = 0.003 \); SVL: two-factor ANOVA, \( \ln(\text{estimate}) \) for infection presence = 0.254 ± 0.087, \( t = 2.921, p = 0.006 \)) (Figs. 4.3B, 4.4B and 4.4D). While infected tadpoles held at 23°C appeared to have reached higher developmental stages than uninfected tadpoles (Fig. 4.5), the effect of infection, or an interaction between temperature and infection, did not appear to be important for model parsimony (Table A3.2).

<table>
<thead>
<tr>
<th>Table 4.3 Summary of minimal adequate model (quasi-Poisson GLM) for developmental stage.</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Factor [level]</strong></td>
</tr>
<tr>
<td>Stage ³</td>
</tr>
<tr>
<td>Temperature [23]</td>
</tr>
<tr>
<td>Temperature [27]</td>
</tr>
</tbody>
</table>

³Stage model is reported in the GLM format as displayed in R. The intercept represents temperature [15], and the estimate for this level represents the mean intercept of this level ± one standard error (SE). The estimates of subsequent levels represent the mean difference with temperature [15]. Residual deviance = 15.633 on 44 degrees of freedom, dispersion = 0.355.
4 Thermal ecology of Bd in tadpoles

<table>
<thead>
<tr>
<th>Tadpole measure</th>
<th>Comparison</th>
<th>Difference between means</th>
<th>95% CI</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Weight</td>
<td>27°C – 15°C</td>
<td>0.755</td>
<td>0.232 – 1.278</td>
<td>0.003**</td>
</tr>
<tr>
<td></td>
<td>23°C – 15°C</td>
<td>0.938</td>
<td>0.416 – 1.461</td>
<td>&lt; 0.001***</td>
</tr>
<tr>
<td></td>
<td>23°C – 27°C</td>
<td>0.184</td>
<td>-0.330 – 0.663</td>
<td>0.698</td>
</tr>
<tr>
<td>SVL</td>
<td>27°C – 15°C</td>
<td>0.319</td>
<td>0.116 – 0.522</td>
<td>0.001**</td>
</tr>
<tr>
<td></td>
<td>23°C – 15°C</td>
<td>0.365</td>
<td>0.153 – 0.559</td>
<td>&lt; 0.001***</td>
</tr>
<tr>
<td></td>
<td>23°C – 27°C</td>
<td>0.037</td>
<td>-0.163 – 0.896</td>
<td>0.237</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Comparison</th>
<th>Difference between means ± SE</th>
<th>t value</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stage&lt;sup&gt;§&lt;/sup&gt;</td>
<td>23°C– 15°C</td>
<td>0.114 ± 0.024</td>
<td>4.748</td>
</tr>
<tr>
<td></td>
<td>27°C– 23°C</td>
<td>0.044 ± 0.023</td>
<td>1.924</td>
</tr>
</tbody>
</table>

**Table 4.4** Summary of contrasts of mean weight, SVL and developmental stage between temperature treatments (weight and SVL: Tukey HSD, stage: orthogonal treatment contrasts within quasi-Poisson GLM).

**Figure 4.3** Gosner (1960) developmental stages of tadpoles after being held at 15°C ($n = 15$), 23°C ($n = 16$) and 27°C ($n = 16$) for 41 days after exposure to Bd. Infected and uninfected tadpoles are shown together in the same box in graph A. Uninfected (denoted as 15a, 23a and 27a, 15°C $n = 8$, 23°C $n = 11$, 27°C $n = 16$) and infected (denoted as 15p and 23p, 15°C $n = 7$, 23°C $n = 5$, 27°C $n = 0$) tadpoles are shown separately in graph B. Boxes labelled with different letters were significantly different ($p < 0.05$).
4.3.4 Results summary: linking it all together

The thermal response of infection prevalence and abundance followed a different pattern from Bd growth, tadpole growth and tadpole development (Table 4.5). At the two temperatures where tadpoles became infected, intensity appeared to follow a similar pattern to Bd growth, being higher at 23°C than at 15°C (Table 4.5). However, whereas Bd growth peaked at 23°C, mean infection abundance and prevalence was intermediate in comparison to the values observed at 15°C and 27°C (Table 4.5). Tadpole growth and development was
similarly high at 23°C and 27°C; however there was a marked difference in infection characteristics at these two temperatures (Table 4.5). Therefore the patterns in infection response to temperature were not correlated with either Bd growth or tadpole responses.

**Table 4.5** Summary of results showing response patterns of Bd, tadpoles and infection to temperature. Bd growth and tadpole responses are grouped into ‘High’ and ‘Low’ based on significant differences ($p < 0.05$) between groups. Abundance and intensity are reported as geometric means.

<table>
<thead>
<tr>
<th>Temperature</th>
<th>Bd growth</th>
<th>Tadpole</th>
<th>Infection</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>SVL</td>
<td>Weight</td>
<td>Stage</td>
</tr>
<tr>
<td>15°C</td>
<td>Low</td>
<td>Low</td>
<td>Low</td>
</tr>
<tr>
<td>23°C</td>
<td>High</td>
<td>High</td>
<td>High</td>
</tr>
<tr>
<td>27°C</td>
<td>Low</td>
<td>High</td>
<td>High</td>
</tr>
</tbody>
</table>

### 4.4 Discussion

The data appears to support the overall hypothesis that the observed infection patterns in *L. raniformis* tadpoles are a product of an interaction between host and pathogen thermal responses. While temperature affected infection characteristics, Bd growth, and tadpole growth and development, the thermal response of infection differed from those observed in host and pathogen. Therefore it is unlikely that Bd growth responses to temperature would be a good predictor for thermal patterns in chytridiomycosis in this system. It also appears unlikely that host tadpole thermal preference could accurately predict patterns in Bd infection, unless it is considered in tandem with Bd thermal biology. Therefore it appears that temperature, host and Bd all have a bearing on the epidemiology of chytridiomycosis. But, how did each node in this framework, and the interactions between them, contribute to the observed thermal patterns in infection? And, what does this imply for our current understanding of how temperature affects chytridiomycosis? However, before addressing these questions, it would be helpful to first consider the results from this study in the context of the amphibian and chytridiomycosis literature.
4.4.1 Responses of Bd, tadpoles and infection to temperature

The observed effect of temperature on Bd growth was similar to the findings of a previous study on Bd growth in vitro. Piotrowski et al. (2004) found that rapid growth of Bd strain JEL197 in culture occurred at 17°C, 23°C and 25°C, whereas cultures grew slowly at 10°C and 4°C and growth ceased at 28°C. Bd infection was restricted by the warm sub-optimal temperature, 27°C, in this study. This is similar to the pattern in chytridiomycosis observed in experimentally infected Great Barred frogs, *Mixophyes fasciolatus*. While all frogs exposed to Bd at 17°C and 23°C died, only 50% of frogs exposed at 27°C died and three of the four surviving frogs held at 27°C eliminated infection (Berger et al. 2004). In the study by Berger and colleagues, the retardation of infection at 27°C could be explained by its being a sub-optimal temperature for Bd, whereas the other two temperatures were within the Bd thermal optimum range. However in this study, observed infection prevalence could not be explained by the Bd thermal optimum range. Growth of Bd in culture was not statistically different between the sub-optimal temperatures (15°C and 27°C); however 46.7% of tadpoles became infected at 15°C and none at 27°C. Prevalence decreased with temperature even though growth of Bd in culture was significantly higher at 23°C. Thus high Bd prevalence was maintained at temperatures beneath the optimum range, despite slower growth. This result is similar to that from a transmission experiment by Rachowicz and Briggs (2007) where similar proportions of naïve *Rana muscosa* tadpoles became infected at 17°C and 4°C. Similarly, Carey et al. (2006) found no significant difference in the survivorship of experimentally infected *Bufo boreas* toadlets at 12°C and 23°C. However, experimental infection of salamanders *Plethodon metcalfi* found that death from Bd infection was around 7 × higher at 8°C than at 16°C (Vazquez et al. 2009). The observed discrepancy between Bd growth and prevalence in this study suggests that the thermal growth response of Bd alone is not an adequate explanation for observed infection prevalence. This is supported by an experimental infection trial of *R. muscosa* tadpoles, where the fate of infected tadpoles held at 17°C and 22°C was followed through metamorphosis (Andre et al. 2008). Significantly more tadpoles died from infection at 17°C than 22°C, even though both temperatures were within the optimal thermal range of Bd, suggesting survivorship was influenced by host thermal response rather than Bd thermal response (Andre et al. 2008).

Temperature had the expected effect on tadpole growth and development; tadpoles held at the coolest temperature grew and developed less. Reduced developmental and growth rates at cool temperatures has been previously shown in tadpoles from a range of species (Berven 1982, Harkey and Semlitsch 1988). Of the temperatures trialled, 23°C and 27°C are
considered to be optimal for *L. raniformis* tadpoles, given that tadpoles developed rapidly and attained larger sizes at these temperatures. Attainment of large size and rapid development as tadpoles are important fitness correlates that promote post-metamorphic survival and adult mating success (Berven 1981, Smith 1987, Semlitsch et al. 1988).

This conclusion is partially supported by a previous temperature experiment with *L. raniformis* tadpoles, where *L. raniformis* tadpoles displayed high mortality at 15°C in comparison to 23°C (Cree 1984). However, this pattern was not apparent in this study and may suggest local adaptation to cool temperature in the Alexandra source population.

### 4.4.2 How could interactions between host, pathogen and temperature explain the observed infection patterns?

How were the observed patterns in Bd infection produced? One possibility is that another aspect of Bd thermal biology other than growth response may be responsible for the observed infection patterns. A recent paper by Woodhams and colleagues (2008) found that Bd zoosporangia are capable of increasing zoospore production at cool temperatures. This was suggested as a strategy by which Bd could compensate for reduced growth rate at cool temperatures, and predicts that more tadpoles would become infected at cooler temperatures (Woodhams et al. 2008). The observed patterns in infection prevalence, abundance and intensity in this study appear to conform to the predictions arising from Woodhams et al. (2008). Prevalence was higher at 15°C than at 23°C but abundance was similar at 15°C and 23°C. However, infected tadpoles at 23°C carried on average 4.38 times more zoospore equivalents than infected tadpoles at 15°C, although this difference should be interpreted cautiously because it could not be statistically tested. The lower average infection intensity in tadpoles at 15°C is possibly explained by the observed lower growth rates at this temperature and may signify slower replication rates within the host at this temperature. However, the pattern in abundance suggests that Bd is capable of maintaining high abundance within tadpole populations at this cool, sub-optimal temperature. The maintenance of abundance during low Bd growth rates at cool temperatures is probably facilitated by the increase in prevalence, which would be expected under conditions of greater zoospore production (Woodhams et al. 2008).

Why then, did no tadpoles become infected at 27°C? This temperature appeared to be similarly detrimental to Bd growth as 15°C, however, zoospore production was markedly higher at cool suboptimal temperatures than at optimal and high sub-optimal temperatures (Woodhams et al. 2008). Reduced growth combined with lower zoospore production could
have contributed to the failure of Bd to establish at 27°C. However, this pattern may also point to a host-pathogen interaction. As discussed previously, 23°C and 27°C were likely to be the most optimal temperatures for L. raniformis tadpoles. While Bd growth was equally handicapped at 15°C and 27°C, at 15°C Bd would have encountered tadpoles in a sub-optimal environment, whereas at 27°C tadpoles were in an optimal environment. Adult frogs of the tropical to sub-tropical species M. fasciolatus or Litoria chloris have been shown to be capable of clearing Bd infections at the high temperatures 27°C and 37°C (Woodhams et al. 2003, Berger et al. 2004), possibly due to a combination of optimal temperature for the host and the reduced growth rate of Bd. Conversely, some components of immunity appear to be compromised at low temperatures in ectotherms such as amphibians, reptiles and fish (Wright and Cooper 1981, Maniero and Carey 1997, Bowden 2007). If host immunity is reduced at cool temperatures, they are presumably easier to infect and exploit (Lafferty and Kuris 1999), thus a trade-off allowing for increased infectivity may be favourable. However, if low temperatures are stressful to the host, as they appear to be to L. raniformis tadpoles, they may be a poorer quality resource for the parasite (Seppälä et al. 2008). Therefore it may be beneficial for Bd to “bet-hedge” and infect more hosts at cool temperatures. The effect of cool temperature on amphibian immunity and condition may in part explain why Bd has evolved a trade-off strategy for cool sub-optimal temperatures, but does not appear to display this response when challenged with warm sub-optimal temperatures.

4.4.3 Correlations between host size and Bd infection

A striking finding of these experiments was the tendency of infected hosts to be larger and the correlation between host size and Bd infection intensity. Smith et al. (2005) also found that Bd positive tadpoles were larger and more developed in wild populations of South African Heleophryne natalensis and Strongylopus hymenopus. This pattern, Smith and colleagues (2005) reasoned, could be explained by larger and more developed tadpoles being older, thus having been exposed to Bd for longer. However the results of this study demonstrated that the size-dependent relationship was independent of time, as all tadpoles had been exposed to Bd for the same amount of time. This size effect was consistent over both temperatures at which tadpoles became infected, with a tendency for the largest hosts available to be infected. However, it appeared that small tadpole size per se did not restrict infection, since the sizes and developmental stages of infected hosts at 15°C were similar to that of uninfected hosts at 23°C. Larger hosts tend to consume more resources; with the
increased risk of encountering parasites present in the consumed substrate (Hall et al. 2007). Many tadpoles respire via active water uptake, and oxygen consumption rate increases with body size (Duellman and Trueb 1986). Therefore the mouthparts of larger tadpoles would be more likely to encounter waterborne zoospores. A similar effect has been demonstrated in other filter feeding aquatic species, for example a parasitic fungus reached higher prevalence in larger size classes of Daphnia (Hall et al. 2007). Similarly, larger individuals of a filter feeding bivalve, Macomona liliana, accumulated more trematode metacercariae (Leung and Poulin 2008b). An alternative hypothesis is that smaller tadpoles may be more resistant to Bd. Barribeau et al. (2008) found that Xenopus laevis tadpoles with MHC genotypes resistant to the bacterial pathogen Aeromonas hydrophila tended to be smaller, highlighting a potential trade-off between disease resistance and growth.

Higher infection intensities of Bd infection were achieved in larger and more developed hosts. This relationship has not been previously documented in a tadpole-Bd parasitism; however this is observed in other host-parasite systems. For example, spore loads of the microsporidian parasite Nosema whitei are higher in larger larvae of its beetle host (Blaser and Schmid-Hempel 2005). A positive host-size effect on parasite abundance has also been demonstrated for ecto- and endo-parasites infecting individuals of the same fish species (Lo et al. 1998, Cable and van Oosterhout 2007). The positive effect of host size on parasite abundance is presumably caused by larger hosts providing more resources and niche space for parasites (Poulin and Rohde 1997, Poulin and George-Nascimento 2007).

### 4.4.4 Implications of this research and conclusions

The results found in this study have two main implications. Firstly, Bd growth alone did not adequately explain infection patterns; instead, the data suggests that interactions between host and Bd thermal responses may be a better predictor for infection patterns. Several studies that have formulated hypotheses about Bd distribution, spread and climatic affiliations have based their models and hypotheses upon the Bd thermal optimum of 17°C – 25°C (Ron et al. 2003, Pounds et al. 2006) found by the in vitro study of Bd growth by Piotrowski et al. (2004). However, increasing evidence suggests that Bd thermal growth responses alone cannot explain infection patterns (Carey et al. 2006, Rachowicz and Briggs 2007, Andre et al. 2008, Rödder et al. 2008, Vazquez et al. 2009). This suggests that either the thermal patterns in infection are driven by another feature of Bd biology (Woodhams et al. 2008), or that the role of host thermal responses need to be addressed in order to make correct
predictions about infection epidemiology in different host species across different environmental gradients (Andre et al. 2008, Rödder et al. 2008). Tadpoles are thought to play important roles in transmission and act as reservoirs for chytridiomycosis (Daszak et al. 1999, Rachowicz and Vredenburg 2004, Woodhams and Alford 2005, Rachowicz and Briggs 2007). They are relatively easy to obtain and keep in large numbers (Browne et al. 2003), and they display marked and measurable responses to temperature (Harkey and Semlitsch 1988). Therefore further experimental exposures, particularly of tadpole stages with differing thermal tolerances, may be helpful in elucidating the role of host-pathogen interactions in determining the effect of temperature on Bd infection.

The second implication is that it appears that larger tadpoles tend to be more likely to become infected with Bd (also see Smith et al. 2005), and to be infected with higher amounts of Bd. Given that the attainment of large size and mass during the tadpole phase is a life-long fitness correlate (Berven 1981, Smith 1987, Semlitsch et al. 1988), it must be considered whether the introduction of Bd into a system could have population-wide effects by selecting against large tadpoles which may become the fittest adults. While tadpoles do not usually die from Bd infection, individuals infected as tadpoles often die upon, or after, the onset of metamorphosis (Berger et al. 1998, Blaustein et al. 2005, Andre et al. 2008). However, studies have found that larger metamorphs take longer to succumb to Bd infection (Carey et al. 2006), and that larger adults in the wild tend to be infected with less zoospores (Kriger et al. 2006b), or are more likely to survive a Bd epizootic (Bell et al. 2004). This is an interesting observation, given that evolutionary trade-offs often exist between immunocompetence and other fitness traits, including large body size (Zuk and Stoehr 2002, Schmid-Hempel 2003). However, hosts do not always carry the maximum amount of parasite biomass possible (Poulin and George-Nascimento 2007). To be exploited by the same number of parasites per unit of tissue, a large host would have to be infected with a greater number of parasites than a smaller individual. In large hosts, it may take longer for Bd infection intensity to reach a critical threshold of exploitation (i.e. number of cells per unit host tissue) in order to cause mortality, giving the host more time to mount an immune response. Therefore while larger tadpoles may be more predisposed to infection with higher Bd loads, they may ultimately be able to avoid death after metamorphosis. Further work involving tracking the progression of infection in the tadpole populations through metamorphosis would be required to address this issue.

In conclusion, the response of Bd growth to temperature does not explain patterns in Bd infection or abundance in Litoria raniformis tadpoles. It is possible that the observed patterns could be better explained by another aspect of Bd thermal biology or by an
interaction between host and pathogen thermal responses. Increasing evidence in the literature also points to host thermal biology affecting infection. Furthermore, positive associations between host size and weight and Bd infection and intensity suggested a relationship between host phenotype and infection dynamics. This suggests that future predictive studies for Bd epidemiology in the wild need to consider the role of host thermal biology.
CHAPTER 5: General Discussion
CHAPTER 5

General Discussion

Several techniques for research on the amphibian-pathogenic fungus, *Batrachochytrium dendrobatidis* (Bd), were designed in this thesis. The development of these techniques was geared towards resolving two current knowledge gaps in how the infection dynamics of chytridiomycosis may be driven by host, pathogen, and environmental characteristics. The first of these gaps concerns whether, and how, co-infecting Bd strains interact. The second knowledge gap concerns how temperature drives chytridiomycosis. In other host-parasite systems, disease characteristics are shaped by the interplay between host and parasite genotypes and/or phenotypes, and the environmental context (Thomas and Blanford 2003, Wolinska and King 2009). However, little is currently known about how host thermal biology affects chytridiomycosis, or how it interacts with Bd thermal biology. These knowledge gaps led to the three approaches used in this study: (1) to reduce the cost of qPCR detection of Bd in amphibian tissue; (2) labelling groups of Bd for discrimination in mixed-strain experiments; and (3) the experimental exposure of tadpoles to evaluate how host and pathogen thermal responses influence infection characteristics.

5.1 Tools for detecting Bd in amphibians: should we standardize or diversify?

The validation performed in Chapter 2 demonstrated that both a SYBR green-based and the standard Taqman qPCR protocol had similar power to detect Bd in amphibians. This raises the point that despite reduced sensitivity, an alternative detection technique can perform as well as the standard assay in some situations. Establishing a standard assay for the detection of Bd in amphibian samples is important for comparison of results between studies. Taqman qPCR is the best candidate for becoming the standard assay, given its outstanding sensitivity, specificity, reproducibility, and the extent to which it has been validated (Boyle et al. 2004, Kriger et al. 2006b, Hyatt et al. 2007, Walker et al. 2007). However, it is perhaps too rigid to suggest that Taqman qPCR should be used in all studies involving detection of Bd (Kriger et al. 2006a, Kriger
et al. 2006b, Kriger et al. 2007a), particularly when the high costs involved in Taqman qPCR (Speare et al. 2005, Kriger et al. 2006a) may be restrictive to some research groups. Rather, it can be argued that maintaining a range of detection techniques would be of benefit for Bd research, as different detection techniques may be more suitable in different situations (Smith 2007). For example, a less sensitive technique may be more suitable for experimentally exposed amphibians where there is a higher chance of detecting false positive results. Furthermore, the use of qPCR has drawbacks for parasitological research, because it merely detects the presence of parasite DNA and cannot actually confirm infection (Smith 2007, Burreson 2008). Perhaps a more reasonable approach would be to recommend the use of Taqman qPCR where possible and suitable, but to also use it as the standard against which alternative detection protocols can be validated in order to provide Bd researchers with a variety of robust assays.

5.2 How does temperature regulate chytridiomycosis?

The optimal range for Bd growth, 17-25°C (Piotrowski et al. 2004), is often used to explain Bd distribution in the wild (Ron 2005, Pounds et al. 2006, Muths et al. 2008). Perhaps the most important example of this is the way that climate change has been postulated to drive Bd infection. The observation that Atelopus and Alytes montane species decline events are increasing in altitude has led to the formulation of a climate-linked epidemic hypothesis (Pounds et al. 2006, Bosch et al. 2007). This hypothesis states that global warming increases the altitude of cloud layers, which compresses daily temperature fluctuation more tightly around the Bd thermal optimum in clouded areas (Pounds et al. 2006). Thus it is postulated that Bd and climate change are synergistically driving declines of montane amphibians, and that Bd-induced mortality should peak after warm periods (Pounds et al. 2006, Laurance 2008). However, this prediction relies on the assumption that the virulence and infectivity of Bd is highest at optimal temperatures, where growth in culture is highest (Piotrowski et al. 2004). Sub-optimal warm temperatures do appear to limit the virulence and infectivity of Bd (Woodhams et al. 2003, Berger et al. 2004, Kriger and Hero 2006b, Puschendorf et al. 2009), but there is reason to question that these characteristics are limited by cold temperatures. Firstly, several authors have reported higher Bd prevalence in cold seasons and regions (Bradley et al. 2002, Woodhams and Alford 2005, Kriger and Hero 2006a, Kriger et al. 2007b). Secondly, empirical evidence suggests that Bd can be of equal (Carey et al.
2006) or higher (Vazquez et al. 2009) virulence at cool sub-optimal temperatures. Thirdly, Bd can maintain comparable transmission rates (Rachowicz and Briggs 2007), and increase production of the infective zoospore stage at sub-optimal cool temperatures (Woodhams et al. 2008). Fourthly, the results from Chapter 4 suggest that the growth response of Bd to temperature is a poor predictor of infection prevalence and abundance. However, the thermal responses of infection intensity and Bd growth were similar.

If virulence is indicated by infection intensity, the prediction that Bd is more virulent at optimal temperatures may hold true. However it appears that the infectivity and reproductive success of Bd is not limited by reduced growth at cool temperatures, as predicted by Woodhams et al. (2008). Possible reasons for this observation are discussed in Chapter 4. This picture of chytridiomycosis leads to an interesting scenario. If cold periods elevate Bd prevalence but constrain infection intensity, this may allow Bd to gain high prevalence in some amphibian populations. However, if a cold period is followed by a period of warm (Bd-optimal) weather, then would infection intensity increase, causing the large number of hosts that became infected in the cold period to die? This idea would seem congruent with the hypothesis that warm periods act in synergy with Bd to cause mass amphibian mortalities (Pounds et al. 2006, Laurance 2008). However, the problem with this hypothesis is that the responses of the host to temperature are ignored.

The observed thermal pattern in prevalence could be explained by an interaction between host and pathogen thermal optima. There appeared to be a mismatch between the thermal optimal ranges of Bd and the model tadpole host. Infection occurred at temperatures that either 1) limited both Bd and host or 2) were optimal for Bd and host. Infection did not occur at a temperature that was optimal for the host, but limiting to Bd. Thus it is postulated that the response of prevalence to temperature will differ in amphibians that have different thermal tolerances. Furthermore, within the thermal tolerance ranges of both host and pathogen (below 29°C; Johnson et al. 2003, Piotrowski et al. 2004), infection prevalence should be limited at temperatures where suitability for the host supersedes that of Bd. Preferred thermal temperatures of amphibians are usually closer to the upper than lower limit of thermal tolerance (Duellman and Trueb 1986), which possibly explains observations of higher virulence at colder temperatures (Berger et al. 2004, Andre et al. 2008, Vazquez et al. 2009). However, it should be noted that there are two potential problems with this framework. Firstly, thermal fluctuation may produce a different effect from those predicted under constant temperature scenarios (Thomas and Blanford 2003). Secondly, the
A useful framework for host species-specific prediction of the thermal envelope for chytridiomycosis could be to consider the thermal patterns in chytridiomycosis as a product of the interaction between temperature, host and Bd. It is recommended that further experimental infection and transmission studies in other amphibian species, particularly those whose thermal tolerance has been characterised, be carried out in similar multi-faceted contexts. This should enable researchers to generate a better picture of how temperature mediates chytridiomycosis.

5.3 Further work

The latest Amphibian Conservation Action Plan has prioritised an increase in field surveying for Bd as a step toward gaining a better understanding of this important threat to amphibian biodiversity (Gascon et al. 2007). However, 90% of the 20 most diverse countries for amphibians are developing nations (Stuart et al. 2008) where research funding is likely to be limited. Therefore further validation of SYBR green qPCR is recommended to determine whether this method could be used to reduce the cost of detecting Bd in field survey samples. The 20 µL qPCR volume protocol is recommended for use on field samples due to higher sensitivity. The ability of SYBR green qPCR to correctly identify Bd in environmental samples suggests that this assay could perform well in amphibian samples from the field (Kirshtein et al. 2007). However, some concerns need to be addressed before its use is recommended for wild amphibian samples. Firstly, SYBR green qPCR may have reduced specificity (Bustin 2004) and secondly, it may differ in resistance to PCR inhibition.

Identifying the nature of interaction between co-infecting strains of Bd is important for making management decisions for this disease. In other pathogens, competitive interactions that arise between co-infecting strains often cause an increase in pathogenicity, due to selection for increased pathogen virulence (Read and Taylor 2001). At present, the long-distance spread of Bd is rapid and can be attributed to anthropogenic movements of amphibians (Daszak et al. 2003, Morehouse et al. 2003, James et al. 2009), meaning that it is likely that more than one strain could be present in an area. If genetically diverse infections are more harmful than single-strain infections (as found in other host-parasite/pathogen systems), this would be a strong reason to
limit the further spread of Bd. BODIPY® fluorescent fatty acid analogues were demonstrated to be potentially useful tools for discriminating between different Bd isolates in mixed-strain experiments (Chapter 3). This can enable the monitoring of the relative abundance of each strain over several generations, which would allow the nature of inter-strain interactions to be revealed. For example, a reduction in the relative abundance or reproductive output of one, or both, strains decreased in the presence of another would be suggestive of intraspecific competition (Gower and Webster 2005). The abundance of each strain would presumably be quantified as the percentage composition of each strain in a sub-sample, multiplied by the absolute abundance of Bd in each replicate (determined in a host by qPCR or in culture by cell counting with a haemocytometer). However, this would rely upon the assumption that each probe is equally heritable by all daughter cells, which is presently unknown. While the results of Chapter 3 are essentially a proof of concept, several steps need to be taken before BODIPY probes can be deemed reliable for use in experimental infection. The strength of fluorescence emitted from BODIPY dyes is light-sensitive (Haugland 2005). In Chapter 3, the longevity of fluorescence was trialled on cultures kept in the dark. Because keeping experimentally infected frogs in continual darkness may cause adverse effects, it would be helpful to evaluate the effect of prolonged light exposure on the longevity of BODIPY fluorescence. Additionally, it would be helpful to determine the absolute maximum time that fluorescence can persist in Bd. A method for visualising and quantifying stained strains in infected hosts also needs to be devised. Possibilities for this are discussed in Chapter 3. However, if the BODIPY dyes prove unsuitable for use with experimentally infected frogs, it could be interesting to examine competitive interactions between strains in culture as a starting point until another method to discriminate strains infecting frogs can be devised.

Resolving whether sexual processes can occur in Bd is important for our understanding of the epidemiology of chytridiomycosis. In the Chytridiales, sex is always followed by the formation of resting spores (Idnurm et al. 2007). Presence of a resting spore would facilitate long-term survival outside the host, thus freeing Bd from obligate dependence upon the host and allowing Bd to drive host populations to extinction (Mitchell et al. 2008). CelltrackerTM cytosolic probes have previously been used for the detection of cell-cell electrofusion (Jaroszeski et al. 1994). Given that sexual reproduction in the Chytridiales involves plasmogamy, the fusion of cytosol, (Idnurm et al. 2007), this system could be applicable for the detection of sexual behaviour in Bd. In order to provide a rigorous test of reproductive mode, it would be wise to
hold Bd under stressful, as well as optimal conditions. This is because some predominately asexual chytrid fungi, as well as other organisms such as *Daphnia* can switch to sexual reproduction under conditions of environmental stress (Zhang and Baer 2000, Deacon 2006).

Experimental exposure of *L. raniformis* tadpoles has been shown to be a good system to test the interaction between Bd and host thermal responses (Chapter 4). Both tadpole growth and development were strongly influenced by temperature. In addition, a large amount of research already exists on the thermal responses of tadpole growth and developmental rates (e.g. Berven 1982, Cree 1984, Harkey and Semlitsch 1988). Furthermore, these larval phenotypes are well-characterised as fitness correlates (e.g. Berven 1981, Smith 1987, Semlitsch et al. 1988, Rosenberg and Pierce 1995). Therefore the thermal optima for the host could be identified with some confidence. However, the outcome of this work raised several questions. Why were no *L. raniformis* tadpoles infected at 27°C? The correlation between large tadpole size and infection is also of interest. At present it is not known why this association existed; did size affect infection, or did infection affect size? Also, what are the potential fitness consequences of this association?

It is unknown why *L. raniformis* tadpoles exposed to Bd at 27°C were not infected at the end of the experiment. Two possibilities exist: (1) Bd may not have established a patent infection or (2) an infection may have become established, but was cleared by the tadpoles during the course of the experiment. Adult frogs of some species can clear themselves of infection at warm temperatures (37°C or 27°C, Woodhams et al. 2003, Berger et al. 2004). In a follow-up experimental exposure (Appendix 4), at least one tadpole held at 27°C that died during the experiment was positive for Bd, suggesting that *L. raniformis* tadpoles can become infected at this temperature. However, to answer this question, the infection status of tadpoles would need to be assessed at multiple points over a period of time.

A positive relationship between tadpole size and infection presence was observed, however it is not known why this existed. Exposure to Bd could have affected tadpole growth and development, as this has been observed in other tadpole species (Parris and Beaudoin 2004, Parris and Cornelius 2004). Alternatively, larger tadpoles could have been more predisposed to infection, as this has been observed in other host-pathogen systems (Hall et al. 2007, Leung and Poulin 2008b). I attempted to address this question with a further experimental trial, where individual tadpoles were held separately allowing pre- and post-exposure measurements to be taken from each tadpole (Appendix 4). However, this trial was not successful because few tadpoles became infected and their mortality rate was high at the highest temperature. Thus it
appeared that holding *L. raniformis* tadpoles in groups was a better experimental design. A system for tagging *L. raniformis* tadpoles has recently been developed, making the group design possible (White 2009).

While infected tadpoles do not usually die from Bd infection (Berger et al. 1998, Blaustein et al. 2005), mortality of Bd-infected metamorphosing, or post-metamorphic, individuals is high (Andre et al. 2008, Garner et al. 2009). The positive correlation between infection presence and tadpole size suggests that larger tadpoles, which usually metamorphose at larger sizes (Berven 1982), would be disproportionately affected by the introduction of Bd. Given that the achievement of large size at the larval stage is an important determinant of fitness in amphibians (Berven 1981, Smith 1987, Semlitsch et al. 1988, Reading 2007), it is important to determine whether the presence of Bd would select for smaller individuals. It is recommended that further studies track both the body size and infection status of Bd-exposed tadpoles through metamorphosis to determine if large individuals are more likely to become infected and die from chytridiomycosis. In addition, it would be wise to contrast the sizes and weights of surviving metamorphs in groups where Bd is present against groups where Bd is absent.

### 5.4 Final note

Two important themes emerge in the literature on parasitic interactions; that the outcomes of parasitism are both highly plastic and strongly context-dependent (Leung and Poulin 2008a, Wolinska and King 2009). Both temperature and co-infection are examples of ‘environmental context’, which encompasses both abiotic and biotic factors within the immediate (host) and wider environment (e.g. Thompson and Cunningham 2002, de Roode et al. 2005a, Mitchell et al. 2005). Investigating the influence of the environment is important, as it can heavily influence infection characteristics such as parasite virulence, pathogenicity for the host, and host-pathogen coevolution (Brown et al. 2000, Read and Taylor 2001, Thomas and Blanford 2003, Wolinska and King 2009). Chytridiomycosis is highly plastic and a multitude of host, pathogen and environmental characteristics have been demonstrated to affect its dynamics (e.g. Berger et al. 2004, Berger et al. 2005b, Woodhams et al. 2007, Harris et al. 2008). Perhaps a timely challenge for Bd researchers is to test and understand how complex networks formed by the interactions between these characteristics affect this infectious disease.
The increasing rate at which infectious diseases are emerging presents a new challenge for the conservation of the world’s biodiversity (Daszak and Cunningham 1999, Harvell et al. 2002, Daszak et al. 2004b). Disease emergence is often tied to anthropogenic change and is rarely caused by a single, or easily identifiable, driver (Harvell et al. 2002, Daszak et al. 2004b). Several authors advocate the use of interdisciplinary approaches for addressing the ecology of infectious diseases (Lafferty and Gerber 2002, Daszak et al. 2004b, Plowright et al. 2008), and the development and integration of a range of approaches is likely to empower research into this field. It is hoped that the approaches developed in this thesis will contribute to the growing body of scientific tools that can be used to further our understanding of amphibian chytridiomycosis and ameliorate the severity of this significant threat to amphibian biodiversity.
References


References


References


References


References


References


APPENDIX 1: Supplementary protocols
APPENDIX 1

Supplementary protocols

A1.1 Sourcing, culture and manipulation of *Batrachochytrium dendrobatidis*

Cultures of Bd type isolate JEL197 were obtained from the cryo-archive of Margi Butler and Russell Poulter stored in the Biochemistry Department of the University of Otago. Strain JEL197 was initially isolated by Joyce Longcore from a captive *Dendrobates azureus* from the National Zoological Park, Washington D.C. and was originally sourced from Joyce Longcore’s chytrid culture collection at the University of Maine (Longcore et al. 1999). Methods of cryo-archiving and thawing were as described in Boyle et al. (2003). Culture and harvesting methods are based on routine procedures (Longcore et al. 1999), with the following modifications. Cultures were maintained at 23°C (Piotrowski et al. 2004) on 1% tryptone agar plates (T-plates) (Rachowicz and Vredenburg 2004) and cultured every 7-8 days. However, the cultures were transferred to storage at 4°C on the 30th March 2008 and cultured every three to four weeks thereafter (Longcore et al. 1999). Bd was transferred to new plates using a sterile cell scraper (BD, Franklin Lakes, New Jersey) or flame-sterilized wire loop. Zoospores were harvested by flooding plates with 6 mL of 1% tryptone broth (T-broth) and agitating gently for 30 minutes to encourage zoospores to enter suspension (Nichols et al. 2001, Rachowicz and Vredenburg 2004). The broth was then collected and zoospore concentration estimated by staining a sub-sample with Lugol’s solution (5 g iodine and 10 g potassium iodide to 85 mL distilled water) and counting with the leukocyte squares of a haemocytometer under 400× magnification. All manipulation of Bd in culture was done in a class II biosafety cabinet to maintain sterility.
A1.2 Standard Taqman qPCR performed by M. Butler, Biochemistry Department, University of Otago for validation of tadpole mouthparts

**DNA extraction:** Using a clean, sterile toothpick, re-hydrated mouthparts were transferred to a 2 mL ‘Green Beads’ tube (Roche Applied Science, Auckland, New Zealand) containing 1.4 mm ceramic beads and 100 μL PrepMan Ultra. The mouthparts in the tubes were homogenized in a Roche MagNa Lyser Instrument twice at 45,000 rpm for 45 seconds each and centrifuged for 1 minute after each homogenising. The tubes were then placed in a boiling water bath for 10 minutes, cooled for 5 minutes, then centrifuged at 12,500 rpm for 3 minutes. 50 μL of supernatant was removed to a fresh tube. A 4 μL sample of this was removed and diluted 1:10 in PCR-grade water (Roche) and stored at 4°C for < 48 hours prior to PCR. The undiluted supernatant was archived at -20°C.

**Taqman qPCR:** Taqman qPCR analysis of the complementary half-mouthparts was performed on a Roche LC2 Lightcycler. Reactions were of a 20 μL volume containing 4 μL 5× HybrProbe Master Mix (Roche), ITS1-3 Chytr and 5.8S Chytr primers at a final concentration of 1000 nM each (Sigma-Genosys, St. Louis, Missouri, USA), the Chytr MGB 2 probe (Applied Biosystems, Foster City, California, sequence described in Boyle et al. 2004) at 250 nM and 5 μL of the 1:10 diluted DNA. Two types of negative control were included in each reaction. These were 1) an extraction control created by adding a small volume of water in place of a mouthpart to an extraction tube and 2) a no-template control where 5 μL water was substituted for 5 μL template DNA in the PCR reaction. The amplification conditions were 2 min at 50°C, 10 min at 95°C, followed by 15 seconds at 95°C and 1 min at 60°C for 46 cycles. A standard curve for quantification was made in advance using the protocol in Boyle et al. (2004) and calibrated against a DNA standard extracted from $2 \times 10^5$ zoospores provided by A. Hyatt at AAHL. This standard curve can be applied to quantify zoospores in any reaction as long as at least one sample containing a known amount of zoospore equivalents is included in each assay run. For this work, two positive controls containing $10^3$ and $10^2$ zoospore equivalents were included in each reaction. Zoospore equivalents for unknown samples are essentially calculated via the ‘threshold value’ ($C_t$) system introduced previously, but termed the ‘crossing point value’ ($C_p$) by the Roche Light Cycler system.
A1.3 References


APPENDIX 2: Details of cost calculation for alternative qPCR protocols
APPENDIX 2

Details of cost calculation for alternative qPCR protocols

A2.1 Notes on calculation methods

All costs for the different assays are calculated as if the analyses were performed by the author with the facilities available in the Genetics laboratory in the Zoology Department and the Botany Department (for PrepMan Ultra extraction protocol) at the University of Otago. All prices are current for 2008 and are in New Zealand Dollars. The costs of sample preparation were not included, since this would be the same for all assays. Prices for the CTAB-based extraction and SYBR green qPCR are based on the products actually used for these protocols where possible. Prices of generic chemicals such as isopropanol or NaCl are estimated from New Zealand prices found on the Sigma-Aldrich website. Consumables for the PrepMan Ultra DNA extraction and Taqman qPCR reagent prices are sourced from the suppliers (named in the protocols by Boyle et al. (2004)), and plasticware prices are based on what was used for the SYBR green qPCR protocols. Biospec prices were converted using 2008 current exchange rates from the currency conversion website www.xe.com. An arbitrary figure of $20 gross was assigned for labour costs, because this approximates technician hourly rates at the University of Otago and the New Zealand-based scientific company Landcare Research (Manaaki Whenua).

A2.2 List of suppliers

**ABgene** Limited, Epsom, UK, website: www.abgene.com

**Applied Biosystems**, Foster City, California, USA, website: www.appliedbiosystems.com

**Axygen Scientific Inc.**, Union City, California, USA, website: www.axygen.com

**Biospec Products**, Bartlesville, Oklahoma, USA, website: www.biospec.com

**Invitrogen**, Carlsbad, California, USA, website: www.invitrogen.com

### Table A2.1 Total (labour + consumables) qPCR costs in order of expense

<table>
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<tr>
<th>Protocol (sample type)</th>
<th>Consumables (C)</th>
<th>DNA extraction</th>
<th>PCR set up + interpretation</th>
<th>Total time (T)</th>
<th>Labour cost per sample (T / 25)</th>
<th>Total cost per sample (C + T)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PrepMan + 10 µL SYBR (swab)</td>
<td>$4.27</td>
<td>1.5</td>
<td>2.5</td>
<td>4</td>
<td>$3.20</td>
<td>$7.48</td>
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<td>1.5</td>
<td>2.5</td>
<td>4</td>
<td>$3.20</td>
<td>$8.23</td>
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<td>PrepMan + 20 µL SYBR (swab)</td>
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<td>2.5</td>
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<td>$3.20</td>
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<td>2.5</td>
<td>4</td>
<td>$3.20</td>
<td>$10.91</td>
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<td>CTAB + 10 µL SYBR (tadpole)</td>
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<td>2.5</td>
<td>9.5</td>
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<tr>
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<td>1.5</td>
<td>2.5</td>
<td>4</td>
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**Notes:** *‘time’ excludes the time taken to run the qPCR, since active labour is not required for this task*
### Table A2.2 Full calculation of costs for CTAB-based DNA extraction

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<th>Consumable</th>
<th>Components</th>
<th>Manufacturer details</th>
<th>Molar weight (g/mol)</th>
<th>Amount (mol)</th>
<th>Total consumable quantity</th>
<th>Price for total quantity</th>
<th>Quantity per sample</th>
<th>Price per sample</th>
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</thead>
<tbody>
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<td>TE buffer (10 mM Tris &amp; 1 mM EDTA)</td>
<td>Tris EDTA</td>
<td>Sigma, 93363</td>
<td>$160.14</td>
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<td></td>
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<td>$30.60</td>
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</tbody>
</table>

**Notes:** - calculation not necessary; * UOZD: University of Otago, Zoology Department; Genetics lab reagent price, Dr. Tania King, pers. comm.; † Sigma biotech grade, >99.8%; ‡ Sigma biotech grade, > 99%.
Table A2.3 Full calculation of costs for PrepMan Ultra extraction

<table>
<thead>
<tr>
<th>Consumable</th>
<th>Manufacturer details</th>
<th>Code</th>
<th>Price</th>
<th>Amount</th>
<th>Quantity per sample</th>
<th>Price per sample</th>
</tr>
</thead>
<tbody>
<tr>
<td>PrepMan Ultra</td>
<td>Applied Biosystems</td>
<td>4318930</td>
<td>$150</td>
<td>20 mL</td>
<td>50 µL (swabs)</td>
<td>$0.375</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>100 µL (tadpole mouthparts)</td>
<td>$0.75</td>
</tr>
<tr>
<td>Zirconium / silica beads</td>
<td>Biospec products</td>
<td>11079105z</td>
<td>$55.25*</td>
<td>454 g</td>
<td>0.035 g**</td>
<td>$0.004259</td>
</tr>
<tr>
<td>Homogenising tubes</td>
<td>Biospec products</td>
<td>522S</td>
<td>$182.12</td>
<td>500 tubes</td>
<td>0.002 of a box of 500</td>
<td>$0.36424</td>
</tr>
<tr>
<td>1.5 ml tubes</td>
<td>Axygen MCT-150-C</td>
<td></td>
<td>$0.09</td>
<td>1 tube</td>
<td>1 tube</td>
<td>$0.09</td>
</tr>
<tr>
<td>Tip yellow 200 µL</td>
<td>Axygen T-200-Y</td>
<td></td>
<td>$0.015</td>
<td>1 tip</td>
<td>1.033 tips ***</td>
<td>$0.0155</td>
</tr>
</tbody>
</table>

| SUM reagent costs (swabs) | $0.379259 |
| SUM reagent costs (tadpole mouthparts) | $0.754259 |
| SUM plastics              | $0.46974  |
| SUM total consumables (swabs) | $0.848999  |
| SUM total consumables (tadpole mouthparts) | $1.603259 |

Notes: * beads are quoted at $42 USD; ** average of recommended 30-40 mg; *** one tip can be used to load PrepMan Ultra into tubes prior to adding the swab or mouthpart.
### Table A2.4 Full calculation of costs for triplicate 20 μL SYBR green qPCR

<table>
<thead>
<tr>
<th>Consumable</th>
<th>Manufacturer details</th>
<th>Amount (nmol)</th>
<th>Total quantity reagent (μL)</th>
<th>Price for total quantity</th>
<th>Quantity per sample</th>
<th>Price per sample</th>
</tr>
</thead>
<tbody>
<tr>
<td>ITS1-3 Chytr (25 uM/L)</td>
<td>Sigma (100M)</td>
<td>390.1 ug</td>
<td>1760</td>
<td>$30.45</td>
<td>3.6864 μL</td>
<td>$0.063779</td>
</tr>
<tr>
<td>5.8S Chytr (uM/L)</td>
<td>Sigma (100M)</td>
<td>294.1 ug</td>
<td>1744</td>
<td>$23.10</td>
<td>3.6864 μL</td>
<td>$0.048828</td>
</tr>
<tr>
<td>SYBR green master mix (100 x 50 μL reactions)</td>
<td>Invitrogen 11733-038</td>
<td>2500</td>
<td>$342.00</td>
<td>38.4 μL</td>
<td>$5.25312</td>
<td></td>
</tr>
<tr>
<td>PCR plates, 96 well</td>
<td>AB gene AB-0900</td>
<td>25 plates</td>
<td>625</td>
<td>$0.28</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Plate cover strips</td>
<td>AB gene AB-0866</td>
<td>120 strips</td>
<td>250**</td>
<td>$0.34</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tips yellow 200 μL</td>
<td>Axygen T-200-Y</td>
<td>0.015</td>
<td>0.48***</td>
<td>$0.0072</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tips white 10 μL</td>
<td>Axygen T-300</td>
<td>0.025</td>
<td>1</td>
<td>$0.025</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.5 ml tubes</td>
<td>Axygen MCT-150-C</td>
<td>0.09</td>
<td>1 tube</td>
<td>$0.09</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Notes:**
* Assuming that the price to analyse each sample will include the negative, no-template (NTC), positive controls plus DNA standard curve that are run on each 96-well plate (i.e. every 25 samples).
** 625 samples per box of plates, assuming that one plate will hold an average of 25 samples after the no-template, negative and positive controls plus the standard curve have been added to the plate.
***One tip is used to add qPCR master mix for every column of eight wells.
# Table A2.5: Full calculation of costs for triplicate 10 µL SYBR green qPCR

<table>
<thead>
<tr>
<th>Consumable</th>
<th>Manufacturer details</th>
<th>Code</th>
<th>Price</th>
<th>Amount (nmol)</th>
<th>Total quantity reagent (µL)</th>
<th>Price for total quantity</th>
<th>Quantity per sample</th>
<th>Price per sample</th>
</tr>
</thead>
<tbody>
<tr>
<td>ITS1-3 Chytr</td>
<td>Sigma (100M)</td>
<td>ITS1</td>
<td>$30.45</td>
<td>390.1 ug</td>
<td>1760</td>
<td>$30.45</td>
<td>1.8432 µL *</td>
<td>$0.031889</td>
</tr>
<tr>
<td>5.8S Chytr</td>
<td>Sigma (100M)</td>
<td>5.8S</td>
<td>$23.10</td>
<td>294.1 ug</td>
<td>1744</td>
<td>$23.10</td>
<td>1.8432 µL *</td>
<td>$0.024414</td>
</tr>
<tr>
<td>SYBR green master mix (100 x 50 µL reactions)</td>
<td>Invitrogen 11733-038</td>
<td>$342</td>
<td>2500</td>
<td></td>
<td>$342.00</td>
<td>19.2 µL *</td>
<td>$2.62656</td>
<td></td>
</tr>
<tr>
<td>PCR plates</td>
<td>AB gene AB-0900</td>
<td></td>
<td>$175</td>
<td>25 plates</td>
<td></td>
<td></td>
<td>625 samples**</td>
<td>$0.28</td>
</tr>
<tr>
<td>Plate cover strips</td>
<td>AB gene AB-0866</td>
<td></td>
<td>$85</td>
<td>120 strips</td>
<td></td>
<td></td>
<td>250**</td>
<td>$0.34</td>
</tr>
<tr>
<td>Tips yellow 200 µL</td>
<td>Axygen</td>
<td></td>
<td>0.015</td>
<td></td>
<td></td>
<td></td>
<td>0</td>
<td>$0.00</td>
</tr>
<tr>
<td>Tips white 10 µL</td>
<td>Axygen</td>
<td></td>
<td>0.025</td>
<td>1 tip</td>
<td></td>
<td></td>
<td>1.48***</td>
<td>$0.037</td>
</tr>
<tr>
<td>1.5 ml tubes</td>
<td>Axygen</td>
<td></td>
<td>0.09</td>
<td>1 tube</td>
<td></td>
<td></td>
<td>1</td>
<td>$0.09</td>
</tr>
<tr>
<td><strong>SUM reagents per sample</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>$2.682863</td>
</tr>
<tr>
<td><strong>SUM plastics per sample</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>$0.747</td>
</tr>
</tbody>
</table>

**Notes:**
- * Assuming that the price to analyse each sample will include the negative, no-template (NTC), positive controls plus DNA standard curve that are run on each 96-well plate (i.e. every 25 samples).
- ** 625 samples per box of plates, assuming that one plate will hold an average of 25 samples after the no-template, negative and positive controls plus the standard curve have been added to the plate.
- ***One tip is used to add qPCR master mix for every column of eight wells.
Table A2.6 Full calculation of costs for triplicate 25 µL and 20 µL Taqman qPCR

<table>
<thead>
<tr>
<th>Consumable</th>
<th>Manufacturer details</th>
<th>Amount (nmol)</th>
<th>Total quantity reagent (µL)</th>
<th>Price for total quantity</th>
<th>Quantity per sample</th>
<th>Price per sample</th>
</tr>
</thead>
<tbody>
<tr>
<td>Taqman 25 µL</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Taqman Master Mix</td>
<td>Applied Biosystems 4304437</td>
<td>$740</td>
<td>400x 25 µL reactions</td>
<td>$740</td>
<td>48 µL</td>
<td>$7.104</td>
</tr>
<tr>
<td>ITS1-3 Chytr†</td>
<td>Sigma (100M)</td>
<td>$30.45</td>
<td>390.1 ug</td>
<td>$30.45</td>
<td>44 nmol</td>
<td>$0.047834</td>
</tr>
<tr>
<td>5.8S Chytr†</td>
<td>Sigma (100M)</td>
<td>$23.10</td>
<td>294.1 ug</td>
<td>$23.10</td>
<td>43.6 nmol</td>
<td>$0.036621</td>
</tr>
<tr>
<td>MGB probe</td>
<td>Applied biosystems 4316034</td>
<td>$590</td>
<td>6000 pmol</td>
<td>$590.00</td>
<td>6000 pmol</td>
<td>$2.36</td>
</tr>
<tr>
<td>PCR plates</td>
<td>AB gene AB-0900</td>
<td>$175</td>
<td>25 plates</td>
<td></td>
<td>625 samples**</td>
<td>$0.28</td>
</tr>
<tr>
<td>Plate cover strips</td>
<td>AB gene AB-0866</td>
<td>$85</td>
<td>120 strips</td>
<td></td>
<td>250**</td>
<td>$0.34</td>
</tr>
<tr>
<td>Tips yellow 200 µL</td>
<td>Axygen 0.015</td>
<td></td>
<td></td>
<td></td>
<td>0.48***</td>
<td>$0.0072</td>
</tr>
<tr>
<td>Tips white 10 µL</td>
<td>Axygen 0.025</td>
<td></td>
<td></td>
<td></td>
<td>1</td>
<td>$0.025</td>
</tr>
<tr>
<td>1.5 ml tubes</td>
<td>Axygen 0.09</td>
<td></td>
<td></td>
<td></td>
<td>1</td>
<td>$0.09</td>
</tr>
<tr>
<td>SUM reagents per sample</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>$9.548455</td>
</tr>
<tr>
<td>PCR plates</td>
<td>AB gene AB-0900</td>
<td>$175</td>
<td>25 plates</td>
<td></td>
<td>625 samples**</td>
<td>$0.28</td>
</tr>
<tr>
<td>Plate cover strips</td>
<td>AB gene AB-0866</td>
<td>$85</td>
<td>120 strips</td>
<td></td>
<td>250**</td>
<td>$0.34</td>
</tr>
<tr>
<td>SUM plastics per sample</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>$0.7422</td>
</tr>
<tr>
<td>SUM consumables per sample</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>$10.29066</td>
</tr>
<tr>
<td>Taqman 20 µL</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SUM reagents per sample</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>$7.638764</td>
</tr>
<tr>
<td>SUM plastics per sample</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>$0.7422</td>
</tr>
<tr>
<td>SUM consumables per sample</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>$8.380964</td>
</tr>
</tbody>
</table>

Notes: † primer concentration is 900 nM. * Assuming that the price to analyse each sample will include the negative, no-template (NTC), positive controls plus DNA standard curve that are run on each 96-well plate (i.e. every 25 samples). ** 625 samples per box of plates, assuming that one plate will hold an average of 25 samples after the no-template, negative and positive controls plus the standard curve have been added to the plate. ***One tip is used to add qPCR master mix for every column of eight wells. ‡ Calculated by multiplying the sum of the 25 µL qPCR reagent costs by 0.8, as plasticware costs are the same for both protocols.
APPENDIX 3: Supplementary information for Chapter 4
For $t \in [0, 330.5]$,

At $15 \degree C$,

$OD = 3 \times 10^{-6} t^2 - 0.0003 t - 0.0017, R^2 = 0.9867$
d$\frac{dOD}{dt} = 6 \times 10^{-6} t - 0.0003$

At $23 \degree C$,

$OD = -2 \times 10^{-8} t^3 + 10^{-5} t^2 - 0.0004 t - 0.0059, R^2 = 0.9961$
d$\frac{dOD}{dt} = -6 \times 10^{-8} t^2 + 2 \times 10^{-5} t - 0.0004$

At $27 \degree C$,

$OD = 3 \times 10^{-6} t^2 - 0.0002 t - 0.007, R^2 = 0.9775$
d$\frac{dOD}{dt} = 6 \times 10^{-6} t - 0.0002$

**Figure A3.1** (Top) Growth rate ($\frac{dOD}{dTime}$) of Bd cultures at $15 \degree C$, $23 \degree C$ and $27 \degree C$ plotted as a function of time (0 to 330.5 hours). (Bottom) Equations describing observed growth of Bd cultures and growth rate. The equations for growth rate at $15 \degree C$ and $23 \degree C$ intersected at time = 7.38 and 225.96 hours. The equations for growth rate at $23 \degree C$ and $27 \degree C$ intersected at time = 15.29 and 218.05 hours.
Table A3.1 GLMMs considered for infection prevalence. All models included the random effect tank(temperature). Chi squared values, df and $p$ describe the outcome of model comparisons between the model in the same row with the model immediately above it in the table. $I =$ infection (response), $C =$ composite SD, $T =$ temperature. Model in bold is the minimum adequate model.

<table>
<thead>
<tr>
<th>Model</th>
<th>DF</th>
<th>AIC</th>
<th>$\Delta$AIC$^\dagger$</th>
<th>Log likelihood</th>
<th>Chisq</th>
<th>Chi DF</th>
<th>$p$</th>
</tr>
</thead>
<tbody>
<tr>
<td>$I \sim T^2+C$</td>
<td>8</td>
<td>46.820</td>
<td>-15.410</td>
<td>2.1893</td>
<td>2</td>
<td>0.3347</td>
<td></td>
</tr>
<tr>
<td>$I \sim T^*C$</td>
<td>6</td>
<td>45.009</td>
<td>-1.811</td>
<td>-16.505</td>
<td>2</td>
<td>0.3347</td>
<td></td>
</tr>
<tr>
<td>$I \sim T+C$</td>
<td>5</td>
<td>43.628</td>
<td>-3.192</td>
<td>-16.814</td>
<td>0.6191</td>
<td>1</td>
<td>0.4314</td>
</tr>
<tr>
<td>$I \sim C^\dagger$</td>
<td>4</td>
<td>49.962</td>
<td>+3.142</td>
<td>-20.981</td>
<td>8.3339</td>
<td>1</td>
<td>0.0039</td>
</tr>
<tr>
<td>$I \sim T^\dagger$</td>
<td>4</td>
<td>53.425</td>
<td>+6.605</td>
<td>-22.713</td>
<td>11.797</td>
<td>1</td>
<td>0.0006</td>
</tr>
<tr>
<td>$I \sim 1^\dagger$</td>
<td>3</td>
<td>55.369</td>
<td>+8.549</td>
<td>-24.684</td>
<td>15.741</td>
<td>2</td>
<td>0.0004</td>
</tr>
</tbody>
</table>

Notes: $^\dagger$ Compared to minimum adequate model.

Figure A3.2 Snout-vent length in mm of tadpoles by temperature and tank compared with infection status (not infected or infected). Each point represents an individual tadpole. Infected individuals are labelled with the estimated Bd load in their half mouthpart in zoospore genomic equivalents.
Figure A3.3 Weight in mg of tadpoles by temperature and tank compared with infection status (not infected or infected). Each point represents an individual tadpole. Infected individuals are labelled with the estimated Bd load in their half mouthpart in zoospore genomic equivalents.
Figure A3.4 Developmental stage (after Gosner 1960) of tadpoles by temperature and tank compared with infection status (not infected or infected). Each point represents an individual tadpole. Infected individuals are labelled with the estimated Bd load in their half mouthpart in zoospore genomic equivalents. The Bd load values 849, 1376 and 40 all refer to infected individuals in tank B that were stage 26. Most tadpoles held at 15°C were at stages 25 and 26 at the end of the experiment, therefore several of these points are masked by others.
Table A3.2 Summary of stepwise simplification of models for tadpole SVL, weight and Stage after being held at either 15°C, 23°C or 27°C for 41 days and exposed to Bd. The most parsimonious model for each measure is shown in bold text. T = temperature, I = infection status (infected or not infected with Bd).

<table>
<thead>
<tr>
<th>Measure</th>
<th>Model</th>
<th>AIC</th>
<th>ΔAIC</th>
</tr>
</thead>
<tbody>
<tr>
<td>SVL</td>
<td>T * I</td>
<td>-131.301</td>
<td></td>
</tr>
<tr>
<td></td>
<td>T + I</td>
<td>-133.202</td>
<td>-1.901</td>
</tr>
<tr>
<td></td>
<td>T</td>
<td>-126.694</td>
<td>+6.508*</td>
</tr>
<tr>
<td></td>
<td>I</td>
<td>-113.134</td>
<td>+20.068*</td>
</tr>
<tr>
<td>Weight</td>
<td>T * I</td>
<td>-42.369</td>
<td></td>
</tr>
<tr>
<td></td>
<td>T + I</td>
<td>-44.369</td>
<td>-2</td>
</tr>
<tr>
<td></td>
<td>T</td>
<td>-36.813</td>
<td>+7.556*</td>
</tr>
<tr>
<td></td>
<td>I</td>
<td>-24.495</td>
<td>+19.874*</td>
</tr>
<tr>
<td>Stage</td>
<td>T * I</td>
<td>268.172</td>
<td></td>
</tr>
<tr>
<td></td>
<td>T + I</td>
<td>267.088</td>
<td>-1.084</td>
</tr>
<tr>
<td></td>
<td>T</td>
<td>266.251</td>
<td>-0.837</td>
</tr>
<tr>
<td></td>
<td>I</td>
<td>272.653</td>
<td>+5.565**</td>
</tr>
</tbody>
</table>

† The change in AIC is compared with the model immediately above in the table. However, if the change in AIC value is flagged with an asterisk it is compared with the model in bold. The model flagged with two asterisks for the stage model is compared against the model T + I.
APPENDIX 4: Further research on the thermal ecology of Bd in tadpoles: does size affect infection, or does infection affect size?
APPENDIX 4

Further research on the thermal ecology of Bd in tadpoles: does size affect infection, or does infection affect size?

A4.1 Introduction

The research in Chapter 4 of this thesis found that temperature had a strong impact upon Bd growth, tadpole growth and development, and on Bd infection of *Litoria raniformis* tadpoles. In addition, this trial found that infected tadpoles were larger than uninfected tadpoles and that there was a positive relationship between tadpole size and the amount of Bd carried. However, it is not known why the relationship between tadpole size and infection occurred. Exposure to Bd could have affected tadpole growth and development, as this has been observed in other tadpole species (Parris and Beaudoin 2004, Parris and Cornelius 2004). Alternatively, larger tadpoles could have been more predisposed to infection, as this has been observed in other host-pathogen systems (Hall et al. 2007, Leung and Poulin 2008b). The aims of this study were to 1) determine whether the initial size of tadpoles was correlated with infection presence and intensity, to 2) determine if exposure to Bd affected *L. raniformis* tadpole size, development or survival. To achieve this, the experimental exposure of tadpoles at 15°C, 23°C and 27°C was repeated. Non-exposed control groups were included to examine the effects of Bd exposure at each temperature on tadpole growth. Tadpoles were housed individually in order to be able to examine the relationship between both the initial and final size and development of each tadpole with their infection status and intensity.

A4.2 Methods

A4.2.1 Experimental exposure

Two hundred and eleven *Litoria raniformis* tadpoles were collected in February-March 2008 at stages 25-36 from the Sweet Koura Inc. farm, Alexandra and used immediately for experimental exposure. Tadpoles were individually housed in 200 mL filtered tap water in cylindrical aquaria (94 mm diameter × 47 mm height). The containers had loosely fitting lids to
allow them to be stacked within the incubators. Tadpoles were anaesthetised with 0.02% MS-222 and measured (SVL, weight and stage as for 4.2.3). Tadpoles were randomly assigned to six treatment groups in a fully factorial design; exposed or not exposed to Bd at 15°C, 23°C and 27°C. This resulted in 35 tadpoles per treatment and 70 tadpoles per temperature. Dataloggers (iButton®, Maxim Integrated Products Inc., Sunnyvale, California) were placed on a shelf and also in mock aquaria in each incubator to monitor air and water temperature fluctuation. Air temperatures in the incubators were: 15°C: mean = 14.08°C, range = 13.5 – 17.0°C; 23°C: mean = 23.38°C, range = 21.5 – 24.5°C, and 27°C: mean = 26.31°C, range = 24.0 – 29.0°C. Temperatures in the aquaria were: 15°C: 14.32°C, range = 14.0 – 15.0°C, 23°C: mean = 23.58°C, range = 20.0 – 24.5°C; 27°C: mean = 26.54°C, range = 19.0 – 28.5°C.

Tadpoles were acclimatized for seven days prior to the first infection. Exposed group tadpoles received a total of $2 \times 10^6$ zoospores in T-broth over three repeated exposures on days 8, 12 and 15 of the experiment (average $3.3 \times 10^3$ zoospores/mL/infection). Control tadpoles received equal amounts of sterile T-broth instead of Bd-containing broth. Aquaria water was not changed until 24 hours post-infection.

Tadpoles were fed 1 mL of a blended mixture of spirulina, fish flakes, boiled lettuce and filtered water ad libitum (Helen White, pers. comm.). However, the blended mixture caused rapid fouling of the aquaria, so on day 19 the food was changed to 2 × 2 cm squares of boiled lettuce. Aquaria water was changed every day. Waste water was decontaminated prior to disposal by boiling (Johnson et al. 2003) or with TriGene (Webb et al. 2007). Any tadpoles that died during the experiment were placed in 70% ethanol. Stringent hygiene protocols were in place to ensure that non-exposed treatment tadpoles were never exposed to Bd. Separate equipment was used for control and exposed groups and decontaminated with TriGene after each use.

This experiment was prematurely terminated on days 30-31 due to high mortality of tadpoles in the 27°C incubator. Surviving tadpoles were euthanized with 0.1% MS-222, their SVL, weight and development was measured, and the whole tadpole was placed in 70% ethanol. Tadpoles (control and exposed) that died after day 15 (after three Bd exposures) or survived to the end of the experiment were tested for Bd with qPCR as for the group trial. The whole mouthpart was used for diagnosis. An additional qPCR run was performed on all tadpoles that were identified as Bd-positive to re-quantify the Bd loads in order to cut out any between-PCR run bias.

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6 Except the 23°C exposed group, as an extra tadpole was accidentally included, thus $n = 36$ for this group and $n = 71$ for 23°C.
A4.2.2 Statistics

Very few tadpoles became infected in this trial; therefore correlations between tadpole initial or final size and infection were unable to be examined. However, differences in infection prevalence between temperatures were examined with a Chi-squared test in SPSS (version 16 for Windows, SPSS Inc., Chicago, Illinois, USA). Several expected frequencies were < 5, but the average expected frequency was always > 6, signifying the chi-squared statistic would be robust for $\alpha = 0.05$ (Zar 1999).

The effect of temperature and exposure on tadpole survival was examined with a Kaplan-Meier survival analysis (Therneau and Lumley 2008), using the survival package in R 2.8.0 (R Development Core Team 2008). The maximal model included temperature and exposure to Bd and an interaction term, and best fitted a Weibull distribution. Maximal models were simplified using the automated step function in R, which uses a penalised log-likelihood value, the Akaike Information Criterion (AIC) to evaluate models. The most with the lowest AIC was selected as the minimal adequate model. Post-hoc comparisons between marginal means for temperature were performed in SPSS. The Surv function was used to perform Chi square based log-rank tests to test for the effects of fixed factors on tadpole survivorship. Kaplan-Meier curves were plotted with the survfit function.

The effect of Bd exposure and temperature on post-treatment (final) tadpole weight and SVL was analysed using ANCOVA. Pre-treatment (initial) weight or SVL was used as a covariate to control for initial differences in size. Factors with a chance of influencing the initial or final measurements were also included in the maximal models. These were: date of initial and final measure and the initial and final measurer. Maximal models included each of these terms plus a three-way interaction between the initial measure, temperature and exposure, to check if the slopes of the response to initial measure differed between experimental treatments. This maximal model was simplified using step.

The effect of temperature and exposure on developmental stage was analysed with a Poisson GLM (log link). The minimal adequate model for stage was underdispersed, therefore was re-fitted using quasi-likelihood. Helmert contrasts were used for post-hoc comparisons between temperatures.
A4.3 Results

A4.3.1 Bd infection

Infection prevalence was very low in all groups exposed to Bd. Of the tadpoles that survived to the end of the experiment, two of the 15°C group were infected (6.25% prevalence, $n = 32$), one of the tadpoles at 23°C was infected (3.57% prevalence, $n = 28$) and none were infected at 27°C group ($n = 6$). These differences in prevalence were not significant ($\chi^2 = 0.196$, df = 2, $p = 0.906$). However, four tadpoles from the 27°C exposed group that died after day 15 (after three exposures to Bd, $n = 25$) were Bd-positive and one was equivocal for Bd. Three of these tadpoles died on day 16 (the day after the third exposure) and one on day 26. None of the tadpoles that died after day 15 of the experiment in the 23°C exposed group ($n = 8$) were positive for Bd. No tadpoles in the 15°C group died after day 15. The two infected tadpoles at 15°C carried 186 and 54 ZE. The infected tadpole in the 23°C group carried 27 ZE. The number of zoospore equivalents estimated from tadpoles in the 27°C exposed group ranged between 9 and 83, with a mean intensity of 46.0 ZE. None of the tadpoles tested from the non-exposed groups were positive for Bd (15°C $n = 29$, 23°C $n = 23$, 27°C $n = 27$).

A4.3.1 Effects of Bd exposure and temperature on tadpole survival, growth and development

Survival. Of the 211 tadpoles initially included in this experiment, only 134 survived until the termination. Percent survival to the end of the experiment was 94.3% for both non-exposed and exposed tadpoles held at 15°C, 68.6% for non-exposed and 77.8% for exposed tadpoles held at 23°C, 28.6% for non-exposed and 17.1% for exposed tadpoles held at 27°C. Differences in survivorship were best explained by temperature only (Table A4.1). Temperature strongly affected survivorship (log-likelihood (model) = -349.1, $\chi^2 = 87.61$, df = 2, $p << 0.01$). The mean time to death predicted by the survival model was lowest at 27°C (27.3 days), followed by 23°C (51.8 days) then 15°C (107.0 days) (Fig. A4.1).
A4 Further research on the thermal ecology of Bd in tadpoles

**Table A4.1** AIC values for Kaplan-Meier survival models. Factors considered include temperature (15°C, 23°C, 27°C) and exposure (exposed to Bd, not exposed). The minimal adequate model is shown in bold font.

<table>
<thead>
<tr>
<th>Model</th>
<th>AIC</th>
<th>Δ AIC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Surv (death, status) ~ temperature * exposure</td>
<td>709.18</td>
<td>0</td>
</tr>
<tr>
<td>Surv (death, status) ~ temperature + exposure</td>
<td>708.25</td>
<td>-0.93</td>
</tr>
<tr>
<td><strong>Surv (death, status) ~ temperature</strong></td>
<td>706.25</td>
<td>-2.93</td>
</tr>
<tr>
<td>Surv (death, status) ~ exposure</td>
<td>791.77</td>
<td>+82.59</td>
</tr>
<tr>
<td>Surv (death, status)</td>
<td>789.82</td>
<td>+80.64</td>
</tr>
</tbody>
</table>

**Figure A4.1** Kaplan-Meier survival curves for tadpoles held at 15°C, 23°C, and 27°C. Bd-exposed and unexposed tadpoles were not significantly different, therefore have been pooled for each temperature. A cross at the end of the line shows that the survival model predicted that the mean time to survival in a group was longer than the experimental time period.
Measurements from three tadpoles were excluded from the data set due to measurer error; therefore measurements from 131 tadpoles in total were used for the following analyses. One more outlier was removed from the 15°C exposed group (Stage \( \text{initial} = 31; \text{SVL Initial} = 18.61 \text{ mm}; \text{Weight Initial} = 999 \text{ mg}; \text{Stage Final} = 37, \text{SVL Final} = 19.03 \text{ mm}, \text{Weight Final} = 1101 \text{ mg} \)). There were no pre-existing differences between the six treatment groups for the measurements SVL (ANOVA: \( F_{5,205} = 1.497, p = 0.196, n = 211 \)), weight (ANOVA: \( F_{5,205} = 1.433, p = 0.217, n = 211 \)), developmental stage (ANOVA: \( F_{5,205} = 1.318, p = 0.261, n = 211 \)).

**SVL.** The model with the lowest AIC for tadpole final SVLs included the following factors: initial date, initial SVL, temperature, Bd exposure treatment and the interactions between initial SVL and temperature and initial SVL and exposure (Table A4.2). However, only initial date and initial SVL significantly affected final SVL (Table A4.3). Taking these factors into account, Bd exposure did not have a significant effect on SVL, but the effect of temperature approached significance (Table A4.3).

**Weight.** For final weights, the model with the lowest AIC value included the following factors: initial date, initial measurer, initial weight, temperature, exposure and the interaction between temperature and exposure (Table A4.2). Initial date, initial measurer and initial weight significantly affected final weight (Table A4.3). Exposure to Bd did not have a significant effect on weight, nor was there a significant interaction between temperature and exposure (Table A4.3). Temperature had a strongly significant effect on final weight (Table 4.5). Final weights were significantly lower at 27°C than at 15°C (mean difference = 147.23 ± 29.92 mg, \( p < 0.001 \), 95\% CI = 74.49 – 219.96) and at 23°C (mean difference = 129.25 ± 30.63 mg, \( p < 0.001 \), 95\% CI = 54.80 – 203.71).

**Developmental stage.** The model with the lowest AIC for developmental stage only included the factors initial date and temperature (Table A4.2). Taking initial stage into account, final stage was significantly higher at 23°C and 27°C than at 15°C (Table A4.3). There was not a significant difference in final stage between 23°C and 27°C (estimate = 0.010 ± 0.006, \( t = 1.540, p = 0.126 \)).
**Table A4.2** Models considered by the step function in R for tadpole final (post-treatment) snout-vent lengths (SVL), weights, and developmental stages. Resulting AIC values after deletion of each term are shown. The maximal model included the terms $Di =$ initial date of measurement, $Mi =$ initial measurer, $Df =$ date final, $Mf =$ final measurer, $Wi$, $SVLi$ or $DSi =$ initial weight, $T =$ temperature (15°C, 23°C or 27°C), $E =$ exposure treatment to Bd (exposed or not exposed). The minimal adequate model is shown in bold text. The model column shows which term was deleted in each step.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Model</th>
<th>AIC</th>
<th>ΔAIC†</th>
</tr>
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<tr>
<td>SVL</td>
<td>Maximal ‡</td>
<td>42.61</td>
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</tr>
<tr>
<td></td>
<td>- Df</td>
<td>42.61</td>
<td>0.00</td>
</tr>
<tr>
<td></td>
<td>- SVLi×T×E</td>
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<td>-2.59</td>
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<tr>
<td></td>
<td>- Mi</td>
<td>37.81</td>
<td>-4.80</td>
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<tr>
<td></td>
<td>- T×E</td>
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</tr>
<tr>
<td></td>
<td>- Mf</td>
<td>33.79</td>
<td>-8.82</td>
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<tr>
<td></td>
<td>- SVL×E</td>
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<td>+0.02*</td>
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<tr>
<td></td>
<td>- SVLi×T</td>
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<tr>
<td></td>
<td>- Di</td>
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<tr>
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</tr>
<tr>
<td></td>
<td>- Df</td>
<td>1216.91</td>
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</tr>
<tr>
<td></td>
<td>- Mf</td>
<td>1215.22</td>
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</tr>
<tr>
<td></td>
<td>- Wi×T×E</td>
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<td></td>
<td>- Wi×E</td>
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<td></td>
<td>- T×E</td>
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<tr>
<td></td>
<td>- Di</td>
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<td>+9.79*</td>
</tr>
<tr>
<td></td>
<td>- Wi</td>
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<td>+84.79*</td>
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<td>- DSi×T×E</td>
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<tr>
<td></td>
<td>- T</td>
<td>729.27</td>
<td>+7.31*</td>
</tr>
<tr>
<td></td>
<td>- DSi</td>
<td>733.02</td>
<td>+11.06*</td>
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</tbody>
</table>

† AIC is compared with that of the model immediately above, except for figures marked with an asterisk, as these are compared with the minimal adequate model.

Maximal models were all of the formula: final measure = $\mu + Di + Mi + Df + Mf + \text{initial measure} \times T + \text{initial measure} \times E + T \times E + \text{initial measure} \times T \times E$
<table>
<thead>
<tr>
<th>Measure</th>
<th>Factor</th>
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<th>$F$</th>
<th>$p$</th>
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<td>SVL</td>
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<td>4.54</td>
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</tr>
<tr>
<td></td>
<td>Initial SVL</td>
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<tr>
<td></td>
<td>Temperature</td>
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<td>2.81</td>
<td>0.065</td>
</tr>
<tr>
<td></td>
<td>Bd exposure</td>
<td>1</td>
<td>0.07</td>
<td>0.797</td>
</tr>
<tr>
<td></td>
<td>Initial SVL × Temperature</td>
<td>2</td>
<td>1.75</td>
<td>0.178</td>
</tr>
<tr>
<td></td>
<td>Initial SVL × Bd exposure</td>
<td>1</td>
<td>1.76</td>
<td>0.187</td>
</tr>
<tr>
<td>Weight</td>
<td>Initial date</td>
<td>9</td>
<td>4.60</td>
<td>$&lt;0.001^{***}$</td>
</tr>
<tr>
<td></td>
<td>Initial measurer</td>
<td>3</td>
<td>2.91</td>
<td>0.038*</td>
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<tr>
<td></td>
<td>Initial weight</td>
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<td>130.00</td>
<td>$&lt;0.001^{***}$</td>
</tr>
<tr>
<td></td>
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<td>2</td>
<td>11.39</td>
<td>$&lt;0.001^{***}$</td>
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<td></td>
<td>Bd exposure</td>
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<td>1.14</td>
<td>0.289</td>
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<td>Temperature × Bd exposure</td>
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<td>2.53</td>
<td>0.084</td>
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</table>

<table>
<thead>
<tr>
<th>Stage</th>
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<th>$t$ value</th>
<th>$p$</th>
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<tbody>
<tr>
<td>(Intercept)</td>
<td>2.930 ± 0.058</td>
<td>50.37</td>
<td>$&lt;0.001^{***}$</td>
</tr>
<tr>
<td>Initial stage</td>
<td>0.018 ± 0.002</td>
<td>9.07</td>
<td>$&lt;0.001^{***}$</td>
</tr>
<tr>
<td>Temperature [23°C]</td>
<td>0.107 ± 0.013</td>
<td>8.22</td>
<td>$&lt;0.001^{***}$</td>
</tr>
<tr>
<td>Temperature [27°C]</td>
<td>0.083 ± 0.020</td>
<td>4.17</td>
<td>$&lt;0.001^{***}$</td>
</tr>
</tbody>
</table>

Notes:
SVL whole model: $F_{16,113} = 14.34$, $p < 0.001$, adjusted R squared = 0.623
Weight whole model: $F_{18,111} = 11.62$, $p < 0.001$, adjusted R squared = 0.597
Stage: residual deviance = 20.093 on 126 degrees of freedom, dispersion = 0.159. Temperature [15°C] is taken as the intercept.

Out of the tadpoles that survived the experiment, those held at 27°C had slightly lower initial SVLs and weights (ANOVA, SVL: 15°C and 23°C, estimate= 0.340 ± 0.496, $t = 0.686$, $p = 0.494$; 15°C and 27°C, estimate = -1.46 ± 0.734, $t = -1.989$, $p = 0.049$; weight: 15°C and 23°C, estimate = 23.28 ± 37.30, $t = 0.624$, $p = 0.534$; 15°C and 27°C, estimate = -111.00 ± 55.24, $t = 2.009$, $p = 0.047$). However, overall temperature did not have a significant effect on the weights or SVLs of surviving tadpoles (ANOVA, weight: $F_{2,127} = 2.870$, $p = 0.063$; SVL: $F_{2,127} = 2.834$, $p = 0.060$). Temperature had no effect on the initial developmental stages of tadpoles surviving to the end of the experiment, with the null model (i.e. no factors) being the most parsimonious (GLM, AIC null model = 724.03, AIC all other models ≥ 725.34).
A4.4 Discussion

Very few tadpoles became infected in this experiment, therefore it was not possible to compare infection with tadpole characteristics. Exposure to Bd did not have a significant effect on final *L. raniformis* tadpole SVL, weight, developmental stage or survival. However, the responses of tadpole size, survival and development to temperature observed in this study were very different from those observed in a previous trial (Chapter 4 of this thesis). It is therefore necessary to evaluate which experimental exposure provided the most realistic results.

In the experimental exposure trial detailed here (‘individual trial’), tadpoles were held in individual 200 mL aquaria. Infection prevalence was very low (≤ 6.25%) at all temperatures. Tadpole weight was lowest at 27°C and developmental stage was higher at 23°C and 27°C than at 15°C. Temperature did not have a significant effect on SVL. Survival was strongly affected by survival, with survivorship decreasing with increasing temperature.

In the experimental exposure trial performed in Chapter 4 (‘group trial’), tadpoles were held in groups of ten in 3 L aquaria. Experimental infection of tadpole groups showed a clear negative linear trend in infection prevalence. Tadpole growth and development were significantly higher at 23°C and 27°C than at 15°C. Tadpole survival was consistent between temperatures in the group trial.

The most likely explanation for differences observed in the effect of temperature on tadpole phenotype and survival, was that tadpoles had insufficient oxygen in the individual aquaria. Tadpoles in the group aquaria had 50% more water per tadpole than those held in individual aquaria (group aquaria: 3 L / 10 = 300 mL; individual aquaria: 200 mL). Despite changing the water every day, it appears that at this lower water volume, this regime may not have been enough to keep tadpoles at the highest temperatures adequately oxygenated. Amphibian metabolism increases exponentially with increasing temperature (Duellman and Trueb 1986, Browne et al. 2003), therefore it is expected that oxygen consumption would have also increased with temperature. Survival decreased with increasing temperature, suggesting that tadpoles may have been under increasing oxygen stress as the temperature increased. Furthermore, the tadpoles that survived the experiment at 27°C had slightly lower initial weights and SVLs than tadpoles held at 23°C or 15°C. Under conditions of oxygen stress, it would be expected that larger animals with a higher oxygen demand (Duellman and Trueb 1986) would be most susceptible to oxygen stress.
Therefore it is likely that the patterns seen in tadpole final weight, SVL and development in the individual trial were confounded by increasing oxygen stress with temperature. Generally, within the tolerance range, tadpole growth and developmental rates are expected to increase with temperature (Berven 1982, Harkey and Semlitsch 1988). In this individual trial, developmental stage followed this pattern, but weight and SVL did not. Therefore, the responses of tadpole phenotype in this individual trial were probably not realistic. This causes uncertainty about the result that *L. raniformis* tadpole size, development or SVL is not affected by Bd exposure, as the responses measured in this trial may not have been accurate. Another concern is that because so few tadpoles became infected, the dose of Bd may have also been too low to induce an exposure response.

Very few tadpoles became infected in this experimental exposure trial. This may have been due to the altered dose regime (Carey et al. 2006), as tadpoles received a total of 5 million zoospores each in the previously described group trial (Chapter 4 of this thesis) and 2 million zoospores each in the individual trial. Although both of these zoospore amounts were well within the range needed for this strain to infect adult frogs (Shaw et al. in review), tadpoles may be harder to infect (Russell Poulter, pers. comm.). Alternatively, transmission between tadpoles may be altered by temperature (Woodhams et al. 2008, although see Rachowicz and Briggs 2007) or be the mechanism through which high prevalence is reached.

Therefore for future trials examining the relationships between temperature, tadpole size and Bd exposure, keeping *Litoria raniformis* tadpoles in groups is recommended. Holding tadpoles individually was a preferable statistical design, as each tadpole was an independent replicate. However, the pseudoreplication caused by grouping tadpoles into aquaria can be handled easily by using mixed-effects modelling, as demonstrated in Chapter 4 of this thesis. Furthermore, grouping tadpoles into aquaria did not contribute to any of the random variation observed, suggesting that the tadpole and temperature responses are relatively consistent between groups. Holding tadpoles individually may cause unrealistic results, for example alterations in movement and feeding rate can result in lower weights (Browne et al. 2003). It is also possible that patterns in Bd infection could be different if it cannot transmit between individuals (Rachowicz and Vredenburg 2004, Rachowicz and Briggs 2007). However, *Bufo bufo* tadpoles have previously been successfully held individually in small (75 cm$^2$) containers for experimental infection with Bd (Garner et al. 2009), therefore success of individual housing probably differs between tadpole species.
The advantage of holding each tadpole in a separate aquarium was that individual tadpoles were easily identifiable, allowing multiple measures to be taken for each individual. However, a method for long-term marking has recently been developed on *L. raniformis* tadpoles, allowing for accurate identification of individuals within groups (White 2009). Therefore, it would be entirely possible to examine the effects of the pre-exposure and post-exposure phenotypes of individual tadpoles on Bd infection, while holding tadpoles in groups.

A4.5 References


