Novel tools to reduce the virulence of *Batrachochytrium dendrobatidis*

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In memory of Rick Speare

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Statement of the Contribution of Others

This thesis was sparked by Tiffany Kosch, Lee Berger and Lee Skerratt who first suggested that RNAi might be useful against amphibian chytridiomycosis. Alex Roberts then wrote and received a Morris Animal Foundation pilot grant to develop RNAi methods, and the rest is history. The majority of the project conception, experimental design, laboratory work and writing presented in this thesis was conducted by myself (RW), with guidance and editorial support from my supervisors (AR, LB, LS & CR). The only exception is Chapter 5, in which some of the preliminary laboratory work, such as fungal isolation and nucleic acid extraction, was conducted by collaborators including Tiffany Kosch (TK), Marcia Merces (MM) and Anthony Waddle (AW), with additional field help from Deborah Bower (DB) and Benjamin Cuff (BC). Illumina sequencing and analysis for this chapter was performed by Steve Wylie (SW).

The laboratory work has primarily occurred at James Cook University (JCU). Some of the pilot experiments in Chapter 4 were conducted at the University of Massachusetts- Amherst, with assistance from Lillian Fritz-Laylin and Andrew Swafford.

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Chapter 2- Viability assay

RW conceived the project, conducted the laboratory work and wrote the manuscript. AR, LB, LS and CR provided advice and editorial support. This project was funded by **RW**'s James Cook University SSA funds.

Chapter 3- Role of glutathione

RW conceived the project, in consultation with AR. **RW** wrote the grant, designed the experiments, conducted the laboratory work, analysed the results and wrote the manuscript. AR provided support for data analysis. AR, LB and CR provided advice and editorial support. LS provided statistical

support. This project was funded by the Australasian Mycological Society (Research Award) "Glutathione biosynthesis in the amphibian fungus, *Batrachochytrium dendrobatidis*" with **RW** as PI.

Chapter 4- RNAi

AR and **RW** conceived the project and designed the experiments. **RW** conducted the laboratory work, analysed the data and wrote the manuscript. CR provided software and data analysis support. AR, LB and CR provided advice and editorial support. This project was funded by the Morris Animal Foundation "Saving endangered frogs: Using RNA Interference to reduce the virulence of the frog-killing fungus" with AR as PI, as well as The Ecological Society of Australia (Holsworth Wildlife Research Endowment) "Using RNA interference as a novel treatment for amphibian chytridiomycosis" with **RW** as PI. Travel costs for training and conference attendance were funded by the European Molecular Biology Laboratory (short-term travel grant), University of Tennessee (travel grant) and James Cook University (HDRES), all with **RW** as PI.

Chapter 5: Mycovirus

LB and **RW** conceived the project and designed the experiments. **RW** wrote the grants, applied for the wildlife collection permits, conducted most of the laboratory work and wrote the manuscript. **RW,** TK and MM conducted the preliminary laboratory work. SW conducted the illumina sequencing and analysis. **RW** collected the majority of new fungal isolates, with field assistance from BC and DB. SW, LB and AW contributed the remaining fungi isolates. AR, LB and CR provided advice and editorial support. LS provided statistical support. This project was funded by the Australian Academy of Science (The Margaret Middleton Fund for endangered Australian native vertebrate animals) "A novel conservation tool for controlling chytridiomycosis in Australian amphibians", and James Cook University (HDRES), both with **RW** as PI.

Abstract

The amphibian chytrid fungus, *Batrachochytrium dendrobatidis*, causes chytridiomycosis, a disease responsible for the decline and extinction of hundreds of amphibian species worldwide. This skin infection compromises epidermal function, resulting in electrolyte imbalance and death via cardiac arrest. Since its discovery in the late 1990's, strategies such as vaccination, antifungal therapeutics and skin biome augmentation have been trialled. However, none provide broadly applicable or sustained mitigation, and amphibians continue to decline, with many species reliant on captive husbandry to prevent extinction. The purpose of this thesis is to explore the feasibility of horticultural antifungal techniques as a novel tool against chytridiomycosis. Gene silencing using RNA interference (RNAi), and viral biocontrol using mycoviruses are two techniques that can reduce pathogen virulence and increase host survival, and my thesis investigates application of these methods to chytridiomycosis. To enable this work, I also assessed a putative virulence gene target, and developed an assay to measure the effect of these treatments.

Chapter 2: Quantifying the reduction in *B. dendrobatidis* growth and viability is necessary to assess novel antifungal treatments. Existing methods to measure *B. dendrobatidis* growth include optical density, which lacks sensitivity, or live cell dye exclusion, which may not be suitable for zoosporangia with porous discharge tubes. In Chapter 2, I developed a rapid viability and growth assay using methylene blue, a cheap and commonly used dye. Methylene blue does not rely on membrane exclusion, but instead on the reduction of the blue dye into a colourless substance and was reliable in determining the viability of both adherent and suspended zoosporangia. Since the amount of dye retained is proportional to the volume of dead cells, I also developed a new assay using eluted methylene blue to estimate cell growth. By measuring the absorbance of the eluted methylene blue, I could detect subtle differences in cell growth that were not apparent using the traditional optical density method. This assay provided the basis for validating molecular studies in the subsequent chapters.

Chapter 3: The first step in the development of RNAi techniques is the selection of a target gene. I chose to target glutathione production. Glutathione is an antioxidant that can protect fungal pathogens from host immune response. As such, the glutathione biosynthesis gene (glutamate cysteine ligase, *GCL*) is a known virulence factor in many pathogenic fungi, and a suspected virulence factor in *B. dendrobatidis*. To explore the role of glutathione synthesis in *B. dendrobatidis* I focussed on its importance during tolerance of hydrogen peroxide (H_2O_2), as a proxy for host immune

generated oxidative stress. I tested the glutathione response to H_2O_2 exposure, as well as other stressors such as cadmium and heat. Although glutathione levels change in response to H_2O_2, chemical depletion of glutathione did not impact tolerance to H_2O_2 suggesting that glutathione may not be essential for evading the host immune response. In contrast, glutathione is important for cadmium tolerance, as cadmium exposure strongly induced glutathione production, and inhibition of glutathione synthesis increased the sensitivity to cadmium stress. Therefore, since glutathione depletion produces a measurable phenotypic response (cadmium sensitivity), *GCL* is an attractive target to determine the feasibility of RNAi in *B. dendrobatidis*.

Chapter 4: RNAi is a tool used to silence gene expression using double stranded RNA. In horticulture, interfering RNA designed to target essential fungal genes can be sprayed directly onto host plants, protecting them from fungal disease. RNAi based therapeutics could be a novel tool in the treatment of chytridiomycosis, but very little is known about the RNAi system in *B. dendrobatidis*. In Chapter 4, I conducted a series of experiments to determine if RNAi can be used to reduce gene expression in *B. dendrobatidis.* I designed two types of short interfering RNA (siRNA) to target the glutathione biosynthesis gene, *GCL*. I found that both types reduced gene expression, with ~50% less mRNA in siRNA treated cells, peaking at 36-42 h after treatment. However, mRNA knockdown did not correlate with an observable phenotypic effect, suggesting that either siRNA design, or siRNA delivery methods require further optimisation. The discovery that the RNAi pathway appears operational in *B. dendrobatidis* is a significant advancement in the use of RNAi for both functional genetics studies and as a potential treatment against chytridiomycosis.

Chapter 5: Mycovirus biocontrol is another antifungal approach that has been successful in horticulture. Mycoviruses infect fungi, and in some cases can reduce virulence and improve host survival. Mycoviruses are an attractive control option due to their host specificity and potential for transmission through wild populations with minimal intervention. However, no mycoviruses have been detected in *B. dendrobatidis*. In Chapter 5, I screened *B. dendrobatidis* isolates for evidence of mycoviral infection. I used a combination of Illumina sequencing and cellulose chromatography with gel electrophoresis to screen 38 isolates, most of which were collected from Australia, but also included some Korean and Brazilian isolates. I did not find mycoviruses in any of the isolates. This may be a founder effect, due to limited *B. dendrobatidis* introductions in Australia, or perhaps the species is generally free of mycoviruses. This research prompts further questions on how *B. dendrobatidis* can evade viral infection.

Overall, my thesis provides a novel platform for antifungal therapies to manage chytridiomycosis. I found that manipulation of gene expression via RNAi appears possible in *B.*

dendrobatidis, although further optimisation is required. Although I did not detect mycovirus infection in Australian *B. dendrobatidis*, potential remains for mycovirus mediated biological control and I suggest further screening of isolates in other countries. In addition, I developed an easy growth and viability assay and characterised the role of glutathione in stress response, which contributes to further understanding of this interesting pathogen.

Publications and presentations in support of this thesis

Publications

Webb, R. J., Berger, L., Skerratt, L. F., & Roberts, A. A. (2019). A rapid and inexpensive viability assay for zoospores and zoosporangia of Batrachochytrium dendrobatidis. *Journal of Microbiological Methods, 165*, 105688. doi:https://doi.org/10.1016/j.mimet.2019.105688

Webb, R. J., Roberts, A. A., Wylie, S., Kosch, T., Toledo, L. F., Merces, M., Skerratt, L.F., Berger, L. (2022). Non-detection of mycoviruses in amphibian chytrid fungus (Batrachochytrium dendrobatidis) from Australia. *Fungal Biology, 126*(1), 75-81. doi:https://doi.org/10.1016/j.funbio.2021.10.004

Conference presentations

RJ Webb, L Berger L, L.F. Skerratt, A.A Roberts **"**RNA interference: a novel treatment against fungal pathogens of amphibians?" Poster presented at EMBL "From Functional Genomics to Systems Biology" Heidelberg. 10th-13th November, 2018 (Chapter 4)

RJ Webb, L Berger L, L.F. Skerratt, A.A Roberts "Wanted dead or alive: A simple viability assay for the amphibian chytrid fungus". Talk presented at "Amphibian Pathogens Annual Meeting. Arizona State University, USA. 15th-16th November, 2019 (Chapter 2)

RJ Webb, L Berger L, L.F. Skerratt, A.A Roberts "A simple viability assay for all life stages of the amphibian chytrid fungus, *Batrachochytrium dendrobatidis*". Poster presented at the World Congress of Herpetology. University of Otago, New Zealand. 5-10th January, 2020 (Chapter 2)

RJ Webb, C Rush, L Berger L, L.F. Skerratt, A.A Roberts. "Glutathione in the amphibian chytrid fungus". Talk presented at Australasian Mycological Society annual meeting, Online. December 1st, 2021 (Chapter 3)

RJ Webb, L Berger L, C Rush, L.F. Skerratt, A.A Roberts "The development of siRNA mediated mRNA knockdown in *Batrachochytrium dendrobatidis*". Poster presented at The 31st Fungal Genetics Conference. Pacific Grove, California. March 15th-20th, 2022 (Chapter 4)

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Chapter 1 **General Introduction**

"It's not easy being green" Kermit the frog famously said (Kermit 1970), and he was right. Amphibians all over the world are suffering due to the fungal disease, chytridiomycosis. This disease has caused declines and even extinctions in hundreds of amphibian species, with these impacts continuing in the three decades since its discovery. My thesis aims to help mitigate the effects of chytridiomycosis with two approaches: understanding virulence and reducing virulence. In this review, I will outline our current understanding of virulence factors in the chytrid fungus and discuss why methods to quantify virulence are required. Next, I will explore two approaches used to successfully control fungal disease in plants and explore their potential application in mitigating chytridiomycosis.

Mitigating Chytridiomycosis: What can we learn from horticulture?

Abstract

Fungal diseases of wildlife pose a threat to biodiversity. None more so than the amphibian chytrid fungus, *Batrachochytrium dendrobatidis*, the cause of chytridiomycosis. Mitigation of wildlife fungal diseases often relies on the use of traditional antifungal chemicals, which can be labour intensive and only offer a short-term, small-scale solution. Furthermore, application of disinfectants and antifungal drugs into wildlife habitats raises environmental and health concerns. Fungal diseases of agricultural plants have received more attention because of their economic impact. Advances in the treatment of plant diseases have revealed novel and targeted treatment options extending beyond antifungals, which could be implemented in the field of wildlife conservation. This review assesses the potential for virus biocontrol and RNA interference as novel interventions in the fight against chytridiomycosis and appraises various methods from discovery through to implementation.

Introduction

Pathogenic fungi have long been a major threat to crop production. Historically, plant fungal diseases have caused famine and economic ruin (Fisher et al. 2012), and the threat to crop plants and global food security still persists today (Dean et al. 2012). Currently, wildlife are experiencing a rise in emerging fungal diseases that are predicted to cause significant biodiversity loss and are already the leading cause of pathogen-driven extinctions (Fisher et al. 2012). Most notable is the amphibian chytrid fungus, the cause of chytridiomycosis, which is one of the worst wildlife diseases due to its devastating impact on biodiversity (Skerratt et al. 2007).

Due to the long history and commercial implications of fungal plant diseases, there has been investment in developing novel control approaches for plant pathogens. Fungicides are still heavily relied on as the major source of pathogen control in crops (Gianessi and Reigner 2006). However, horticultural fungicides have environmental and human health implications (Margni et al. 2002, Wightwick et al. 2010) and there is increasing concern about the development of fungicide-resistant strains (Mercier et al. 2010). To overcome the disadvantages of fungicides, horticultural science has begun to develop alternative (non-chemical) therapeutics in the fight against fungal diseases.

In this review, I focus on chytridiomycosis and the current mitigation strategies for this disease. I then assess the potential application of novel horticultural techniques and provide recommendations on their implementation for the control of chytridiomycosis.

Fungal diseases of wildlife and their impacts

Fungal infectious diseases are a global problem and affect a broad range of wildlife species from diverse environments. Pathogenic fungi often thrive in lower temperatures and tend to invade the epidermis of ectothermic animals (Berger et al. 1998, Martel et al. 2013, Lorch et al. 2016) but can also infect the epidermis of mammals that hibernate (such as bats) (Blehert et al. 2009) or have naturally lower body temperatures (such as platypus) (Connolly 2009). The physiological processes of ectotherms are governed by environmental temperature, so it is not surprising that outbreaks of fungal disease are often seasonal (Ruggeri et al. 2015, McCoy et al. 2017). Pathogenic fungi of wildlife include filamentous and non-filamentous species, in contrast to horticultural pathogens, which are predominantly filamentous (Dean et al. 2012). The impacts of wildlife fungal diseases range from isolated mortality events (Adrian et al. 1978), to devastating large-scale species declines (Skerratt et al. 2007, Blehert et al. 2009). Outbreaks can occur due to the spread of disease into naïve populations (Wibbelt et al. 2010), environmental changes (Shinn et al. 2000), or human activities (Friend 1999).

Amphibian Chytrid Fungus

The worst wildlife disease is chytridiomycosis (Skerratt et al. 2007), caused by the amphibian chytrid fungus *Batrachochytrium dendrobatidis* (Longcore et al. 1999). This fungus belongs to the Chytridiomycota, an ancient and early diverging phylum (Heckman et al. 2001) (James et al. 2006). The pathogen likely originated in East Asia, as this region harbours the highest genetic diversity of *B. dendrobatidis* (Bataille et al. 2013, O'Hanlon et al. 2018, Byrne et al. 2019), and endemic amphibians from this region appear resistant to chytridiomycosis, with no reports of mass die-off events (Rahman 2020). However, within the last few decades, *B. dendrobatidis* has spread around the world to susceptible populations (Skerratt et al. 2007, O'Hanlon et al. 2018) causing the extinction of at least 90 amphibian species, and remains an ongoing threat to hundreds more species (Carvalho et al. 2017, Scheele et al. 2019). The host range of *B. dendrobatidis* is incredibly broad; it can infect over 700 amphibian species (Lips 2016) and at least 500 species have experienced significant population declines due to chytridiomycosis (Scheele et al. 2019). The fungus has two life stages, the flagellated motile zoospore, and the zoosporangium (Figure 1.1). The zoospore infects the host epidermis (Berger et al. 1998) via a germ tube, before developing into a zoosporangium in the stratum granulosum and the keratin rich stratum corneum (Berger et al. 2005a, Greenspan et al. 2012, Van Rooij et al. 2012). The zoosporangia reproduce asexually, releasing zoospores via a discharge tube, which can reinfect the same host, or infect a new host through direct contact or indirectly via a moist substrate. Only the mouthparts of larval amphibians (tadpoles) are keratinised (Marantelli et al.

2004), therefore, infection is limited to the mouthparts and is usually not fatal. As the larvae begin to metamorphose however, keratinised skin develops on the growing limbs and the infection spreads (McMahon and Rohr 2015). In juveniles and adults, severe chytridiomycosis presents as lethargy, excessive skin sloughing, redness, and loss of righting reflex. The damage caused by the pathogen compromises the physiological function of the epidermis, and the resulting electrolyte loss leads to eventual death due to cardiac arrest (Voyles et al. 2009).

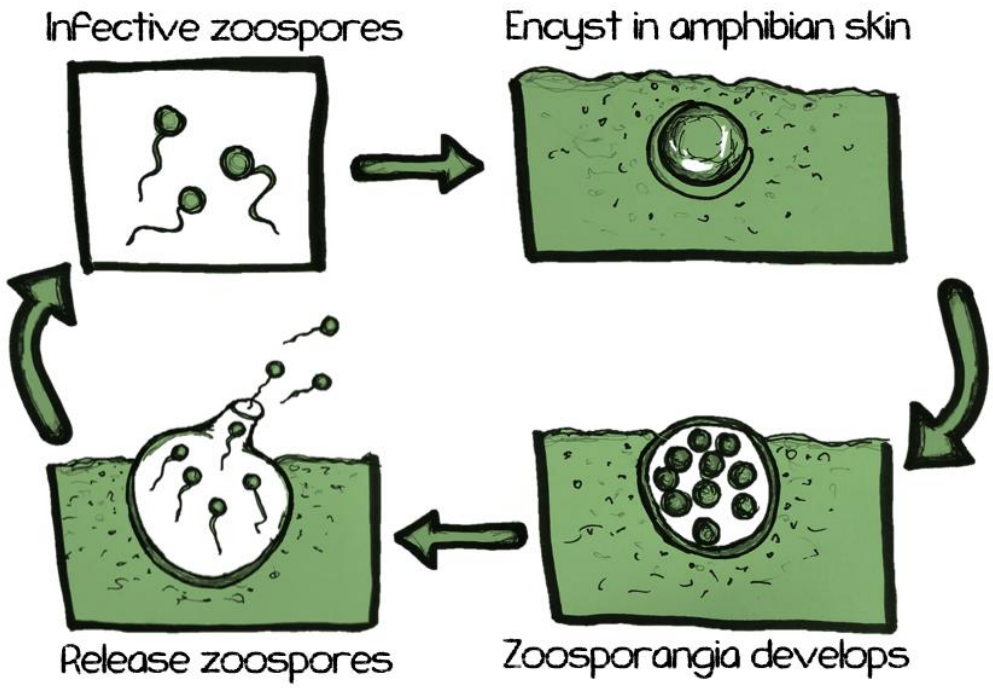


Figure 1.1 *Batrachochytrium dendrobatidis* life cycle. The flagellated motile zoospore infects the host epidermis, then develops into a zoosporangium, which asexually produces more zoospores. Diagram by R. Webb

Host factors impacting susceptibility to chytridiomycosis

With such a broad host range, it is not surprising that hosts differ in their susceptibility to *B. dendrobatidis* (Figure 1.2). Iconic species such as the Australian southern corroboree frog (*Pseudophryne corroboree*) (Hunter et al. 2010, Brannelly et al. 2015) and the Panamanian golden frog (*Atelopus zeteki)* (Bustamante et al. 2010) are incredibly susceptible to chytridiomycosis. These species die rapidly in the laboratory, and their wild populations have declined to near extinction. Conversely, the common froglet (*Crinia signifera*) co-exists with the corroboree frog but appears tolerant of chytridiomycosis, as evident by the low mortality in the field and laboratory (Brannelly et al. 2018b). Some highly susceptible species manage to persist in the wild despite heavy mortality

due to life history strategies, such as the alpine tree frog (*Litoria verreauxii alpina*) (Scheele et al. 2014, Scheele et al. 2015), which relies on high annual turnover for survival (Scheele et al. 2016). Even within species, susceptibility to chytridiomycosis can vary amongst individuals (Savage and Zamudio 2011). Understanding what drives these differences is an important field of research and will greatly assist amphibian conservation. Genetic variation (in immune genes such as MHC) (Savage and Zamudio 2011, Bataille et al. 2015), host body size (Burrow et al. 2017), host epidermis characteristics (Van Rooij et al. 2012), life history (eg: tadpole vs direct development) (Mesquita et al. 2017) and age (Brannelly et al. 2018a) have been shown to contribute to disease susceptibility, but in many cases the reason for variation in susceptibility is unknown.

Pseudophryne corroboree	***Atelopus zeteki***
	
Crinia signifera	***Litoria verreauxii alpina***
	

Figure 1.2 Frog species variably impacted by chytridiomycosis. Both *Pseudophryne corroboree* and *Atelopus zeteki* are examples of highly susceptible species that have experienced severe population declines. *Litoria verreauxii alpina* is also highly susceptible but populations have managed to persist in the wild due to high reproduction rates. *Crinia signifera* can tolerate infection without mortality. Photos by R. Webb

Environmental factors limiting *B. dendrobatidis*

With susceptible hosts on every continent (except Antarctica), the main factor limiting *B. dendrobatidis* distribution is temperature. In general, the thermal optimum of *B. dendrobatidis* is 17 –25°C (Piotrowski et al. 2004), but temperature tolerance differs between isolates (Stevenson et al.

2013, Sheets et al. 2021). Continual exposure to 28°C (Stevenson et al. 2013) or acute exposure to >37°C is lethal (Johnson et al. 2003). In addition, *B. dendrobatidis* is also very susceptible to desiccation, with diurnal temperature and annual precipitation the strongest predictors of disease presence (Murray et al. 2010). Thus, landcover and vegetation density are extremely important proxies for *B. dendrobatidis* persistence in the environment (Becker and Zamudio 2011).

Salinity is another environmental factor that can influence *B. dendrobatidis* distribution. The fungus only occurs in fresh water environments, as relatively low concentrations of sodium chloride inhibit growth *in vitro* (Stockwell et al. 2012). Amphibians living in salty habitats have reduced infection loads and increased host survival (Heard et al. 2014, Stockwell et al. 2015).

The perplexing observations that vulnerable green and golden bell frogs (*Litoria aurea*) thrive in suboptimal habitats, such as disused quarries (Mahony 1996), and that amphibian species richness is higher in polluted urban areas (Lane and Burgin 2008), prompted the suspicion that *B. dendrobatidis* might be sensitive to heavy metals (Threlfall et al. 2008). Copper inhibits *B. dendrobatidis* growth *in vitro* (Threlfall et al. 2008, Boisvert and Davidson 2011), and can lessen disease in infected hosts (Parris and Baud 2004). Zinc was also found to inhibit growth (Threlfall et al. 2008), but the effect of other metals is unknown.

Salamander chytridiomycosis

Within the same genus is *Batrachochytrium salamandrivorans*, which infects urodeles (salamanders and newts). Described in 2013, *B. salamandrivorans* has caused dramatic declines in fire salamander (*Salamandra salamandra*) populations in Europe (Martel et al. 2013). The clinical presentation of *B. salamandrivorans* chytridiomycosis is slightly different to that of *B. dendrobatidis*, with multifocal deep erosive skin lesions (Martel et al. 2013). In addition, *B. salamandrivorans* prefers a lower temperature (15°C) than *B. dendrobatidis*, and produces a resting spore stage when grown in culture (Stegen et al. 2017). Despite these differences, it is likely that developing methods to mitigate *B. dendrobatidis* would also be applicable to *B. salamandrivorans*.

Understanding virulence

Virulence factors impact chytridiomycosis severity

The severity of chytridiomycosis not only depends on host characteristics and environmental conditions, but is also influenced by pathogen virulence. Virulence is defined as the ability of a microorganism to cause disease in the host; a quantitative measure of the "disease producing power" (Casadevall and Pirofski 1999, Shapiro-Ilan et al. 2005). Virulence factors are the distinct properties or characteristics that allow the organism to damage host fitness. For example, the adhesion or invasion of host tissue, evading host defenses, and surviving and reproducing in the hostile host environment. Traditionally, virulence factors are defined as those that specifically relate to host infection and not those necessary for basic survival (Casadevall and Pirofski 1999). However, for the purpose of increasing host survival, antifungal therapeutics could either target true virulence factors, or an essential gene, with a similar outcome.

It was recognised early on that virulence differs between various *B. dendrobatidis* isolates (Berger et al. 2005b). Of the five known lineages, BdASIA-1, BdASIA-2/BdBRAZIL, BdASIA-3, BdGPL, BdCAPE (O'Hanlon et al. 2018, Byrne et al. 2019), the BdGPL "global panzootic lineage" has been shown to be hypervirulent (Farrer et al. 2011, Greenspan et al. 2018, Belasen et al. 2022). This increased virulence likely explains how this lineage recently emerged from the endemic lineages, to cause an epizootic disease with global amphibian mortality (O'Hanlon et al. 2018). Furthermore, even within the BdGPL lineage, virulence can vary widely (Greener et al. 2020). For example, BdGPL isolates collected from amphibian mortality events demonstrated higher virulence in the lab than those collected from stable populations (Piovia-Scott et al. 2015).

Detecting differences in virulence

Detecting differences in virulence requires methods to quantify virulence. Traditionally, this is achieved by infection experiments, in which amphibians are infected with a standardised number of zoospores, and host survival is quantified to determine virulence (Berger et al. 2005b, Greenspan et al. 2018). As well as mortality rate and time to death, other disease characteristics that relate to virulence include host body condition, onset of clinical signs, and disease burden (zoospore load) determined by qPCR (Greenspan et al. 2018, Fu and Waldman 2019). *In vitro* immune cell assays using human lymphocytes or amphibian splenocytes have also been used to measure virulence, avoiding lengthy *in vivo* experiments (Fites et al. 2013, Piovia-Scott et al. 2015, Rollins-Smith et al. 2022). Finally, fecundity is associated with virulence (Fisher et al. 2009, Langhammer et al. 2013, Greener et al. 2020), which means characteristics such as zoosporangia size, zoospore density, growth and viability can be compared without the need for a host or immune cells (Piovia-Scott et al. 2015). These *in vitro* growth assays are an attractive option due to their simplicity. However, there

are still challenges with developing these *in vitro* assays. For example, growth assays can be time consuming (Lindauer et al. 2019) and current methods to determine cell viability are only valid for the zoospore life stage, but not for zoosporangia (McMahon and Rohr 2014). Therefore, there is a need to develop new *in vitro* methods that could be used as a proxy for *B. dendrobatidis* virulence.

Searching for virulence factors

Identifying virulence factors will help uncover the mechanism behind the observed differences in virulence. Efforts to elucidate virulence factors in *B. dendrobatidis* have mostly focused on analysis of the secretome, as well as comparative transcriptomics and genomics approaches.

Secretions from *B. dendrobatidis* can inhibit lymphocytes (Fites et al. 2013) and disrupt skin cells (Brutyn et al. 2012), so the analysis of its secretome compared to non-pathogenic relatives is one method used to identify virulence factors. Another approach is to use comparative transcriptomics to identify differential gene expression. In initial transcriptomics studies, *in vitro* gene expression was compared between the free-living zoospore stage and the parasitic zoosporangial stage to identify genes associated with host tissue invasion (Rosenblum et al. 2008). Cultures of *B. dendrobatidis* are usually maintained *in vitro* in a simple peptide media, so subsequent studies investigated differential expression when exposed to host tissue (Rosenblum et al. 2012) or during active host infection (Ellison et al. 2017, Farrer et al. 2017, Verbrugghe et al. 2019). Comparative genomic approaches have also been used to identify expanded gene families in *B. dendrobatidis* compared to non-pathogenic fungal relatives (Joneson et al. 2011), or to compare between hypervirulent and hypovirulent isolates (Piovia-Scott et al. 2015). Genes undergoing directional selection (Farrer et al. 2013) or that have been acquired by horizontal gene transfer (Sun et al. 2011, Sun et al. 2016) may also be important for virulence.

Candidate virulence factors

Analysis of the secretome identified factors that can inhibit the host's immune system. The metabolites methylthioadenosine (MTA), tryptophan, and kynurenine inhibit lymphocyte survival and proliferation, and these are not produced by a non-pathogenic relative (Rollins-Smith et al. 2015). The polyamine spermidine is also secreted by *B. dendrobatidis* and recently it was discovered that it too inhibits lymphocyte proliferation (Rollins-Smith et al. 2019). In addition to these metabolites, the *B.dendrobatidis* secretome includes several types of proteases (Brutyn et al. 2012), which are common virulence factors in other fungi (Monod et al. 2002, Chandrasekaran et al. 2016). There is a strong consensus among the transcriptomic and genomic studies that proteases, specifically serine type proteases and metalloproteases, are candidate virulence factors in *B.*

dendrobatidis. These have been shown to be upregulated in the presence of the host (or host tissue) (Rosenblum et al. 2012), differentially expressed across life stages (Rosenblum et al. 2008), and show expansion compared to non-pathogenic relatives (Joneson et al. 2011). These proteases may play a role in germ tube invasion and subsequent degradation of host skin (Rosenblum et al. 2008, Rosenblum et al. 2012, Farrer et al. 2017). Since epidermal dysfunction leads to electrolyte loss and then cardiac arrest (Voyles et al. 2007, Voyles et al. 2009), these proteases are likely to be very important for the disease outcome.

Comparison of gene expression between cells grown in normal media vs media containing host tissue also detected upregulation of "crinkler and necrosis" (CRN) genes (Rosenblum et al. 2012, Farrer et al. 2017), and expression of these genes was found to be higher in more virulent isolates (Greener et al. 2020). CRN genes are common in pathogenic oomycetes (Stam et al. 2013) and are named for the necrotic crinkling effect they have on infected leaves (Torto et al. 2003). In *B. dendrobatidis*, CRN expression appears to be correlated with the ability to invade cells both *in vitro* and *in vivo* (Greener et al. 2020).

The serine type proteases and CRN genes also appear to have been acquired by horizontal gene transfer (HGT), indicating they may provide an adaptive advantage to the pathogen (Sun et al. 2011, Sun et al. 2016). Other genes acquired by HGT have been identified as putative virulence factors, including two genes related to glutathione (Sun et al. 2011). Glutathione is an antioxidant, and an important virulence factor in many other pathogenic fungi as it offers protection from oxidative stress generated by host immune cells (Baek et al. 2004, Yadav et al. 2011). The role of glutathione in the virulence of *B. dendrobatidis* is unknown. However, since the synthesis of glutathione is governed by a single pathway that can be chemically inhibited, its importance could be determined experimentally.

Identifying and confirming the function of virulence factors is an important component of disease mitigation. A thorough understanding of virulence factors might help to explain the variations in host susceptibility to *B. dendrobatidis*. Virulence factors can be also targets for antifungal drugs (Nosanchuk et al. 2001) or vaccines (De Bernardis et al. 2012), and the encoding genes could be targeted by new techniques, such as gene silencing or mutagenesis.

Current mitigation strategies and limitations for treating chytridiomycosis

Antifungals

Recommendations for the intervention of wildlife fungal diseases have mostly focussed on biosecurity measures to contain any further spread (Fisher et al. 2012, Bower et al. 2019). However, methods to control disease *in situ* are urgently required for populations that are already infected. Traditional antifungal drugs transferred from human and veterinary medicine (such as terbinafine, voriconazole and itraconazole) have been the primary treatment option for treating chytridiomycosis (Forzán et al. 2008, Bowerman et al. 2010, Brannelly et al. 2012). Administration of antifungal drugs usually involves repeated chemical bath immersion over several days (Brannelly et al. 2012), and therefore is chiefly used for individuals in captivity rather than in the wild. However, there are some promising examples of treating wild amphibians with antifungal drugs. *In situ* treatment of tadpoles has been attempted via addition of aquarium antifungals to pond water (Geiger et al. 2017), or via capture and bathing of individuals with itraconazole (Bosch et al. 2015, Hudson et al. 2016). This approach only offers a short-term solution and prevalence eventually returns to original levels as animals become re-infected. Thus, while antifungals have had some limited success *in situ*, they are only effective if the animals can be isolated from re-infection (Bosch et al. 2015), and high clearance efficiencies are required for success (Canessa et al. 2018). There is also increasing concern about the development of resistance to antifungal drugs (Sanglard 2003), a process already occurring in fungal pathogens of humans (Fisher et al. 2020), mammals (Connolly et al. 1998) and birds (Beernaert et al. 2009).

Vaccination

Vaccination of amphibians against *B. dendrobatidis* has been trialled with mixed results. Several early studies found a single prior exposure to be ineffective at reducing disease in frogs (Stice and Briggs 2010, Cashins et al. 2013), or salamanders (Kocher et al. 2019), suggestive of a lack of adaptive immunity against *B. dendrobatidis* infection. It was later discovered that *B. dendrobatidis* inhibits the host immune system via the production of immunosuppressant molecules (Fites et al. 2013), which may explain why vaccination was not successful in single-exposure experiments. However, repeated exposures to *B. dendrobatidis* can result in decreased pathogen burden and increased lymphocytes in Cuban tree frogs (McMahon et al. 2014), suggesting that repeated exposures may allow frogs to overcome pathogen immune suppression and gain some protection against disease. Recent research has found that inoculation with a less virulent *B. dendrobatidis* isolate provided protection against subsequent infection with a more virulent isolate (Waddle et al. 2021). A hypovirulent isolate could serve as a “transmissible vaccine”, reducing some of the logistical challenges of vaccinating wild animals (Waddle et al. 2021). Creating hypovirulent isolates by

inhibition of key virulence genes (such as those that suppress the immune system), or via endogenous mycoviruses could increase the efficiency of vaccination.

Alternative therapies against chytridiomycosis

Other therapies, such as inoculation with probiotic bacteria, have been explored as a prophylactic protection from fungal infection. Augmentation of individual frogs with probiotic bacteria can reduce disease in the short term in the laboratory (Harris et al. 2009) and field (Vredenburg et al. 2011). However, this strategy is unlikely to have broad scale success because probiotics lack comprehensive inhibition against all strains of *B. dendrobatidis* (Antwis and Harrison 2018), and probiotics sourced from one individual or species do not necessarily grow well on other amphibians (Becker et al. 2011), or the effects are too short lived (Knapp et al. 2022). Genetic engineering of naturally occurring skin biota to boost antifungal production has been trialled, but unfortunately did not increase host survival (Becker et al. 2021). Therefore, this probiotic approach has promise, but is not a definitive prophylaxis for preventing death from chytridiomycosis.

Heat therapy can clear infection in captive frogs. Adult frogs can be cleared after only 16 h at 37°C (Woodhams et al. 2003), and heat therapy can also work for tadpoles (Heuring et al. 2020). However, results of heat therapy are inconsistent (Woodhams et al. 2012), and this method is not appropriate for amphibians adapted to cold climates. In the wild, naturally occurring hot microclimates are correlated with reduced infection (Forrest and Schlaepfer 2011). This observation has prompted studies on environmental modifications to provide heat refuges, eg: removing tree branches or providing heat absorbing bricks (Roznik et al. 2015, Garner et al. 2016, Greenspan et al. 2017). The success of this approach will depend on the physical characteristics of the environment as well as the thermoregulatory behaviour of the target species.

In summary, despite numerous approaches towards mitigating chytridiomycosis, protecting wild amphibians from chytridiomycosis still remains a major challenge.

Novel antifungal strategies from horticulture.

Due to the social and economic impacts of fungal disease in crops, horticultural science is at the forefront of implementing novel strategies to reduce the virulence of fungal pathogens. Two strategies that may be transferable to wildlife are discussed below. Firstly, **RNA interference (RNAi)**, which is a method to reduce gene expression and could be used to target pathogen virulence genes. And secondly, **mycovirus-mediated hypovirulence**, which is a form of biocontrol where viral infection reduces pathogen virulence.

RNA interference

RNA interference (RNAi) is a natural defence mechanism and regulator of gene expression in eukaryotes, and has been harnessed by molecular biologists to investigate gene function (Hannon 2002). The term RNAi was first coined after the injection of double stranded RNA (dsRNA) into the nematode *Caenorhabditis elegans* silenced a homologous target gene via the reduction of messenger RNA (mRNA) (Fire et al. 1998), although the mechanism was unknown at the time. It is now understood that RNAi-mediated post transcriptional gene silencing (PTGS) typically involves the enzymes Dicer (Bernstein et al. 2001) and the multi-protein "RNA-induced silencing complex" (RISC) (Hammond et al. 2001). Dicer is triggered by the presence of exogenous dsRNA (Figure 1.3), which it then cleaves into small interfering RNA (siRNA) (Zamore et al. 2000, Bernstein et al. 2001). These siRNAs are relatively stable, double stranded and approximately 20-24 base pairs long (Hamilton and Baulcombe 1999, Ding and Lu 2011), and their unique size avoids a nonspecific interferon response, while still allowing recognition by RISC (Watts and Corey 2012). Adenosine triphosphate (ATP) facilitates the siRNA unwinding (Nykänen et al. 2001), and the antisense strand (Martinez et al. 2002) is loaded onto an Argonaute protein (Hammond et al. 2001), forming RISC. The siRNA then guides RISC to its complementary mRNA, which is cleaved by Argonaute (Liu et al. 2004), preventing translation (Zamore et al. 2000). The degraded mRNA is amplified by an RNA dependent RNA polymerase (RdRP) which is subsequently processed by Dicer into additional secondary siRNA (Nishikura 2001). This amplification of siRNA enables potent and sustained RNAi-induced gene silencing (Nishikura 2001).

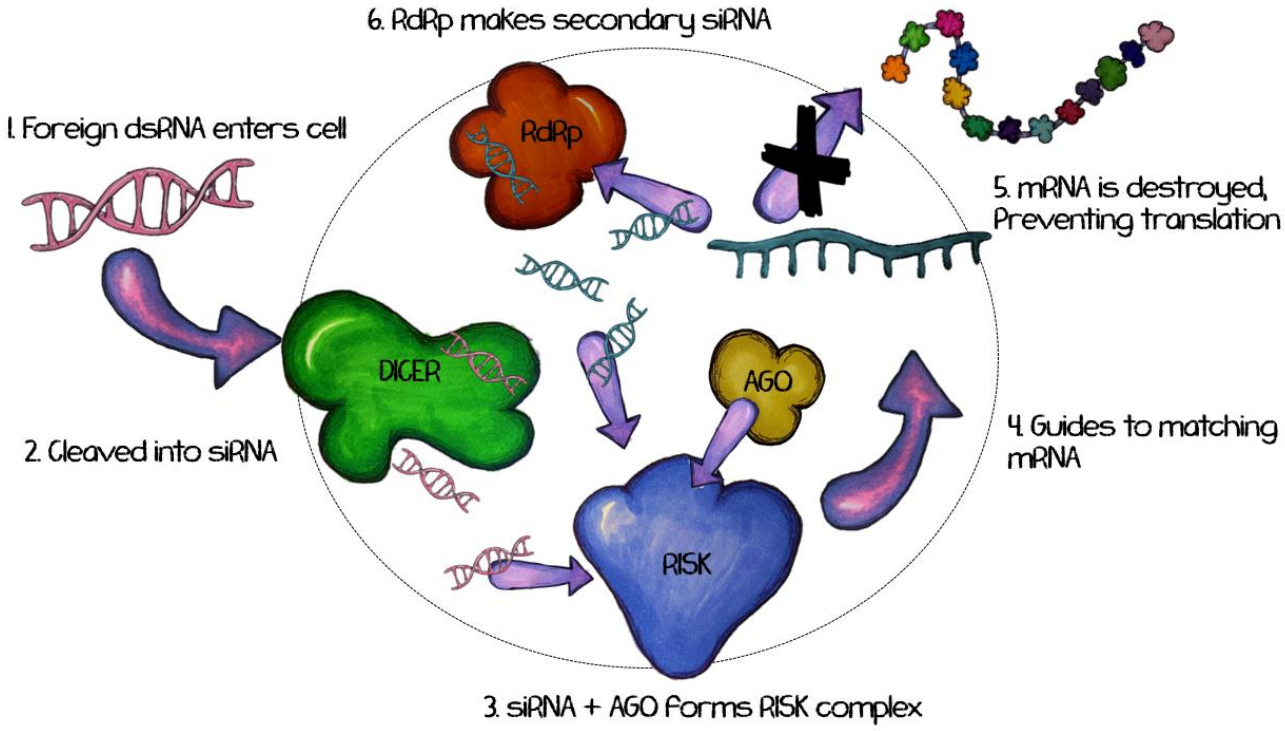


Figure 1.3 Simplified RNAi pathway. Gene knockdown is initiated by dsRNA cleavage, producing siRNA, which is loaded onto Argonaute to form the RNA-induced silencing complex (RISC). The resulting complex then guides the destruction of homologous mRNA. The RNA-dependent RNA polymerase, RdRp, synthesises additional siRNA molecules to propagate gene silencing.

RNAi in fungi

The first observation of fungal RNAi was in the model fungus *Neurospora crassa*, predating the discoveries of Fire et al 1998, and was originally termed "quelling" (Romano and Macino 1992). The components of RNAi are highly conserved across fungi, but the number of genes encoding Dicer, Argonaute and RdRP (or their homologs) vary across species (Nuss 2011, Choi et al. 2014), and some taxa lack some, or all, the machinery (Nakayashiki and Nguyen 2008). Foreign dsRNA is usually an indication of viral replication, so the RNAi pathway is likely a protective antiviral response (Karpala et al. 2005). The hypovirulence-associated mycovirus CHV-1 in *Cryphonectria parasitica* (discussed in the next section) supports this virus defence theory, as mutants lacking RNAi machinery were severely debilitated by mycovirus infection (Segers et al. 2007). In contrast, RNAi machinery can be lost if mycovirus infection confers an advantage. This is the case with RNAi-deficient “killer yeast” that can kill their competitors via a toxin produced by their mycovirus symbionts (Drinnenberg et al. 2011).

In addition to an antiviral defence system, RNAi can naturally regulate endogenous gene expression via micro RNA (miRNA) although these were long thought be absent in fungi (Torres-Martínez and Ruiz-Vázquez 2017). Recently, micro RNA-like RNA (milRNAs) have been detected in some fungi species (Lee et al. 2010, Jin et al. 2019). The milRNAs arise from endogenously produced hairpin structures called long primary-milRNA (pri-milRNA) which then from RISCs and initiate RNAi to control endogenous gene expression (Torres-Martínez and Ruiz-Vázquez 2017). Exogenous synthetic hairpin RNA (hpRNA) can mimic endogenously produced pri-milRNAs and initiate RNAi in fungi (Brody and Maiyuran 2009, Leng et al. 2011). As a result, dsRNA, siRNA and hpRNA can all be used by researchers to manipulate RNAi and knockdown homologous genes of interest.

Manipulation of RNAi occurs naturally between host and pathogen, and this phenomenon is termed "cross kingdom RNAi" (Weiberg et al. 2013). The interaction between the fungus *Botrytis cinerea* and the plant *Arabidopsis thaliana* demonstrates the remarkable trafficking of RNAi molecules between host and pathogen. The fungal pathogen can hijack the host RNAi pathway to reduce the host's immune response (Weiberg et al. 2013, Wang et al. 2017). However, cross kingdom RNAi can also be harnessed by the host to manipulate RNAi in the fungal pathogen. The plant host can secrete siRNAs within exosomes, which are taken up by the pathogen, reducing virulence (Cai et al. 2018). This naturally occurring use of RNAi as an antifungal defence provides hope for manipulating this system for use against chytridiomycosis.

RNAi as a therapeutic?

Like many fungal pathogens of wildlife*, B. dendrobatidis* infects the epidermis and hence is amenable for direct topical treatment. A topical spray technique, termed spray-induced gene silencing (SIGS) has shown enormous potential in horticulture and could be adapted for animals to replace antifungal drugs. Koch *et al* (2016) demonstrated the usefulness of SIGS by using dsRNA and siRNA spray pre-treatment on barley leaves to reduce the effect of the fungal pathogen *Fusarium graminearum* (Koch et al. 2016). The protective effect of the spray extended to unsprayed parts of the leaf, indicating that the host RNAi machinery processed the dsRNA for systemic silencing throughout its tissues. This important observation demonstrates the complexity of the system when both host and pathogen RNAi pathways are activated. Since then, SIGS has been utilised to control *Sclerotinia sclerotiorum* (McLoughlin et al. 2018) and *B. cinerea* (Duanis-Assaf et al. 2022). This demonstrates the potential of using an RNAi spray to control fungi, however, the mode of delivery is important to ensure nucleic acid stability and effectiveness of RNA. Exogenous interfering RNA can be delivered naked (Koch et al. 2016), but due to the fragile nature of RNA it will degrade quickly. In horticulture, clay nanoparticles have been used to adhere dsRNA to the host, and as a result the effect duration was doubled (Mitter et al. 2017). Polyethyleneimine nanoparticles have also been

used for RNA protection, but these may cause inflammation or apoptosis in an animal host (Shahabipour et al. 2017). Another consideration is optimising RNA infiltration through the host epidermis. Encasing RNA in a similar substance to the epidermis, such as liposomes or solid lipid nanoparticles (SLNs), can sometimes aid in topical delivery (Aldawsari et al. 2015).

RNAi virulence targets and siRNA design for control of B. dendrobatidis

Rational design of interfering RNA is now feasible due to the availability of the *B. dendrobatidis* genome on public databases. RNAi targets could include genes essential for fungal growth and fecundity (Fisher et al. 2009), or virulence factors, such as those responsible for tissue degradation or immune evasion. The putative protease virulence genes, discussed earlier, likely contribute to epidermal damage (Brutyn et al. 2012) so targeting these genes could allow increased host survival. Another virulence mechanism of *B. dendrobatidis* is its ability to evade the host immune system by the production of the metabolites spermidine, kynurenine and methylthioadenosine that inhibit lymphocyte proliferation (Fites et al. 2013). Restricting production of these metabolites could possibly allow the host immune system to develop an adaptive immune response to the pathogen and help clear the disease (Rollins-Smith et al. 2015).

Creating a transgenic host that expresses siRNA to initiate RNAi

Although flexible in the face of changing resistance or types of pathogens, therapeutic use of RNAi has similar drawbacks as traditional antifungal drugs in that the effect is not long lasting and requires frequent reapplication. Host induced gene silencing (HIGS) overcomes this hurdle, using transgenesis to create hosts that stably express the interfering RNA. In animals, HIGS has been used to reduce the effect of viruses in diseases such as foot and mouth in mice (Jiao et al. 2013) and pigs (Hu et al. 2015), anaemia virus in chickens (Hinton and Doran 2008) and porcine reproductive and respiratory syndrome in pigs (Li et al. 2014). Current HIGS approaches in animals are directed towards viral diseases, but HIGS has been used to target fungal pathogens in plants. Transgenic barley strains have been created that have reduced susceptibility to *F. graminearum* (Koch et al. 2013), and *Blumeria graminis* (Nowara et al. 2010) infection.

A variety of methods could create transgenic amphibians that resist fungal infection. Random stable insertion of interfering RNA into the host genome could be achieved through DNA microinjection into fertilised eggs, or introduction into the sperm head (Bradford et al. 2017). Microinjection techniques (DeLay et al. 2016) have been established that could enable transgenesis in amphibian eggs, and the development of RNAi systems in amphibians is already underway (Li and Rohrer 2006, Flynt and Lai 2011). Caution, however, must be taken to avoid overexpression of the

interfering RNA in the transgenic animals, as it can saturate the RNAi pathway (Flynt and Lai 2011) and even cause mortality (Grimm et al. 2006).

Potential applications and limitations of RNAi for amphibian conservation

In the short term, the introduction of exogenous siRNA is probably the most viable option for using RNAi approaches against fungal diseases in amphibians, as dsRNA is more likely to initiate an immune response in the host due to its similarity to viral particles (Karpala et al. 2005). Application of siRNA could be achieved via a spray (Koch et al. 2016), however, SIGS will probably have similar logistical constraints and short half-life as traditional antifungal pharmaceuticals (Bradford et al. 2017). The advantage of SIGS is that the target sequence can be updated as pathogens change, and can be adapted for newly emerging pathogens. In contrast, stable expression of siRNA via HIGS will avoid the need for constant reapplications, but fungal pathogens may become resistant via mutations. A HIGS approach could be useful for captive breeding programs. For example, the green and golden bell frog (*L. aurea*) breeds well in captivity but succumbs to *B. dendrobatidis* when released into the wild (Stockwell et al. 2008). Releasing HIGS individuals that are resistant to *B. dendrobatidis* would increase the success of captive re-introductions, however, there are ethical limitations and public perception issues that may make the release of genetically modified organisms controversial. Initial proof of concept experiments are required to first ascertain the feasibility of using siRNA as a novel treatment against chytridiomycosis.

Mycoviruses

Another promising treatment approach includes biological control via the use of mycoviruses. Mycoviruses are viruses that infect fungi, and more than 250 mycoviral species have been identified (Xie and Jiang 2014) from 16 families (Kotta-Loizou and Coutts 2017). While mycoviruses are obligate parasites, in most cases they cause little or only cryptic effects on their fungal hosts (Nuss 2005, Pearson et al. 2009). However, some mycoviral species have a detrimental effect, and if the host is a pathogenic fungus, this effect is termed “hypovirulence” (Grente 1965).

Mycovirus-mediated hypovirulence as an antifungal strategy?

Harnessing mycovirus-mediated hypovirulence is a novel intervention for fungal diseases and has been against variety of fungal plant pathogens. The mycovirus CHV-1 was used to control the devastating “chestnut blight”, caused by *C. parasitica,* in European chestnuts (Heiniger and Rigling 1994, Ghabrial et al. 2015, Rigling and Prospero 2018), and this widespread success led to increased investment in the use of mycovirus-mediated hypovirulence. Further laboratory trials have demonstrated that mycoviruses can reduce the virulence of root rot (*S. sclerotiorum*) (Yu et al. 2013),

white root rot (*Rosellinia necatrix*) (Chiba et al. 2009), Phomopsis rot (*Phomopsis* G-type) and valsa canker (*Valsa ceratosperma*) (Sasaki et al. 2002), and stem canker (*Cryphonectria cubensis*) (Van Heerden and Wingfield 2001) in a variety of host plants.

One important consideration in adapting mycoviruses as biocontrol agents is finding the balance between reduction in virulence and fungal host fitness. If the virus reduces fungal reproduction and growth, the infected fungal strains will be outcompeted by the pathogenic wildtype. In horticulture, effective mycoviruses reduce the size of the lesions, but not the reproduction output (spore production) of the host fungus (Nuss 2005). This balance can be fine-tuned by creating transgenic viruses using cDNA. Chen et al (2000) achieved this by using two strains to create a chimeric hypovirus incorporating the desired traits of small lesion size without suppression of spore production (Chen et al. 2000).

Approaches for identifying and assessing mycoviruses

The observation of healing cankers in chestnuts affected by chestnut blight led to the opportunistic discovery of a dsRNA virus from a hypovirulent fungal strain. In contrast, the search for a mycovirus that would confer hypovirulence to the white root rot pathogen (*R. necatrix*) was only successful after extensive screening of >400 fungal isolates (Arakawa et al. 2002, Ikeda et al. 2005, Chiba et al. 2009). These different approaches to discovering mycoviruses with biocontrol potential highlight the advantage of targeting strains with naturally reduced virulence. Less virulent fungal strains can be screened for mycoviruses by deep sequencing (Wylie and Jones 2011) or by dsRNA extraction and enrichment using phenol/chloroform (Morris and Dodds 1979), or via a commercial kit (Castillo et al. 2011), followed by cloning and sequencing. When screening large numbers of fungal strains, commercial kits are less time consuming than traditional extraction methods, but can be less sensitive due to lower nucleic acid extraction yields (Fanson et al. 2000). The fungal culture quality also affects mycoviral detection. Freeze/thawing, as well as repeated passing can cure fungi of their mycovirus (Marquez et al. 2007, Springer et al. 2013, Zamora-Ballesteros et al. 2021) so freshly isolated fungal cultures should be used if possible.

Assessing the effect of mycoviruses on fungal virulence

Once mycoviruses are identified, their ability to confer hypovirulence must be determined via infection studies. Horticultural trials to evaluate hypovirulence typically involve inoculating plants with fungal strains compared to a virus free control. Virus free control fungal strains can be naturally occurring, or artificially created by 'curing' infected fungi with polyethylene glycol (Thapa et al. 2016), cycloheximide (Bhatti et al. 2011), ribavirin (Herrero and Zabalgogeazcoa 2011), or by freezing (Marquez et al. 2007). In plants, reduction in virulence is quantified by host survival rate or lesion

size (Li et al. 2003, Yu et al. 2013). Amphibian infection studies to quantify the virulence of mycovirus-infected fungi should follow standard protocols, with survival rate and pathogen load as indicators of virulence, as discussed earlier.

Delivery of mycovirus-associated hypovirulent strains

The delivery and dissemination of hypovirulent strains remains a major challenge to adopting this technique for mitigating wildlife diseases (Root et al. 2005). Wild amphibians could be inoculated with hypovirulent fungal strains, following the same approach used for chestnut trees (Milgroom and Cortesi 2004). Alternatively, the virus could be purified and directly applied (Yu et al. 2013) to infect the endogenous fungi on amphibians; however, this approach is likely to be less feasible. Although it is possible to artificially introduce mycoviruses into related fungal species (Lee et al. 2011), mycoviruses tend to be very host specific, and they are generally unable to exist outside their host. This specificity is an advantage for biological control, as non-target species will be unaffected. However, their limited infectivity (Ghabrial 1998) also limits their dissemination into target populations. Mycoviruses can only be transferred into virus free strains horizontally (by hyphal fusion or anastomosis), or vertically (by spores). This is a major challenge in horticulture, as the filamentous fungal pathogens require vegetative compatibility for horizontal transfer. Vegetative incompatibility likely contributed to the failure of mycovirus control of chestnut blight in America (due to the high diversity of compatibility types compared to Europe) (Nuss 2005), although some studies disagree (Milgroom and Cortesi 2004). The issue of vegetative incompatibility is unlikely to be a barrier to chytridiomycosis mitigation however, as *B. dendrobatidis* reproduces via zoospores, meaning vertical transmission of mycovirus is essential. Reliance on vertical transmission is likely to be a feasible approach as suggested by the 50% vertical transmission rates in *F. graminearum*, with half the daughter spores containing the mycovirus (Chu et al. 2002).

Potential of using mycoviruses for amphibian conservation

If a hypovirulence-inducing mycovirus can be found in *B. dendrobatidis*, it offers a novel approach to protecting wild amphibians. Using a natural mycovirus to mediate fungal hypovirulence would be a relatively environmentally friendly approach. In addition, the logistical constraints of catching and treating wild amphibians would be lessened as the less virulent mycovirus-infected fungi could spread naturally through the amphibian population. Although thorough validation and government approval would need to occur before deliberately releasing virus-infected *B. dendrobatidis* isolates into the wild, the idea of a natural biocontrol might be less controversial than releasing genetically modified organisms via the HIGS approach.

Aims of this thesis

Chytridiomycosis is a deadly disease that continues to threaten the world's amphibians. The broad aim of this thesis is to develop tools that can reduce the virulence, and hence impact, of the amphibian chytrid fungus. I focussed on exploring the feasibility of two approaches that have been useful in horticulture: RNAi and mycovirus-mediated hypovirulence. This required the development of an assay to quantify growth and viability, as well as investigation of a possible RNAi target (Figure 1.4).

Aim 1: To develop an effective growth and viability stain for *B. dendrobatidis*

A rapid, simple, and inexpensive viability assay is essential to detect the impact of RNAi treatments. Therefore, my first aim was to develop and validate this assay as a platform for subsequent experiments.

Aim 2: To determine the role of glutathione in *B. dendrobatidis*

Glutathione is a virulence factor in many other pathogenic fungi and glutathione associated genes have been acquired by *B. dendrobatidis* via HGT. It is an ideal target for RNAi as its synthesis and function has been studied in other fungi, and it is amenable to chemical inhibition by buthionine sulfoximine (BSO). However, little is known about the role of glutathione in *B. dendrobatidis*, thus I explored the function of this antioxidant via stress treatments. Chemical inhibition via BSO allowed me to characterise phenotypes arising from suppression of glutathione synthesis.

Aim 3: To develop RNAi methodologies in *B. dendrobatidis* to inhibit gene expression

There are no molecular techniques reported to inhibit gene expression in *B. dendrobatidis*. Therefore, the aim of this chapter was to develop and optimise RNAi methodologies in this species, using various transformation techniques and siRNA designs. I designed siRNA to target the glutathione biosynthesis gene, and the phenotypic results from Aim 2 were used to assess the effectiveness of siRNA mediated knockdown, as compared to chemical inhibition.

Aim 4: To screen for the presence of mycoviruses in *B. dendrobatidis*

An alternative method to reduce fungal virulence is via mycovirus infection. Mycoviruses have not yet been reported in *B. dendrobatidis* but have been identified across many fungal genera. My fourth aim was to complete a thorough search for mycovirus presence in Australian *B. dendrobatidis*, as well as from selected isolates from Asia and South America.

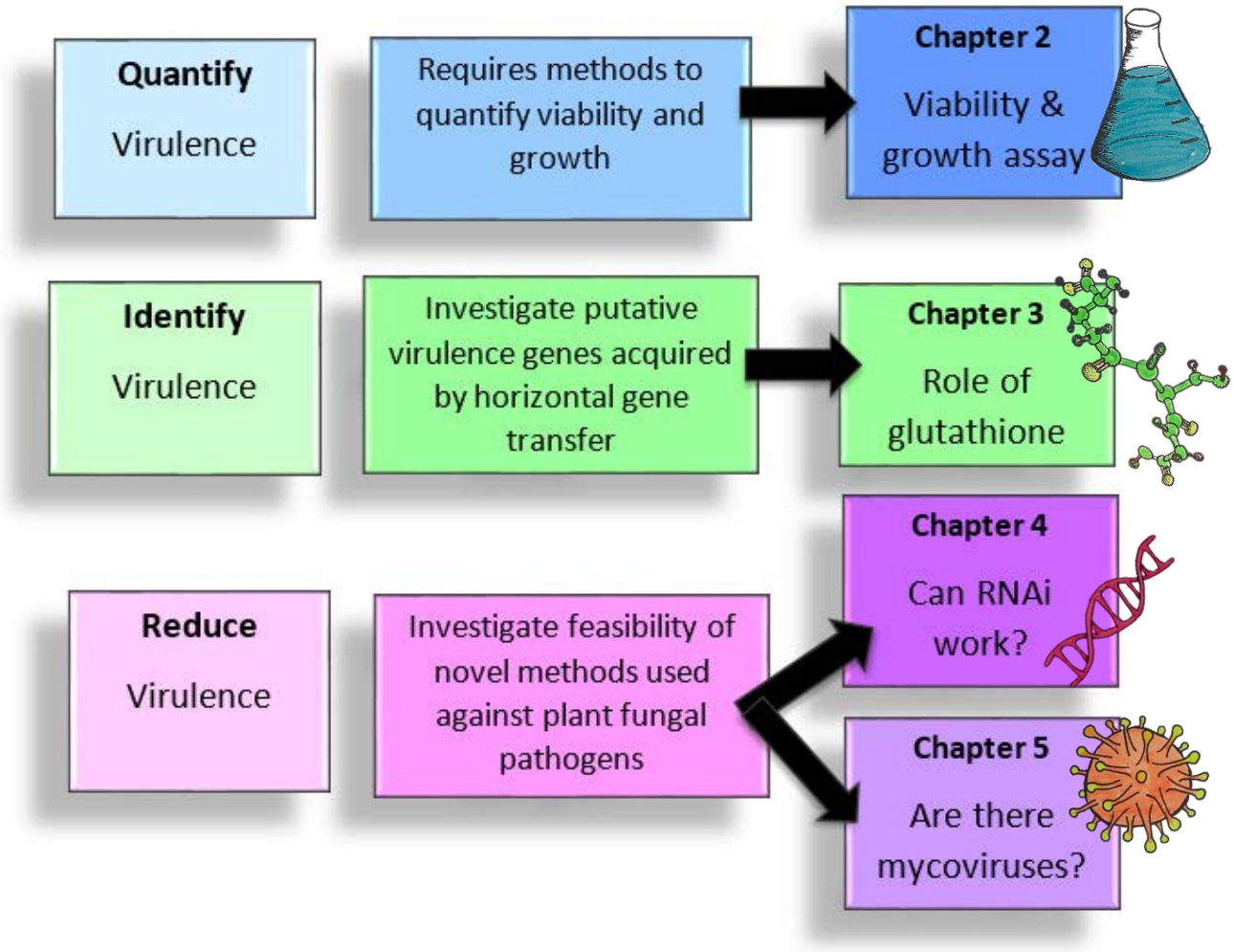


Figure 1.4 Thesis outline. In Chapter 2 I develop a viability and growth assay. Then, I investigate glutathione as a potential virulence factor in *B. dendrobatidis* and use glutathione biosynthesis as a target for RNAi. Finally, I screen for mycovirus presence in *B. dendrobatidis*.

Chapter 2 **Viability Assay**

A major hurdle in the quantification of virulence in *B. dendrobatidis* is quantifying viability and growth. In this chapter, I developed a simple and rapid viability stain for *B. dendrobatidis* zoosporangia using the commonly available dye, methylene blue. I evaluated the stability of methylene blue over time and across zoosporangial stages, and validated its use for quantification of viable cells. In addition I developed a growth assay using fixation and elution of stain from dead cells. The ability to quantify viable zoosporangia and measure relative growth was an essential technique that was utilised in Chapters 3 to estimate growth of *B. dendrobatidis* in the presence of different stressors, and in Chapter 4 to ensure viability post-siRNA delivery.

The results from this chapter have been published in Journal of Microbiological Methods:

Webb, R.J., Berger, L., Skerratt, L.F., Roberts, A.A., 2019. A rapid and inexpensive viability assay for zoospores and zoosporangia of *Batrachochytrium dendrobatidis*. Journal of Microbiological Methods 165, 105688. https://doi.org/10.1016/j.mimet.2019.105688

This chapter has 4 aims.

1: Determine whether methylene blue differentially stains live and dead zoosporangia of *B. dendrobatidis*

2: Determine consistency of staining over time and between different manufacturers

3: Validate the assay by measuring the proportion of viable zoosporangia

4: Develop a colorimetric assay on eluted methylene blue to measure growth

Development of a viability stain for *Batrachochytrium dendrobatidis*

Abstract:

Despite its pathogenicity, *Batrachochytrium dendrobatidis* is a sensitive organism in culture, and it is often necessary to perform viability estimates to confirm that cells are alive. Existing protocols to determine the viability of zoospores of *B. dendrobatidis* rely on the intact membranes of live cells to exclude the dye. This approach, however, may not be suitable for the zoosporangial lifestage as their discharge tubes may allow dye to enter the cell. I assessed staining by methylene blue, which is be rendered colourless by enzymes on live cells, and stains dead cells blue. I found that 0.1mg/mL methylene blue stains dead zoosporangia bright blue, whereas live zoosporangia remain unstained regardless of discharge tubes. Methylene blue staining can provide reliable estimates of viability by counting stained and unstained cells using standard microscopy techniques. Elution of methylene blue from fixed cells can provide a comparison of cell growth via colorimetric assay. Accurate quantification of *B. dendrobatidis* survival can aid amphibian conservation efforts by allowing rapid testing for susceptibility to antifungal drugs and other treatments. This assay can also be adapted to measure growth and detect sub lethal effects of treatments.

Introduction

Advancing our understanding of *Batrachochytrium dendrobatidis* requires laboratory experiments. Despite its pathogenicity, *B. dendrobatidis* is easily killed by many factors, including heat (Johnson et al. 2003), desiccation (Johnson et al. 2003), disinfectants (Webb et al. 2012), and salt (Stockwell et al. 2012). Therefore, it is often necessary to perform viability estimates on laboratory cultures to determine the proportion of live cells before commencing experimental work. Viability estimates are also essential in fungal susceptibility trials, such as evaluating suitable disinfectants and therapeutics.

The flagellated zoospores of *B. dendrobatidis* are motile and can be easily classified as alive if they are moving. However, zoospores will occasionally cease movement, for example when they begin to encyst. Therefore, researchers cannot definitively classify an individual zoospore as dead based on the absence of movement. Two viability stains have been validated for use on *B. dendrobatidis* zoospores: trypan blue (McMahon and Rohr 2014), which is quick and easy but requires manual counting; and SYBR green/propidium iodide, which is less subjective, but more expensive and requires specialised equipment (Stockwell et al. 2010). Both of these techniques rely on the exclusion of dye by intact zoospore membranes, so that viable cells remain unstained. For this reason, they may be unsuitable for the zoosporangial life stage, because the discharge tubes could allow the dye to enter the cell regardless of cell viability. This theory is supported by

McMahon and Rohr (2014), who found that trypan blue stained 100% of zoosporangia in a healthy culture, indicating falsely that they were all dead (McMahon and Rohr 2014).

Methylene blue differs from other viability stains in that it relies on the ability of viable cells to reduce the stain to a colourless substance via enzymatic activity, rather than relying on membrane impermeability (Painting and Kirsop 1990, Bapat et al. 2006). Methylene blue has a long history as a viability stain (Borzani and Vairo 1958), and is commonly used to determine fungal viability at a concentration of 0.1 mg/mL (Kwolek-Mirek and Zadrag-Tecza 2014, Parker et al. 2020). Here I assess whether methylene blue will function as a viability stain in *B. dendrobatidis,* despite the presence of discharge tubes.

In addition to viability, it is often necessary to quantify sub-lethal effects, such as reduced growth rate, as measured by the number and size of cells. Growth of *B. dendrobatidis* can be estimated by measuring optical density (OD) using a spectrophotometer (Rollins-Smith et al. 2002, Stevenson et al. 2013, Voyles et al. 2017). Increased cell growth will result in a higher absorbance of the emitted light. This is a comparatively cheap and easy method; however, the accuracy decreases if the cells are unevenly distributed within the suspension or growth surface. Dent *et al* (1995) developed a growth assay for mammalian tumour cells in which the cells were fixed, stained with methylene blue, and then the dye eluted and quantified spectrophotometrically (Dent et al. 1995). This method may also work for *B. dendrobatidis.*

Methylene blue is inexpensive, stable and readily available in most laboratories. In this study, I aimed to determine whether methylene blue is suitable as a viability stain for *B. dendrobatidis* zoosporangia through a series of small studies. I examined whether methylene blue can be used on both adherent and suspended zoosporangia, and measured the stability of the stain over time and between stain manufacturers. In addition, I explored whether measuring eluted methylene blue in a colorimetric assay can quantify differences in growth, and compare this to the traditional method of growth measurement.

Methods

B. dendrobatidis culture

B. dendrobatidis was grown using standard laboratory protocols (Longcore et al. 1999). Cultures were maintained with in a tryptone, gelatin hydrolysate and lactose broth (TGhL) or agar at 20°C. The isolates used in this Chapter were: Mitta Mitta-L. spenceri-2018-LB, Yanchep-L.moorei-2019-RW, Frenchmans Creek-L.sp-2020-RW, and Ethel creek-L.nannotis-2013-LB. Pure zoospore suspensions were obtained by flooding mature cultures on solid agar, or by removing the broth from mature flasks and incubating the zoosporangia monolayer with fresh broth (Robinson et al. 2019).

Zoospore solutions were filtered to remove any detached zoosporangia with a sterile isopore PC 10 μM filter (TCTPO2500 Millipore).

Evaluation of methylene blue suitability for zoosporangia and comparison to trypan blue

Live and dead zoosporangia were used in an initial trial to evaluate the suitability of methylene blue as a viability stain for *B. dendrobatidis*. Sterilised glass slides were placed in individual 50 mL centrifuge tubes and filled with 25 mL TGhL broth. A concentrated zoospore solution (1 mL) was added to each tube and incubated at 20°C. Each day, two slides were removed for staining with methylene blue and trypan blue. The lower half of each slide was killed by dipping in 100% ethanol for 1 min. The other half of the slide remained untreated. The slides were rinsed and then stained with either 0.4% trypan blue in PBS for 1 min (McMahon and Rohr 2014), or 0.1 mg/mL methylene blue for 1 min (Kwolek-Mirek and Zadrag-Tecza 2014). Slides were examined under a compound microscope to determine if live and dead cells could be clearly differentiated. Special attention was paid to the "transition zone" between the ethanol treated and untreated sections, where there was a mixture of live and dead cells. This process was repeated for 3 days to capture zoosporangia at all stages of growth. An additional slide was prepared with a mixture of cultures from different age classes and stained with methylene blue to ensure that the method worked when discharge tubes are present.

Evaluation of consistency of methylene blue staining over time and between manufacturers

Individual stained and unstained cells were monitored to assess whether methylene blue staining is consistent over time. A concentrated suspension of actively growing culture was divided into two aliquots, one of which was killed by heating to 50°C for 10 min and then combined to create a mixed population of live and dead cells. This suspension was added to an equal volume of 0.2 mg/mL methylene blue, stained for 1 min, centrifuged to remove excess stain, and then resuspended in water. The cells were viewed under a compound microscope and the same field of view was photographed every 10 min to determine if staining of individual cells changed over time.

Methylene blue from two manufacturers (Australian Biostain "ADMB" and Sigma "M9140") was compared to ensure consistent staining. Both dyes were dissolved in water to a 2x stock solution of 0.2 mg/mL. A concentrated actively growing suspension containing a mix of life stages was split equally into two tubes. One tube was killed via heat (10 min at 50°C). Aliquots from suspensions of live or heat killed cells were stained with each brand of methylene blue, and compared using microscopy.

Validation of methylene blue to estimate viability.

The applicability of methylene blue as a viability assay for cells in suspension was investigated by preparing suspensions with known proportions of viable cells. A concentrated, actively growing suspension containing a mix of life stages was split equally into two tubes. One tube was killed via heat (10 min at 50°C). Live and dead cells were mixed to prepare suspensions that contained either 0%, 25%, 50%, 75% and 100% dead zoosporangia. Methylene blue (0.2 mg/mL) was added at a ratio 1:1 to the suspensions to give a final concentration of 0.1 mg/mL and samples were incubated for 2 min. The proportion of stained and unstained cells in each suspension was determined at least twice via manual counting using a haemocytometer, and corrected using the 100% live treatment. The experiment was repeated using flash freezing (McMahon and Rohr 2014) to kill the cells in order to confirm that the staining efficacy wasn't influenced by the killing method. Paired T-tests (Graph pad Prism 8.1.2) were performed to compare the observed counts with the known proportion of dead cells.

Development of a colorimetric growth assay using eluted methylene blue

To evaluate whether a methylene blue colorimetric assay (Dent et al. 1995) could be developed to detect differences in growth in *B. dendrobatidis*, cells were exposed to different levels of oxidative stress to create a gradient of reduced growth. Zoospores (100 µL) were used to inoculate a 96 well culture plate at three densities; High (1×10^5), medium (5×10^4) and low (2.5×10^4). The cells were incubated at 20°C for 24 h to allow them to adhere. Hydrogen peroxide (H_2O_2) was added to the wells at concentrations of 0 mM, 0.1 mM, 0.2 mM, 0.3 mM, and 0.4 mM with three replicates per treatment, and incubated at 20°C for 72 h. Absorbance was measured at 492 nM (Rollins-Smith et al. 2002) as a comparison between quantification methods. The methylene blue assay was then performed as follows: Excess media was removed from the wells, and cells were fixed with 70% ethanol for 1 min. The cells were stained with 0.1 mg/mL methylene blue for 1 min, followed by gentle washing with water to remove traces of dye. The dye was eluted using Hydrochloric acid (HCL), with 45 µL 0.1 M HCL added per well and incubated for 15 min, then transferred to a new 96 well plate and absorbance measured at 650 nM using an Omega Polarstar spectrophotometer (using 0.1 M HCl as a blank). The amount of growth was determined as relative to the untreated control using the equation below. Linear regression (Graphpad Prism 8.1.2) was performed on each concentration separately to determine the goodness of fit for methylene blue vs optical density. A one way ANOVA (GraphPad) was performed on each cell density to determine which method could distinguish between sub lethal levels of H_2O_2.

$$Relative\ growth = \frac{Absorbance\ killed\ treated}{Absorbance\ killed\ control}$$

To establish the optimal concentration of methylene blue for colorimetric growth assays, cells were stained with a 10-fold range of methylene blue concentrations. Zoospores (100 µL) were added to a 96 well plate to give a final number of zoospores of either, 1.5 x 10^4, 3 x10^4, 6 x 10^4 incubated at 20°C for 2 days. Excess media was removed from the wells, and the cells were then killed with 70% ethanol, and stained with either 0.1 mg/mL, 0.5 mg/mL or 1 mg/mL methylene blue for 1 min. The cells were washed, and the dye eluted in 40 µL of 0.1 M HCl. The supernatant was measured using the colorimetric assay was conducted as described earlier.

Results

Methylene blue as a viability stain for zoosporangia

Methylene blue clearly differentiated between live and dead cells. Cells were grown on a glass slide and half dipped in ethanol before staining the entire slide. Examination using a compound microscope revealed that all ethanol-killed zoosporangia were stained bright blue, while the untreated control (viable) half of the slide remained colourless. The transition zone in the middle of the slide contained a mixture of live and dead zoosporangia, which could clearly be differentiated (Figure 2.1a). Live zoosporangia with discharge tubes remained unstained (Figure 2.1b). In addition, empty, effete zoosporangia that had released zoospores were easily distinguishable from killed zoosporangia, as their walls, but not their contents stained light blue (Figure 2.1b). When compared to an established stain (trypan blue) methylene blue produced a clearer distinction between live and dead zoosporangia of various ages (Appendix A, Figure 8.1).

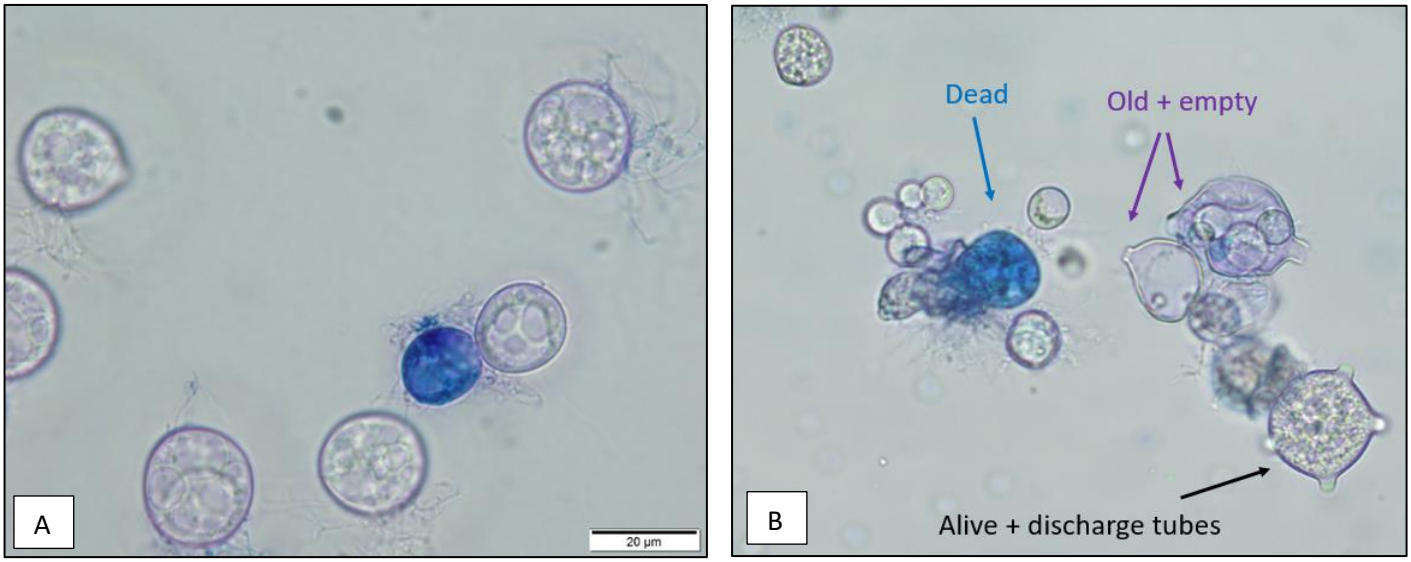


Figure 2.1 Live and dead zoosporangia are distinguishable with methylene blue. A: Dead zoosporangium stained bright blue, which is clearly differentiated from live colorless cells. B: The methylene blue stain is not taken up by live cells with discharge tubes.

Consistency of staining over time and between manufacturers

Further tests demonstrated stability of the stain over time and consistency across manufacturers. The colour of zoosporangia after methylene blue exposure did not change over the 1 h of observations (Appendix A, Figure 8.2). An additional brand of methylene blue was tested to confirm the consistency of this methodology. Both Australian Biostain and Sigma branded methylene

blue produced similar results on microscopic examination (Appendix A, Figure 8.3), suggesting that this assay will work regardless of the source of dye.

Estimating viability

Methylene blue staining can be used to estimate the proportion of live and dead cells in a suspension. Suspensions with known proportions of live and dead cells were manually counted after methylene blue staining and confirmed that methylene blue can accurately estimate viability. As the original inoculum for this experiment was from a mixed culture, there was a background level of 2-15% dead cells in the untreated "0% dead" control. This background was subtracted from the final percentage of cells observed in the assays to determine the corrected percentage. There was a strong linear relationship between the percentage of stained zoosporangia counted and the percentage of dead cells added (R^2= 0.964) (Figure 2.2). A paired T-test indicated no difference between the known proportion and counted dead cells (T-test p=0.3155, t=1.013, df=55).

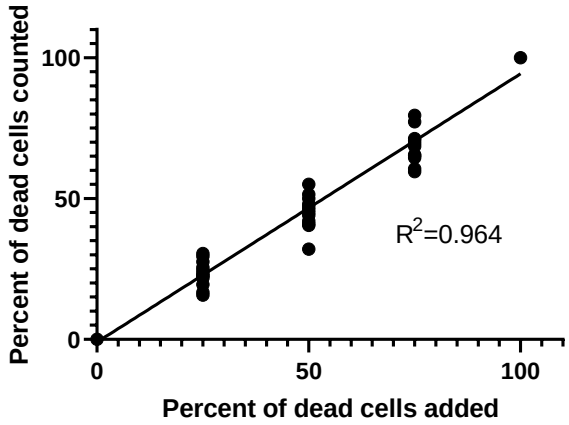


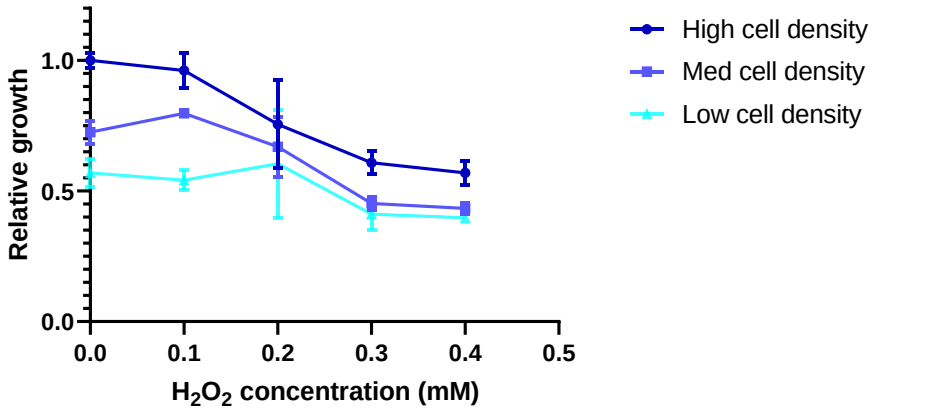
Figure 2.2 Counts of zoosporangia from suspensions of known viability. The percentage of dead cells counted correlated with the percentage of dead cells added to the suspension.

Estimating growth

The methylene blue assay can also be used to measure growth to allow comparisons between varying severities of sub-lethal treatments. Sub lethal concentrations of H_2O_2 were used to create a spectrum of subtle growth responses to test the sensitivity of the methylene blue assay compared with measuring optical density. Growth was calculated as relative to the untreated (0 mM) control. The methylene blue colorimetric assay was more sensitive than the traditional optical density assay, especially at low cell densities (Figure 2.3). Linear regression analysis indicated that the methylene blue assay could detect subtle growth changes with various (0.1-0.4 mM) concentrations of H_2O_2 (R^2 High=0.90, medium=0.90, low=0.79) compared to the optical density assay (R^2 High=0.81, medium=0.72, low=0.43). When total cell numbers were less than 2.5×10^4 per well, the optical density method could not distinguish between cells subjected to 0.1 – 0.4 mM H_2O_2 ($p=0.0824$, $F= 14.21$ [3,7]), whereas the methylene blue method could ($p=0.0018$, $F= 0.1779$ [3,8]).

The absorbance of eluted methylene blue is impacted by the concentration initially used to stain the cells. The absorbance values were higher when 0.5 mg/mL methylene blue was used instead of 0.1 mg/mL, indicating that the higher concentration may enable more sensitive measurement of low cell growth. Increasing the methylene blue concentration to 1 mg/mL did not further improve the sensitivity (Appendix A, Figure 8.4).

A



B

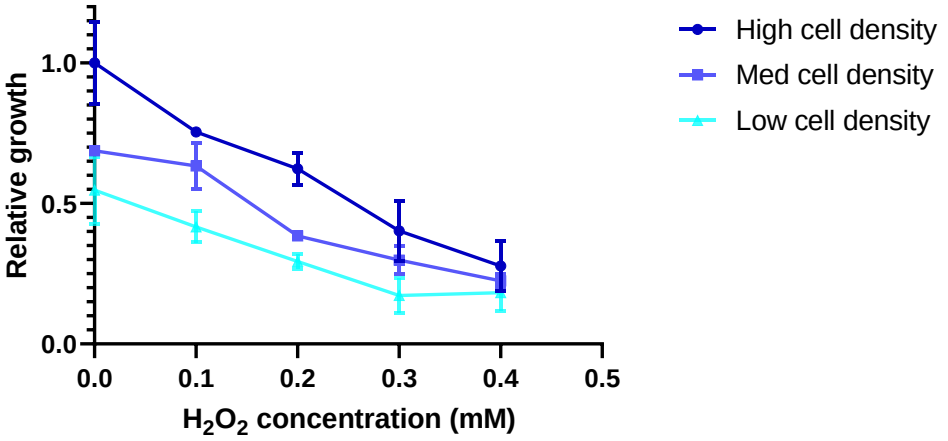


Figure 2.3 Comparison of growth measurements using A) Optical density method and B) Methylene blue colorimetric assay. Cells at three densities were treated with various levels of H_2O_2, and growth estimated first using the optical density method and then the methylene blue method. The methylene blue measurements remained linear over a wider range of growth, and were more sensitive at low cell concentrations.

Discussion

This study demonstrates for the first time that methylene blue is an effective viability stain for zoosporangia of *B. dendrobatidis*. Methylene blue stains dead zoosporangia bright blue, leaving live zoosporangia unstained, and this was validated using three methods to kill the cells (ethanol, heat, and snap freezing). Methylene blue can be used to estimate proportion of viable cells by manual counting or colorimetric assay.

Other viability dyes, such as trypan blue, rely on exclusion by permeable membranes. This dye exclusion method is hypothesised to have limited use in *B. dendrobatidis* due to the presence of leaky discharge tubes that enable live cells to be stained, leading to false negative results (McMahon and Rohr 2014). In this Chapter, I directly compared trypan blue with methylene blue and found the latter to be superior (Figure 8.1). The advantage of methylene blue is that it relies on the enzymatic reduction of the dye by live cells. A redox reaction occurs in which the blue methylene blue is reduced to colourless leucomethylene blue. The mechanism of action of methylene blue within the context of cell viability is still not completely understood. It is hypothesised that the reduction of the methylene blue occurs at the cell membrane, after which the reduced leucomethylene blue diffuses across the cell membrane (Bongard et al. 1995, Merker et al. 1997, May et al. 2003). It is likely that due to the positive charge of methylene blue in the oxidised (blue) state, its permeability is excluded in live cells (Chilver et al. 1978). Leucomethylene blue however is uncharged, and therefore may diffuse across the cell membrane (May et al. 2003). In some cells, this intracellular leucomethylene blue can be reoxidised (for example by haemoglobin-containing proteins) (Metz et al. 1976), leading to the re-appearance of the blue colour (Bapat et al. 2006). If the reduction of methylene blue indeed occurs at the cell surface, then the observation that live zoosporangia remain unstained suggests that their discharge tubes are tightly sealed. If they were leaky, the dye could enter the zoosporangia before the cell surface reductases could reduce it. This raises the possibility that the sub-optimal performance of trypan blue may be more complicated than simply discharge tube porosity.

Methylene blue is a fungicide, and mildly toxic to *B. dendrobatidis*. However, the stain concentration is ten-fold lower than the minimum concentration needed to cause zoospore death in 30 min (Berger et al. 2009). To address the possibility of re-oxidation or cytotoxic effects in *B. dendrobatidis*, live cells were exposed to methylene blue, and the assay was shown to be stable for at least 1 h.

The absence of reductive enzyme activity in dead cells means that methylene blue can enter the cell and stain the basophilic cellular contents blue. After washing, the amount of oxidised, blue

dye remaining is proportional to the volume of stained basophilic cellular contents. When the bound dye is eluted using dilute HCl, the intensity of the blue colour is, therefore, proportional to the biomass of dead cells the sample (Pelletier et al. 1988, Dent et al. 1995). This study demonstrates that this principle can also be used to estimate growth in *B. dendrobatidis.* Traditionally, the simplest method to estimate growth is to measure OD using a spectrophotometer (Rollins-Smith et al. 2002, Stevenson et al. 2013, Voyles et al. 2017). The absorbance of the cells themselves are measured without the addition of dye. This method is quick and non-destructive, which allows the repeated measurement of the same sample over multiple days. However, this method relies on the homogenous distribution of cells. When grown in circular wells, *B. dendrobatidis* often grows heterogeneously across the growth surface, with concentrated growth on the outer edge of the well and sparse growth in the centre (personal observation). Therefore, spectrophotometric measurements can provide erroneous measurements, and have poor sensitivity at low cell concentrations. The colorimetric methylene blue assay circumvents the issues of heterogenous growth by measuring the eluted dye. However, the limitation of this method is that the sample is destroyed during the assay and requires additional steps than spectrophotometry alone.

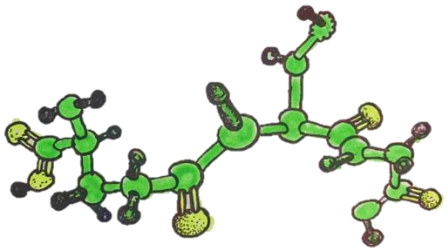
The traditional concentration of methylene blue is 0.1 mg/mL, but increasing the concentration to 0.5 mg/mL may enable a lower limit of detection for low cell concentrations. The observation that absorbance values vary with methylene blue concentration highlights the importance of using the same batch of dye throughout an experiment.

Since publication, two new cell vitality assays (CellTiter-Glo and MTT) have been developed for *Batrochochytrium* species (Lindauer et al. 2019, Carter et al. 2021). While the methylene blue assay determines cell viability (percentage of live cells), assays such as the CellTiter-Glo® (Promega) and MTT provide a measure of the physiological state of the cells (cell vitality). Vitality assays quantify metabolic activity, as cells that are alive may differ in their metabolism (Liao et al. 1999, Kwolek-Mirek and Zadrag-Tecza 2014, Gaálová et al. 2019). The CellTiter-Glo® luminescence assay has been validated to determine vitality in *B. salamandrivorans* by measuring ATP levels (Carter et al. 2021), and has recently been adapted for determining viability of *B. dendrobatidis* zoospores (Jiménez et al. 2022). The MTT assay has been established for use in *B. dendrobatidis* (Lindauer et al. 2019), and measures cell activity by the reduction of the yellow tetrazolium salt MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) into purple MTT-formazan crystals in metabolically active cells. Used in conjunction, these two assays along with the methylene blue assay described here, could provide in depth data on *B. dendrobatidis* viability and vitality.

Summary

Methylene blue is a readily available, stable, inexpensive and rapid viability stain for zoosporangia of *B. dendrobatidis*. The dye can be used on either adherent or suspended cells, and viability can be calculated by counting stained cells. The live/dead assay described here can also be modified into a colorimetric assay to detect subtle changes in growth or sub-lethal effects of treatments.

Chapter 3 **Role of Glutathione**



Glutathione is an important antioxidant for most eukaryotes, including fungal pathogens. The role of glutathione in *B*. *dendrobatidis* pathogenesis is unknown, but it has been identified as a candidate virulence factor. In this chapter, I used multiple approaches to explore the function of glutathione in *B*. *dendrobatidis,* including quantification of gene expression of a glutathione synthesis gene (glutamate cysteine ligase, *GCL*) during the transition from free-living zoospore to parasitic sporangial life stage. I also investigated whether glutathione levels affect the tolerance to various stressors (oxidative, heavy metal, fungicidal, osmotic and heat stress) using the viability assay developed in Chapter 2. This chapter required extensive development of RT-qPCR protocols to measure gene expression, which are described in Appendix B.

These studies showed that glutathione is important for tolerating cadmium. Thus, the *GCL* gene is an attractive target for developing RNAi methodologies in *B. dendrobatidis,* as heavy metal susceptibility is a measurable phenotype for assessing gene knock down (Chapter 4). In addition, the observation that background *GCL* expression is stable during cell development indicates that the RT-qPCR protocol developed in this chapter is a valid approach to detect gene knockdown.

<u>This chapter has 3 aims</u>

1: To determine the *GCL* expression patterns during early cell development in *B. dendrobatidis*, both *in vitro* and in an amphibian infection model.

2: To observe the effect of various stressors on glutathione levels and gene expression in *B. dendrobatidis*.

3: To determine whether glutathione replete or deplete conditions affect the sensitivity of *B. dendrobatidis* to various stressors.

The role of glutathione in the virulence of *Batrachochytrium dendrobatidis*

Abstract:

In many pathogenic fungi, the antioxidant glutathione is a virulence factor that helps evade oxidative stressors generated from host immune cells. Here I explore the role of glutathione in *Batrachochytrium dendrobatidis,* through gene expression, glutathione quantification and stress exposure studies. Expression of a glutathione biosynthetic gene (glutamate cysteine ligase, *GCL*) remained stable during cell development, and did not appear to be upregulated in a host infection model. Buthionine sulfoximine (BSO)-mediated depletion of glutathione prevented the development of discharge tubes and release of zoospores, indicating that glutathione is important for life cycle completion in *B. dendrobatidis*. Sub-lethal hydrogen peroxide exposure lowered total cellular glutathione levels by 42%, with a slight increase in the reduced:oxidised glutathione ratio, possibly due to the upregulation of glutathione reductase. In contrast, exposure to 0.04 mM cadmium increased total cellular glutathione by 93%. Glutathione-depleted cells had an increased sensitivity to cadmium stress, and this effect was reversed by glutathione supplementation. Heat treatment decreased the reduced:oxidised glutathione ratio, while the sensitivity to heat increased with glutathione supplementation. Overall, this study shows that glutathione levels are impacted by oxidative (peroxide) stress, but that it may not be an important component of host invasion. Glutathione plays an important role in cadmium tolerance, likely through irreversible sequestration of the heavy metal. The impact of glutathione levels on heat sensitivity may help explain differences in host susceptibility to chytridiomycosis and may provide opportunities for synergistic therapeutics.

Introduction:

Batrachochytrium dendrobatidis is a chytridiomycete pathogen that has decimated amphibian species worldwide (Scheele et al 2019). The success of this epidermal pathogen is likely due to its ability to evade the host immune system, mediated via production of immunosuppressant molecules (Fites et al. 2013, Rollins-Smith et al. 2015). However, identification of other virulence factors has been limited by the lack of molecular biology tools developed for this species (Rosenblum et al. 2010). Identification of potential virulence factors is an important step in the development of antifungal interventions such as targeted drugs (Nosanchuk et al. 2001), vaccines (De Bernardis et al. 2012) and beneficial microbes (Mayer and Kronstad 2017); which have limited efficacy in the current treatments for chytridiomycosis.

Chapter 3: Role of glutathione

One potential fungal virulence factor is the glutathione antioxidant system, as *in silico* studies suggest that *B*. *dendrobatidis* acquired several glutathione-associated genes via horizontal gene transfer (Sun et al. 2011). Glutathione is a thiol tripeptide containing three amino acids: glutamate, cysteine and glycine (Pirie and Pinhey 1929). Glutathione exists in either a reduced (GSH) state or as oxidised dimer (glutathione disulfide, GSSG). The synthesis of glutathione is a two-step process (Figure 3.1). The first reaction is catalysed by glutamate-cysteine ligase (GCL, also known as γ-glutamylcysteine synthetase), which is the rate limiting step of glutathione synthesis and is transcriptionally regulated by yAP-1p (Wu and Moye-Rowley 1994), and Met4 (Wheeler et al. 2002). Excess glutathione creates a feedback loop that inhibits Met4, allowing precise control over glutathione levels (Wheeler et al. 2002). In the second step, the enzyme glutathione synthetase (GS) forms glutathione by adding glycine to γ-glutamylcysteine (Meister and Anderson 1983, Pócsi et al. 2004, Sofyanovich et al. 2019).

Glutathione has antioxidant properties that are not only important for normal cellular metabolism (Pócsi et al. 2004), but also play a role in pathogen virulence by neutralising oxidative stressors generated by host immune systems (Wojtaszek 1997, Missall et al. 2004). As such, pathogenic fungi often show higher tolerance to oxidative stress compared to non-pathogenic species (Jamieson et al. 1996). Phagocytic immune cells such as macrophages and neutrophils produce reactive oxygen species (ROS), including hydrogen peroxide, to destroy fungal pathogens within the phagosome (Brown 2011). Glutathione, however, can protect some fungal species from this oxidative stress, allowing them to survive phagocytosis (Erwig and Gow 2016). Phyto-pathogenic fungi also encounter defensive hydrogen peroxide from the host (Apostol et al. 1989), and the ability to detoxify hydrogen peroxide is a virulence factor in plant fungal diseases such as smut (Molina and Kahmann 2007, Lin et al. 2009).

Although the host response to *B. dendrobatidis* infection is generally poor (Grogan et al. 2018), *B. dendrobatidis* could encounter oxidative stress during infection, as amphibian macrophages (Johnson et al. 2000) and neutrophils (Froese et al. 2005) produce hydrogen peroxide. The function of the glutathione system in *B*. *dendrobatidis* has not been elucidated, although hydrogen peroxide has been shown to alter the pattern of glutathione modifications on proteins (Claytor 2020). In other species, glutathione can neutralise hydrogen peroxide directly (Pócsi et al. 2004), or in a reaction catalysed by glutathione peroxidase (GPx) (Galiazzo et al. 1987). Both processes result in the oxidisation of glutathione into the dimerised glutathione disulfide (GSSG), which is then recycled back into GSH by glutathione reductase (GR) (Grant et al. 1996, Sato et al. 2009) (Figure 3.2).

Chapter 3: Role of glutathione

Disruption or inhibition of GCL, GR, or GPx can result in decreased tolerance of oxidative stress and decreased fungal virulence, highlighting the importance of this system for pathogenicity. For example, GCL is essential in many fungi, including in the human pathogens *Candida albicans* and *Candida glabrata*, (Baek et al. 2004) with *C. albicans GSH1* (*GCL*) mutants demonstrating increased susceptibility to macrophage attack and lowered virulence in a mouse host (Yadav et al. 2011). The ability to recycle oxidised GSSG back into GSH is also important for the virulence of many fungal pathogens of both plants and animals. In the rice blast fungus *Magnaporthe oryzae,* deletion of the gene encoding GR (*GTR1*) rendered the mutant more susceptible to hydrogen peroxide and less virulent (Fernandez and Wilson 2014). Similarly, GR deletion in *C. albicans* increased the sensitivity to hydrogen peroxide and macrophage attack, and reduced virulence in both moths and mice (Tillmann et al. 2015). Finally, glutathione peroxidase (*GPx*) is also important for fungal virulence. There are multiple *GPx* homologs in fungi (Inoue et al. 1999), with *GPx3* likely the major contributor to virulence (Missall et al. 2005). *S. cerevisiae GPx3* mutants were more sensitive to oxidative stress (Thorpe et al. 2004, Kho et al. 2008), and *GPx3* mutants were less virulent in the plant pathogens *Alternaria alternata* (Yang et al. 2016a) and *M. oryzae* (Huang et al. 2011).

Other functions of glutathione include the detoxification of endogenous metabolites, such as formaldehyde, and sequestration of heavy metals (Pócsi et al. 2004). In particular, glutathione is associated with tolerance of the heavy metal cadmium (Cd). Glutathione can remove Cd by either forming a complex with Cd directly or by serving as a precursor for a phytochelatin complex, before exportation to a vacuole (Li et al. 1996, Cobbett and Goldsbrough 2002). Exposure to Cd increases *GCL* expression and a corresponding increase in glutathione in *S. cerevisiae* (Vido et al. 2001) and *Laccaria bicolor* (Khullar and Sudhakara Reddy 2019). *GCL* mutants display increased susceptibility to Cd (Mutoh and Hayashi 1988, Glaeser et al. 1991, Gutiérrez-Escobedo et al. 2013, Khullar and Sudhakara Reddy 2019), as do fungi exposed to buthionine sulfoximine (BSO), a chemical inhibitor of GCL (Clemens et al. 1999, Prévéral et al. 2006). Glutathione also helps to protect fungi against heat shock (Sugiyama et al. 2000, Sato et al. 2009), and increases resistance to antifungals such as fluconazole (Maras et al. 2014). Thus, the glutathione pathway is an attractive target for the development of novel antifungal therapies. In this study, I explored the function of glutathione and its potential as a virulence factor in *B. dendrobatidis* through *GCL* expression analysis and glutathione quantification; and by examining the effect of glutathione levels on tolerance of oxidative, heavy metal, fungicidal, osmotic and heat stress.

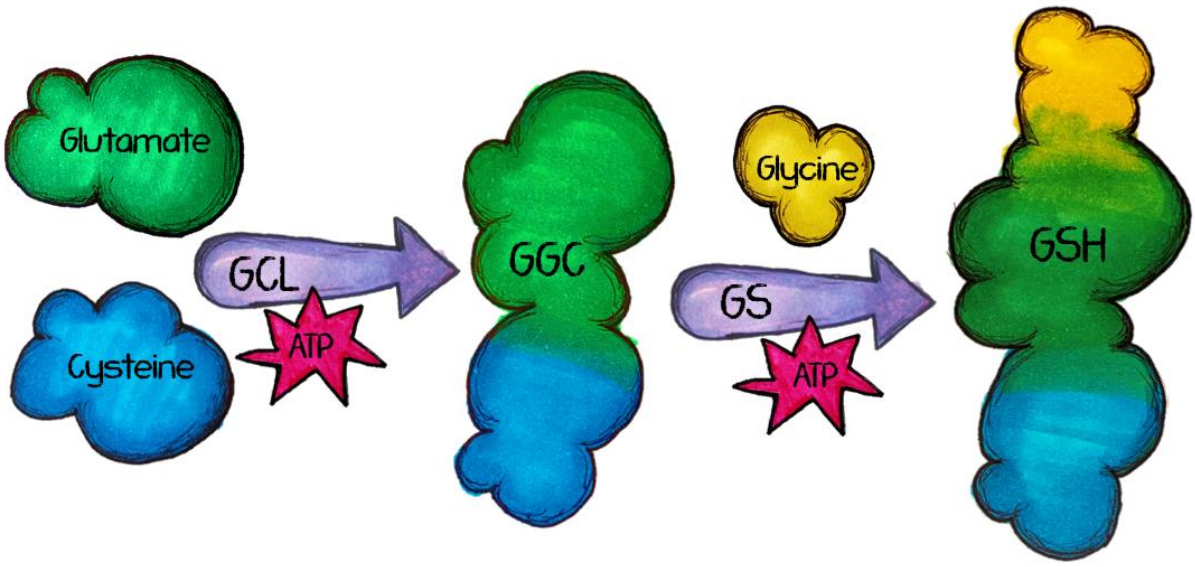


Figure 3.1 Simplified synthesis of glutathione. Synthesis of glutathione (GSH) involves two enzymes: glutamate cysteine ligase (GCL) and glutathione synthase (GS)**.** The GCL catalyses the formation of γ-glutamylcysteine (GGC) from glutamate and cysteine and is the rate-limiting step of GSH synthesis. The GS catalyses the formation of GSH from γ-glutamylcysteine via the addition of glycine. Both reactions require ATP.

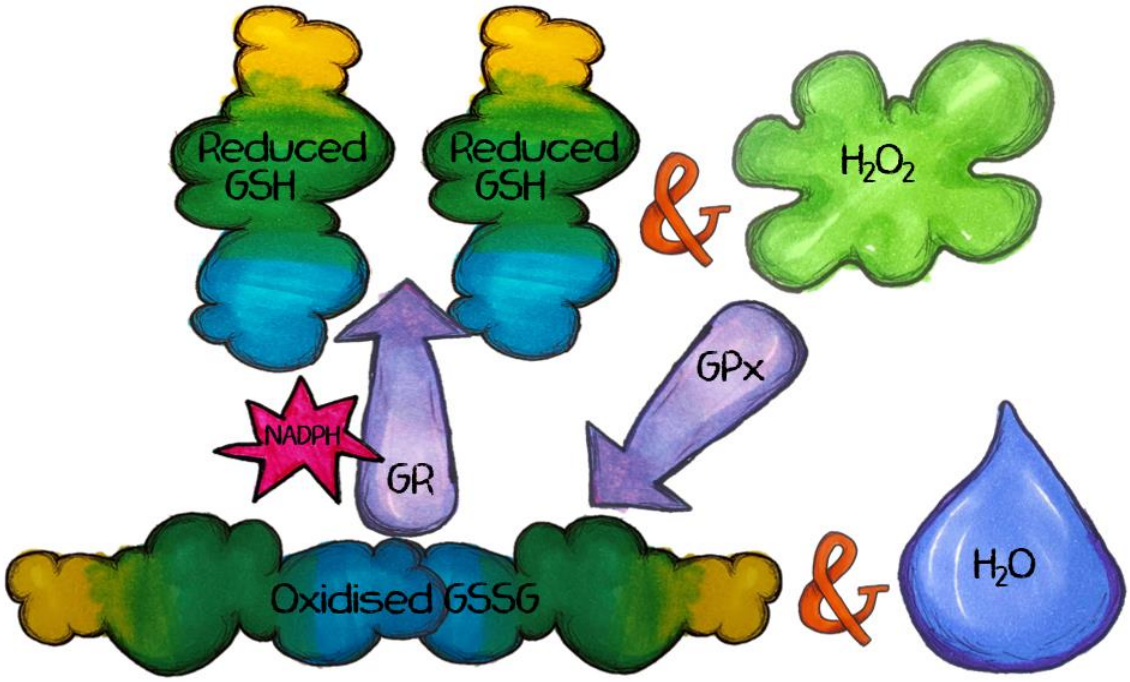


Figure 3.2 Glutathione-mediated neutralisation of hydrogen peroxide (H_2O_2). Reduced glutathione (GSH) is oxidised to the dimer glutathione disulfide (GSSG) during exposure to H_2O_2, resulting in detoxification of peroxide to water. This process can occur independently, or via glutathione peroxidase (GPx). Oxidised GSSG can be reduced to GSH via glutathione reductase (GR).

Methods:

Culture of B. dendrobatidis

B. dendrobatidis was grown in TGhL broth, and synchronised zoospores were collected as described in Chapter 2. The isolates used in this chapter were Yanchep-L.moorei-2019-RW, Werribee-L.dumerilli-2021-LB.

Quantification of glutathione gene expression via RT-qPCR

The relative expression of glutamate cysteine ligase (*GCL*), the first enzyme in glutathione synthesis, was determined by RT-qPCR. Total RNA was extracted using the Quick RNA Micro extraction kit (Zymo). The cells were pelleted, treated with 90 µL of RNA/DNA shield (Zymo), and subjected to 1 min of bead beating with 0.05 g of 0.1 mm and 0.5 mm silica beads (Daintree Scientific) to break up the chitin cell walls. RNA was extracted from the homogenised solution following manufacturer's instructions (Zymo), including the optional 15 min DNase treatment. RNA concentration and purity was measured using a Nanodrop spectrophotometer (Thermo Scientific), and cDNA synthesis was performed using the QuantiTect reverse transcription kit (Qiagen). The cDNA was diluted to 10 ng/µL before use. RT-qPCR was performed in a 20 µL reaction containing 1 ng cDNA template, 1x Rotor-Gene SYBR green master mix and 1 µM of each forward and reverse primer. The reactions were run in triplicate on a Rotorgene 6000 (Qiagen) with an initial activation step of 95°C for 5 min, followed by 35 cycles of a 5 s 95°C denaturation step and a 10 s 60°C annealing/extension step.

The *B. dendrobatidis* mRNA sequences for GCL (BATDEDRAFT_35498) and GR (BATDEDRAFT_21097) were obtained from the NCBI database using a protein blast search against well-characterised fungal species. Primers were designed in Geneious (version 8) based on the following criteria: similar melting point, small (<150 bp) amplicons, and low homology to non-target sequences. The *GCL* primer set (Forward: 5'-TCGTACTCATGGCATCGCTC and Reverse: 5'-AGTGCGAGCATCCTTGTTGA), produced a 127 bp product in cDNA and a 358 bp product in DNA. The *GR* primer set (Forward 5'-CTGGGCAGTAGGACGGAATG and Reverse 5'-CCCAAGAGCGTAAACACCCT) produced a 139 bp product in both cDNA and DNA (Appendix B, Figure 8.6). Reference gene primers *α-centractin* (Farrer et al. 2017), *APRT* (Verbrugghe et al. 2019), and *5.8s* (A. Starr personal communication) were used for normalisation.

Prior to use, the efficiency of each primer set was determined for *GCL*, *α-centractin* and *5.8s* using a four-point standard curve and calculated using Rotorgene software (Appendix B, Table 8.2). Primer efficiency for *APRT* was obtained from the literature (Verbrugghe et al. 2019). Each reference gene was also evaluated for stability using the algorithms: BestKeeper (Pfaffl et al. 2004), Ref Finder

(Xie et al. 2012), Delta Ct (Silver et al. 2006) and NormFinder (Andersen et al. 2004) (Appendix B, Table 8.3). The specificity of the primers was confirmed by low homology in BLAST searches and by the absence of amplification in cDNA from control uninfected cane toad skin (Appendix B, Section 1). The reference genes *α-centractin* and *APRT* were chosen for *in vitro* expression analysis. The reference genes *α-centractin* and *5.8s* were chosen for expression analysis in the cane toad infection model because *APRT* primers produced non-specific amplification in control (uninfected) cane toad tissue (Appendix B, Section 1).

GCL expression in early life cycle

The pattern of *GCL* expression during encystation and early sporangial development was determined by harvesting mRNA 0, 20, 32 and 44 h after zoospore release. For *in vitro* expression, synchronised zoospores were obtained from 7 d flask cultures following protocols for *B. salamandrivorans* (Robinson 2019). The zoospore solution was syringe filtered using a 10 µm isopore filter (Millipore) to remove any zoosporangia, and concentrated by centrifugation at 2500 x *g* for 5 min. A total of 10^6 zoospores in 500 µL broth were added per well in a 12 well culture plate (Sarstedt), and incubated at 20°C. An additional 3 x 10^6 zoospores were directly preserved in RNA/DNA shield for the 0 h time point. At the 20 h, 32 h and 44 h time points, cells were harvested from three wells. The zoosporangia were scraped from the well surface, centrifuged, and the pellet resuspended in 90 µL RNA/DNA shield and zirconia/silica beads. RNA extraction, cDNA synthesis and RT-PCR was performed as described above.

The pattern of *GCL* expression during initial host infection was determined by infecting juvenile cane toads (*Rhinella marina*) with *B. dendrobatidis*, and extracting mRNA from infected skin at 20, 32 and 44 h. Newly metamorphosed toads, measuring between 9-15 mm were collected from Anderson Park, Townsville, Australia (-19.292402, 146.787219) (James Cook University Ethics approval: A2702). For each experiment, six toads were held together in a shallow container and exposed to 2 x 10^7 zoospores in 3 mL TGhL at 21°C for 5 h (Appendix B, Figure 8.5). After inoculation, the animals were gently rinsed with water to remove any unencysted zoospores, and placed in a new plastic enclosure with damp paper towel, at 20°C. At each time point (20, 32, and 44 h), two animals were euthanised by 30 min exposure to 1% tricaine methanesulfonate (MS222) (Acros Organics). The ventral pelvic patch skin (thighs and lower abdomen) was removed (Appendix B, Figure 8.5) and cut into small pieces. The skin from both animals was combined in a 1.5 mL tube containing 0.1 mm and 0.5 mm zirconia and silica beads (Daintree scientific) and 150 µL DNA/RNA shield (Zymo), then subjected to 3 min bead beating (Biospec mini beadbeater). The homogenised tissue in DNA/RNA shield was then stored at -80°C until RNA extraction. RNA extraction, cDNA synthesis and RT-PCR was

performed as described above. The experiment was conducted three times to give a sample size of n=9.

The expression of *GCL* relative to the reference genes was calculated using the Pfaffl method (Pfaffl 2001). Relative quantity (RQ) was calculated using the equation $RQ= E^{\Delta CT}$, and the geometric mean of the two reference genes was used to determine fold change in GCL expression (Vandesompele et al. 2002). Fold change calculations were calculated as relative to the 20 h time-point, and fold change values converted to log2. One way ANOVA analysis (GraphPad Prism version 8) was used to determine if expression changed significantly during development.

Depletion of cellular glutathione by buthionine sulfoximine

GCL can be chemically inhibited using buthionine sulfoximine (BSO) (Griffith and Meister 1979). BSO can inhibit glutathione synthesis in other fungi at concentrations ranging from 2-6 mM (Courbot et al. 2004, Prévéral et al. 2006, Patsoukis and Georgiou 2007, Lis et al. 2012) but its effectiveness in *B. dendrobatidis* is unknown. As a pilot study to evaluate whether BSO depletes cellular glutathione, a mixed population (zoospores and zoosporangia) of *B. dendrobatidis* cells were added to a sterilised white microtiter plate and incubated for 8 h to allow adherence. BSO (Sigma) was prepared as a filter-sterilised 100 mM solution in water. The cells were exposed to BSO at concentrations ranging from 2-30 mM, with two replicates per concentration. After 20 h BSO exposure, the total glutathione levels were estimated using a luminescent assay (Promega GSH:GSSG Glo V6611), following the manufacturer's instructions for adhered cells. For all experiments, the relative amount of total glutathione in the BSO treated wells was calculated compared to the control. The strength of the linear relationship between BSO concentration and relative glutathione levels was calculated using GraphPad Prism (version 8). BSO at 30 mM resulted in the greatest depletion of total glutathione and therefore chosen for the remaining experiments.

The duration of glutathione depletion was established by measuring total glutathione after 6, 24 and 48 h of exposure to BSO. Zoospores (100 µL) were added to a 96 well plate (Nunc) at 8.5×10^4 zoospores per well and incubated overnight. Excess TGhL broth was removed, and cells were incubated with fresh broth containing either 30 mM BSO or a corresponding volume of water (control). At each time point, total glutathione was measured in three control and three BSO wells. Growth at each time point was estimated from the two-dimensional cell surface area in three representative images using Image J (Baviskar 2011). Relative glutathione was calculated relative to the control for that time point and adjusted for differences in cell growth caused by BSO exposure.

An additional experiment was conducted to determine if BSO inhibits glutathione synthesis in zoospores. A concentrated zoospore solution (500 µL) was added to two sterile 1.5 mL tubes. To

one tube, BSO was added to give a final concentration of 30 mM, and a corresponding amount of water was added to the control tube. After incubation at 20°C for 10 h, zoospores were pelleted by centrifugation and re-suspended in 50 µL water. Each sample was split into two 25 µL replicates and total glutathione levels in the BSO treated zoospores compared to the control were measured using the GSH Glo luminescent assay (Promega), following the protocol for cell suspension.

The effect of BSO treatment on *B. dendrobatidis* growth and development was monitored by incubating zoospores with BSO. Zoospores (100 µL) were added to a 96 well plate (Nunc) at 8.5 x 10^4 zoospores per well. Two replicate wells were treated with either 5 mM or 30 mM BSO, and an additional two wells with no BSO (control). After 5 days, the wells were observed and photographed. Additional replicates were included in which the BSO was removed on day 2 and replaced with TGhL broth to determine if cells recovered after BSO treatment.

Stress assays:

Sublethal levels of various stressors were first determined by MIC experiments (Appendix B, Figure 8.9). Chemical stressors were freshly prepared in water and filter sterilized (see Appendix B, section 3). To evaluate changes in the glutathione pool in response to stress, zoospores (100 uL) were added to 96 well plates (Nunc) at 8.5 x 10^4 zoospores per well and incubated at 20°C. After 18 h, cells were exposed to each stressor (Table 3.1). After 24 h of stress exposure, cells were photographed to estimate growth before lysis and glutathione measurement. Growth of cells exposed to each stressor was estimated from the two-dimensional cell surface area in three representative images using Image J (Baviskar 2011). The GSH Glo luminescent assay (Promega) was used to measure reduced and oxidised glutathione in three replicate wells. Cells were lysed and transferred to a white luminescent plate following the manufacturer's instructions for adherent cells. Glutathione levels in stressed cells were calculated as relative to the untreated control, after adjusting for differences in cell growth caused by stress exposure. The ratio of oxidised and reduced glutathione was calculated using the relative luminescence units following manufacturer's instructions. Glutathione levels in stressed cells were compared the control using T-tests (GraphPad Prism).

To investigate the mechanisms underpinning changes in total glutathione and glutathione ratio, expression of the key genes involved in glutathione synthesis (GCL) and glutathione recycling (GR) were monitored in cells exposed to stress. Zoospores (500 µL) were placed in 12 well plates (Nunc) at approximately 1 x10^6 zoospores per well and incubated at 20°C. After 24 h to allow encystation, cells were exposed to stress for 12-18 h. RNA extraction, cDNA synthesis and qRT-PCR were performed as described earlier.

Chapter 3: Role of glutathione

To evaluate the role of glutathione for stress tolerance, glutathione was either depleted with BSO or supplemented with additional GSH in cells before stress exposure. Zoospores were placed in 96 well plates at 8.5 x 10^4 zoospores per well and incubated at 20°C. After 18 h, cells were treated with either 30 mM BSO for 6 h, or 2 mM glutathione for 3 h before stressor exposure. GSH (Sigma) was prepared fresh as a filter-sterilised 100 mM stock in water, and the appropriate concentration for supplementation was determined by MIC experiments (Appendix B, Figure 8.11). Growth and viability of cells was measured 48 h after stress exposure using methylene blue as described in Chapter 2. Two-way ANOVA analysis (GraphPad Prism) was used to determine if the interaction between stress and BSO or GSH was significantly different to the control.

Table 3.1 Sub-lethal levels of stressors

Stress	Conditions	Rationale
Oxidative	0.2 mM Hydrogen peroxide (H_2O_2)	Proxy for host immune response (Froese et al. 2005)
Heavy metal	0.04 mM Cadmium (Cd)	Some heavy metals inhibit *B. dendrobatidis* growth (Threlfall et al. 2008, Boisvert and Davidson 2011)
Fungicidal	0.1 µg/mL Terbinafine hydrochloride (TBF)	Used as a chytridiomycosis treatment at 0.1 mg/mL (Bowerman et al. 2010)
Osmotic	0.1 M Sodium chloride (NaCl)	*B. dendrobatidis* is sensitive to NaCl (Stockwell et al. 2012), and increasing habitat salinity has been suggested to provide refugia from chytridiomycosis (Stockwell et al. 2015)
Heat	30°C for 4 h (Heat)	*B. dendrobatidis* occurs in temperate climates, with optimal growth at 15-25°C, and frogs subjected to daily 4 h pulses of 29°C can clear infection (Greenspan et al. 2017)

Results

Patterns of GCL expression

In the *in vitro* experiments, *GCL* expression remained stable as *B. dendrobatidis* zoosporangia developed (Figure 3.3) (p=0.275, F=0.7668 [2,6], R2=0.3492). The mRNA levels (of both *GCL* and the reference genes) in zoospores was very low and has been excluded from this analysis due to issues with the limit of detection (see Appendix B-Section 2), therefore the fold change was calculated relative to the 20 h time-point. Expression of *B. dendrobatidis GCL* did not appear to be upregulated during infection of cane toads compared to *in vitro* (Appendix 3-Table 8.4), although the accuracy of the results was limited by the very low abundance of *B. dendrobatidis* mRNA in the sample (Linear CT >29).

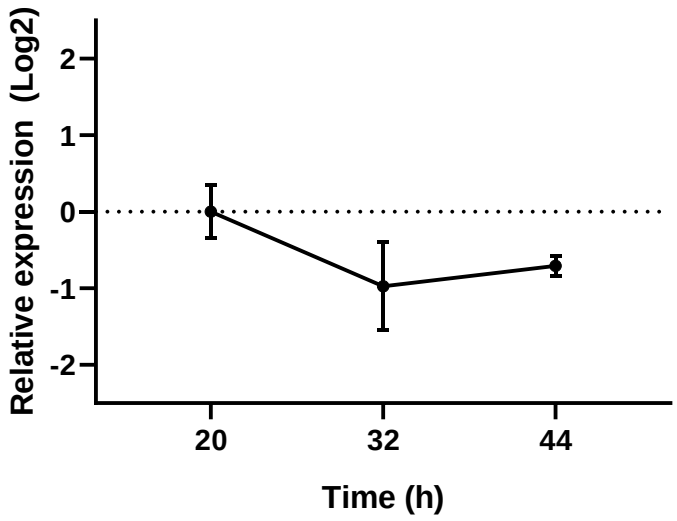


Figure 3.3 GCL expression *in vitro*. Relative *GCL* was stable during early *B. dendrobatidis* development *in vitro* (p=0.275, F=0.7668 [2,6], R^2=0.3492). Fold change was calculated as relative to the 20 h time-point N=9, mean and SEM

Depletion of cellular glutathione via BSO

The addition of BSO to growth media caused a dose dependent decrease in total glutathione levels after 20 h. The greatest decrease was seen in cells exposed to 30 mM BSO (70% decrease in total glutathione) (Figure 3.4), and this concentration was chosen for further experiments. The effect of BSO on zoosporangia lasted for at least 48 h (Figure 3.5). However, BSO appeared to have only a minor effect on zoospores, with <20% decrease in total glutathione after 10 h (Figure 3.6). Incubation with BSO did not prevent zoospore encystation. Exposure to 5 mM BSO only had a minor effect, whereas exposure to 30 mM slowed growth and prevented maturation of the zoosporangia.

Removal of the BSO partially restored growth and ability to form discharge tubes and release zoospores (Figure 3.7).

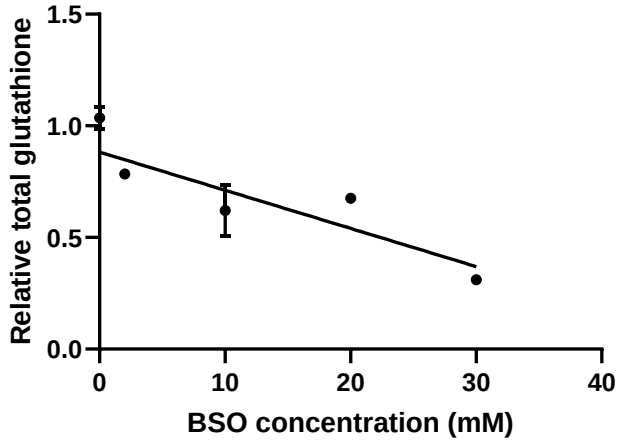


Figure 3.4 Total glutathione levels in a mixed stage culture after BSO treatment. BSO causes a dose-dependent decrease in total glutathione levels in zoosporangia after 20 h incubation. Levels are relative to the untreated control, but not adjusted for growth. N=9, mean and SD.

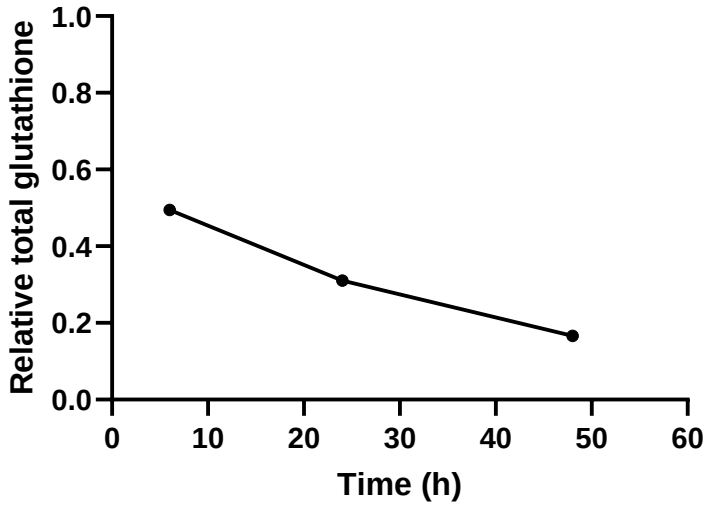


Figure 3.5 Total glutathione levels in zoosporangia after 6, 24 and 48 h 30 mM BSO treatment. BSO exposed cells had 51% less total glutathione at 6 h (p=0.0018, t=7.398, df=4), 69% less at 24 h (p=0.0001, t=55.62, df=4), and 84% less at 48 h (p=0.0004, t=10.70, df=4) compared to the untreated control at the same timepoint after adjustment for growth. N=18, mean and SD.

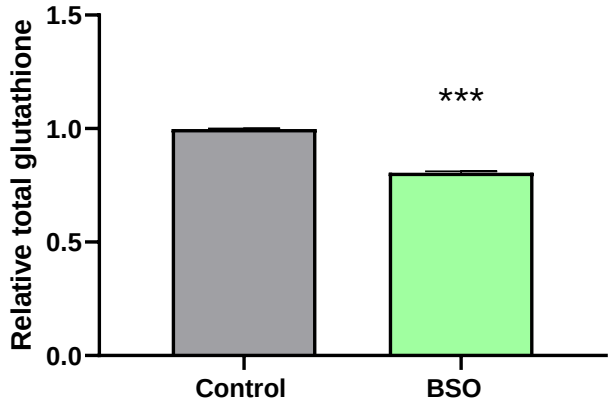


Figure 3.6 Total glutathione in zoospores after 10 h 30 mM BSO treatment. Treatment with 30 mM BSO caused a 20% depletion in total glutathione levels in zoospores after 10 h (p=0.008, t=35.40, df=2). N=4, mean and SD.

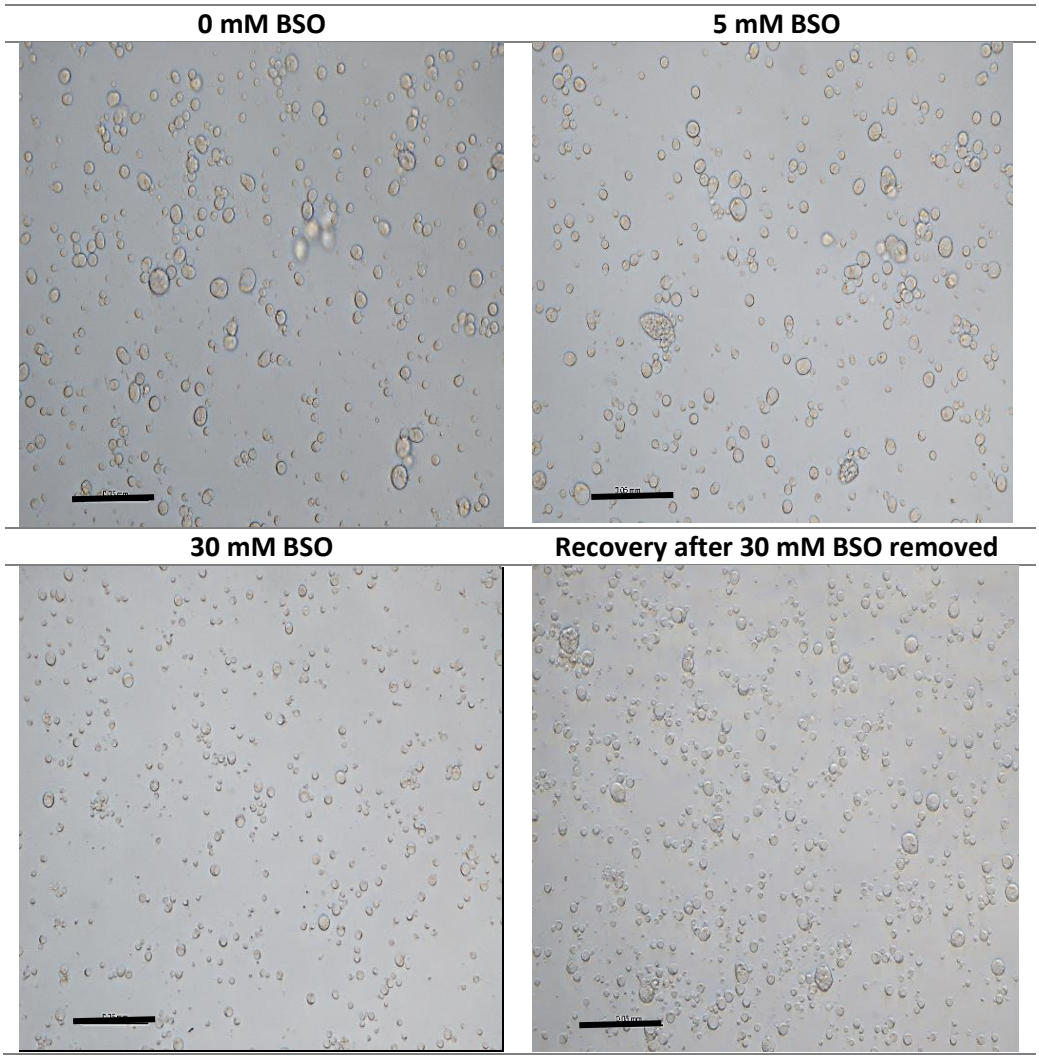


Figure 3.7 Effect of BSO on *B. dendrobatidis* lifecycle. These representative images illustrate the differences in cell size and coverage between the treatments. The refractile circular bodies are the zoosporangia. Continual exposure to 30 mM BSO prevented maturation of zoosporangia. This effect was reduced when BSO was removed from growth media. The scale bar is 0.05 mm and magnification is 400x.

Glutathione levels in response to stress exposure.

Glutathione levels varied after exposure to different stressors (Figure 3.8). Treatment with H_2O_2 decreased total glutathione levels by 42% ($p=0.0008$, $t=8.992$, $df=4$), whereas cells exposed to Cd had a 93% increase in glutathione levels compared to the control ($p= 0.0001$, $t=20.20$, $df=4$). Exposure to TBF, NaCl and heat caused slight changes to the total glutathione pool. The ratio of reduced (GSH) and oxidised (GSSG) glutathione also changed depending on the type of stress (Figure 3.9). Control untreated zoosporangia had an average GSH:GSSG ratio of 6.44. Cells exposed to H_2O_2

(p=0.016, t=2.894, df=16), Cd (p=0.0004, t=4.438, df=16) and NaCl (p=0.0001, t=7.322, df=16) had a slight, but significant increase in GSH to GSSG ratio, whereas cells exposed to heat stress had significantly lower GSH to GSSG ratio (p=0.0001, t=14.68, df=16). The GSH:GSSG ratio of cells exposed to TBF fungicide was not significantly different to the control. Unstressed zoospores had a GSH:GSSG ratio of 5.99, which is slightly lower than that of zoosporangia.

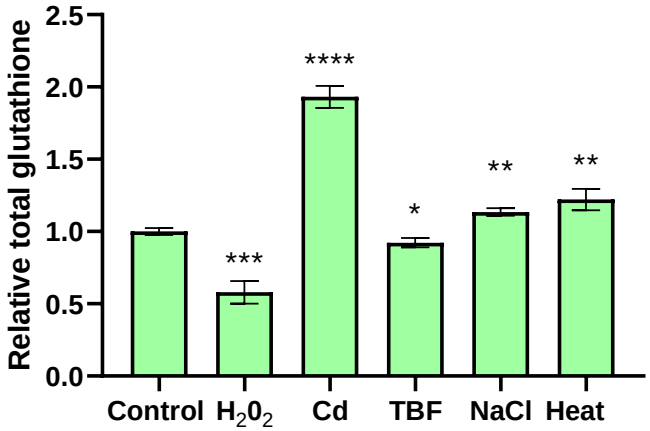


Figure 3.8 Relative total glutathione (GSH + GSSG) levels in *B. dendrobatidis* 24 h after stress exposure. Cells exposed to 0.2 mM H_2O_2, had 42% less total glutathione (p=0.0008), whereas cells exposed to 0.04 mM Cd had 93% more total glutathione (p= 0.0001). Treatment with 0.01 µg/mL TBF, 0.1 M NaCl and heat slightly altered the total glutathione levels compared to the control (8% less, 13% more and 12% more P=0.0279, 0.0027, 0.0077 respectively). Total glutathione was measured using a commercial luminescent assay. N=18, mean and SD.

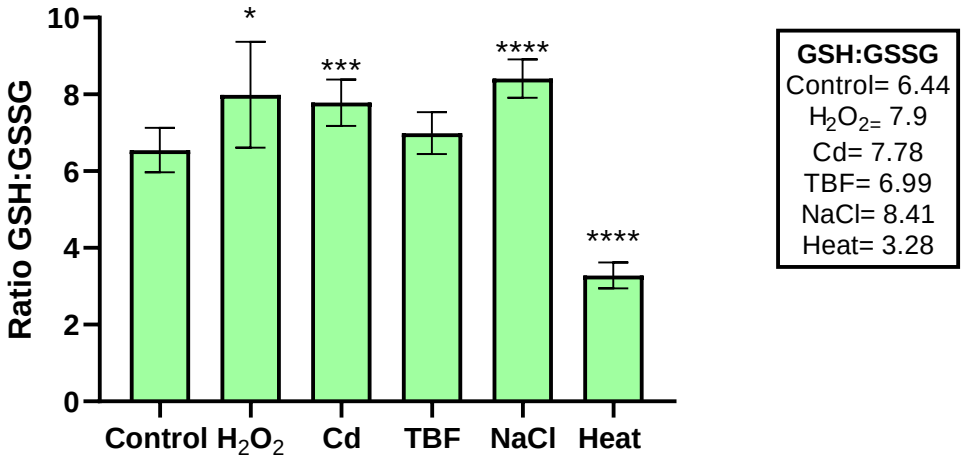


Figure 3.9 GSH:GSSG ratios in *B. dendrobatidis* after 24 h stress exposure. Heat exposure reduced the GSH:GSSG ratio by approximately 50% (p=0.0001). Treatment with 0.2 mM H_2O_2 (p=0.016), 0.04 mM cadmium (0.0004) and 0.1 M NaCl (p=0.0001) slightly increased the GSH:GSSG ratio compared to the control. N= 18, mean and SD.

Glutathione gene expression after stress exposure

The expression of glutathione-associated genes (*GCL, GR*) was measured in cells exposed to H_2O_2, Cd and heat. Cells exposed to heat had a slight but significant downregulation in expression of the glutathione synthesis gene, *GCL* (p=0.024, t=9.601, df=3) (Figure 3.10). There was no significant effect of Cd or H_2O_2 exposure on the expression of *GCL*. In terms of glutathione recycling, cells exposed to both H_2O_2 (p=0.0025, t=6.737, df=4) and Cd (p=0.0119, t=5.485, df=3) had slightly higher glutathione reductase (*GR*) expression compared to the control. There was no significant change in *GR* expression in cells exposed to heat (Figure 3.11).

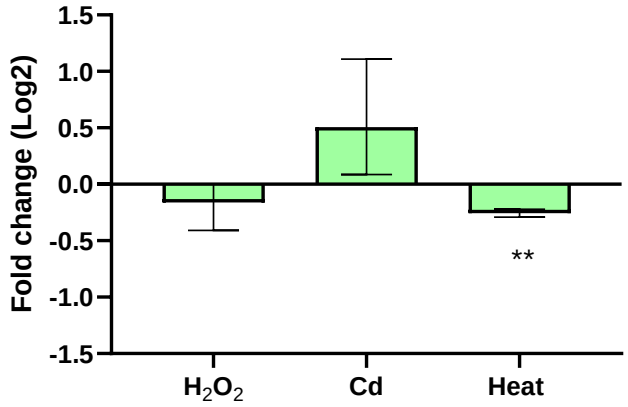


Figure 3.10 Fold-change glutamate cysteine ligase (*GCL*) expression after stress exposure compared to untreated control. There was a slight but significant reduction in *GCL* expression in cells exposed to heat (p=0.024). There was no significant difference in the expression of *GCL* in cells exposed to H_2O_2 or Cd. N=11, mean and range.

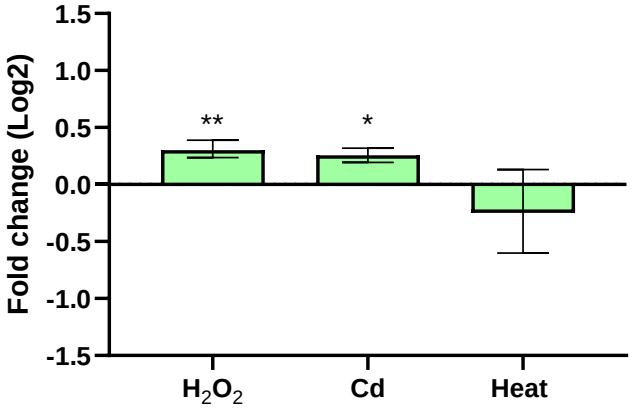


Figure 3.11 Glutathione reductase (*GR*) expression after stress exposure compared to untreated control. There was a slight but significant increase in *GR* expression in cells exposed to H_2O_2 (p=0.002) and Cd (p=0.011). There was no significant difference in *GR* expression in cells exposed to heat. N=11, mean and range.

Chapter 3: Role of glutathione

Stress tolerance with glutathione depletion or supplementation

The role of glutathione in stress tolerance was further examined under conditions of glutathione depletion (via BSO) or glutathione (GSH) supplementation. First, growth studies were performed with a range of BSO and GSH concentrations to determine the background effect on *B. dendrobatidis* growth (Appendix B-Section 4). Cells exposed to 30 mM BSO experienced minor growth inhibition (Appendix B-Figure 8.10), but this concentration was necessary for efficient glutathione depletion. Exposure to glutathione at 2.5 mM and above caused growth inhibition (Appendix B, Figure 8.11). Therefore, depletion and supplementation experiments were performed with 30 mM BSO and 2 mM GSH.

Glutathione depletion further attenuated the growth of cells exposed to 0.04 mM Cd (63% less growth than cadmium alone) (Figure 3.12). Taking into account the negative effects of BSO, the interaction between BSO and Cd stress was significantly different to the control ($p=0.017$, $F=8.538$ [1,9]). Methylene blue staining indicated the cells were still viable after Cd stress and BSO treatment (Appendix B, Figure 8.12), but microscopy revealed that the cells were small and contained unusually large vacuoles (Figure 3.14). Supplementation with 2 mM glutathione prior to the addition of the stressor partially rescued Cd stressed cells exposed to BSO (Figure 3.14).

Compared to the untreated control, depletion of available glutathione via BSO did not affect the sensitivity to H_2O_2 ($p=.829$, $F=0.049$ [1,8]), TBF ($p=0.543$, $F=0.403$ [1,8]), NaCl ($p=0.570$, $F=0.350$ [1,8]) or heat stress ($p=0.060$, $F=0.297$ [1,8]) (Figure 3.12).

Supplementation with 2 mM glutathione did not cause a significant change in growth in the control ($p=0.433$, $t=0.811$, $df=12$). Glutathione supplementation increased the tolerance to cadmium stress ($p=0.033$, $F=5.014$ {1,26]), but decreased the tolerance of NaCl ($p=0.045$, $F=4.396$ [1,26] and heat ($p=0.011$, $F=7.438$ [1,26]) (Figure 3.13). Most striking was the effect of glutathione on heat tolerance, with obvious inhibition in cells that were pre-treated with glutathione before heat exposure (Figure 3.15).

Additional results can be found in Appendix B, including the response of stressed cells to various BSO (Figure 8.13) and glutathione concentrations (Figure 8.14).

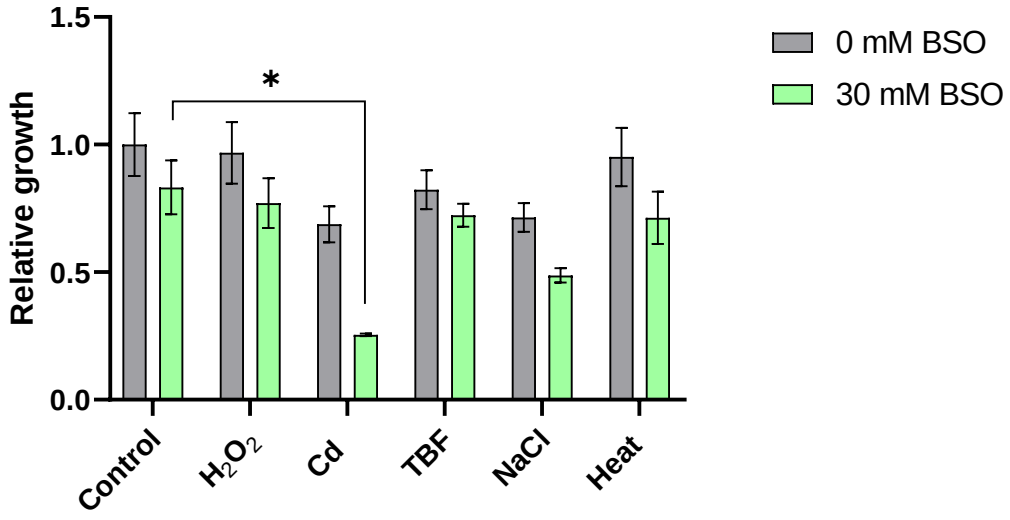


Figure 3.12 Relative growth of stressed *B. dendrobatidis* zoosporangia with and without glutathione deprivation (via 30 mM BSO). The interaction between glutathione depletion (via BSO) and Cd stress was significantly different to control cells (p=0.017, F=8.538 [1,9]), whereas glutathione depletion had no impact on sensitivity to H_2O_2, TBF, NaCl or heat (30°C). Cell growth was measured using the methylene blue assay, and reported relative to the untreated control. N=36, mean and SD.

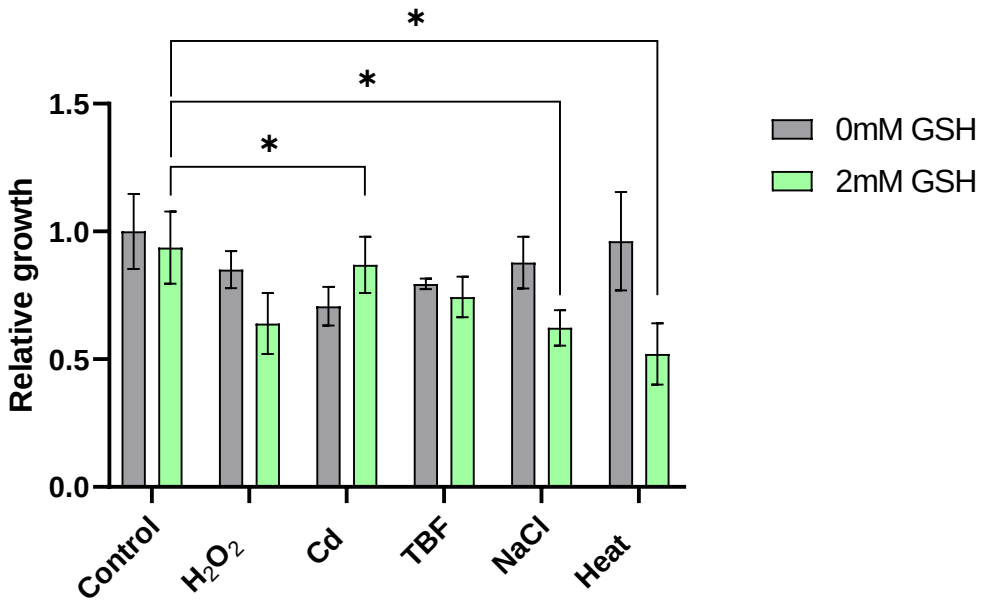


Figure 3.13 Relative growth of stressed *B. dendrobatidis* zoosporangia under GSH supplementation compared to stress alone. Glutathione supplementation increased growth in 0.04 mM Cd stressed cells (p=0.033, F=5.014 {1,26]), but decreased growth in 0.1 M NaCl (p=0.045, F=4.396 [1,26]) and 30°C heat (p=0.011, F=7.438 [1,26]) stressed cells. Cell growth was measured using the methylene blue assay and reported relative to the untreated control. N=48, mean and SD.

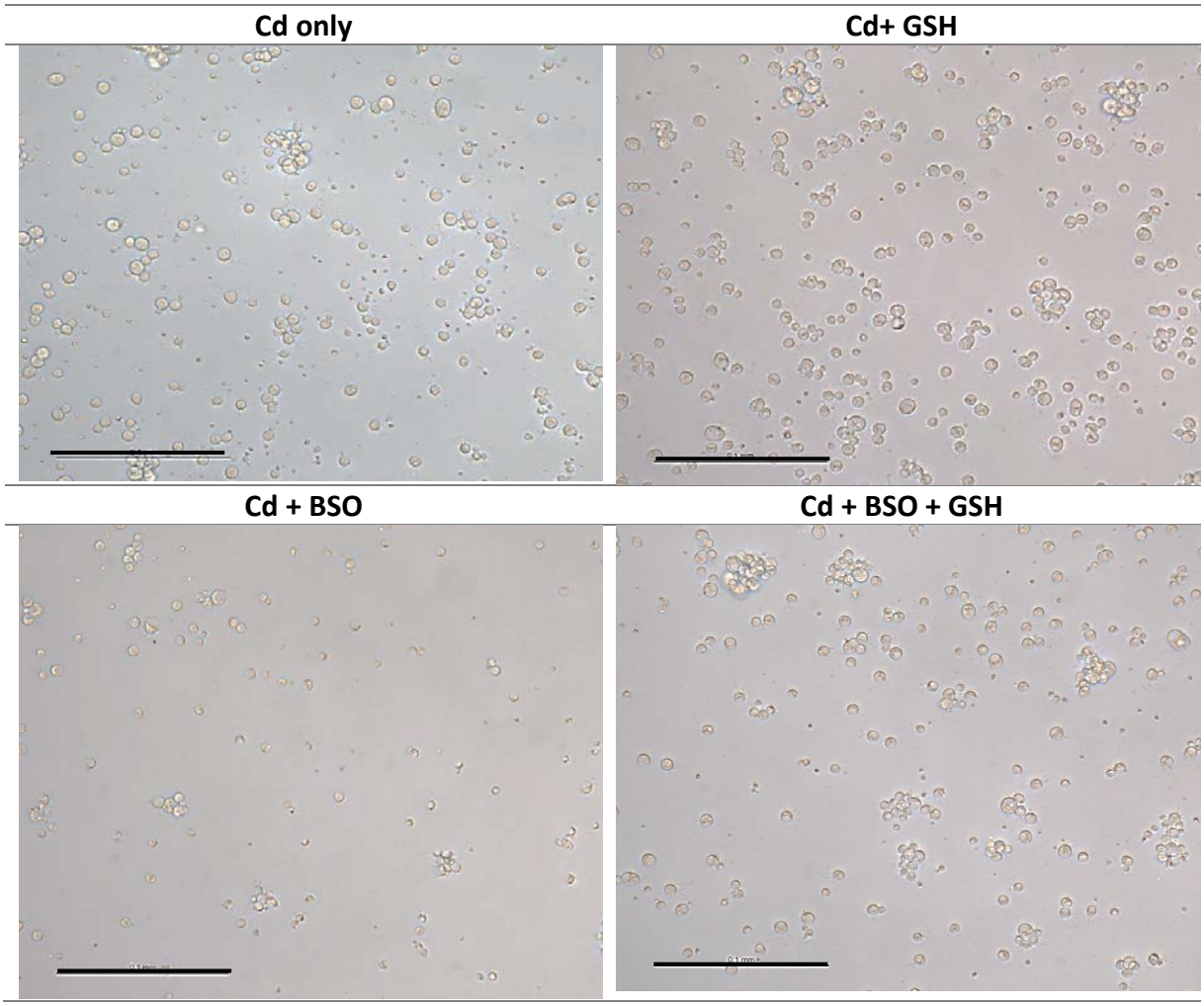


Figure 3.14 Microscopy of Cd stressed *B. dendrobatidis* zoosprorangia under GSH depletion (30 mM BSO) or supplementation (2 mM GSH), or a combination of both. These representative images illustrate the differences in cell size and coverage between the treatments. The refractile cicular bodies are the zoosporangia. Zoosprorangia treated with BSO before Cd exposure had visibly impaired growth compared to the cadmium-only control and glutathione supplementation. Glutathione supplementation partially rescues the inhibitory effect of BSO. The scale bar is 0.1 mm and magnification is 400x.

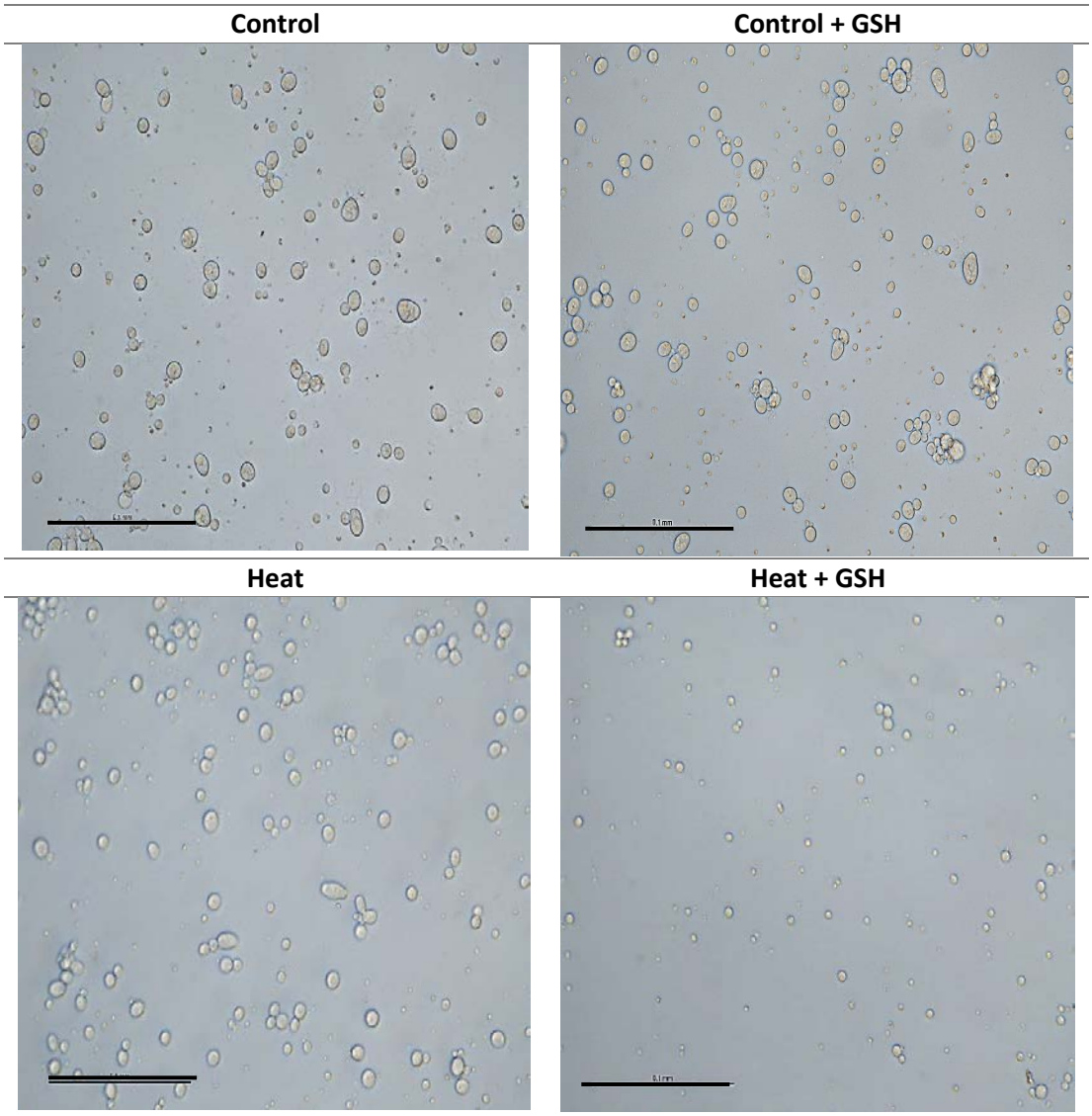


Figure 3.15 Microscopy of heat-stressed *B. dendrobatidis* zoosporangia under glutathione supplementation (2 mM GSH). These representative images illustrate the differences in cell size and coverage between the treatments. The refractile circular bodies are the zoosporangia. Zoosporangia treated with GSH before heat exposure had visibly impaired growth compared to the heat-only control. This inhibition by GSH was not observed in the absence of heat (control). The scale bar is 0.1 mm and magnification is 400x.

Discussion:

The GCL inhibitor, BSO, effectively depleted glutathione levels in *B. dendrobatidis* zoosporangia by 84% after 48 h. However, a concentration of 30 mM was required to achieve sufficient glutathione depletion, which is much higher than the 2-6 mM commonly used in other fungi (Courbot et al. 2004, Prévéral et al. 2006, Lis et al. 2012). Exposure to 30 mM BSO did not prevent zoospores from encysting and developing into zoosporangia, however, these zoosporangia grew slowly and failed to develop discharge tubes and produce more zoospores during the timeframe they were monitored. This finding suggests that glutathione is important for completion of the lifecycle in *B. dendrobatidis*; and establishes glutathione biosynthesis as a potential virulence factor and target for future gene knock down approaches.

It is interesting that BSO appeared mostly ineffective against the infective, free-living zoospore life stage. Preliminary experiments also suggested that the GSH:GSSG ratio in zoospores is lower than zoosporangia, meaning they contain proportionally more oxidised glutathione. Another interesting finding was the very low levels of mRNA in zoospores. The levels of *GCL*, *α-Centractin*, *APRT* and *Cystn1* were almost undetectable in zoospores compared to zoosporangia (Appendix B, Figure 8.8). Conversely, levels of the ribosomal subunit 5.8s remained stable between the two life stages (Appendix B, Figure 8.7), indicating adequacy of RNA extraction. Due to the lack of transcriptional complexity and the ineffectiveness of transcription blockers on zoospores (Rosenblum et al. 2008), it has been suggested that chytrid zoospores are not transcriptionally active and instead they may contain dormant ribosomes and provisioned (pre-packaged) mRNA (Léjohn and Lovett 1966, Rosenblum et al. 2008, Laundon et al. 2022). Further work is required to understand the complexity of zoospores and the importance of the glutathione system in this infective life stage.

This study also explored the various functions of glutathione in *B. dendrobatidis* with particular emphasis on the role of glutathione in the tolerance of oxidative, heavy metal and heat stress.

Hydrogen peroxide tolerance

Total glutathione levels decreased in response to H_2O_2, suggesting an involvement of glutathione in oxidative stress tolerance in *B. dendrobatidis.* However, as H_2O_2 did not impact *GCl* expression, the decrease in total glutathione is likely not due to changes in glutathione synthesis and may rather reflect consumption during peroxide detoxification. The decrease in total glutathione is consistent with the oxidative stress response of other fungi such as *Candida albicans* (Maras et al. 2014) and *Ashbya gossypii* (Kavitha and Chandra 2014). However, in these species, oxidative stress induced a corresponding decrease in the GSH:GSSG ratio, which was not observed in *B.*

dendrobatidis. This difference may be due to the limited H_2O_2 concentrations used in this experiment, or could also be due to an increased GR activity, resulting in rapid conversion of GSSG (produced during H_2O_2 neutralisation) back into GSH. In other fungi species, exposure to H_2O_2 has been shown to upregulate GR activity (Jamieson et al. 1996) or *GR* expression (Tillmann et al. 2015). I found a slight increase in *GR* expression, which may allow maintenance of the GSH:GSSG ratio in *B. dendrobatidis* under oxidative stress.

Unexpectedly, glutathione depletion (via BSO treatment) did not increase the sensitivity of *B. dendrobatidis* to 0.2 mM H_2O_2, unlike reports from other fungi (Grant et al. 1996, Baek et al. 2004, Yadav et al. 2011). It is possible that the inhibition of GCL by BSO is partially mitigated by the H_2O_2-mediated release of GSH from protein reservoirs, such as enolase (Claytor 2020). Alternative antioxidant mechanisms, such as superoxide dismutases, catalases, or the thioredoxin system, may also act without glutathione to detoxify H_2O_2 (Grant et al. 1998, Sato et al. 2009, Fréalle et al. 2013, Brown and Goldman 2016). In addition, expression of *GCL* (first enzyme in glutathione biosynthesis) did not increase during the early development of zoosporangia, nor did it appear to be upregulated *in vivo*. Together these results suggest that glutathione might not be an important component of the ability of *B. dendrobatidis* to evade host oxidative stress. Animal infection studies with glutathione deficient cells are required to confirm whether glutathione plays any role in virulence. Since glutathione depletion via BSO is not very effective on the infective zoospore stage, the development of other methods (such as RNAi) to target glutathione biosynthesis in *B. dendrobatidis* could test the function of glutathione during infection.

Cadmium tolerance

In contrast, glutathione is important for Cd resistance in *B. dendrobatidis.* Exposure to Cd doubled the relative total glutathione pool compared to unstressed cells. This response is consistent with that seen in other fungal species (Vido et al. 2001, Khullar and Sudhakara Reddy 2019). In other fungi, Cd exposure increases *GCL* expression, although a significant increase in GCL expression was not detected in this study. It may be that the increased glutathione levels under Cd stress creates a feedback loop in *B. dendrobatidis*, which further inhibits glutathione synthesis (Wheeler et al. 2002). Glutathione depletion (via BSO) attenuated growth in Cd-treated cells, an effect that was reversed by glutathione supplementation. Unlike oxidative stress, Cd detoxification removes GSH from the available pool when the glutathione conjugates irreversibly with Cd and is exported to the vacuole (Li et al. 1996, Li et al. 1997), making it more susceptible to glutathione depletion. Therefore, BSO-treated *B. dendrobatidis* cells are more sensitive to Cd than H_2O_2, as GSH is consumed rather than replenished from GSSG. Further immunohistochemistry studies could localise Cd in *B. dendrobatidis*, to confirm sequestration within the vacuole, as seen with studies in wheat (Gao et al. 2015).

Heat

In other fungi, heat stress can increase cellular respiration, resulting in ROS accumulation and an oxidative stress response (Sugiyama et al. 2000, Abrashev et al. 2008). In *B. dendrobatidis*, while heat slightly increased the relative level of total glutathione, it halved the GSH:GSSG ratio. The low GSH:GSSG ratio suggests that GSH oxidation was part of the heat stress response, as also seen in *Aspergillus nidulans* (Sato et al. 2009). Unlike cells under H_2O_2 stress, there was no increase in *GR* expression. This might explain why the GSH:GSSG ratio remains low in heat stressed compared to H_2O_2 stressed cells, as there is no compensatory increase in GR activity to replenish GSH from GSSG. Interestingly, the addition of 2 mM exogenous glutathione increased the sensitivity of *B. dendrobatidis* to heat. Amphibian skin contains glutathione and other antioxidants thought to protect against environmental stressors such as UV (Yang et al. 2016b). Thus, glutathione might have a protective effect against *B. dendrobatidis* infection in amphibian skin. Concentrations of exogenous glutathione over 2.5 mM were strongly inhibitory to *B. dendrobatidis*, similar to reports of toxicity in *A. nidulans* (Bakti et al. 2017). Future work should examine the glutathione content of amphibian skin to determine if differences in concentration correlate with disease susceptibility. Of particular interest could be the freeze tolerant wood frog, *Lithobates sylvaticus,* which contains elevated glutathione levels (to protect against freezing) (Joanisse and Storey 1996) and has not undergone widespread chytridiomycosis-related declines (Gahl et al. 2011).

Tolerance of other stressors

NaCl stress slightly increased the GSH:GSSG ratio and the total glutathione levels in contrast to a previous study in the thermophilic fungus *Thermomyces lanuginosus,* where NaCl decreased the total glutathione pool (Jepsen et al. 2008). Surprisingly, supplementation with glutathione increased the susceptibility to NaCl. There is limited literature on the role of glutathione in osmotic stress in fungi, but NaCl treatment upregulated *GR* in *S. pombe* (Lee et al. 1997). In the plant *Arabidopsis*, the addition of exogenous glutathione improved NaCl tolerance, while NaCl exposure increased glutathione levels (Cheng et al. 2015), and both caused global changes in gene expression. Thus, further studies could assess the effect of varying NaCl concentrations on glutathione levels and growth, to assess whether synergistic glutathione and NaCl exposures could be used as a treatment against chytridiomycosis.

Finally, although total GSH levels slightly decreased after treatment with the antifungal drug terbinafine (TBF), there was no impact the GSH:GSSG ratio nor was susceptibility impacted by varying GSH levels. These results indicate that the sensitivity of *B. dendrobatidis* to TBF is not modulated by glutathione, unlike that reported for the antifungal drug fluconazole in *C. albicans (Maras et al. 2014).*

Chapter 3: Role of glutathione

Summary:

Glutathione is an important molecule for the life cycle of *B. dendrobatidis,* and for resistance to H_2O_2 and Cd. However, *GCL* expression did not increase during early zoosporangia development, and does not appear to be upregulated *in vivo*, suggesting a limited role in host invasion. Tolerance of oxidative, heavy metal, osmotic and heat stress all appear to involve the glutathione system, albeit in different ways. H_2O_2 decreased total glutathione levels, indicating a role for glutathione in peroxide clearance; although glutathione depletion did not increase H_2O_2 susceptibility, suggesting it may not be an essential virulence factor for evasion of host immune cells. Cd exposure increased total glutathione levels, and the susceptibility of *B. dendrobatidis* to Cd increased with glutathione depletion. Thus, glutathione likely reduces Cd toxicity by irreversibly binding the heavy metal and enabling its sequestration. Exogenous glutathione unexpectedly increased susceptibility to osmotic and heat stress. Modulating glutathione levels may be an attractive target for future therapeutics against chytridiomycosis, and exogenous glutathione could also have potential as a synergistic treatment with heat or salt.

Chapter 4 **Development of RNAi**

The overall goal of this thesis is to develop methods to reduce the virulence of *B. dendrobatidis*. Chapter 3 investigated glutathione as a putative virulence factor and glutathione synthesis as a candidate target for knockdown. Chapter 4 builds on this knowledge to explore whether RNA interference (RNAi) techniques can be used to knockdown the expression of the glutathione biosynthesis gene. RNAi is a tool that has been widely used to reduce pathogen virulence by targeting the expression of virulence genes. However, the utility of RNAi in modulating gene expression in *B. dendrobatidis* remains unexplored. In this Chapter, I optimised the design and delivery of siRNA to *B. dendrobatidis* cells and confirmed RNAi-mediated gene knockdown via RT-qPCR. The viability assay and cadmium phenotypes described in Chapters 2 and 3 were used to assess the efficacy of the gene knockdown methodology. Additional results from method optimisation experiments are included in Appendix C.

This Chapter has 3 aims:

1: Develop the RNAi methodology in *B. dendrobatidis* using siRNA for gene knockdown

2: Assess the efficacy of glutamate cysteine ligase (*GCL)* knockdown via RNAi, by measuring target mRNA, protein and glutathione levels

3: Explore the effect of *GCL* knockdown on the sensitivity of *B. dendrobatidis* to growth inhibition by cadmium.

Development of RNAi-mediated gene knockdown in *Batrachochytrium dendrobatidis*

Abstract

RNA interference (RNAi) is a valuable tool to manipulate and understand gene function. The insertion of rationally designed small interfering RNA (siRNA) leads to the destruction of homologous messenger RNA (mRNA), resulting in the "knockdown" of target gene expression. The development of RNAi technologies in *Batrachochytrium dendrobatidis* could be applied to elucidate virulence mechanisms, identify therapeutic targets, and may present a novel antifungal treatment option for chytridiomycosis. Here, I investigated whether siRNA can be used to manipulate gene expression in *B. dendrobatidis.* I designed two siRNA strategies with 3'-UU or 3'-dTdT modifications to target gamma-glutamylcysteine synthetase, the first rate-limiting step in glutathione synthesis. The siRNA was delivered to zoospores by electroporation, and mRNA levels were monitored over a 48 h period. Both types of siRNA triggered a ~50% reduction in target transcripts, with a maximal knockdown at 36-42 h. My results show that RNAi is possible in *B. dendrobatidis*, and that gene expression can be manipulated in this pathogen. However, knockdown of mRNA did not produce robust phenotypic changes, highlighting the need for further optimisation of siRNA delivery and target gene selection.

Introduction

RNA interference (RNAi) is a powerful gene silencing tool. It is especially valuable for species that are difficult to genetically modify, such as *B. dendrobatidis* (Rosenblum et al. 2010). RNAi harnesses a widespread natural phenomenon, found in most eukaryotes, which regulates both host and pathogen gene expression (Hannon 2002). The process is initiated by the introduction of double stranded RNA (dsRNA) sequences into the cytoplasm, resulting in transient reduction or "knockdown" of corresponding messenger RNA (mRNA). The core components of RNAi are the enzyme Dicer (Bernstein et al. 2001), Argonaute (Hammond et al. 2001) and RNA dependent RNA polymerase (RdRp) (Nishikura 2001). The presence of exogenous dsRNA in the cytoplasm triggers Dicer to cleave the dsRNA into smaller fragments, termed "small interfering RNA" (siRNA) (Zamore et al. 2000, Bernstein et al. 2001). ATP facilitates the siRNA unwinding (Nykänen et al. 2001), and the antisense strand (Martinez et al. 2002) is loaded onto an Argonaute protein (Hammond et al. 2001), forming an "RNA-induced silencing complex" (RISC) (Hammond et al. 2000). The siRNA then guides RISC to its complementary mRNA, which is cleaved by Argonaute (Liu et al. 2004), preventing translation (Zamore et al. 2000). RdRp creates additional siRNA from the cleaved mRNA, amplifying the RNAi process (Nishikura 2001) (

Figure ***1.3***). The RNAi pathway can be manipulated to knockdown target genes by introducing either dsRNA or siRNA that are complementary to a gene of interest. Gene silencing via RNAi can provide important functional genomic information (Kamath et al. 2003, Prawitt et al. 2004, Travella et al. 2006), and in addition it can be used as a therapeutic strategy to control infectious diseases. RNAi techniques have been developed in a variety of pests and pathogens, ranging from viruses (Krishnan et al. 2009) to insects (Vogel et al. 2018). In fungal pathogens, RNAi can reduce virulence and increase host survival. For example, siRNA-mediated knockdown of an important glyoxylate cycle gene reduced the virulence of *Penicillium marneffei*, and led to increased mouse survival (Sun et al. 2014). Plant hosts can be protected from fungal diseases by an innovative technique termed spray induced gene silencing (SIGS), where the siRNA or dsRNA is simply sprayed onto the plant surface (Koch et al. 2016). Hence RNAi could potentially be a valuable tool to protect amphibians from chytridiomycosis.

The RNAi pathway is widespread in fungi. The Chytridiomycota, including *B. dendrobatidis*, are the only fungal phyla in which RNAi has not yet been demonstrated (Moore 2009, Cairns et al. 2016). Importantly, the genome of *B. dendrobatidis* appears to lack one of the core RNAi components, RdRP (Farrer et al. 2017), so it is not known whether the RNAi pathway is functional in this species. Establishing if RNAi mediated knockdown can be manipulated in *B. dendrobatidis* will facilitate functional genomic studies and may even lead to novel antifungal strategies.

Here, I explore whether siRNA can induce gene silencing in *B. dendrobatidis* as evidence of a functional RNAi pathway in this organism. I used siRNA to target the glutathione synthesis gene (glutamate cysteine ligase, *GCL*); the first enzyme required for the biosynthesis of glutathione, and confirmed knockdown via RT-qPCR. A time course experiment was used to establish the duration of *GCL* mRNA knockdown. I then assessed the efficacy of knockdown by quantifying changes in GCL protein levels, glutathione levels, and tolerance to cadmium stress.

Methods

B. dendrobatidis culture

B. dendrobatidis was grown in TGhL broth and synchronised zoospores were collected as described in Chapters 2 & 3. The isolate used in this Chapter was Yanchep-L.moorei-2019-RW.

siRNA design

Glutamate cysteine ligase (*GCL*, BATDEDRAFT_35498) was targeted using two siRNA strategies. The first strategy, “siRNA #1” was a 19 bp molecule designed using the siDesign Center (Dharmacon), and contained a 3’-UU sequence overhangs (sense strand: 5’-CCAAAGAAGACCACATTTAUU). The second strategy, “siRNA #2” was a pool of 3 different 19 bp

siRNA molecules designed by the Rosetta algorithm (Sigma), all of which contained 3'-dTdT overhangs (sense strands: 5'-CUUAAUUUCUACAUUCCUU, 5'-CUCCUAUCAUGCUUGCUCU, 5'-CGCUAUUUACAUUCACCAA). The siRNA was reconstituted to 100 µM using Horizon (B-002000-UB-100) siRNA buffer (siRNA #1) or water (siRNA#2). Each siRNA had a corresponding negative control siRNA with no homology to *B. dendrobatidis* genes. A 19 bp scrambled sequence with UU sequence overhangs (5`UAAAUAGAGGUCUGCGAAAUU) was used as a negative control for siRNA#1, and the proprietary universal negative control #1 (Sigma SIC001) was used for siRNA#2 experiments.

siRNA delivery

Extensive optimisation experiments identified electroporation as the preferred method to deliver siRNA to *B. dendrobatidis* cells (Appendix C- Sections 1&2). Zoospores were electroporated in the presence of siRNA using protocols adapted from Swafford *et al* (2020). One million zoospores in 200 µL SM media (5 mM KCl, 15 mM sodium phosphate buffer (pH 7.2), 15 mM $MgCl_2$, 25 mM sodium succinate dibasic hexahydrate, 25 mM D-Mannitol) were transferred to a 2 mm cuvette (BioRad) with 3 µM siRNA. The cuvettes were chilled on ice, electroporated with 2 x 3 ms square wave pulses at 1000 V (Swafford et al. 2020). After ten minutes recovery on ice, zoospores were gently pipetted from the cuvette to a 24 well culture plate (Nunc) and 200 µL ice cold TGhL broth was added. The plate was sealed with parafilm and incubated at 20°C.

Relative GCL mRNA quantification

GCL mRNA was quantified over 48 h to determine the optimal time course for knockdown. At various time points, *B. dendrobatidis* cells were carefully detached from the replicate well surface using a cell scraper and harvested for RNA extraction, cDNA synthesis, and RT-qPCR using methods and primers described in Chapter 3. Relative *GCL* mRNA was calculated by normalising the CT value to the reference genes *α-centractin* (Farrer et al. 2017), and *APRT* (Verbrugghe et al. 2019) using the Pfaffl method. Relative quantity (RQ) was calculated using the equation $RQ= E^{\Delta CT}$, and the geometric mean of the two reference genes was used to determine the fold change in *GCL* expression in the treatment compared to the control (Vandesompele et al. 2002) for each experiment separately. For each time point, a T-test was used to determine if the fold change of the target siRNA treated cells was significantly different to that of the control siRNA (GraphPad Prism).

To confirm the qRT-PCR results, a subsample of cDNA was re-tested using semi-quantitative agarose gel PCR. Additional *GCL* primers were designed (forward: 5'-ACTTTTATCTTTGGGTACTCC and reverse: 5'-ATCCAGAGCCACAGAAGC) to produce a 223 bp product in cDNA. The reference gene *α-centractin* was also included for normalisation. The PCR was performed as a 25 µL reaction containing 15 ng of template, 2.5 µL 10X ThermoPol Reaction Buffer, 10 mM dNTP's, 0.625 U Taq

polymerase (NEB), with 0.2 µM of each forward and reverse primer. The reactions were performed on a Biorad S1000 thermocycler with an initial activation step of 95°C for 30 s, followed by cycles of 30 s 95°C denaturation, 30 s 55°C annealing, and 40 s 68°C extension. With these PCR conditions, the optimum number of cycles before plateau was determined to be 28 for *α centractin* and 35 for *GCL*. The PCR product (10 µL) was run on a 1.2% agarose gel with gel red (Biotium) and visualised under UV light.

Protein quantification

The effect of mRNA knockdown on transcribed proteins was determined by western blot using protocols developed after extensive optimisation (Appendix C, Section 3 Figure 8.20 & Figure 8.21). Zoospores were electroporated as described above, using pooled siRNA#2. After the required incubation time (48 h and 56 h), excess broth was removed from the wells, and the adherent zoosporangia were washed with cold PBS. Cells were scraped from the wells, centrifuged, and the pellet frozen at -80°c until lysis. Cells were lysed in 90 µL of buffer containing 20 mM Tris pH 7.6, 1 mM EDTA, 100 mM NaCl and the protein inhibitor cocktails P8215 (Sigma) and P2714 (Sigma). The solution was subjected to 4 cycles of 1 min bead beating with 0.05 mm and 0.1 mm zirconia silica beads, interspersed with 1 min incubation on ice. The solution was centrifuged to remove cellular particulates, and the protein concentration was measured using a Micro BCA Protein Assay Kit (Thermo Scientific). Protein samples (5.5 µg) were heated at 95°C for 5 min with Laemmli buffer (containing 5% beta-mercaptoethanol), cooled on ice, and run on two replicate Mini-PROTEAN® TGX 4-20% precast gels (Biorad) in Tris-glycine-SDS buffer (Biorad) at 100 V for 1.5 h. The PageRuler™ Plus Prestained Protein Ladder (Thermo Scientific 26619) was run as a size marker. One gel was stained with Instant Blue™ Ultra Fast Protein Stain (Merck) for visualisation of protein bands and the replicate gel was transferred to a 0.45 µm pore polyvinylidene difluoride (Merck) membrane that had been activated in 100% methanol for 30 s. Proteins were transferred in ice cold buffer (20% methanol, 25mM Tris, 192 mM glycine) at 100 V for 1 h. The membrane was blocked in 5% skim milk in PBST (PBS with 0.1% Tween 20) for 1 h, then washed with PBST, and cut horizontally at the 60 kDa mark. The upper half of the membrane (>60kDa proteins) was incubated overnight at 4°C with 1:1000 rabbit GCLC polyclonal antibody (Invitrogen PA5-44189) in 5% skim milk in PBST. The lower half of the membrane (<60kDa proteins) was stored in PBST overnight, and then incubated with 1:2000 rabbit anti-actin antibody (Sigma A2066) in 5% skim milk PBST for 1 h at room temperature to serve as a loading control. Both membranes were washed with PBST, then incubated with 1:2000 Goat anti-Rabbit lgG (H+L) Superclonal secondary antibody HRP conjugate (Invitrogen A27036) in 5% skim milk in PBST for 1 h at room temperature. After washing with PBST and PBS, the membranes were incubated for 5 min with 1 mL chemiluminescence reagent (SuperSignal™ West Femto

Maximum Sensitivity Substrate, Thermo Scientific). Proteins were visualised using a on a G:Box system (SYNGENE) using the Genesys® software.

Glutathione quantification

The effect of *GCL* mRNA knockdown on the biosynthesis of glutathione was determined by measuring total glutathione levels after siRNA treatment. Relative total glutathione levels were measured using a commercial kit (GSH:GSSG GLO, Promega) as described in Chapter 3. Briefly, 1 x 10^6 zoospores were electroporated in the presence of siRNA#2 or control siRNA, as previously described. Electroporated zoospores were added (1 x 10^5 zoospores per well) to a sterilised white 96 well plate and incubated for 42-76 h in 200 µL TGhL broth. Total glutathione (reduced GSH + oxidised GSSG) was measured in duplicate wells using the Promega GSH:GSSG GLo assay and compared relative to the control.

Stress tolerance

The effect of GCL knockdown on the ability to tolerate heavy metal stress was determined by exposing cells to cadmium (Cd) after buthionine sulfoximine (BSO) and siRNA treatment. BSO is a chemical inhibitor of GCL and serves as a positive control for the Cd phenotype as described in Chapter 3. Zoospores were electroporated in the presence of siRNA#2 or control siRNA as described above, and then plated at approximately 8.5 x 10^4 zoospores per well in a clear 96 well plate (Nunc). Cells were exposed to 0.03 mM Cd at 24 h or 48 h post siRNA treatment. Control wells for each treatment included one well without Cd (TGhL only), and one well with 30 mM BSO and 0.03 mM Cd added at 24 h. After three days, viability was estimated by staining the wells with methylene blue and taking two representative images under 200x microscope. The cells were then fixed with ethanol and total growth was measured using the methylene growth assay described in Chapter 2.

Results

mRNA knockdown

The time course experiment confirmed the successful knockdown of *GCL* in *B. dendrobatidis*. RT-qPCR results showed a significant reduction in *GCL* expression from 36 h post electroporation ($p=0.0007$, $t=9.626$, $df=4$) and persisting until at least 48 h ($p=0.0005$, $t=43.00$, $df=2$) (Figure 4.1). Maximum knockdown occurred at 36-42 h post electroporation, with an average of 56% less *GCL* mRNA in cells treated with siRNA#1 compared to controls.

There are many different algorithms and modifications available for siRNA design. To confirm consistency of the knockdown results, mRNA quantification experiments were repeated using a second siRNA strategy, siRNA#2. The siRNA#2 was a pool of three different siRNA molecules, each with dTdT overhangs. This siRNA also resulted in significant reduction of mRNA levels at 36 h

(p=0.045, t=2.871, df=4) and 42 h (p=0.008, t=4.856, df=4). Overall, there was a 36-51% decrease in *GCL* mRNA at these time points (Figure 4.2).

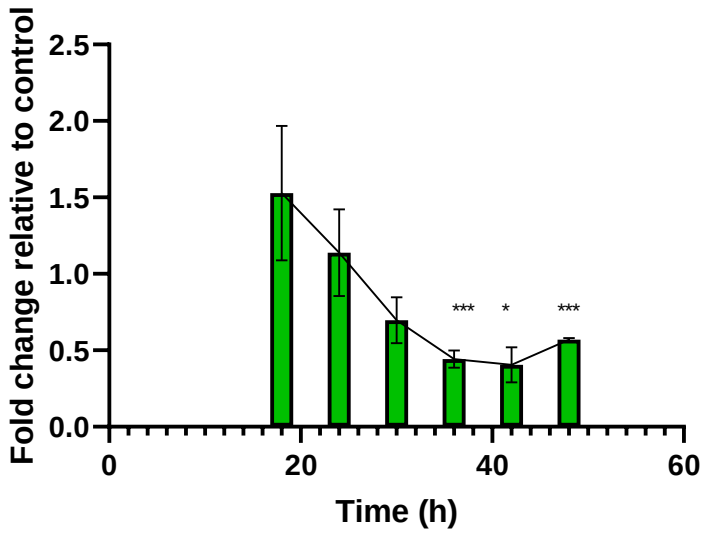


Figure 4.1 Time course of *GCL* mRNA levels in cells treated with siRNA#1 compared to cells treated with control scrambled siRNA, normalised to both *α centractin* and *APRT*. A significant reduction in *GCL* mRNA was detected in cells treated with siRNA#1 at 36 (p=0.0007), 42 (p=0.0354) and 48 h (p=0.0005) post siRNA delivery. N= 20, mean and SEM.

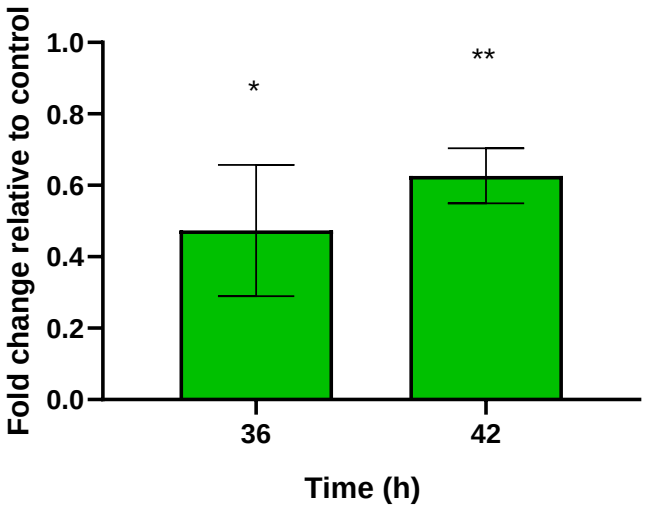


Figure 4.2 *GCL* mRNA levels in cells treated with pooled siRNA#2 and control siRNA, normalised to *α centractin* and *APRT*. A significant reduction in *GCL* mRNA was detected in cells treated with GCL siRNA#2 at both 36 h (p=0.045) and 42 h (p=0.008). N= 9, mean and SEM.

To confirm the RT-qPCR results, a subset of cDNA was also analysed via semi-quantitative PCR and gel electrophoresis. The *GCL* band was visibly less intense in the target siRNA treated samples compared to the controls (Figure 4.3) in agreement with the RT-qPCR results.

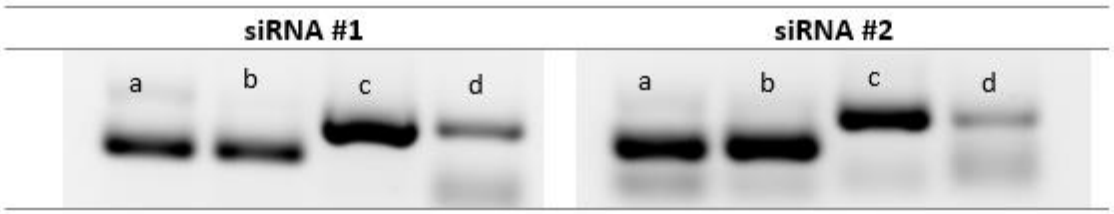


Figure 4.3 Semi-quantitative PCR confirmed reduced *GCL* mRNA at 36 h in cells treated with either siRNA #1 or siRNA #2 compared to control siRNA. The reference gene *α centractin* served as loading control.

Lane a = *α centractin* expression with control siRNA, b = *α centractin* expression with target siRNA, c = *GCL* expression with control siRNA, d = *GCL* expression with target siRNA.

Protein levels after siRNA treatment

The relative levels of GCL protein were quantified with western blot to determine the effect of mRNA knockdown at 48 h and 56 h post electroporation. The actin loading control produced non-specific bands at ~ 25 kDa (Appendix C, Figure 8.23). Despite this, the correct band (at 42 kDa) showed equal protein loading between the control and siRNA treated cells (Appendix C, Figure 8.23 & Figure 8.24). The quantity of protein in the siRNA treated cells was visually similar to the control for both of these time points (Figure 4.4), indicating that there was no significant protein knockdown at the tested time points.

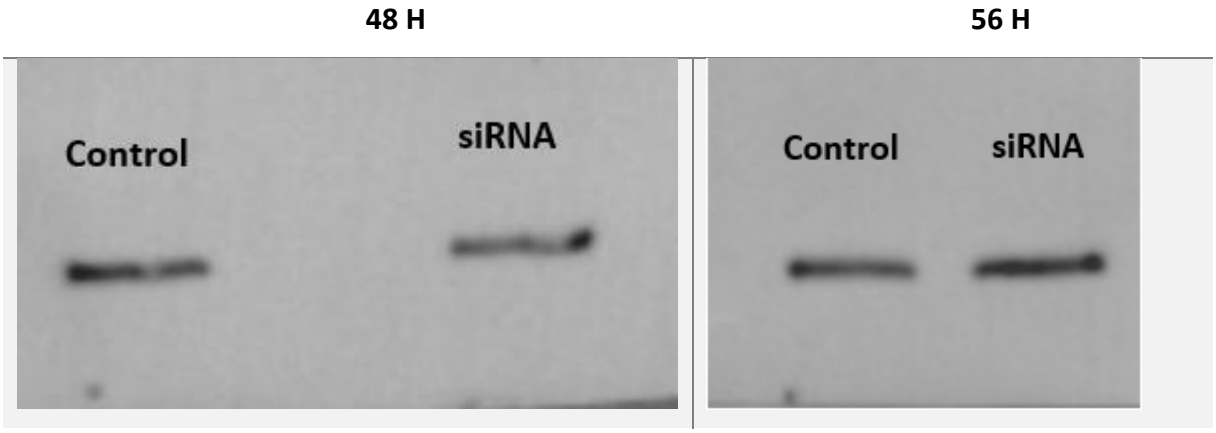


Figure 4.4 Western blot of GCL expression in cells treated with siRNA #2 or control siRNA. GCL protein levels do not differ between treatment and control at either 48 h or 56 h. Full gel image in Appendix C Figure 8.23 and Figure 8.24.

Glutathione levels after siRNA treatment

The relative quantity of total glutathione was compared between target and control siRNA treated cells, via the GSH-GLO luminescence assay (Promega). The time frame from mRNA synthesis to corresponding glutathione production in *B. dendrobatidis* is unknown. Therefore, a range of time points 42 h to 76 h post siRNA delivery were tested to maximise the likelihood of observing differences. There was no significant change in total glutathione levels in the target siRNA treated cells compared to the control siRNA for all time-points tested (Figure 4.5).

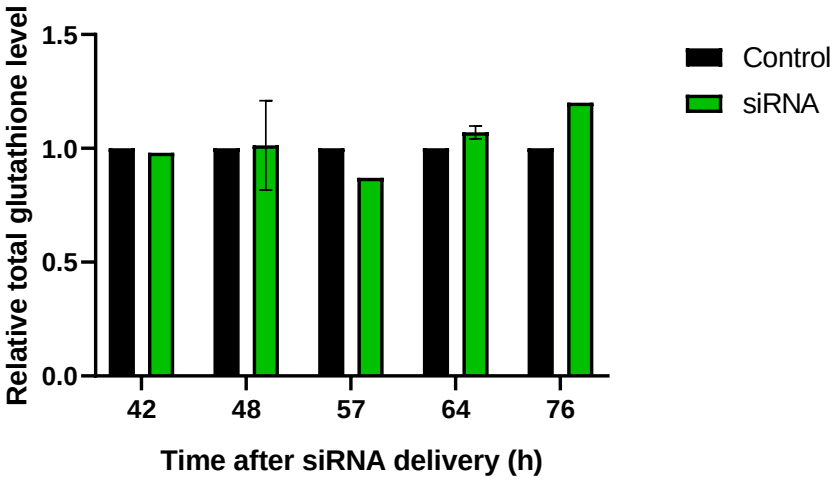


Figure 4.5 Relative levels of total glutathione in cells treated with siRNA #2 compared to cells treated with control siRNA. There was no difference in total glutathione level in siRNA#2 treated cells compared to the control siRNA treated cells, between 42 h to 76 h. N=12, error bars = SD.

Cadmium tolerance after glutathione treatment

Chemical inhibition of GCL with buthionine sulfoximine (BSO) produces a phenotype of increased sensitivity to Cd, as demonstrated by reduced growth (Chapter 3). Therefore, cells treated with siRNA#2 were tested for their ability to tolerate Cd at two time points post siRNA delivery, 24 h and 48 h. There was no difference in the growth of siRNA-treated cells in comparison to the control siRNA when exposed to Cd (Figure 4.6). Methylene blue staining indicated that the viability of siRNA-treated cells was the same as the control (Appendix C, Figure 8.25). The addition of BSO decreased growth, indicating that the Cd concentration was adequate for GSH-dependent inhibition (Figure 4.6).

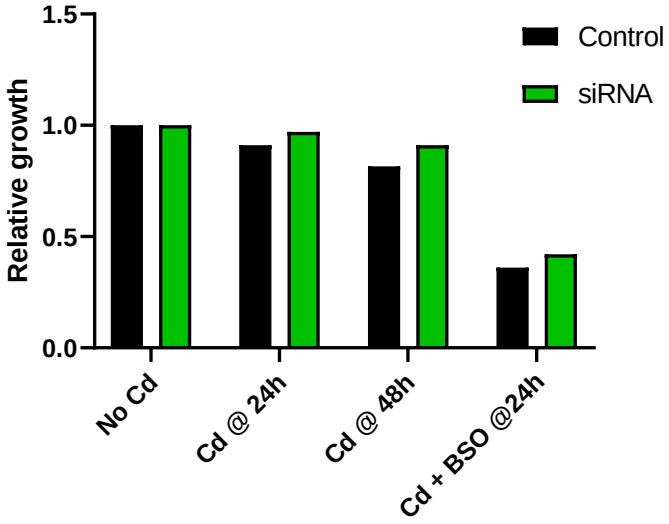


Figure 4.6 Growth in the presence of 0.03 mM Cd in cells treated with siRNA#2 compared to cells treated with control siRNA, as determined by methylene blue assay at 72 h post siRNA delivery. Cells treated with siRNA#2 did not display increased susceptibility to Cd. Cells treated with BSO were more susceptible to Cd. N=8.

Discussion:

The results from this study demonstrate that siRNA can manipulate gene expression in *B. dendrobatidis* and provide the first evidence of a functional RNAi pathway in this species.

Peak mRNA knockdown occurred at 36-42 h, and persisted for at least 48 h. This onset of mRNA knockdown is late compared to other fungal systems, where knockdown can occur as early as 18 h (Khatri and Rajam 2007). However, my failure to detect mRNA knockdown at the early time points may be due to the dynamic nature of gene expression at this stage in the *B. dendrobatidis* life cycle. Data from Chapter 3 indicates that expression of both the target gene, *GCL*, and the reference genes *α centractin* and *APRT* changes dramatically between the zoospore and zoosporangia life stage. It is possible that the variation seen in these early time points is due to the large metabolic shifts associated with encystation (Rosenblum et al. 2008). Therefore, the results from the early time points were reanalysed using *5.8s* rRNA for normalisation, which is a reference gene found to be stable across the life stage transition (Appendix B, Figure 8.7). This normalisation resulted in reduced variation in expression, and suggests that subtle mRNA knockdown (average of 15%) may be occurring at these earlier time-points (Appendix C, Figure 8.22). By 48 h, mRNA knockdown appears to wane, although extended time course studies are required to confirm the precise duration of effect. While the heterogeneous effect of cell cycle was moderated in our experiments by synchronisation of zoospores, longer time course studies are complicated in *B. dendrobatidis* due to the cyclical release of zoospores from zoosporangia. Overall, the duration of RNAi activity in *B. dendrobatidis* appears relatively short lived compared to other species, such as *Sclerotinia sclerotiorum*, where knockdown persisted for at least 96 h (McLoughlin et al. 2018). This is likely attributable to the lack of enzyme RNA dependent RNA polymerase (RdRp) which can amplify and sustain RNAi silencing (Nishikura 2001). RdRp is often described as a "core component" of RNA induced silencing, however there are rare examples of fungi exhibiting RNAi in the absence of RdRp (Goldoni et al. 2004, Calkins et al. 2018). While *B. dendrobatidis* lacks RdRp (Farrer et al. 2017), our results indicate that this does not preclude RNAi activity, but it may help to explain the extent and duration of mRNA knockdown.

Consistent mRNA knockdown was observed across different siRNA designs and validated with two PCR techniques (qPCR and semi-quantitative gel electrophoresis). However, the ~50% decrease in mRNA did not appear to have any corresponding impact on various phenotypic measurements. This effect is unusual (or may appear so due to under-reporting of negative results), but not unprecedented. In *Aspergillus fumigatus*, siRNA treatment achieved 30%-60% mRNA knockdown in two genes, but neither influenced growth (Jöchl et al. 2009). In my study, despite a reduction in *GCL* mRNA, I found no observable difference in GCL protein levels, glutathione levels or

Cd tolerance. A reduction in mRNA should have a corresponding reduction in translated protein, but this effect on *de novo* GCL translation and glutathione biosynthesis could be masked by a long protein half-life of previously translated proteins. Additionally, the lack of impact of siRNA on glutathione levels may also be attributed to the release of glutathione from protein reservoirs, such as enolase (Claytor 2020). In Chapter 3, BSO (a GCL chemical inhibitor) was used to produce phenotypes with reduced glutathione content and reduced tolerance to Cd. RNAi mediated knockdown of *GCL* mRNA should produce a similar phenotypic effect, but presumably the partial and transient mRNA destruction is not as potent as extended inhibition by BSO, which reduced glutathione levels by 50-80% over 6 to 48 h.

Traditionally, effective knockdown is defined by at least a 70% decrease in mRNA (Krueger et al. 2007). As I observed only 50% reduction of *GCL* mRNA, the lack of phenotypic effects may simply be due to insufficient knockdown. Increasing knockdown efficiency is, therefore, an important step in the further development of RNAi techniques in *B. dendrobatidis.* This could be achieved by delivery optimisation and through wider screening of siRNA designs as discussed below.

I tested a range of transformation methods, including lipotransfection, PEG and RNA scavenging, with electroporation being the most effective method tested (Appendix C, Table 8.5). The electroporation protocol used here was originally developed to deliver dextran molecules to zoospores (Swafford et al. 2020). A pilot study (Appendix C, Section 2) found that this protocol could deliver siRNA, albeit at a lower efficiency (~85% for dextrose compared to ~50% for siRNA). Hence, further optimisation of electroporation protocols specifically for siRNA delivery is a logical step to increase mRNA knockdown. Electroporation employs pulses of high electric fields to create pores within cell membranes through which siRNA can enter (Neumann et al. 1982), but there are many parameters that must be optimised for maximum cell viability and transfection efficiency. Optimising the electric field intensity is crucial, as pores will only form above a certain threshold (Rols and Teissié 1998). The field intensity is positively correlated with uptake of nucleic acids, but inversely correlated with cell survival (Li et al. 2017), therefore, a compromise must be sought to achieve the highest transfection efficiency. The waveform (exponential decay or square) is also an important consideration, as well as duration and capacitance (Jordan et al. 2008). All of these parameters could be further optimised for effective electroporation-mediated delivery of siRNA into *B. dendrobatidis*.

Apart from the delivery of siRNA, another variable for consideration is the siRNA design. Although there is vast literature on the rational design of siRNA, a comparison of different designs has not been explicitly tested in fungi. Synthetic siRNA duplexes can include various modifications to increase their efficacy, for example the presence of two extra nucleotide “overhangs” are particularly

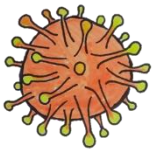
important (Elbashir et al. 2001). The nucleotides are interchangeable, and different types of overhangs have been shown to initiate RNAi in fungi, including dTdT (Chum et al. 2017), TT (Khatri and Rajam 2007) and UU (Calkins et al. 2018). I found similar results using siRNA with UU and dTdT overhangs, suggesting that either approach may be acceptable for use in *B. dendrobatidis.* Further optimisation studies could incorporate other designs to extend the half-life of the siRNA molecules, including modifications in the phosphodiester backbone, sugar and base modifications (Chiu and Rana 2003).

Finally, some genes are inherently difficult to knockdown with siRNA, whether it is because of the low abundance of transcripts (Hu et al. 2004), or other unknown gene features (Krueger et al. 2007). Therefore, future work should also target a variety of genes in *B. dendrobatidis.* Appropriate targets should have low redundancy and produce an obvious phenotype for effective screening, particularly given that mRNA analysis may be inadequate for assessment of RNAi efficacy in this species.

Summary:

This chapter indicates that RNAi may occurs in *B. dendrobatidis* and siRNA could be used to manipulate gene expression. Two siRNA strategies were tested and they both reduced mRNA levels by ~50%. However, this transient reduction in mRNA did not have any measurable effects on protein level or phenotype. This chapter developed the tools to implement RNAi in *B. dendrobatidis,* providing the platform for future studies to harness the opportunities of RNAi in reducing and understanding fungal virulence

Chapter 5 **Mycovirus screen**



The overall goal of this thesis is to develop methods to reduce the virulence of *B. dendrobatidis.* The previous Chapter investigated whether RNAi technologies could be employed to reduce expression of virulence genes, a technique successfully used to tackle fungal pathogens in crop plants. This Chapter investigates virus biocontrol, an alternative approach that has also been used in horticulture to combat fungal disease. Many species of fungi are host to mycoviruses, and their infection can sometimes cause hypovirulence in the fungal host pathogen, providing a tool for disease management. It was unknown whether mycoviruses were present in *B. dendrobatidis*, so I conducted the first widespread screen in this species. A mycovirus with biocontrol potential is most likely to be found in areas where chytrid virulence is low, therefore, I focused on isolates from amphibian populations that had not undergone serious declines (such as Western Australia), with additional screens from South Korean and Brazilian strains. Despite the large sample size and two screening methods (Illumnia sequencing and cellulose chromatography), mycoviruses were not detected. This Chapter revealed that mycoviruses are rare or absent in Australian *B. dendrobatidis*, and future surveys should focus on other regions. If further screens do not detect mycoviruses, then attention should focus on why *B. dendrobatidis* is uniquely free of viruses.

The results from this Chapter have been published in *Fungal Biology*:

Webb, R. J., A. A. Roberts, S. Wylie, T. Kosch, L. F. Toledo, M. Merces, L. F. Skerratt and L. Berger (2022). "Non-detection of mycoviruses in amphibian chytrid fungus (*Batrachochytrium dendrobatidis*) from Australia." Fungal Biology 126(1): 75-81. 10.1016/j.funbio.2021.10.004

This Chapter has 3 aims:

1. To develop a dsRNA extraction method for *B. dendrobatidis* cells.
2. To screen archived *B. dendrobatidis* isolates for the presence of mycovirus
3. To collect and screen fresh *B. dendrobatidis* isolates for mycovirus

Chapter 5: Mycoviruses

Screening for mycovirus presence in *Batrachochytrium dendrobatidis*.

Abstract

Mycoviruses may influence the pathogenicity of disease-causing fungi. Although mycoviruses have been found in some chytrid fungi, mycoviruses have not yet been detected in *Batrachochytrium dendrobatidis*. Here I conducted a survey for mycovirus presence in 38 *B. dendrobatidis* isolates from Australia (n=31), Brazil (n=5) and South Korea (n=2) with a combination of modern high-throughput sequencing and conventional dsRNA cellulose chromatography. Mycoviruses were not detected in any isolates. This result was unexpected, given the long evolutionary history of *B. dendrobatidis*, as well as the high prevalence of mycoviruses in related fungal species. Given my widespread sampling in Australia and the limited number of *B. dendrobatidis* introductions, I suggest that mycoviruses are uncommon or absent from Australian *B. dendrobatidis*. Testing more isolates from regions where *B. dendrobatidis* originated, as well as regions with high diversity or low fungal virulence may identify mycoviruses that could aid in disease control.

Introduction:

The amphibian chytrid fungus, *Batrachochytrium dendrobatidis* (Longcore et al. 1999), is recognised as the worst wildlife pathogen in recorded history (Skerratt et al. 2007, Scheele et al. 2019). An ancient and basal fungi species, *B. dendrobatidis* likely originated in East Asia, the region that harbours the highest genetic diversity (Bataille et al. 2013, O'Hanlon et al. 2018, Byrne et al. 2019), and where endemic amphibians appear resistant, with no reports of mass die-off events (Rahman 2020). However, the pathogen has recently spread around the world to susceptible populations (Skerratt et al. 2007, O'Hanlon et al. 2018), causing the extinction of at least 90 amphibian species, and remains an ongoing threat to hundreds more (Carvalho et al. 2017). Preventing further spread into naïve populations is a high priority (Bower et al. 2019), but strategies are also urgently required to manage the impact of *B. dendrobatidis* in populations that are already infected (Scheele et al. 2014, Skerratt et al. 2016).

A potential novel strategy to mitigate chytridiomycosis is biological control using mycoviruses (Woodhams et al. 2011). A mycovirus is a virus that infects a fungal host. First discovered in 1948 (Ghabrial et al. 2015), there are now more than 250 mycoviral species described (Xie and Jiang 2014), from 16 diverse viral families (Liu et al. 2019). The majority of mycoviruses are double stranded RNA (dsRNA) viruses, but some single stranded RNA (ssRNA) and single stranded circular DNA (ssDNA) viruses have also been reported (Son et al. 2015, Kotta-Loizou and Coutts 2017). A broad range of fungi are host to mycoviruses, including several chytridiomycetes species (Myers et al. 2020).

Chapter 5: Mycoviruses

Mycoviruses are obligate parasites, and in most cases cause few or only cryptic effects to their fungal hosts (Nuss 2005, Pearson et al. 2009). However, some mycoviruses have a detrimental effect, including impairing growth or spore production (van Diepeningen et al. 2006). If the host is a pathogenic fungus, then this negative effect (termed hypovirulence) can reduce the ability of the host fungus to cause disease (Grente 1965). A mycovirus conferring hypovirulence, therefore, presents a promising biological control option for fungal diseases.

Mycoviruses have been used to control plant diseases caused by phytopathogenic fungi. The best-known example being the use of *Cryphonectria hypovirus* 1 (CHV-1) to control chestnut blight, caused by *Cryphonectria parasitica*, which has devastated European chestnut trees (Heiniger and Rigling 1994, Rigling and Prospero 2018). The widespread success of CHV-1 led to increased investment in the study of mycovirus-mediated hypovirulence. Further laboratory trials have demonstrated that mycoviruses can reduce the virulence of disease caused by *Sclerotinia sclerotiorum* in rapeseed (Yu et al. 2013), *Rosellinia necatrix* in Japanese fruit trees (Chiba et al. 2009), and stem canker (*Cryphonectria cubensis*) in eucalypts (Van Heerden and Wingfield 2001). Despite their success in controlling plant fungal pathogens, there has been relatively little investigation of mycoviruses in fungal pathogens of animals (Thapa et al. 2016, Takahashi-Nakaguchi et al. 2020).

A recent survey failed to detect any mycoviruses in nine *B*. *dendrobatidis* isolates collected from North and South America (Myers et al. 2020). Here I conducted a larger survey of *B. dendrobatidis,* including isolates from widely separated regions of Australia (Queensland, New South Wales, Tasmania and Western Australia). In particular, I targeted fungal isolates from Western Australia. Although *B. dendrobatidis* arrived in Western Australia at least 3 decades ago (Murray et al. 2010) there have been no reports of amphibian population declines, despite evidence that local species can become infected (Riley et al. 2013). For example, the motorbike frog (*Litoria moorei*), which is commonly infected (West et al. 2020), has not undergone the dramatic declines seen in other bell frogs such as the green and golden bell frog (*Litoria aurea*), yellow spotted bell frog (*Litoria castanea*) and southern bell frog (*Litoria raniformis*). Targeting the areas where *B. dendrobatidis* appears to be less virulent will maximise the chance of detecting a virus that may be useful for biological control. The screen also included a smaller cohort of isolates from Brazil and Korea. I screened cryo-archived and freshly collected low passage isolates using Illumina next generation sequencing (NGS) and dsRNA cellulose chromatography. The cellulose traditionally used for dsRNA extraction has been discontinued; therefore, I also tested whether an alternative cellulose could be used.

Methods

Isolation of B. dendrobatidis

Isolates of *B. dendrobatidis* were collected from infected larval and adult amphibians (James Cook University Ethics approval A2595) and cultured using standard methods. Adult amphibians were caught by hand, and a ~1 mm piece of webbing removed from the left hind foot. The biopsies were washed by vortexing in successive changes of sterile water and stored in antibiotic water on ice until samples were transferred to the laboratory. Larval amphibians were caught using a dip net from shallow areas, and kept cool until arrival in the laboratory, where they were euthanised with an overdose of 0.1% tricaine methanesulfonate (MS222), and the mouthparts removed. Wet preps of tissue (mouthparts or biopsy) were examined for the presence of *B. dendrobatidis* using microscopy (Appendix D, Figure 8.26). Infected tissue was cleaned by dragging through sterile agar to remove surface contaminants, and then grown in TGhL broth containing antibiotics (penicillin, streptomycin, ciprofloxacin and kanamycin) (Waddle et al. 2018). Once zoosporangia had grown for 3-5 d, cells were transferred to TGhL broth without antibiotics (Longcore et al. 1999, Fisher et al. 2018). The isolates were then either cryo-archived in liquid nitrogen (Boyle et al. 2003) or maintained at 20°C with weekly passaging until the screening for mycovirus was performed. A total of 38 isolates of *B. dendrobatidis* from Australia, Brazil and South Korea were screened for mycovirus presence (Table 5.1). The Brazilian isolates were generously provided by Luís Felipe Toledo (Laboratório de História Natural de Anfíbios Brasileiros), and the Korean isolates were generously supplied by Tiffany Kosch (Seoul National University). Archival isolates (n=21) were screened using NGS (Illumina), and a subset of these (n=5) were further screened using cellulose chromatography to validate the sequencing methodology. Additional fresh isolates (n=17) were collected from around Australia (Table 8.7) and screened using cellulose chromatography.

Sequencing

Isolates of *B. dendrobatidis* were defrosted from storage in liquid nitrogen (Boyle et al. 2003) and grown on TGhL agar plates for 5 d before RNA extraction using either RNeasy (Qiagen) or FavorPrep viral (Favorgen) kits. The RNA was sent to Murdoch University, where Steve Wylie synthesised the cDNA and performed the sequencing and analysis as follows. For cDNA synthesis, approximately 2 µg of RNA from each sample was heat-denatured for 4 min at 94°C, and rapidly cooled in ice water before adding it to a reaction volume of 20 µL, comprised of GoScriptTM RT buffer (Promega), 3 mM $MgCl_2$, 0.5 mM dNTPs, 0.5 mM of random primer (8N, where N is any nucleotide) with a known 5' 16-nt adaptor, and 160 units of reverse transcriptase (GoScriptTM, Promega). The reaction was carried out at 25°C for 5 min, followed by cDNA synthesis at 42°C for 60 min, then incubation at 70°C for 15 min. The cDNA from each sample was labelled with a unique

barcode sequence during PCR amplification using a primer adaptor. Amplification reagents were GoTaq® Green Master Mix (Promega), 1 mM of a uniquely tagged (8-nt) barcode primer (with a 3' end that was complementary to the 16-nt adaptor sequence used to synthesize cDNA), and 2 µL of synthesised cDNA. The reaction was carried out with an initial incubation at 95°C for 3 min, followed by 35 cycles of 95°C for 30 s, 60°C for 30 s, and 72°C for 1 min, followed by a final extension at 72°C for 10 min. The amplicons from the samples were purified using columns of a QIAquick PCR Purification Kit (Qiagen), quantified, and pooled in equimolar amounts. Pooled amplicons (10 µg) were pair-end sequenced over 100 cycles on the Illumina MiSeq platform. Read files were imported into CLC Genomics Workbench 7.5.1 (Qiagen) to trim adapter sequences and filter out low quality sequences.

Mycovirus positive control

An isolate of the fungus *Ceratobasidium* sp. naturally infected with three dsRNA endornaviruses (*Ceratobasidium* endornaviruses B, C and D) was included as the positive control during dsRNA extraction (Ong et al. 2016). Control cultures were grown in 100 mL of oatmeal agar in the dark on a shaker for 7 d at 20°C. Fungal material was then frozen, freeze-dried, and ground into a powder using liquid nitrogen in a mortar and pestle.

dsRNA extraction optimisation

The conventional method of dsRNA extraction requires CF11 cellulose (Whatman) (Morris and Dodds 1979, Marquez et al. 2007), however, this product has been discontinued and an alternative cellulose is required for future studies. Therefore, I first conducted a pilot experiment to compare the standard protocol using CF11 cellulose to an alternative protocol using Sigmacell cellulose type 101 (Sigma) (Peyambari and Roossinck 2018), using the mycovirus-infected *Ceratobasidium* isolate as a positive control. The CF11 and Sigma type 101 protocols were scaled down versions of the protocols described by Marquez et al 2007, and Peyambari & Roossinck 2018, respectively. Briefly, ~100 mg of ground fungal tissue was added to 900 µL extraction buffer (0.1 M, NaCl, 50 mM Tris pH 8, 1 mM EDTA pH 8, 1% SDS, 0.01% 2-mercaptoethanol). The fungal cells were further disrupted by 1 min of bead beating (mini bead beater, Biospec) with silica beads. Two rounds of phenol extraction were performed by adding 900 µL phenol:chloroform:isoamyl alcohol pH 8 (25:24:1). The final aqueous phase was adjusted to 16.5% ethanol and added to 0.03 g cellulose to bind the dsRNA. The cellulose was either packed into a mini-column (CF11), or added directly to the tube (Sigmacell 101). After washing the dsRNA was eluted from the cellulose with 1 mL elution buffer, and precipitated overnight at -20°C with 2.5 mL 100% ethanol with 0.3 mM sodium acetate. Precipitated dsRNA was recovered by high-speed centrifugation, and air drying of the pellet, before resuspension in 50 µL 0.1 mM EDTA. The dsRNA was treated with DNase (New England Biosciences)

to remove traces of DNA (Jacquat et al. 2020). The dsRNA was visualised by running 6 µL on a 1 % agarose gel with gel red (Biotium) at 80 V for 1.5 h. The method producing the clearest dsRNA bands was selected for subsequent fungal isolate screening.

The extract was tested by PCR to confirm that viral dsRNA was present after cellulose extraction. Briefly, 7.5 µL of extract was denatured at 95°C, and then converted to cDNA (Qiagen). The PCR was performed as a 25 µL reaction containing 1 µL of cDNA template, 2.5 µL 10X ThermoPol Reaction Buffer, 10 mM dNTP's, 0.625 U Taq polymerase (NEB), with 0.2 µM of each forward and reverse primer (Ong et al. 2016) (Appendix D, Table 8.8).

The reactions were performed on a Biorad S1000 thermocycler with an initial activation step of 95°C for 30 s, followed by cycles of 30 s 95°C denaturation, 30 s 55°C annealing, and 40 s 68°C extension for 35 cycles. The PCR product (10 µL) was run on a 1.2% agarose gel with gel red (Biotium) and visualised under UV light.

dsRNA extraction of B.dendrobatidis

Freshly collected *B. dendrobatidis* isolates were grown to obtain large volumes of cells. For each isolate, ten 75 cm^2 tissue culture flasks (TPP) were grown for 7 d at 20°C. Cells were collected by centrifugation, and frozen at -80°C. The fungal cells were further disrupted using bead beating before dsRNA extraction as previously described. Each isolate was subjected to dsRNA extraction twice, first individually, and then as a pool of isolates from each region.

Prevalence estimation

Estimates of true prevalence were calculated using EpiTools online epidemiological calculator (https://epitools.ausvet.com.au/trueprevalence) (Rogan and Gladen 1978, Brown et al. 2001, Sergeant 2018), using Blaker's 95% confidence level (95% CL) (Reiczigel et al. 2010) and assuming sensitivity of either 0.5 or 1 and perfect specificity (=1) of the screening methods.

Table 5.1 Location and host species of *B. dendrobatidis* isolates

ID	Location [a]	Host species	Year	Lineage [b]	Method
OHRG 8	Mount Wellington, TAS, Australia	*Litoria ewingii*	2012	GPL	Illumina
OHRG 32	Flinders Island, TAS, Australia	*Litoria ewingii*	2012	GPL	Both
OHRG 20	Corrinna, TAS, Australia	*Crinia tasmaniensis*	2012	GPL	Illumina
OHRG 35	Ethel Creek, QLD, Australia	*Litoria nannotis*	2013	GPL	Illumina
OHRG 5	Paluma, QLD, Australia	*Litoria serrata*	2010	GPL	Both
OHRG 12	Rockhampton, QLD, Australia	*Litoria caerulea*	1999	GPL	Illumina
OHRG 13	Paluma, QLD, Australia	*Litoria serrata*	2012	GPL	Illumina
OHRG 25	Tully, QLD, Australia	*Litoria rheocola*	2012	GPL	Illumina
OHRG 30	Tully, QLD, Australia	*Litoria nannotis*	2012	GPL	Illumina
OHRG 40	Kirrama, QLD, Australia	*Litoria nannotis*	2013	GPL	Illumina
OHRG 43	Cooranbong, NSW, Australia	*Limnodynastes peronii*	2013	GPL	Illumina
OHRG 4	Abercrombie, NSW, Australia	*Litoria booroolongensis*	2009	GPL	Illumina
OHRG 46	Jindabyne, NSW, Australia	*Litoria verreauxii*	2013	GPL	Both
OHRG 47	Melbourne, VIC, Australia	*Litoria lesueuri*	2007	GPL	Illumina
CLFT001	Serra Do Japí, SP, Brazil	*Hylodes ornatus*	2010	BRAZIL/ ASIA-2	Both
CLFT026	Reserva Betary, SP, Brazil	*Boana faber*	2011	GPL	Illumina
CLFT021	Serra Do Japí, SP, Brazil	*tadpole-unidentified*	2010	GPL	Illumina
CLFT044	Serra Da Graciosa, PR, Brazil	*Hylodes cardosoi*	2013	BRAZIL/ ASIA-2	Illumina
CLFT023	Monte Verde, MG, Brazil	*Boana sp.*	2011	GPL	Illumina
KBO 347	Guman-Ri, Gangwon-Do, S Korea	*Bombina orientalis*	2012	ASIA-1	Both
KCL 72	Buam-Ri, Chungcheongnam-Do, S Korea	*Lithobates catesbeianus*	2014	BRAZIL/ ASIA-2	Illumina
OHRG 80	Frenchmans Creek, QLD, Australia	*Litoria sp.*	2020	Unknown	Cellulose
OHRG 81	Kirrama, QLD, Australia	*Litoria sp.*	2020	Unknown	Cellulose
OHRG 82	Tully, QLD, Australia	*Litoria sp.*	2020	Unknown	Cellulose
OHRG 83	Goomburra, QLD, Australia	*Mixophyes fleayi*	2020	Unknown	Cellulose
OHRG 84	Goomburra, QLD, Australia	*Mixophyes fasciolatus*	2020	Unknown	Cellulose
OHRG 85	Cunninghams Gap, QLD, Australia	*Mixophyes fleayi*	2020	Unknown	Cellulose
OHRG 86	Sydney, NSW, Australia	*Litoria fallax*	2020	Unknown	Cellulose
OHRG 88	Armidale, NSW, Australia	*Mixophyes balbus*	2020	Unknown	Cellulose
OHRG 87	Nariel Valley, VIC, Australia	*Litoria spenceri*	2020	Unknown	Cellulose
OHRG 102	Wongungarra, VIC, Australia	*Litoria spenceri*	2021	Unknown	Cellulose
OHRG 103	Bemm River, Vic, Australia	*Litoria aurea*	2021	Unknown	Cellulose
OHRG 74	Augusta, WA, Australia	*Crinia georgiana*	2019	Unknown	Cellulose
OHRG 75	Dunsborough, WA, Australia	*Litoria adelaidensis*	2019	Unknown	Cellulose
OHRG 76	Gwelup, WA, Australia	*Litoria adelaidensis*	2019	Unknown	Cellulose
OHRG 77	Gwelup, WA, Australia	*Litoria moorei*	2019	Unknown	Cellulose
OHRG 78	Nannup, WA, Australia	*Crinia georgiana*	2019	Unknown	Cellulose
OHRG 79	Yanchep, WA, Australia	*Litoria moorei*	2019	Unknown	Cellulose

[a] Australian states: QLD = Queensland, NSW = New South Wales, VIC = Victoria, TAS = Tasmania, WA = Western Australia, Brazil: SP = São Paulo, PR = Paraná, MG= Minas Gerais. [b] Lineage from O'Hanlon et al. 2018.

Results

Isolates

A total of 17 new *B. dendrobatidis* isolates were collected from around Australia (Appendix D, Table 8.7). Combined with 14 cryo-archived samples, a total number of 31 Australian isolates were screened and covered a broad range of species, geographic locations and habitat types (Figure 5.1). In addition, 5 isolates from Brazil and 2 isolates from South Korea were also screened.

Sequencing

A total of 14.2 million paired 100-nt reads were obtained representing the pooled *B. dendrobatidis* samples (data obtained by Steve Wylie). The remaining 11.4 million reads after trimming and filtering were paired and assembled *de novo*. There were 1,001 contigs over 500 nt in length, the largest being 7,095 nt. These contigs were compared to sequences held in the NCBI nr database using Blastn. The majority of hits were of fungal origin, and none showed identity with known viruses. The 41 'orphan' sequences > 500 nt that did not show significant identity to any sequences on GenBank were analysed using Blastx (protein database interrogated using six reading frames), and most were resolved as fungal-like sequences. Again, there were no matches with known viral groups.

dsRNA extraction optimisation

The mycovirus positive control produced clear dsRNA bands when extracted using the Sigmacell 101 cellulose protocol. Sigma 101 cellulose also successfully extracted all three endornaviruses, as confirmed by PCR (Figure 5.2). Therefore, I considered the Sigmacell 101 protocol to be an acceptable alternative to the traditional (now discontinued) CF11 cellulose, and this newly validated method was used to screen the *B. dendrobatidis* isolates.

dsRNA screen of B. dendrobatidis and prevalence estimation

Cellulose chromatography did not detect any dsRNA in any of the *B. dendrobatidis* isolates. Given the non-detection in a sample size of 38, I estimated the true prevalence of mycovirus in *B. dendrobatidis* to be between 0 and 9.1 % (95% CL) given a perfect test. When the test sensitivity was reduced to 0.5, the upper limit of the estimated true prevalence increased to 18.3 %.

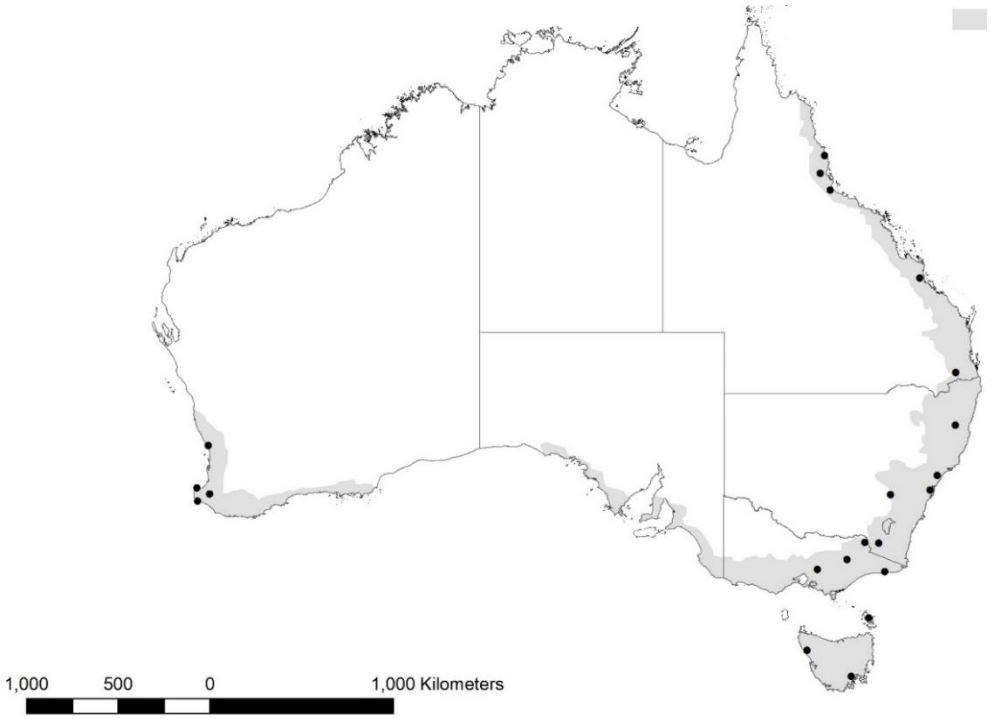


Figure 5.1 Map showing the wide distribution of Australian *B. dendrobatidis* isolates screened. The black dot points indicate the location of isolates collected from Australia. This covers most of the current distribution of *B. dendrobatidis* (shaded region).

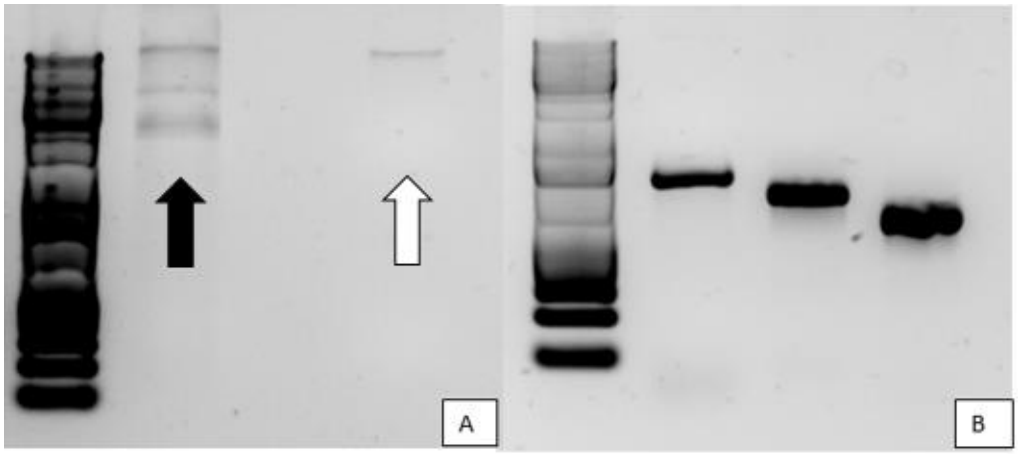


Figure 5.2 Sigma 101 cellulose is an acceptable alternative to CF11 cellulose. **A:** Agarose gel electrophoresis showing dsRNA bands resulting from two types of dsRNA extraction of *Ceratobasidium* sp. harbouring three endornaviruses, Sigmacell 101 cellulose vs CF11 cellulose. From left to right; 1kb marker, Sigmacell extraction (black arrow), CF11 extraction (white arrow). **B:** An agarose gel electrophoresis of PCR product indicating that the dsRNA extracted using Sigma 101 cellulose contains all three viruses known to be present in the positive control; from left to right, 1kb marker, *Ceratobasidium* endornaviruses B, C and D. The PCR was performed using primers from Ong *et al* 2016.

Discussion:

This survey of 38 *B. dendrobatidis* isolates from Australia, Brazil and Korea failed to detect any mycoviruses. The initial screen consisted of 21 archival *B. dendrobatidis* isolates that had been cryo-preserved. However, cryo-preservation can lead to mycovirus loss (Springer et al. 2013), and mycoviruses can also be lost spontaneously during long term passaging (Zamora-Ballesteros et al. 2021). Therefore, I collected an additional 17 fresh isolates from around Australia for screening. With this sample size, I estimated that if *B. dendrobatidis* is indeed host to mycoviruses, the prevalence is likely to be low, below 10% with perfect test sensitivity.

This study used two screening techniques, cellulose-based dsRNA extraction and next generation sequencing (NGS). Cellulose based dsRNA extraction is a robust method for novel virus detection that has been used since 1979 (Morris and Dodds 1979). This method is inexpensive and can be conducted with conventional laboratory equipment, however, it may be less sensitive as it relies on visualisation of dsRNA bands. As most known mycoviruses are either dsRNA, or have dsRNA stages in their life cycle (Son et al. 2015), dsRNA extraction by cellulose chromatography is a commonly used mycovirus screening method (Castro et al. 2003, Castillo et al. 2011). Sigmacell 101 cellulose has been validated for screening plant viruses, and here I show it can also be used to extract

dsRNA viruses in fungi. Recently, the development of NGS has greatly accelerated the identification of new mycoviruses (Donaire et al. 2016, Marzano et al. 2016, Zoll et al. 2018). This method is more sensitive than gel electrophoresis and can detect both RNA and DNA mycoviruses from total RNA extracts (Nerva et al. 2016). Unlike dsRNA extraction, sequencing relies on comparison to known viral sequences, however this technique has been used successfully to detect ancient viruses only distantly related to known taxa (Ng et al. 2014). A direct comparison of sequencing and dsRNA extraction methods found that they were mostly in agreement when used to screen for mycoviruses in early diverging fungi (Myers et al. 2020).

The negative results of this survey were unexpected as mycoviruses are often described as being ubiquitous in fungi (Ghabrial et al. 2015). Indeed, individual fungal isolates are often found to be co-infected with phylogenetically diverse mycoviruses (Ong et al. 2018, Thapa and Roossinck 2019). Although early-diverging fungal lineages are less frequently screened than higher fungi, other members of the chytrid order Rhizophydiales have been found to harbor mycoviruses at a prevalence of 14.3% (Myers et al. 2020). The *Batrachochytrium* genus (Martel et al. 2014) and mycoviruses (Ghabrial 1998) are both ancient lineages, with ample time to form associations.

With the exception of its sister species, *B. salamandrivorans* (Martel et al. 2013), *B. dendrobatidis* is unique in that it is the only chytridiomycete known to infect vertebrates. This should not preclude *B. dendrobatidis* from harbouring mycoviruses, as infections have been detected in other pathogenic fungi of vertebrates. For example, a novel partitivirus was found to be widespread in North American (Thapa et al. 2016) and Czech Republic (Ren et al. 2020) isolates of the bat pathogen, *Pseudogymnoascus destructans*. This pathogen causes white nose syndrome in a variety of bat species (Blehert et al. 2009), a devastating skin disease that has drawn comparisons to chytridiomycosis due to its impact on wildlife.

Like most chytridiomycetes, *B. dendrobatidis* mostly reproduces asexually (Berger et al. 2005a), which theoretically should favour mycovirus transmission (Pearson et al. 2009). However, the monocentric mode of asexual reproduction in *B. dendrobatidis* could help explain the paucity of mycovirus infections. In monocentric fungi, one zoospore produces just one zoosporangia and species with this mode of development have been shown to have significantly fewer mycoviruses than their polycentric counterparts (Myers et al. 2020).

It is also possible that *B. dendrobatidis* is naturally infected with mycoviruses, but the antibiotics used in the isolation process may have inadvertently cleared them. Both kanamycin and streptomycin have been known to eliminate some mycoviruses (but not others) after prolonged exposure (Cao et al. 2019). However, the period of antibiotic exposure during *B. dendrobatidis*

isolation was shorter than what was required to clear mycovirus infections in *Ceratobasidium* (~7 vs. 35 d). Culture independent methods of obtaining *B. dendrobatidis* cells (eg: filtration or metatransciptomics) could help avoid this problem by omitting the antibiotic exposure. This approach may also overcome the potential bias of the culturing process, which selects for the healthiest cells.

The isolates used in this survey were predominantly from Australia. I was unable to detect evidence of mycovirus infection despite sampling from diverse locations and host species, suggesting that perhaps the Australian *B. dendrobatidis* population may be free of mycoviruses. Previous genetic (O'Hanlon et al. 2018) and epidemiological (Murray et al. 2010, Scheele et al. 2017) data suggests *B. dendrobatidis* may have only been introduced once to Australia, and so it is possible that absence of mycovirus infection may be a founder effect. Future surveys should concentrate on testing other *B. dendrobatidis* isolates from more locations, especially the evolutionary origin in Asia, where diversity is highest (Bataille et al. 2013, O'Hanlon et al. 2018, Byrne et al. 2019). In addition, sampling *B. dendrobatidis* isolates with low virulence may increase the chance of detecting a mycovirus with hypovirulence potential. Should viruses be found in *B. dendrobatidis*, studies on how they interact with the fungus and influence virulence will be needed to assess their potential for use as a biocontrol for protecting vulnerable amphibian species.

Mycoviruses tend to be host specific, but it may be possible to artificially infect *B. dendrobatidis* with viruses from closely related fungi (Van Diepeningen et al. 1999, Kanematsu et al. 2010). Infection experiments using mycoviruses from related fungi may shed light on whether *B. dendrobatidis* can be infected, and whether there are any phenotypic impacts of infection.

Viruses are the most abundant life forms on Earth, probably infecting every extant species, and their infections have driven the evolution of all life from ancient times (Paez-Espino et al. 2016). If further screening confirms that *B. dendrobatidis* is not widely infected with mycoviruses, this raises interesting questions on its resistance. For example, *B. dendrobatidis* could be a model to understand how virus infection can be avoided with implications for understanding virus-host specificity, and the evolution of these pathosystems.

Summary:

Unexpectedly, I was unable to find any evidence of mycovirus infection in *B. dendrobatidis.* This inability to detect mycoviruses may be due to the isolation process, or the fact that the majority of isolates were from Australia. These results should prompt investigation into how the Australian *B. dendrobatidis* population has avoided viral colonisation. Future screens for mycoviruses should focus *B. dendrobatidis* in areas of high diversity, and perhaps employ reduced antibiotic use during

isolation. The dsRNA extraction method developed in this Chapter will be useful for studies requiring an alternative extraction method to CF11 cellulose.

Chapter 6 **Discussion**

The amphibian chytrid fungus, *B. dendrobatidis*, continues to cause widespread amphibian declines and extinctions. The loss of amphibian biodiversity has wide-ranging consequences on other species and ecosystems. The disappearance of tadpoles from rainforest streams left behind reduced food web complexity (Schmidt et al. 2017), and the loss of neotropical adult frogs from was followed by snake declines as the chytridiomycosis epidemic depleted their prey (Zipkin et al. 2020). There has been progress in the development of antifungal, probiotic and vaccine therapeutics against chytridiomycosis, however these have not been effective for wild amphibians, and without immediate interventions many species are still on the brink of extinction (Skerratt et al. 2016). Therefore, there is an urgent need to explore novel approaches to treating chytridiomycosis, especially methods for wild amphibians. In this thesis, I explored the feasibility of RNAi (Chapter 4) and mycovirus-based (Chapter 5) therapeutics, both of which have been successful in treating fungal diseases of plants. In doing so, I investigated a candidate virulence factor to target with RNAi (Chapter 3) and developed a method to rapidly assess *B. dendrobatidis* viability and growth (Chapter 2), which enabled me to quantify the success of RNAi optimisation. In this final chapter, I discuss the unique challenges of working with *B. dendrobatidis*, and the prospects for implementing RNAi or mycovirus-mediated hypovirulence as a method to control chytridiomycosis.

Chapter 6: Discussion

General Discussion

The uniqueness and challenges of B. dendrobatidis

The majority of chytrid species are saprobes, although there are a few parasitic species that infect plants, insects and algae (Van den Wyngaert et al. 2018, van de Vossenberg et al. 2022). The *Batrachochytrium* genus (*B. dendrobatidis* and *B. salamandrivorans*) contains the only chytrid species known to parasitise vertebrates and their unique biology differs substantially from other fungal species. Throughout this thesis, I describe several characteristics of *B. dendrobatidis* that highlight the specific challenges of working with this non-model fungus.

Measuring growth and viability in B. dendrobatidis

In Chapter 2, I found that trypan blue is not an effective stain for *B. dendrobatidis*. Trypan blue is a dye that has been used to stain fungi since the 1950s (Boedijn 1956, Phillips and Hayman 1970), and is also commonly used to determine fungal viability, especially in yeast. However, I found that trypan blue resulted in suboptimal staining of *B. dendrobatidis* cells, particularly as a viability stain. These results agree with those of McMahon et al 2014, who suggested that the presence of discharge tube might prevent the use of trypan blue as a viability stain in this species (McMahon and Rohr 2014). I found that an alternative dye, methylene blue, is a superior viability stain for *B. dendrobatidis* and can be used on mature zoosporangia as its performance was not compromised by the presence of discharge tubes. The mechanism of methylene blue staining is still not understood, and it is not clear whether the interaction between the dye and live cell occurs intracellularly or on the cell surface (Bongard et al. 1995, Merker et al. 1997, May et al. 2003). My observation that methylene blue does not stain live *B. dendrobatidis* cells, despite the presence of discharge tubes, suggests that either the reduction from blue to colourless form is occurring within the cell (rather than by reductases on the cell surface), or that the discharge tubes do not permit dye penetration as hypothesised by McMahon et al 2014.

Without using methylene blue, it is difficult to determine the viability of *B. dendrobatidis* zoosporangia using microscopy alone. In Chapter 3, cells exposed to BSO and cadmium possessed large vacuoles and faint cell walls, but it was unclear whether these cells were viable. The methylene blue assay was able to confirm that, despite dysmorphic phenotype, the cells were in fact viable (Figure 6.1a). Thus, future growth experiments in *B. dendrobatidis* should consider the limitations of measuring growth alone, which may underestimate the impact of stressors on cells. An additional measure of cell health may be useful to capture the full response to stress. A vitality assay such as MTT (Lindauer et al. 2019), which measures cellular metabolism, could be used in conjunction with methylene blue to allow a more complete characterisation of the stress response.

Chapter 6: Discussion

RNAi studies often use fluorescently labelled siRNA to assess the efficiency of delivery during methodology optimisation (Calkins et al. 2018). In Chapter 4, I observed that fluorescent siRNA could passively stain the contents of dead zoosporangia, obscuring the quantification of siRNA uptake (Figure 6.1c). I used methylene blue to establish the viability of fluorescing cells after siRNA treatment and confirm the delivery efficiency. Thus, it is important for future RNAi studies to use a viability stain to avoid an overestimation of fluorescent siRNA delivery to *B. dendrobatidis*.

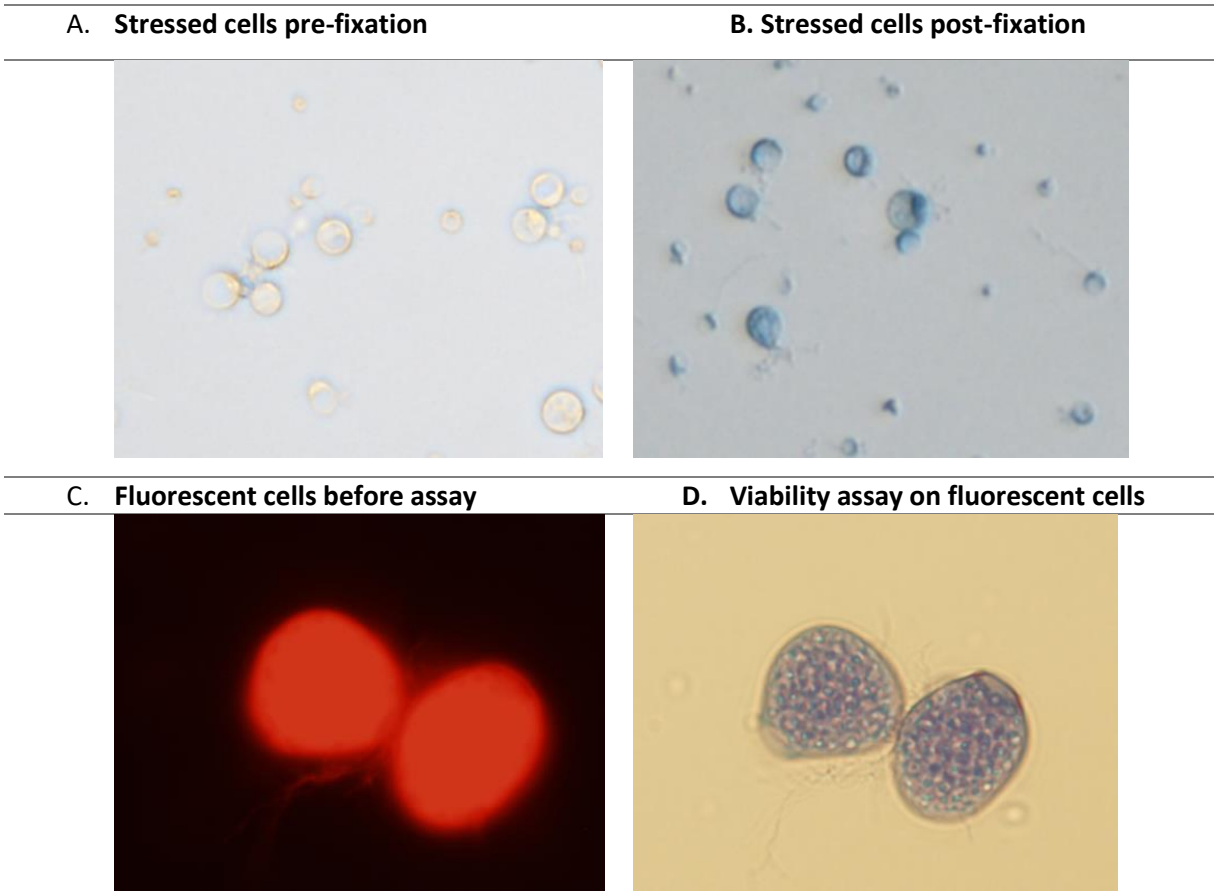


Figure 6.1 Application of the methylene blue viability stain A: Unhealthy, but viable, cells remain unstained after methylene blue assay. B: The same population of cells after ethanol fixation and repeated methylene blue assay indicating that they had been alive prior. C: Two zoosporangia after delivery of fluorescently tagged siRNA. D: Methylene blue assay indicates the zoosporangia were killed in the siRNA delivery process, leading to an overestimation of siRNA uptake by fluorescence measurement alone.

Atypical response of B. dendrobatidis to buthionine sulfoximine and oxidative stress

Chapter 6: Discussion

In Chapter 3, I found that BSO is ineffective against *B. dendrobatidis* at concentrations commonly used in other fungi, and higher levels were needed to inhibit glutathione synthesis. BSO is frequently used to inhibit glutathione synthesis at 2-6 mM (Clemens et al. 1999, Courbot et al. 2004, Patsoukis and Georgiou 2007). However, a 5-fold increase in BSO was required to be effective in *B. dendrobatidis*. At this concentration, glutathione levels were decreased by ~50% after 6 h. This timeframe is in line with experiments with *Saccharomyces cerevisiae*, in which 6 h of BSO exposure resulted in 32% less glutathione, but this was achieved with only 5 mM BSO (Prévéral et al. 2006). BSO acts by inhibiting glutamate cysteine ligase, the first enzyme of the glutathione synthesis pathway, therefore preventing the synthesis of new glutathione (Griffith and Meister 1979). A previous study in *B. dendrobatidis* used BSO at 100 µM (Claytor 2020), a concentration effective in mammalian cells (Chowdhury et al. 2013). Claytor 2020 found that 100 µM BSO did not change glutathionylation (glutathione modifications) in *B. dendrobatidis*. However, my results indicate that this concentration is likely too low to impact cellular GSH levels. The higher levels of BSO required for depletion of GSH in *B. dendrobatidis* highlights the importance of validating any new protocols for use in this species.

Chapter 3 revealed that *B. dendrobatidis* has an atypical response to oxidative stress under glutathione depletion. Although hydrogen peroxide (H_2O_2) lowers total glutathione levels, there is no decrease in the reduced:oxidised glutathione ratio nor is there an increased susceptibility to H_2O_2 with glutathione depletion, which are the hallmarks of the oxidative stress response in other fungi (Grant et al. 1996). These results suggest that *B. dendrobatidis* is able to cope well with oxidative stress, possibly due to glutathione reservoirs on proteins (Claytor 2020). Alternatively, *B. dendrobatidis* may possess other, unknown methods to cope with oxidative stress. This may be an important area of future research, since, for most pathogens, tolerance of host immune-generated oxidative stress is important during infection (Wojtaszek 1997, Missall et al. 2004, Aguirre et al. 2006). However, it is possible that antioxidant systems are not as important for infection by *B. dendrobatidis* as it encounters limited oxidative stress from the host immune system. This is supported by a low level of immune cell recruitment observed in *B. dendrobatidis*-infected hosts (Young et al. 2014), possibly due to immune suppression (Fites et al. 2013), or due to the protection of the superficial and intracellular infection site. While glutathione may not play an important role in the invasion of amphibian skin, my results suggest that it is essential for the completion of the zoosporangial life stage and for zoospore release. Thus, further work should investigate whether glutathione suppression may inhibit *B. dendrobatidis* growth in amphibian skin.

Chapter 6: Discussion

Antiviral mechanisms and the absence of mycoviruses in B. dendrobatidis

Another way in which *B. dendrobatidis* differs from other fungi is that it does not possess RdRp (Farrer et al. 2017), an important amplifying component of the RNAi machinery (Nishikura 2001). The RNAi pathway is present in most fungi and is a defence against viral infection (Segers et al. 2007, Hammond et al. 2008). Fungi lacking a complete RNAi pathway are more susceptible to mycovirus infection, but can be cured of viruses when RNAi is reconstituted (Drinnenberg et al. 2011). Thus, it is interesting that no mycoviruses were detected in *B. dendrobatidis* (Chapter 5), given it does not possess the full complement of RNAi genes. In the chestnut blight fungus, *Cryphonectria parasitica*, experimental mutants lacking either Dicer (Segers et al. 2007) or Argonaute (Sun et al. 2009) display increased susceptibility to mycoviral infection. However, the role of RdRp is less clear. As with Dicer and Argonaute, RdRp is upregulated by the host during viral infection suggesting its involvement in antiviral defence (Zhang et al. 2014, Yu et al. 2018). In addition, host RdRp is targeted and supressed by mycovirus proteins (Suzuki and Nuss 2002, Hammond et al. 2008), presumably to facilitate infection. However, *C. parasitica* mutants lacking RdRp had no observable differences to wildtype strains when infected with a hypovirus (Zhang et al. 2014). Therefore, it is unknown if the incomplete RNAi pathway in *B. dendrobatidis* would result in increased susceptibility to mycoviruses.

An alternative interpretation is that *B. dendrobatidis* may have lost partial RNAi functionality in response to beneficial mycovirus infections. This scenario occurs in fungi hosting mycoviruses that encode so called 'killer toxins', which confer an advantage to their hosts by killing competing fungi (Park et al. 1996, Schmitt and Breinig 2006). All known species that possess the beneficial "killer virus" lack some or all of the RNAi genes, and closely related species that retain RNAi lack the killer virus, suggesting that RNAi capability has been lost in favour of a mutualistic infection with a mycovirus (Drinnenberg et al. 2011). Both of these explanations predict mycovirus presence in *B. dendrobatidis*, however, I found no evidence of mycovirus infection in Australian *B. dendrobatidis* isolates (Chapter 5). This result could be due to a coincidental founder effect, if the single introduction of *B. dendrobatidis* into Australia was free of mycovirus. Alternatively, the absence of mycovirus in *B. dendrobatidis* raises the question of whether this species possesses other antiviral defences to protect it from viral infection. The lack of RdRp in *B. dendrobatidids* may also contribute to suboptimal gene knockdown via RNAi, as discussed later.

Future directions

Novel treatments for chytridiomycosis

Treating chytridiomycosis in wild amphibians remains a major challenge as eradication is impossible, largely because the pathogen can persist in reservoir amphibian hosts (Daszak et al. 2004, Brannelly et al. 2018b) and probably in the environment (Fisher et al. 2021). Similarly, the chytrid *Synchytrium endobioticum*, which causes potato wart disease, is almost impossible to eradicate from infested soil, and it also appears to have reservoir hosts (Cotton 1916). A wide variety of chemical treatments have been tested against *S. endobioticum* but were either ineffective or too toxic for environmental use (Hampson 1988, van de Vossenberg et al. 2022). Using antifungal chemicals against *B. dendrobatidis* is likely to face the same issues. Attempts to treat wild frogs with chemical disinfectants and antifungal drugs had short term success in specific situations (Bosch et al. 2015, Hudson et al. 2016, Geiger et al. 2017), but this approach is unlikely to provide a widespread solution due to environmental reservoirs of *B. dendrobatidis*. Alternative approaches, such as RNAi (Koch et al. 2016, Wang and Jin 2017, Werner et al. 2020) and mycovirus biocontrol (Heiniger and Rigling 1994, Chiba et al. 2009, Yu et al. 2013) are being developed for the control of phytopathogenic fungi and could be useful against chytridiomycosis.

Implementing RNAi

I explored the feasibility of using RNAi in *B. dendrobatidis* and achieved the first steps towards implementation by demonstrating that siRNA can knock down gene expression (Chapter 4). However, mRNA knockdown did not correspond to a robust phenotypic change. As previously mentioned, the effect of RNAi may be inherently low in *B. dendrobatidis* due to the lack of RdRp-driven signal amplification (Farrer et al. 2017).

Along with Dicer and Argonaute, RdRp is a core component of RNAi, as it amplifies and sustains the RNAi response (Nishikura 2001). However, effective RNAi has been demonstrated in other fungi without RdRp, including *Pecoramyces ruminantium*, *Saccharomyces castellii* and *Candida albicans* (Drinnenberg et al. 2009, Moazeni et al. 2012, Calkins et al. 2018). Mammals also have robust RNAi pathways despite an absence of RdRp (Stein et al. 2003, Pinzón et al. 2019), suggesting that RdRp might not be essential. In Chapter 4, I quantified gene knock down over 48 h, however, longer time course studies could determine whether sustained RNAi can occur in *B. dendrobatidis*, despite the lack of RdRp.

Optimising the RNA molecule design, siRNA delivery, target gene or the fungal life stage used for RNAi could help improve knock down efficiency. In Chapter 4, siRNA was investigated as an RNAi trigger; however, most phytopathogenic RNAi systems use dsRNA rather than siRNA to knockdown

gene expression (Werner et al. 2020), and in some cases dsRNA has been found to be more potent that siRNA (Wang et al. 2013). While dsRNA could be explored for RNAi in *B. dendrobatidis*, it may also illicit an immune reaction in animal hosts (Karpala et al. 2005), and therefore may be less suitable as a chytridiomycosis treatment.

siRNA design can have major effects on knockdown efficiency (Strapps et al. 2010), as can the choice between using single or pooled siRNA (Parsons et al. 2010). However, since my two siRNA designs produced similar results, this may not be a limitation on efficacy in *B. dendrobatidis*. On the other hand, the delivery of the siRNA could be a focus area for improved knock down effects. My pilot flow cytometry experiments suggested that electroporation only delivered siRNA to ~50% of cells (Figure 8.17). This electroporation protocol was optimised for delivery of dextran (Swafford et al. 2020), so future work should focus on optimising this system for siRNA. This approach could involve modification of the electroporation parameters, buffers or siRNA concentration. As only 80% of cells were viable after electroporation (Figure 8.18), efforts to increase zoospore survival could also improve knock down efficiency.

The choice of target gene may also be important for RNAi. By targeting a gene essential for glutathione biosynthesis, I aimed to produce a cadmium sensitive phenotype due to glutathione depletion. Chemical inhibition of glutathione synthesis by BSO is rapid and sustained, with ~90% less total glutathione after 48 h. However, siRNA treatment only transiently decreased mRNA by ~50%, and there was no impact on GCL protein levels, total glutathione or cadmium sensitivity, likely due to the long half-life of GCL. My observation that cells returned to normal growth after removal of BSO suggests that a strong and sustained glutathione depletion is required for obvious phenotypic change. Therefore, future work should investigate other target genes, such as ornithine decarboxylase (ODC), which are more likely to produce phenotypic effects with ~50% mRNA knockdown. ODC is a known virulence factor in many fungal pathogens of plants (Bailey et al. 2000, Yan et al. 2022), and has been targeted by RNAi to control *Fusarium* wilt disease (Singh et al. 2020). The *ODC* gene encodes the first and rate limiting enzyme in the production of the polyamine putrescine (Tabor and Tabor 1976), which then form the polyamines spermidine and spermine (Pegg 2006). Spermidine produced by *B. dendrobatidis* can inhibit lymphocyte proliferation (Rollins-Smith et al. 2019), suggesting it may be important for evasion of the host immune system. Like *GCL*, the *ODC* gene can also be chemically inhibited (Metcalf et al. 1978), which produces a phenotype of suppressed growth in *B. dendrobatidis* (Rollins-Smith et al. 2019). Hence, ODC is also an attractive candidate gene for future RNAi knockdown experiments.

Chapter 6: Discussion

For my proof-of-concept experiments in Chapter 4, I delivered siRNA to the zoospore life stage. Zoospores have no cell wall, making them amenable to electroporation, and they can be easily purified and quantified. Zoospores can also be "synchronised" to be the same age to ensure a consistent cell type, which minimises the impact of dynamic gene expression on RT-qPCR analysis (as discussed in Chapter 3). Despite the advantages of using uniform zoospores for siRNA delivery, their reported lack of transcriptional activity (Rosenblum et al. 2008) could be a barrier to initiating RNAi. If transcriptional activity is low in zoospores, then mRNA knockdown will have limited effect. This might explain why I did not detect significant mRNA reduction until 36-42 h after siRNA delivery. Degradation of siRNA can occur quickly, so encouraging rapid encystation using mucin (Robinson et al. 2022) could allow RNAi to occur when siRNA levels are highest.

Alternate siRNA delivery methods.

Delivering siRNA to zoospores via electroporation is feasible in the laboratory for functional genomic experiments, and perhaps also to prepare avirulent isolates for vaccination efforts. However, this approach is not viable for the wide-scale treatment of wild amphibians, and alternative methods will be required.

Some fungi naturally take up exogenous RNA from the environment (known as "RNA scavenging") (Jöchl et al. 2009) or from plant hosts (known as "cross kingdom RNAi") (Wang et al. 2016, Schaefer et al. 2020) via extracellular vesicles (Cai et al. 2020). In these species, RNAi can be initiated by simply incubating the fungi with dsRNA (Wang et al. 2016) or siRNA (Calkins et al. 2018), and spraying siRNA/dsRNA onto the surface of plants can protect them from fungal diseases (Koch et al. 2016). In the fungal pathogen *Sclerotinia sclerotiorum*, uptake of dsRNA occurs at the hyphal tip via endocytosis (Wytinck et al. 2020). The zoosporangia of *B. dendrobatidis* do not possess hyphae, but instead the zoosporangia develop rhizoids, which may enable similar uptake. In other fungi, uptake is greatest during spore germination (Khatri and Rajam 2007, Calkins et al. 2016). Future work should determine whether *B. dendrobatidis* can scavenge exogenous RNA molecules and whether uptake changes during different life stages, using fluorescently tagged RNA constructs and viability testing to enable precise tracking (Whisson et al. 2005). If *B. dendrobatidis* cells take up RNA without electroporation, this would support the development of a spray-based therapeutic approach.

In summary, I found that RNAi-mediated gene knockdown is feasible in *B. dendrobatidis* and, here, I suggest the next steps to optimise this process. Further development of an efficient gene knockdown methodology would allow a wide range of functional genetic studies, vastly increasing

our understanding of this pathogen. This also provides the first step in implementing an RNAi based therapeutic for the treatment of chytridiomycosis.

Mycovirus-mediated hypovirulence

Mycoviral infection can reduce the virulence of some fungal strains, such as in the chestnut blight fungus (*C. parasitica*). Inoculating virus-infected *C. parasitica* onto chestnut trees can protect them from severe disease and has contributed to their conservation (Heiniger and Rigling 1994). In Chapter 5, I screened *B. dendrobatidis* strains for mycovirus infection, with the aim of developing a novel biocontrol method. However, my screen of 38 isolates from Australia, Brazil and South Korea did not identify the presence of mycoviruses. Continued efforts towards mycovirus identification in *B. dendrobatidis* are warranted (Woodhams et al. 2011), due to their great potential for amphibian conservation with minimal intervention. Further screens could be conducted outside Australia, targeting areas where *B. dendrobatidis* virulence is low, and in Asia where the pathogen originated and where strain diversity is highest (Bataille et al. 2013, O'Hanlon et al. 2018, Byrne et al. 2019). However, it is important to note that some areas with perceived low virulence (e.g.: Panama) may be due to increased resistance of the hosts, rather than decreased pathogen virulence (Voyles et al. 2018).

In addition to the use of hypovirulent *B. dendrobatidis* as a treatment approach, mycoviruses could also be used for functional genomic studies and identification of virulence factors. In *Aspergillus fumigatus*, viral infection downregulated stress tolerance genes, and the increased susceptibility to oxidative stress resulted in reduced virulence (Takahashi-Nakaguchi et al. 2020). Other studies have shown that mycovirus-infected fungi stimulate beta interferon levels in a murine host, with implications for increased clearance by macrophages (Applen Clancey et al. 2020). Similar approaches could be used in *B. dendrobatidis*, with either natural or exogenous mycoviruses, to identify potential virulence factors or to increase the host immune response.

Implications for other wildlife diseases

My results may have utility for developing mitigation approaches against other emerging fungal diseases of wildlife. White nose syndrome (*Pseudogymnoascus destructans*), snake fungal disease (*Ophidiomyces ophiodiicola*) and yellow fungus disease (*Nannizziopsis sp*) are also causing concern due to their emergence in wildlife populations.

White nose syndrome is an emerging disease of North American bats, causing spectacular mortality events throughout the Eastern United States and Canada (Blehert et al. 2009, Maher et al. 2012). Since its discovery in 2006, the disease has caused mortality in six bat species, the most affected being the little brown bat (*Myotis lucifugus*) (Turner et al. 2011). The cold adapted

pathogen infects hibernating bats, with mortality rates up to 75% at some hibernacula (Blehert et al. 2009). Increased arousal from torpor and associated dehydration is thought to be the cause of death (Warnecke et al. 2012).

Snake fungal disease is widespread in eastern USA and has been flagged as a potential conservation issue (Sutherland et al. 2014). The causative agent is the ascomycete *O. ophiodiicola*, and has been linked to populations declines in timber rattlesnakes (*Crotalus horridus*) (Clark et al. 2011) and Eastern massasaugas (*Sistrurus catenatus*) (Allender et al. 2016). Clinical signs vary, but include crusty lesions (especially on the head), anorexia and abnormal behaviour. Fungal hyphae invade the epidermis and can persist through moulting (Lorch et al. 2016). A recent review has suggested that snake fungal disease may not be causing widespread mortality, but nevertheless disease monitoring is still necessary (Davy et al. 2021).

Yellow fungal disease is caused by fungal pathogens in the genus *Nannizziopsis.* This disease is primarily found in captive reptiles (Cabañes et al. 2014), and also occasionally occurs in humans (Baggott et al. 2017, Nourrisson et al. 2018). Recently *Nannizziopsis barbatae* has been identified as the agent responsible for mortality in wild lizards in Australia (Peterson et al. 2020). It causes skin colour changes (hence the name), and crusty necrotic skin lesions (Mitchell and Walden 2013). It is contagious, difficult to treat, and often results in death (Peterson et al. 2020).

Although these pathogens belong to other fungal phyla, they share similarities to *B. dendrobatidis*. Like *B. dendrobatidis*, *P. destuctrans*, *O. ophiodiicola* and *N. barbatae* also infect the skin of their hosts. The management of these diseases in the wild also faces similar challenges to chytridiomycosis. For example, the limited feasibility of using antifungal drugs (Chaturvedi et al. 2011, Allender et al. 2015) or probiotic augmentation (Cheng et al. 2017) on wild animals, and the difficulty of eliminating the pathogen from the environment (Vanderwolf et al. 2016). Therefore, the novel antifungal approaches investigated in this thesis may also be applied to other fungal wildlife diseases.

As the first attempt to use RNAi on wildlife fungal disease, my thesis provides a framework for investigating the feasibility of this approach, and how to navigate potential challenges. In Chapter 4, I encountered a range of methodological hurdles (see Appendix C) that required troubleshooting before I could proceed to testing RNAi in *B. dendrobatidis*. The successful use of RNAi in non-model fungi requires the development of methods for the delivery and visualisation of exogenous nucleic acids, assessing cell viability, and accurate quantification of mRNA and protein levels.

Chapter 6: Discussion

To my knowledge, RNAi has never been demonstrated in the Chytridiomycota; and designing siRNA for use in *B. dendrobatidis* was hampered by the lack of literature on RNAi approaches in chytrids. Conversely, RNAi methodologies are well established in the Ascomycota (Romano and Macino 1992), to which *P. destructans*, *O. ophiodiicola* and *N. barbatae* all belong, although RNAi has not yet been reported in these wildlife pathogens. Optimal siRNA length, overhang modifications, G/C ratios and design algorithms have been validated for use in Ascomycete fungi (Table 6.1). Ascomycetes also demonstrate both RNA scavenging (Jöchl et al. 2009) and cross kingdom RNAi (Wang et al. 2016, Schaefer et al. 2020), indicating that the delivery of interfering RNA molecules into these species may be less challenging than for *B. dendrobatidis*. Selection of target genes may also be less challenging for these other wildlife diseases, as several have been characterised in Ascomycetes. Riboflavin (vitamin B2) is a known virulence factor of many ascomycetes (Garfoot et al. 2014, Dietl et al. 2018), including *P. destructans* (Flieger et al. 2016). In infected bats, riboflavin increases skin necrosis and likely leads to increased arousal from hibernation and associated depletion of fat reserves (Flieger et al. 2016). Riboflavin has been successfully targeted in nematodes (Biswas et al. 2013) and mice (Yao et al. 2013) using RNAi against riboflavin transporters. Therefore, RNAi appears a promising antifungal option, not just for chytridiomycosis, but also for other fungal wildlife pathogens.

Table 6.1 Characteristics of successful siRNA designs in Ascomycete fungi

Length	overhang	G/C ratio	Concentration	Reference
21bp	TT	57%	10-25nM	(Khatri and Rajam 2007)
19bp	TT	52%	10µM	(Rehman et al. 2016)
19bp	dTdT	57%	100nM	(Chum et al. 2017)
21bp	UU/GC	36%	5 µM	(Ghag et al. 2014)

Although mycovirus-mediated hypovirulence of animal fungal pathogens has yet to be reported (Nuss 2005), this approach also holds promise for the biological control of wildlife pathogens (Woodhams et al. 2011). The majority of mycovirus-associated hypovirulence has been observed in the Ascomycota (Nuss 2005), which suggests a high probability of finding a useful mycovirus in *P. destuctrans*, *O. ophiodiicola* or *N. barbatae*. In fact, a mycovirus has already been detected in *P. destructans*. A screen of 62 North American and European isolates identified a Partitivirus (named PdPV-pa) in all the North American isolates, but not in the European isolates (Thapa et al. 2016). The presence of PdPV-pa appears to be beneficial to its fungal host, as reproductive output is reduced when it is cured of the virus. The virus occurs in the fungal isolates responsible for mass mortality events in North America, but not in the European isolates (where die offs have not occurred), suggesting it might confer hypervirulence (see (Marquez et al. 2007).

Although PdPV-pa is not suitable for mycovirus mediated hypovirulence, it could potentially be modified using cDNA (Chen et al. 2000) to attenuate the virulence of *P. destuctrans*. The PdPV-pa virus is the only known mycovirus infecting a wildlife pathogen. The lack of reported surveys for mycoviruses in fungi affecting wildlife indicate that this is a neglected topic and useful mycoviruses may remain undetected. Hence, screening pathogenic fungal isolates for the presence of mycoviruses is warranted.

Summary

The chytridiomycosis crisis continues to kill amphibians worldwide. This thesis aims to increase our understanding of *B. dendrobatidis* virulence and investigate potential strategies to reduce virulence (Figure 6.2). The development of a rapid viability and growth assay for *B. dendrobatidis* will assist efforts to quantify the effectiveness of antifungal strategies. By increasing our understanding of glutathione and stress tolerance, we are one step closer to identifying the pathogen's susceptibilities. Because traditional antifungal approaches have not been widely successful, further investigation of novel control methods, such as RNAi and mycovirus mediated hypovirulence, is necessary. *B. dendrobatidis* causes the world's worst wildlife disease and deserves our continued effort to minimise its impact and prevent further loss of amphibian biodiversity.

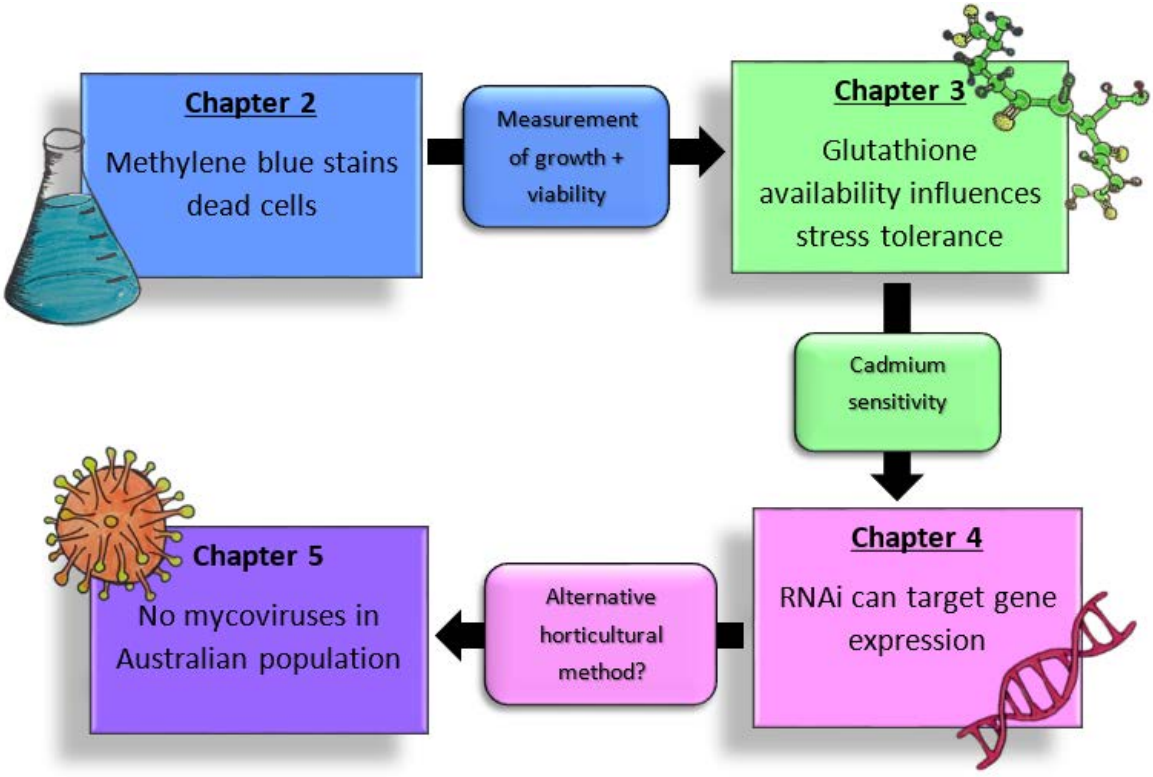


Figure 6.2 Overall thesis findings

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Chapter 8 **Appendices**

Appendix A

This appendix contains additional images during the development of the methylene blue viability and growth assay (Chapter 2).

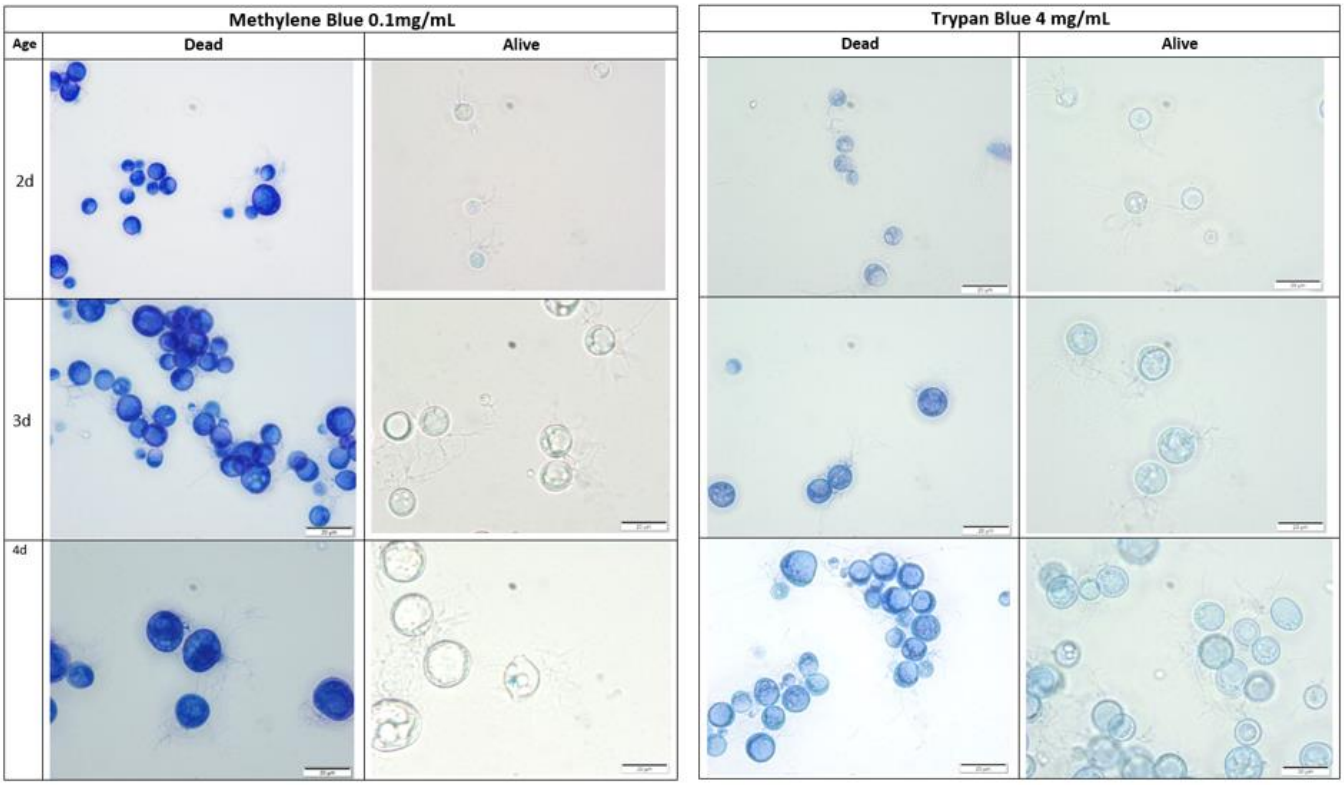


Figure 8.1 Comparison between methylene blue and trypan blue stain on zoosporangia of different ages. Dead cells stained blue with both methods, however, methylene blue provided a clearer distinction between live and dead fungi at all ages. Scale bar= 20 µM.

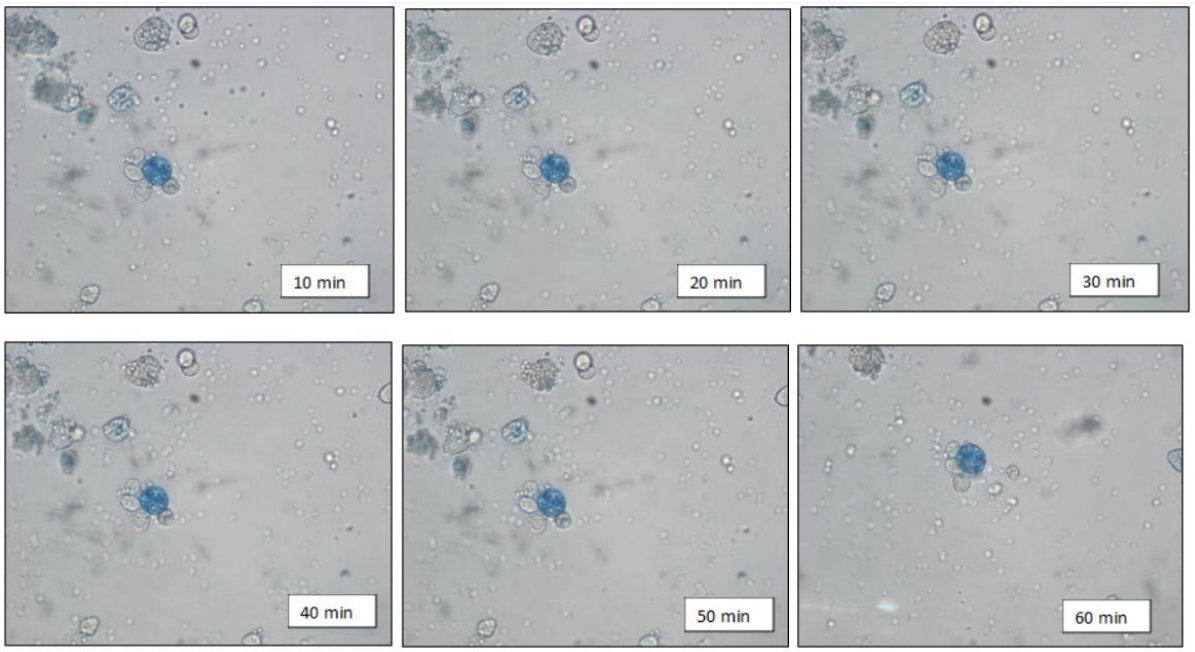


Figure 8.2 Stability of methylene blue staining. Serial images of the same slide were taken over 60 min. The stained zoosporangia remained blue indicating that the stain stable and was not re-oxidised over 60 min.

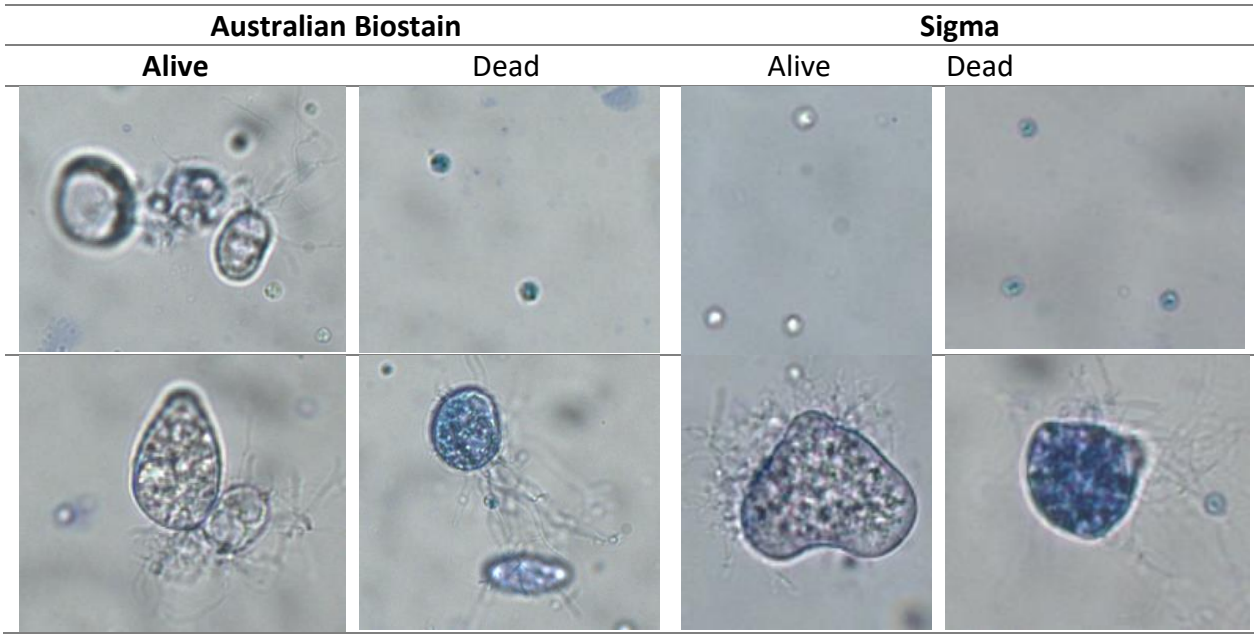


Figure 8.3 Comparison of from methylene blue staining from two stain manufacturers, Australian Biostain and Sigma. Both types of methylene blue stained dead cells blue leaving live cells unstained. The stain works on both the zoospore (upper image) and zoosporangia (lower image) life stage.

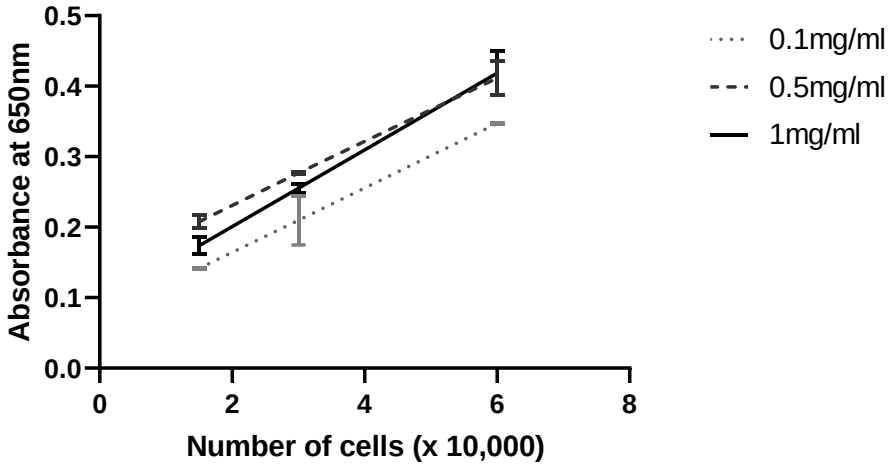


Figure 8.4 Effect of different concentrations of methylene blue on absorbance of eluted dye at different cell densities. Cells were fixed with ethanol before staining with methylene blue, washing and subsequent elution of bound dye. A concentration of 0.5 mg/mL methylene blue increased the absorbance compared to 0.1 mg/mL, although there was no improvement in staining with 1 mg/mL.

Appendix B

Chapter 3 required substantial development and optimization of RT-qPCR protocols and determination of the minimal inhibitory concentration (MIC) for a range of stressors, as described in this Appendix.

Section 1 RT-qPCT optimisation: Methods and results.

Section 2 A note on mRNA expression in zoospores

Section 3 Sub-lethal concentrations for stressors

Section 4 Growth effects of glutathione modulators- BSO and GSH

Section 5 Viability of cadmium + BSO treated cells.

Section 6 Effect of varying BSO concentrations on stressed cells

Section 7 Effect of varying glutathione concentrations on stressed cells.

Appendix B- Glutathione

SECTION 1:

RT-qPCR optimisation methods

Validation of GCL and GR primers

The specificity of primers designed in this study was confirmed by PCR and agarose gel electrophoresis to ensure that they produced the expected product sizes in both DNA and cDNA. The PCR was performed as a 25 µL reaction containing 15 ng of template, 2.5 µL 10X ThermoPol Reaction Buffer, 10 mM dNTP's, 0.625 U Taq polymerase (NEB), with 0.2 µM of each forward and reverse primer. The reactions were performed on a Biorad S1000 thermocycler with an initial activation step of 95°C for 30 s, followed by cycles of 30 s 95°C denaturation, 30 s 55°C annealing, and 40 s 68°C extension for 35 cycles. The PCR products (10 µL) were run on a 1.2% agarose gel with gel red (Biotium) and visualised under UV light.

Table 8.1 primers used for GCL and GR gene expression quantification

Gene	Forward	Reverse	Product size
GCL (BATDEDRAFT_35498)	TCGTACTCATGGCATCGCTC	AGTGCGAGCATCCTTGTTGA	127bp in cDNA 358bp in DNA
GR (BATDEDRAFT_21097)	CTGGGCAGTAGGACGGAATG	CCCAAGAGCGTAAACACCCT	139bp in both

RT-qPCR primer efficiency

The efficiency of each primer pair under the specific PCR conditions is required for expression calculations. The efficiency of *APRT* was obtained from published material. The efficiency of the *GCL*, *α Centractin* and *5.8s* were calculated by a 4 point standard curve as follows. A mixed population of cells from an actively growing *B. dendrobatidis* culture was harvested for RNA extraction, followed by cDNA synthesis, and qPCR. Pilot experiments indicated that a Zymo RNA extraction kit in conjunction with bead beating and DNAse treatment resulted in the best RNA yields, and this protocol was chosen for the remaining experiments. Cells were pelleted, treated with 90 µL of RNA/DNA shield (Zymo), and subjected to 1 min of bead beating with 0.1 mm and 0.5 mm silica beads (Daintree Scientific) to break up the chitin cell walls. RNA was extracted from the homogenized solution using the "Quick RNA micro kit" (Zymo), including a 15 min DNase treatment. RNA concentration and purity was measured using a nanodrop, and RNA synthesised into cDNA using the QuantiTect reverse transcription kit (Qiagen). To produce the standard curve, four x 10-fold dilutions of cDNA were prepared. qRT-PCR was performed in a 20 µL reaction containing 1 µL of template, 1x Rotor-Gene SYBR green master mix and 1 µM of each forward and reverse primer. Two replicates were included for each dilution point. The reactions were run on a Rotorgene 6000 with an initial activation step of

95°C for 5mins, followed by 35 cycles of a 5 s 95°C denaturation step and a 10 s 60°C annealing/extension step. Primer efficiency was calculated using Rotorgene Q software.

***in vitro* reference gene selection**

To evaluate the best reference genes for early life stages of *B. dendrobatidis in vitro*, the stability of each gene was compared over a range of samples. RNA was harvested from synchronized cells at various time points (18-36h), followed by cDNA synthesis and RT-PCR as described earlier. CT values were also retrieved from experiments in which the zoospores had been electroporated, giving a final sample size of n=35. The CT values for each gene were analysed using different algorithms, including "BestKeeper" (Pfaffl et al. 2004), "Ref Finder" (Xie et al. 2012), Delta Ct (Silver et al. 2006) and "NormFinder" (Andersen et al. 2004). NormFinder allows samples to be grouped, therefore additional analyses were run grouping samples together by age (18 h, 24 h, 30 h).

***in vivo* reference gene selection**

To evaluate the best reference genes for *B. dendrobatidis in vivo*, the primer specificity was evaluated using BLAST and uninfected host tissue. Each primer set was assessed for non-specific amplification using the NCBI Primer blast. In addition, RNA was extracted from uninfected host tissue to confirm that the primers were only detecting *B. dendrobatidis,* and not host gene expression. Two juvenile cane toads *Rhinella marina* were euthanized using 0.1% MS222. The ventral pelvic patch skin (thighs and lower abdomen) was removed (Figure 8.5) and cut into very small pieces. The skin was placed in individual 1.5 mL tubes containing 0.1 mm and 0.5 mm zirconia/silica beads (Daintree scientific) as well as 150 µL DNA/RNA shield (Zymo). The tubes were then subjected to 3 x cycles of 1 min bead beating. RNA extraction, cDNA synthesis and RT-PCR was performed as described previously. The target gene primers were also evaluated using this method to ensure that they would only detect *B. dendrobatidis GCL*.

Appendix B- Glutathione

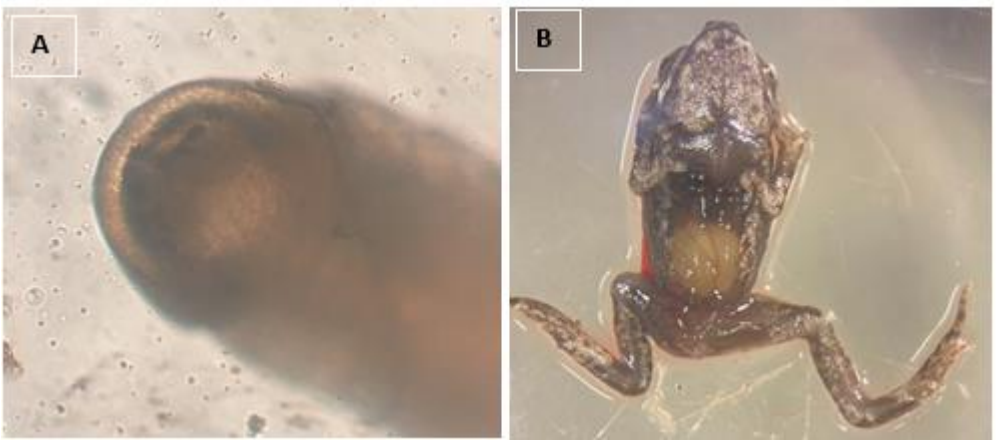


Figure 8.5 Infection and harvesting of host tissue for in vivo gene expression. A. *Rhinella marinia* toe in contact with live zoospores. B. Ventral skin of *R. marinia* removed for RT-PCR.

RT-qPCR optimisation results:

The primers designed in this study produced amplicons of the expected product size (127 bp for *GCl* and 139 bp for *GR*).

Figure 8.6 PCR amplicons. L= ladder (Gene Ruler 1kb+), *GCL* primer set, *GR* primer set in cDNA.

The efficiency for the *GCL* primer pair and reference genes are listed in Table 8.2. Reference gene analysis consistently ranked *α centractin* and *APRT* as the most stable for young zoosporangia *in vitro* (Table 8.3). However, *α centractin* primers produced non-specific amplification when tested on uninfected host tissue. Therefore, *APRT* and *5.8s* were chosen as reference genes for *in vivo* experiments.

Table 8.2 Primer efficiency

Primer set	**Efficiency (%)**
GCL	91.5%
α centractin	96.4%
5.8s	118.4%
APRT	96.9% (Verbrugghe et al. 2019)

Table 8.3 Ranking of *in vitro* reference genes

Program	Best	Second	Third
RefFinder	*α centractin*	*APRT*	*5.8s*
BestKeeper SD	*α centractin*	*5.8s*	*APRT*
BestKeeper R	*α centractin*	*APRT*	*5.8s*
Δ Ct	*α centractin*	*APRT*	*5.8s*
NormFinder	*α centractin*	*APRT*	*5.8s*

Similar ΔCT values indicates that *GCL* is not upregulated in the cane toad infection model compared to the in vitro results (Table 8.4).

Table 8.4 Results from expression analysis ΔCT APRT vs GCL

ΔCT APRT vs GCLC		
	In vitro	***In vivo***
20h	1.34	2.63
32h	2.69	1.99
44h	2.18	3.03

SECTION 2:

mRNA expression in zoospores.

It was noted that although *5.8s* was the lowest ranked housekeeping gene for young zoosporangia (18-36 h), it was clearly the most stable when comparing between zoospores and zoosporangia. Zoospores contained very little mRNA. In these pilot experiments, the levels of *GCL* and the housekeeping genes *α centractin*, *APRT* and *Cystn1* were all very low in the zoospore samples, compared with *5.8s*, even though equal cell numbers were compared (3×10^6) and the spectrophotometric quantification (via Nanodrop, Thermo Scientific) indicated high total RNA concentrations. The Ct value of *5.8s* remained stable across the zoospore and zoosporangia sample, whereas the Ct values of *α centractin*, *APRT*, *Cystn1* and *GCL* all decreased in the zoosporangia sample, correlated to higher expression in zoosporangia (Figure 8.7). When mRNA expression is normalised to *5.8s*, the fold change expression increases dramatically, eg: ~50-400-fold between the zoospore and zoosporangia sample for *α centractin*, *APRT*, *Cystn1* and *GCL* (Figure 8.8).

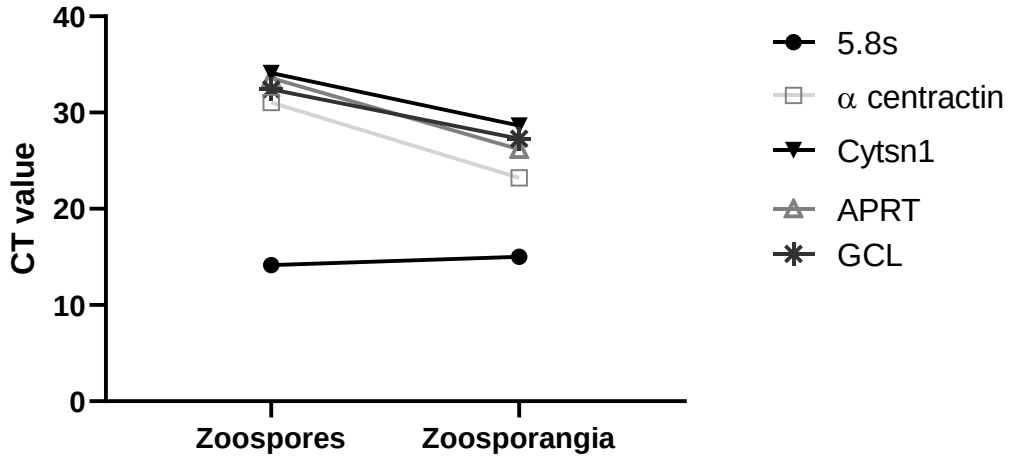


Figure 8.7 The Ct values between zoospore and zoosporangia samples. The Ct values of the rRNA reference gene *5.8s* remain relatively stable between life-stages, whereas the genes encoding mRNA display decreased Ct values, indicating higher expression.

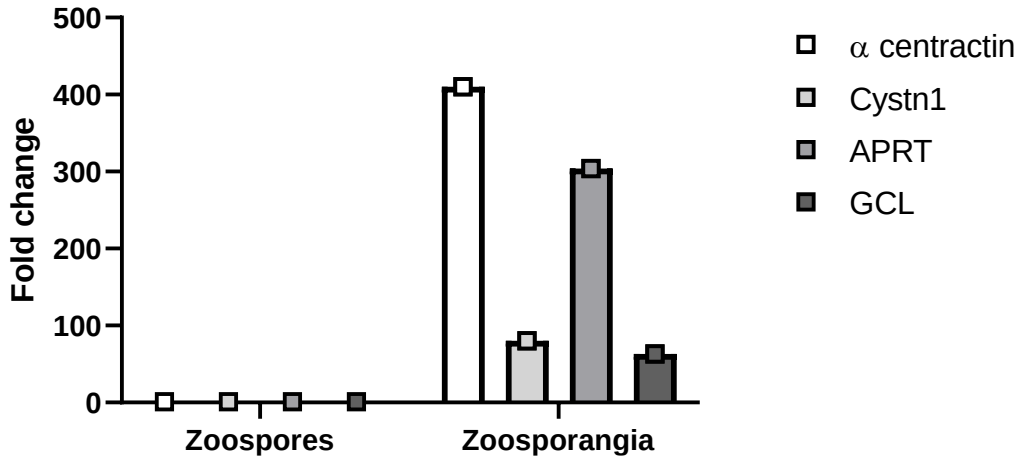


Figure 8.8 Fold-change in mRNA expression normalised to 5.8s rRNA. Expression of reference genes (*α centractin*, *APRT* and *Cystn1*) and gene of interest (*GCL*) increases in zoosporangia compared to zoospores, when normalised to *5.8s.*

Appendix B- Glutathione

SECTION 3

Determining sub-lethal levels of stressors

Methods:

Zoospores (8.5×10^4) were plated in 96 well plates in TGhL broth and allowed to encyst overnight. Various concentrations of stressors were added to the cells. All chemical stressors were dissolved in water and filter sterilized before use. The hydrogen peroxide (H_2O_2) solution was freshly prepared from 30% stock solution (Merck), the cadmium (Cd) solution was prepared from $CdCl_2$ salt, the terbinafine (TBF) solution was prepared from a commercial tinea spray (Ego SolvEasy), and the NaCl solution was prepared using laboratory grade NaCl (Sigma). After 48 h growth was measured using the methylene blue assay, and calculated as relative to the untreated control. For heat tolerance, values were obtained from the literature (Greenspan et al. 2017).

Results:

The ideal stress level for subsequent assays is a concentration that produces only a mild negative effect. This level ensures that the stress levels are adequate, but also allows detection of increased susceptibility. The concentration of each stressor selected is indicated by a dark shaded data point in the following graphs (Figure 8.9).

Appendix B- Glutathione

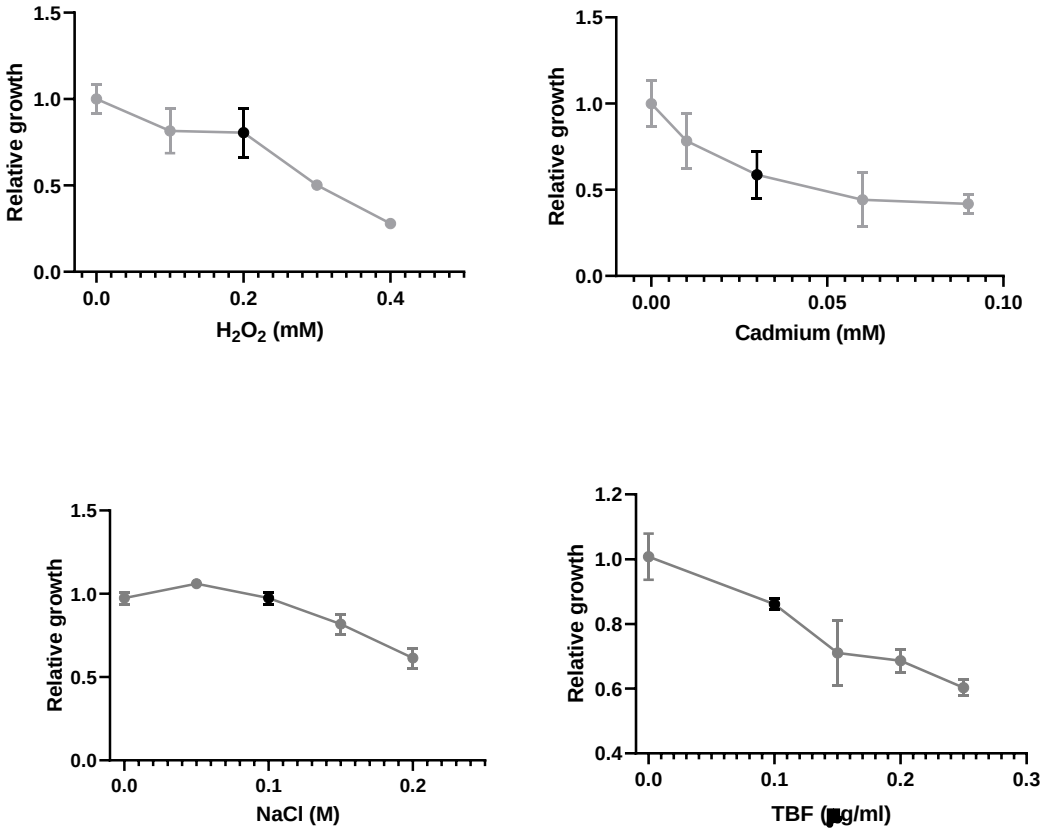


Figure 8.9 Sub-lethal concentrations of stressors. Young zoosporangia were exposed to H_2O_2, cadmium, terbinafine (TBF) or NaCl and growth was measured after 48 h using methylene blue. Relative growth was calculated relative to 0 mM control. The sub-lethal concentrations were: 0.2 mM H_2O_2, 0.04 mM Cd, 0.1 µg/mL TBF, and 0.1 M NaCl.

SECTION 4

Growth effects of glutathione modulators

Methods:

Zoospores (8.5×10^4) were plated in 96 well plates and allowed to encyst overnight. Various concentrations of buthionine sulfoximine (BSO) or glutathione (GSH) were added to the cells. After 48 h growth was measured using the methylene blue assay, and calculated as relative to the untreated control.

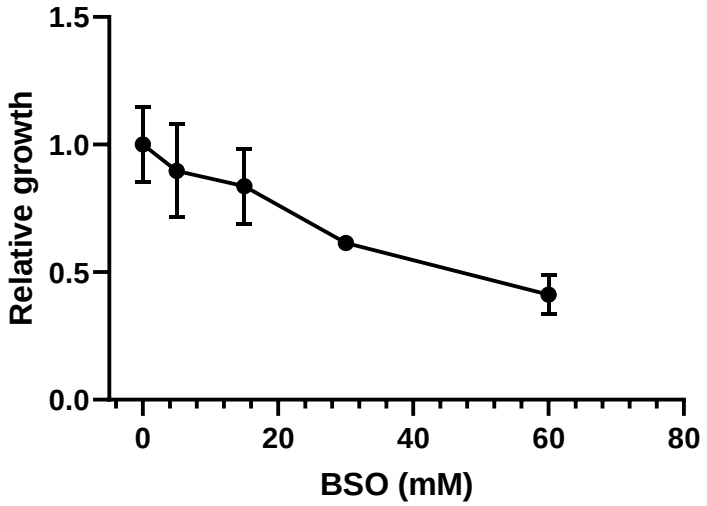


Figure 8.10 Relative growth of *B. dendrobatidis* at various BSO concentrations. The BSO concentrations required for adequate glutathione depletion (30 mM) also caused inhibited cell growth.

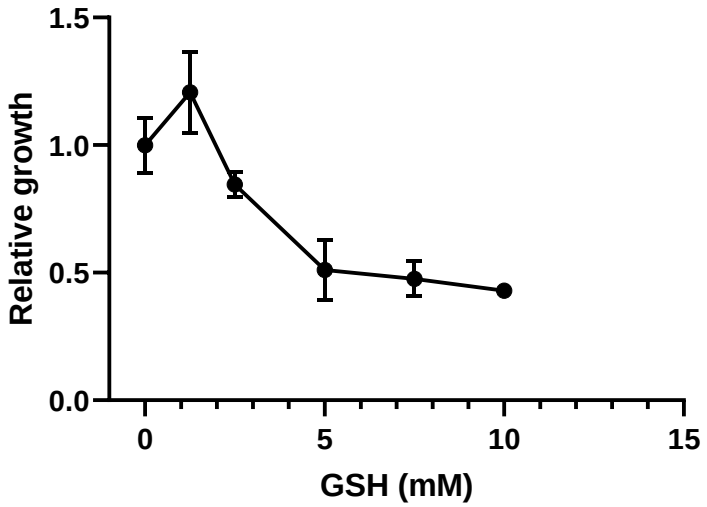


Figure 8.11 Relative growth of *B. dendrobatidis* at various GSH concentrations. Unexpectedly, glutathione concentrations above 2.5mM inhibited cell growth.

Appendix B- Glutathione

SECTION 5

Viability of cadmium stressed cells.

Glutathione depletion and subsequent cadmium (Cd) exposure inhibits cell growth, but the majority of cells are still viable (remain unstained with methylene blue) (Figure 8.12).

0.04mM Cd only	0.04mM Cd + 30mM BSO

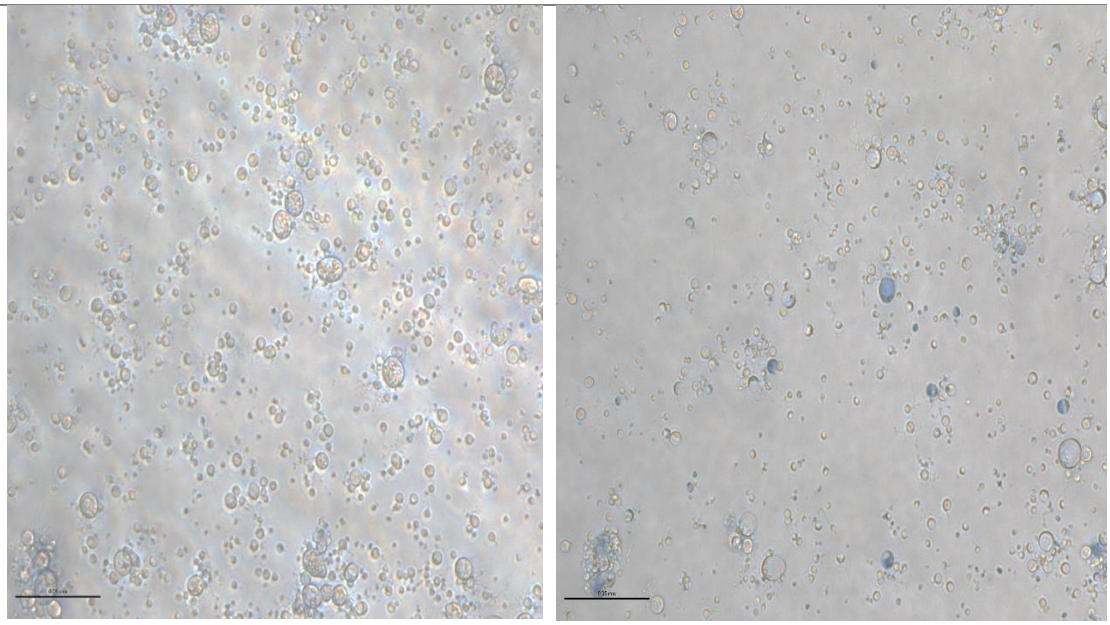


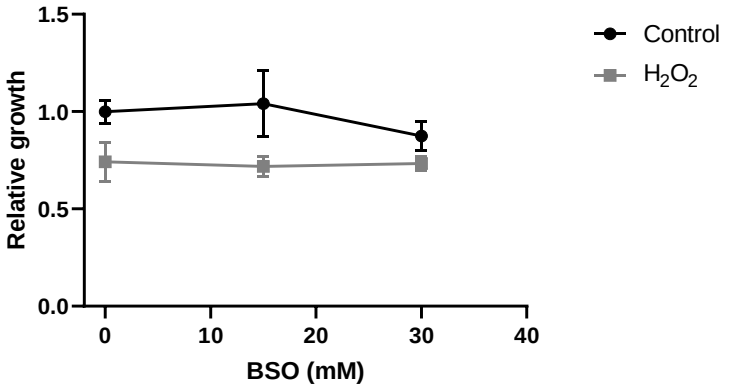
Figure 8.12 Methylene blue staining of cells exposed to BSO and Cd. Cells stained blue are non-viable. The cells in the Cd + BSO treatment are small with large vacuoles, but mostly still viable.

Effect of varying BSO concentrations on stressed cells

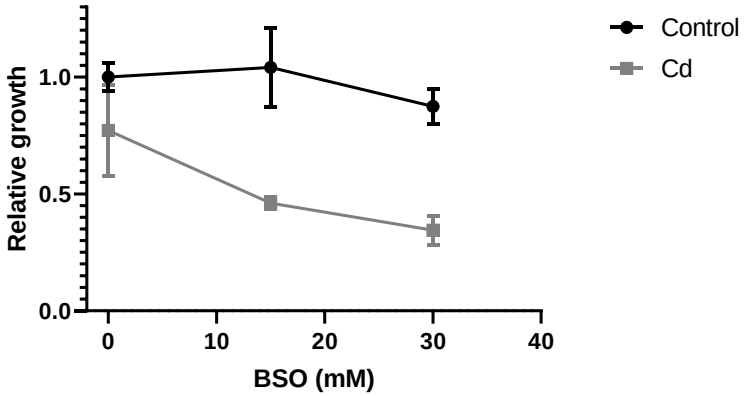
Zoospores (8.5×10^4) were plated in 96 well plates and allowed to encyst overnight. Various concentrations of buthionine sulfoximine (BSO) were added to the cells, followed by addition of the stressor 6 h later. After 48 h growth was measured using the methylene blue assay, and calculated as relative to the untreated control.

Appendix B- Glutathione

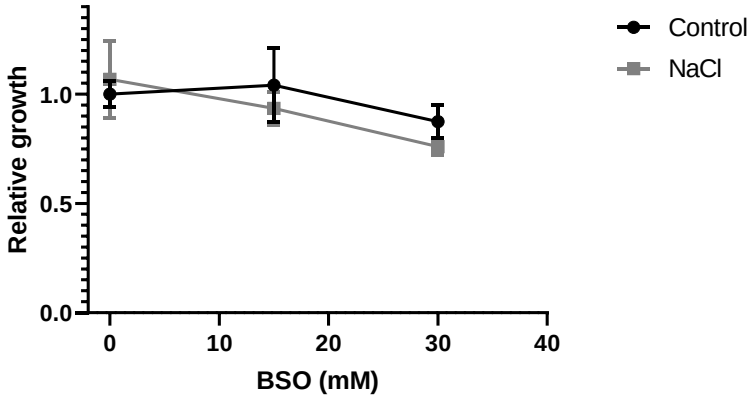
A



B



C



Appendix B- Glutathione

D

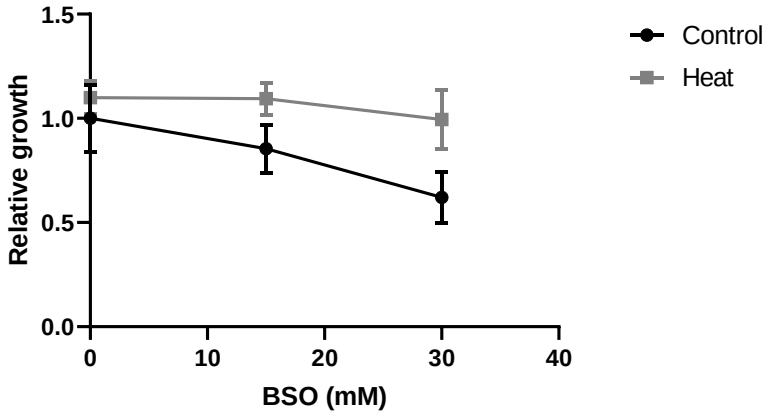
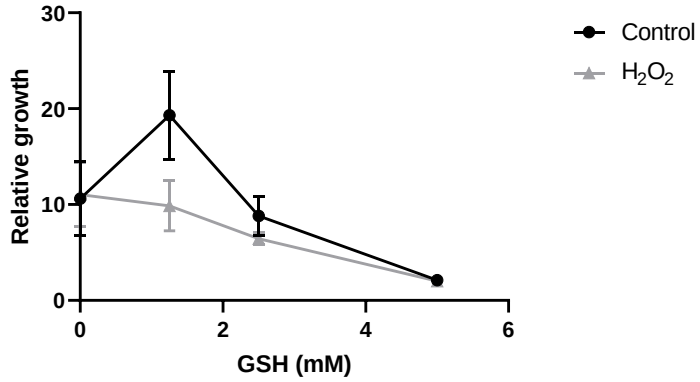


Figure 8.13 Effect of varying BSO concentrations on stressed cells. A=H_2O_2 (oxidative stress), B= Cd (heavy metal stress), C= NaCl (osmotic stress), D= heat stress.

Exposure to various concentrations of glutathione to stressed cells

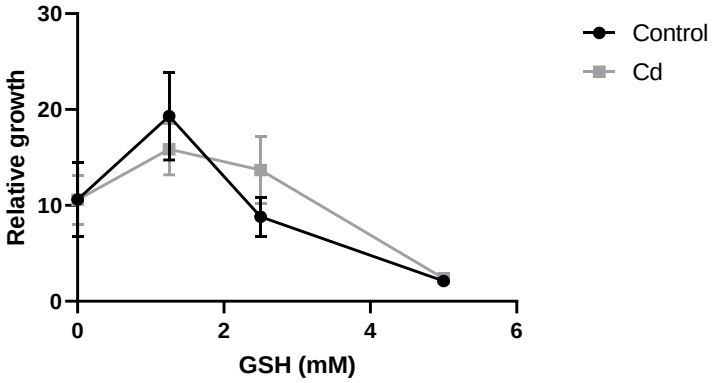
Zoospores (8.5 x 10^4) were plated in 96 well plates and allowed to encyst overnight. Various concentrations of glutathione (GSH) were added to the cells, followed by addition of the stressor 3 h later. After 48 h growth was measured using the methylene blue assay, and calculated as relative to the untreated control.

A

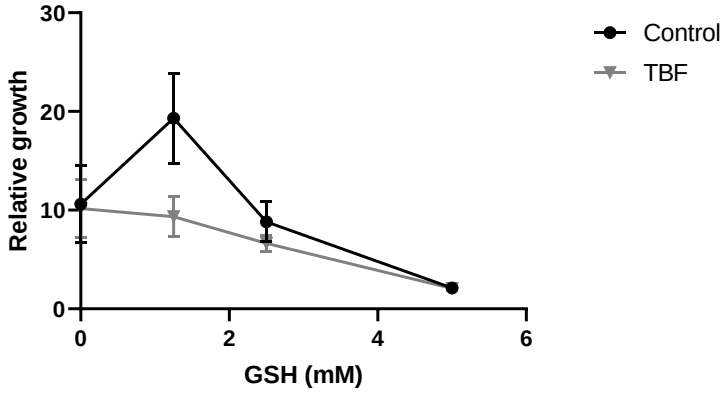


Appendix B- Glutathione

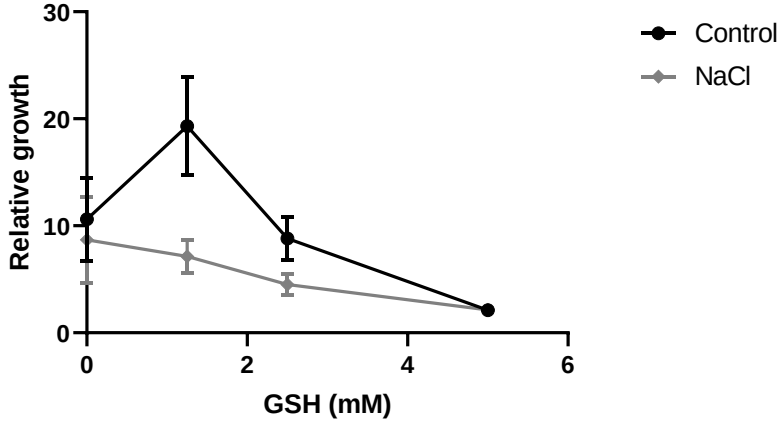
B



C



D



Appendix B- Glutathione

E

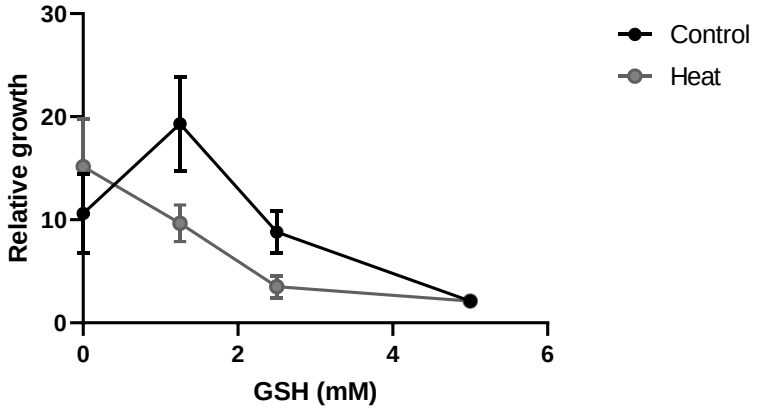


Figure 8.14 Effect of GSH on stressed cells. A=H_2O_2 (oxidative stress), B= Cd (heavy metal stress), C= TBF (Fungicidal stress), D=NaCl (osmotic stress), E=heat stress.

Appendix C

Chapter 3 required substantial optimisation with respect to siRNA delivery and protein quantification and is described in the following section. This Appendix also includes images used to determine viability of cells subjected to cadmium stress.

Section 1: siRNA delivery- Initial trials

Section 2: siRNA delivery-Electroporation optimisation

Section 3: Protein quantification- Western Blot optimisation

Section 4: RT-qPCR normalisation with rRNA

Section 5: Full sized western blot images

Section 6: Viability images of siRNA treated cells under Cd stress.

Appendix C- RNAi

SECTION 1

siRNA delivery- Initial trials.

Methods

There are various methods for delivering genetic material into cells, each with multiple variables to optimise. At the time of experimentation, there were no published methods to deliver genetic material to *B. dendrobatidis* cells. Therefore, it was necessary to trial a variety of methods/variables to determine the most effective delivery methodology. Lipotransfection, electroporation and PEG-mediated transformation were all tested (Table 8.5). Because uptake of dsRNA has been observed in fungi, termed RNA scavenging (Jöchl et al. 2009), I also included a treatment incubating the siRNA directly with the cells without any additional manipulation. All experiments used siRNA#1, modified to include a Cy3 fluorescent tag to visualise siRNA delivery. Lipotransfection experiments generally followed the manufacturer's recommendations, with varied concentrations and siRNA concentrations in both zoospores and zoosporangia. Electroporation experiments used either HEPES buffer (Ozeki et al. 1994) or Petri's buffer (Huitema et al. 2011), and explored different volumes, siRNA concentrations, pulse waveform, voltage and capacitance. PEG experiments were conducted by slowly adding a PEG/LiAc solution to either zoospores or zoosporangia (Thekkiniath 2013), and explored different reagent concentrations, exposure durations and inclusion of a heat shock treatment. For each of the lipotransfection/electroporation or PEG experiments, the control was siRNA added to cells without any further treatment. After each transformation attempt, the cells were transferred to a 12 well plate containing TGhL broth and a sterilised circular glass coverslip. Zoosporangia adhered to the coverslip, which was removed after the appropriate incubation for microscopic observation. A Cy3 filter was used to count fluorescing cells and estimate transformation efficiency. If necessary, extracellular fluorescence was quenched using a drop of trypan blue, and cell viability was determined using a drop of methylene blue and light microscopy.

Appendix C- RNAi

Table 8.5 Summary of different siRNA delivery techniques trialed.

siRNA delivery method	siRNA concentration	Other variables
Lipotransfection – Dharmacon **5 x experiments**	25 nM-100 nM	**Reagent type:** Red, yellow, and blue buffers (Dharmacon) **Reagent concentration:** 0.25-0.5% **Temperature:** 4-21°C **Life stage:** zoospores, zoosporangia.
RNAi max – Thermo **1 x experiment**	12.5 nM	**Reagent concentration:** 3% **Life stage:** zoospores, zoosporangia.
Electroporation **6 x experiments**	1 mM-3 mM	**Wave:** exponential **Voltage:** 400-550 V
PEG/LiAc **6 x experiments**	0.5 µM-2 µM	**Reagent concentration:** 15-40% **Life stage:** zoospores, zoosporangia **Temperature:** 20-30°C
RNA scavenging **4 x experiments**	1 µM	**Growth media:** 10%-100% TGhL **Life stage:** zoospores, zoosporangia. **Washing:** Micrococcal nuclease

Results:

A range of transformation methods were tested for delivering fluorescently tagged siRNA into *B. dendrobatidis* cells. Early attempts were complicated by two factors: the discovery that the siRNA bound to the outside of the zoosporangia wall, and that dead zoosporangia were brightly stained by fluorescent siRNA. Extracellular bound fluorescent siRNA obscured the cell, making it difficult to determine if the contents were also fluorescing. Washing or treating the cells with micrococcal nuclease did not remove the bound siRNA. This problem was eventually overcome by the addition of trypan blue to quench extra cellular fluorescence (Figure 8.15). Trypan blue revealed that intracellular florescence was much less common, indicating that delivery of siRNA had not occurred. Also, the observation that dead sporangia were strongly stained by the fluorescent siRNA raised the difficulty of distinguishing if the siRNA had been successfully delivered, or if the cell had been killed by electroporation. Adding a drop of methylene blue without moving the slide from the stage enabled fluorescing cells to be identified as live or dead (Figure 8.16). Overall, there was no technique that consistently produced high transformation efficiency. In many cases, the siRNA-only control had similar levels of fluorescing cells compared to the treatment cells, along with higher survival. An electroporation protocol to deliver dextran was published during my optimisation experiments (Swafford et al. 2020) hence this protocol was adopted for all further experiments.

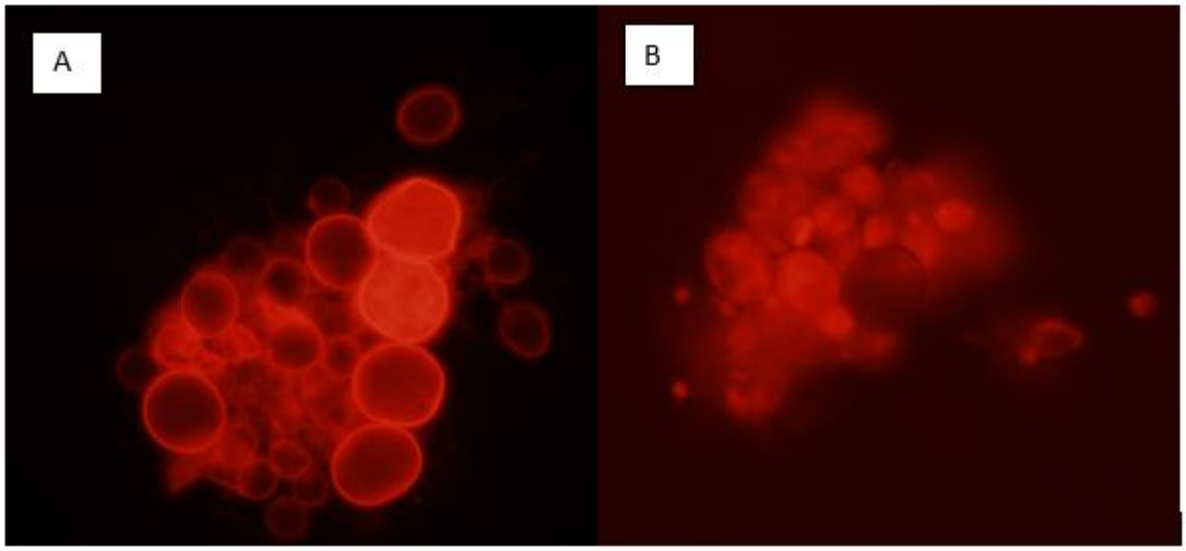


Figure 8.15. Effect of trypan blue. Trypan blue quenches extracellular fluorescence after Cy3-siRNA delivery A: Zoosporangia incubated with fluorescently tagged siRNA which is bound to the cell wall B: Cells from the same sample with trypan blue added to quench cell wall bound fluorescence.

Appendix C- RNAi

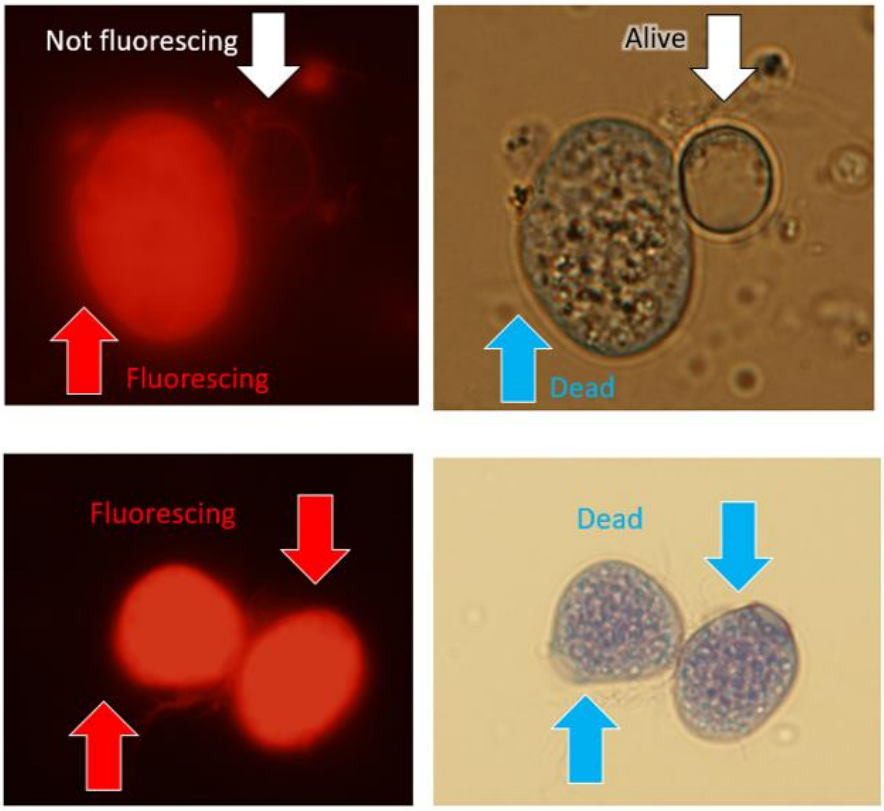


Figure 8.16 Methylene blue staining after siRNA delivery. Methylene blue distinguishes dead fluorescing cells from live cells with successful siRNA delivery.

SECTION 2

Optimisation of siRNA delivery via electroporation

Methods:

Electroporation was chosen as the siRNA delivery method. Zoospores were electroporated following protocols developed for delivering fluorescent dextran (Swafford et al. 2020). Zoospores (1.2×10^7) in 100 µL SM buffer were added to a 4 mm cuvette (Bulldog Bio), along with 100 µL of siRNA (in Horizon buffer). The cuvette was incubated on ice for 10 min before exposure to 2 x 3 ms square wave pulses, with a 5 s interval. Immediately following electroporation, the cuvette was incubated on ice for a further 10 min, after which 200 µL ice cold 1% tryptone was gently added before a final 10 min incubation to allow the zoospores to recover. A range of voltages and siRNA concentrations were tested, with a total of seven experiments (Table 8.6). Each experiment included a control without siRNA to test the effect of electroporation alone (E+R-), a control without electroporation to provide information on background staining due to non-specific uptake of the siRNA (E-R+), as well as a negative no-electroporation and no-siRNA control (E-R-). In addition, a

dextran control was included for comparison. After electroporation and recovery, the entire 400 µL was removed from the cuvette and diluted with 5 mL 1% tryptone, spun at 2500 x *g*/4°C for 5 min and the supernatant removed. This process was repeated for a total of 3 washes with 1% tryptone, and a further 2 washes with Bonners salts. After washing the final pellet was resuspended in 500 µL Bonners salt solution. Flow cytometry (BD LSRFortessa DUEL) was utilised to quantify the percentage of zoospores that successfully received siRNA. From each treatment, 400 µL zoospore solution was fixed 1:1 with fixation buffer (PFA, sucrose and sodium phosphate) on ice for 20 min. Fixation buffer was removed by centrifugation at 2200 x *g* for 5 min, and the pellet resuspended in 200 µL Bonners salts. The zoospore solution was protected from light and stored at 4°C for approximately 1 h until flow cytometry analysis was performed. Prior to analysis, zoospore suspensions were diluted with saline and gently vortexed. A total of 10^4 cells were analysed using forward scatter, side scatter and FITC. Survival and transformation rates were calculated using machine software. The E-R- control from each day was used to identify and gate the population of single, intact, non-fluorescing cells. These gates were then used to quantify percent survival. Percent survival of the experimental treatments was calculated in respect to the untreated control (E-R-). Intact cells displaying FITC fluorescence higher than that of the control were considered to have successfully received siRNA. The background fluorescence in the E-R+ control was subtracted to give a final transformation percentage. Transformation efficiency was calculated by multiplying the percent transformed by the survival rate. Fluorescent intensity data was analysed using FlowJo software (Treestar, BD). Relative fluorescence intensity was obtained by subtracting the intensity of the E-R- control for that experiment.

Table 8.6 Electroporation parameters tested. Combinations of siRNA concentration and voltages tested indicated in green.

	750 V	**1000 V**	**1250 V**	**1500 V**
0.5 µM				
3 µM				
12.5 µM				

Results:

Electroporation successfully delivered siRNA into *B. dendrobatidis* zoospores. Both siRNA concentration and voltage affected the transformation rate (Figure 8.17). Low concentrations and voltages resulted in a lower percentage of transformed zoospores, whereas higher concentrations and voltages increased the transformation rate. Zoospore survival was affected by voltage, with 1500 V resulting in a dramatic decline in survival (Figure 8.18). Survival was generally lower in the presence

of siRNA compared to the electroporation only control, although this difference was not significant (Paired T-test P=0.49). Overall transformation efficiency (survival x percent transformed) was highest in the mid-range voltages (1000-1250 V) and in the mid to high range of tested concentrations (3-12.5 µM) (Figure 8.19). I chose 3 µM siRNA with 1000 V electroporation as the parameters for further experiments.

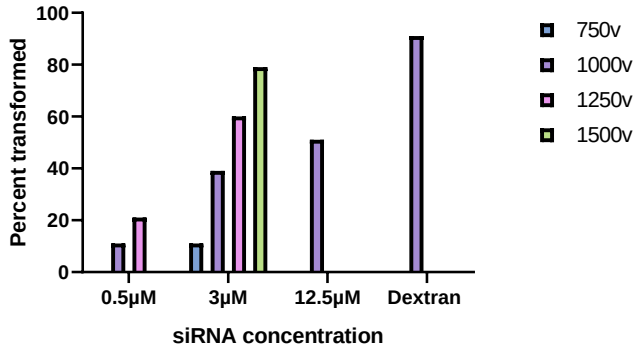


Figure 8.17 Percentage of transformed zoospores after electroporation. Transformation rates increased with siRNA concentration and voltage.

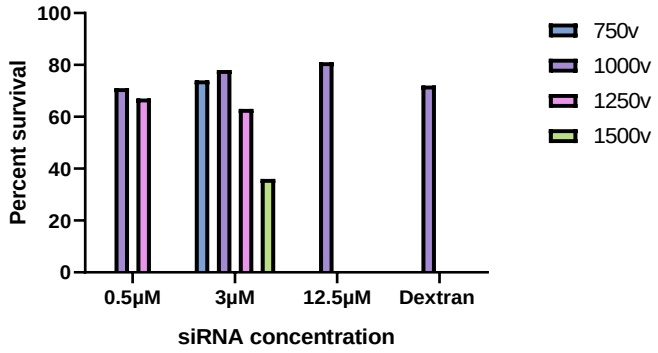


Figure 8.18 Zoospore survival after electroporation. Survival was reduced at 1500v.

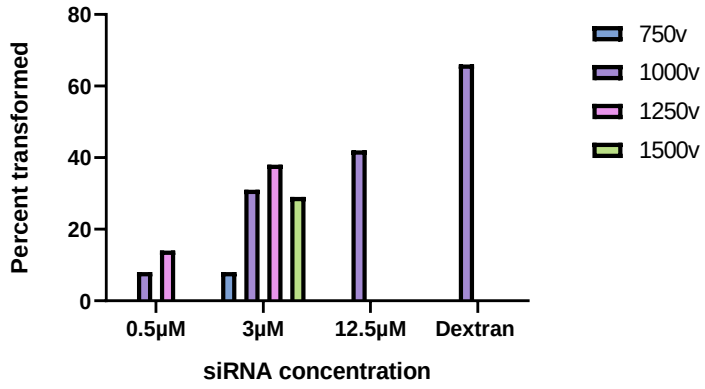


Figure 8.19 Transformation efficiency of different electroporation parameters. Transformation efficiency calculated as survival x percent transformed.

Appendix C- RNAi

SECTION 3

Western blot optimisation

Methods:

As there are no published methods to quantify protein for *B. dendrobatidis* using western blot, I assessed two buffers and cell lysis methods, and determined the minimum amount of protein required for visualisation. A commercial buffer (RIPA ThermoFisher) was compared to a Tris based buffer. Cell lysis via sonication (3 cycles x 20 s 10% amplitude pulses) was compared to bead beating (4 cycles x 30 s with 0.1 and 0.05mm beads). Protein was measured using a Bradford assay, and concentrations of 0.02 µg to 34 µg were trialled. The protein from the pilot trials was run on a PAGE gel and stained with Instant Blue to compare yield and band sharpness. The proteins were then transferred to PVDF membrane for western blot. The optimal buffer, lysis method and protein concentration was determined by visualisation of the western blot under UV light using the G-box software.

Results:

The Tris buffer was clearly superior for protein extraction compared to the commercial RIPA buffer. Cell lysis via sonication produced a higher protein yield, but bead beating produced clearer bands on the page gel (Figure S6). Using the Tris lysis buffer and bead beating, 5 µg of protein was enough to produce a clear band after western blot (Figure 8.20). Hence this protocol used for further experiments.

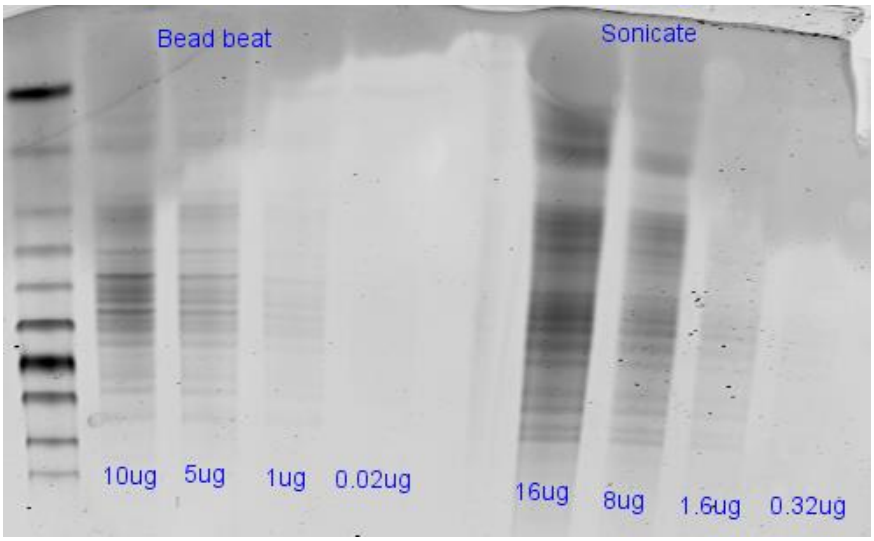


Figure 8.20 SDS-PAGE gel comparing the cell lysis and protein extraction efficiency of bead beating and sonication. Sonication produced higher protein yield, but bead beating produced clearer bands.

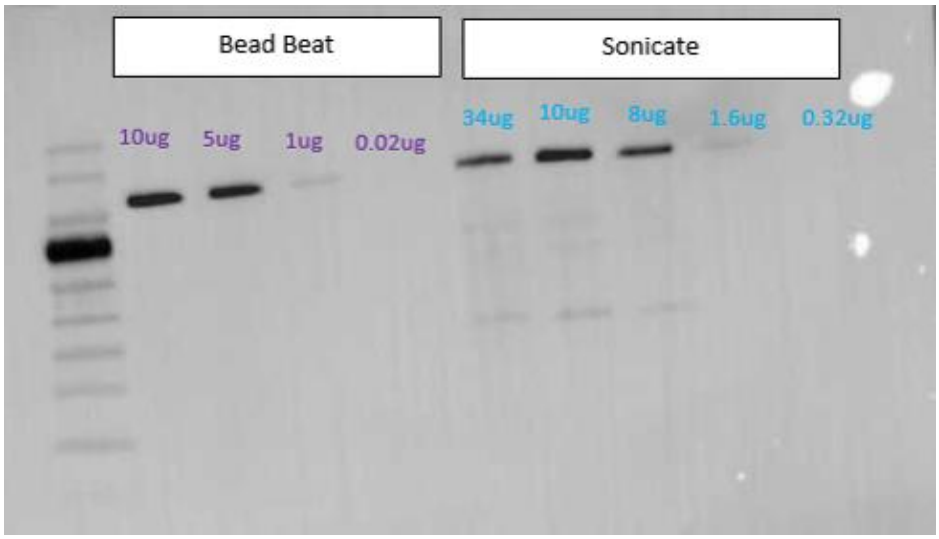


Figure 8.21 Western blot of GCL protein in *B. dendrobatidis* total protein extracts. The minimum total protein quantity required for sufficient detection is 5 µg.

SECTION 4

Additional mRNA analysis of GCL knockdown with rRNA reference gene

In Chapter 3, analysis of reference genes ranked *α centractin* and *APRT* as the most stable. However, the time course experiment had large variation in fold change results using these genes for normalisation at 18-30 h post siRNA delivery. In Chapter 3 it was noted that the ribosomal reference gene *5.8s* was much more stable during the transition from zoospore to zoosporangia. Therefore, the gene expression data from the earlier time points was re-analysed using *5.8s* as the reference gene. This produced less variation and indicated that there was significantly less *GCL* mRNA in the siRNA#1 treated cells compared to the control siRNA treated cells at 18 h (Figure 8.22).

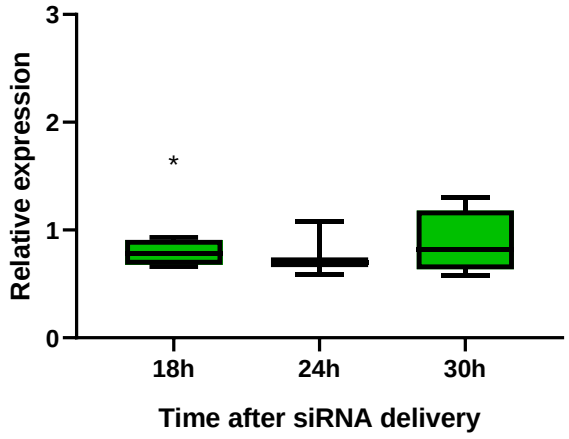


Figure 8.22 Relative gene expression at 18-30 h, normalised to 5.8s. Cell treated with siRNA#1 had significantly less GCL mRNA at 18 h post siRNA delivery (p=0.0124) when normalised to *5.8s*.

SECTION 5

Full size western blot

The western blot images in the main text were cropped for clarity. The full images with loading control and ladder are included below for 48 h (Figure 8.23) and 56 h (Figure 8.24).

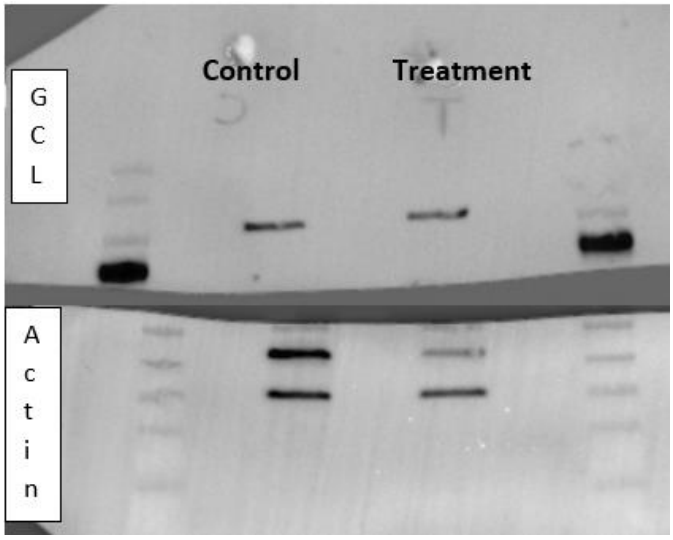


Figure 8.23 Full sized western blot of protein extractions from cells incubated for 48 h after siRNA treatment. The blot was separated just below the 70kDa mark (darkly stained mark on ladder), the top section contains the GCL protein, the bottom section contains the actin loading control and non-specific actin band. A pre-stained ladder is on either side (ThermoFisher-PageRuler).

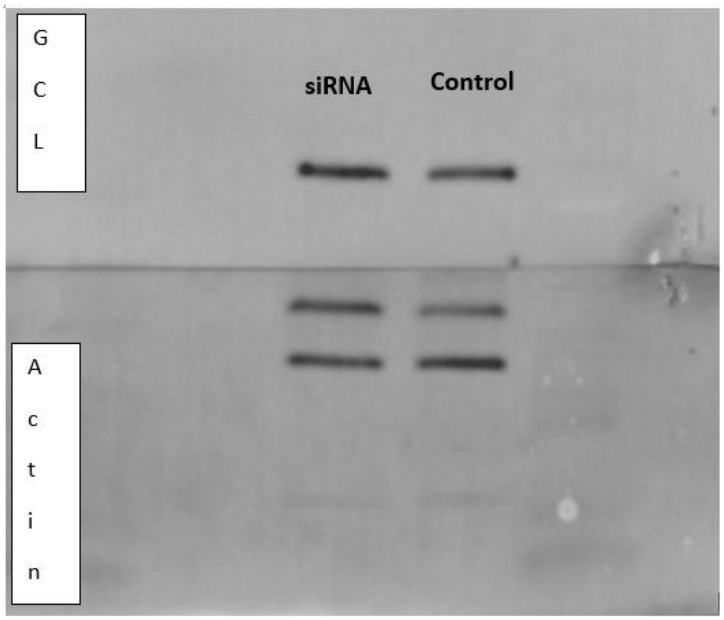


Figure 8.24 Full sized western blot of protein extractions from cells incubated for 56 h after siRNA treatment. The blot was separated just below the 70kDa mark, the top section contains the GCL protein, the bottom section contains the actin loading control and non specific actin band. A faint pre-stained ladder is on either side (ThermoFisher-PageRuler).

Appendix C- RNAi

SECTION 6

Viability of siRNA treated cells

Methylene blue was used to determine cell viability (Chapter 2) of the siRNA treated cells after exposure to Cd stress. There was no obvious difference between control siRNA and GCL siRNA treated cells (Figure 8.25).

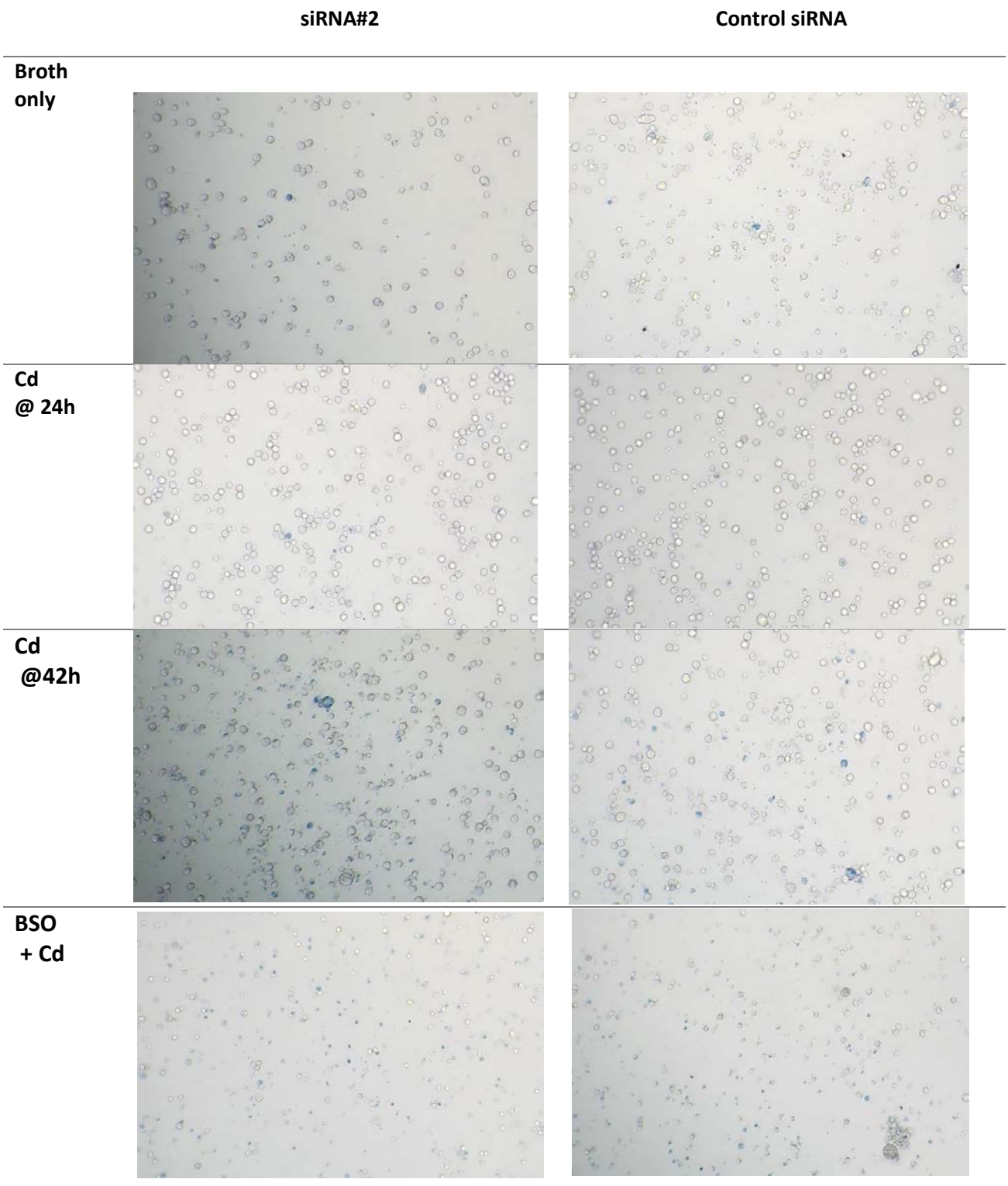


Figure 8.25 Methylene blue staining to estimate viability of siRNA treated cells exposed to cadmium (Cd). There was no difference in viability of siRNA treated cells compared to the control.

Appendix D

This appendix contains extra information in relation to Chapter 5 (Mycovirus screening).

Geographical site characteristics of the freshly collected isolates

New *B. dendrobatidis* isolates were collected from a variety of habitats around Australia

Table 8.7 Site information for new isolates collected by R Webb

Site	Amphibian	Description	Additional information
Yanchep **-31.548299, 115.681952**	Adult *Litoria moorei*	Large lake, National park	Water: 20°C, EC=748, pH=6.70 salt=478
Gwelup **-31.876608, 115.793290**	Adult *Litoria moorei* *Litoria adelaidensis*	Medium lake, Suburban	Water: 16°C, EC=522, pH=7.29 salt=334
Augusta **-34.287352, 115.153576**	Adult *Crinia georgiana*	Roadside seep, farmland	Water: 13°C, EC=652, pH=7.04, salt=417
Dunsborough **-33.634241, 115.120600**	Adult *Litoria adelaidensis*	Roadside seep, farmland	Water: 10°C, EC=790, pH=6.86 salt=505
Nannup **-33.936461, 115.743564**	Adult *Crinia georgiana*	Roadside seep, forest	Water: 10°C, EC=169, pH=6.98 salt=108
Frenchmans creek **-17.304099, 145.916183**	Larvae *Litoria sp*	Lowland fast flowing stream, Rainforest	95 m elevation
Tully River **-17.765604, 145.639052**	Larvae *Litoria sp*	Lowland fast flowing stream, Rainforest	
Kirrama bridge 10 **-18.21087 145.80671**	Larvae *Litoria sp*	Upland fast flowing stream, rainforest	710 m elevation
Gap Creek **-28.049577, 152.383142**	Larvae *Mixophyes fleayi*	Slow flowing stream, Subtropical Rainforest	Water: 17°C, pH=7.22, 720 m elevation
Dalrymple Creek **-27.980320,152.348408**	Larvae *Mixophyes fleayi* *Mixophyes fasciolatus*	Slow flowing stream, Tall open forest	Water 15°C, pH=7.33, 690 m elevation
Armidale **-30.559654, 152.345979**	Larvae *Mixophyes balbus*	Fast flowing stream, Tall open forest	

EC = electrical conductivity, salt = parts per million

Appendix D- Mycovirus

Primers used to validate dsRNA extraction

The dsRNA extraction protocol developed in this thesis was validated using a positive control fungal isolate known to be infected with three mycoviruses. After dsRNA extraction, PCR was performed using the following primers (Table 8.8).

Table 8.8 Primers used to confirm successful extraction of vial dsRNA by modified cellulose protocol

Virus	Forward 5'-3'	Reverse 5'-3'	Product size
CbEVB	CCAATGCGTAGCAGACCTGT	GTTTCCGCACTTGATTVVAT	918 bp
CbEVC	GCAATGAGGAGGCAGAAGAG	GCCAACATGCGAAGGAATAC	809 bp
CbEVD	AGATTTGCAGAAGCGTGGTT	AGCGTGGATGATAAGGTTGG	631 bp

Identification of B. dendrobatidis cells in amphibian tissue.

New *B. dendrobatidis* isolates were collected from naturally infected amphibian tissue. After removal of toe webbing or mouthparts, the tissue was examined for the presence for circular bodies to indicate *B. dendrobatidis* infection (Figure 8.26).

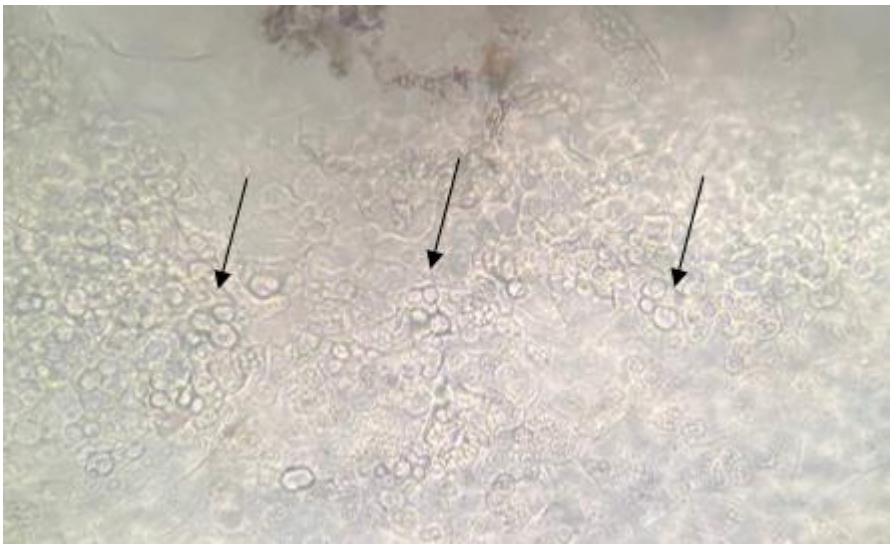


Figure 8.26 Wet preparation of infected amphibian tissue. *B. dendrobatidis* zoosporangia are visible as circular bodies (arrows).