The role of serotonin and glutathione in the pathogenesis of chytridiomycosis

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Abstract

This thesis comprised of six chapters addressing two main aims: 1) To determine the role of serotonin in the pathogenesis of *Batrachochytrium dendrobatidis* (*Bd*) and 2) To determine if glutathione is involved in *Bd*'s ability to evade host immune defences. Chapter 1 is an introduction and literature review of the virulence of the fungus *Bd*. *Bd* causes the disease chytridiomycosis and is a major cause of the global decline and extinction of amphibians. Previous studies of *Bd* susceptible populations of endangered alpine tree frogs (*Litoria verreauxii alpina*) in Australia, found that the levels of the skin metabolite serotonin (5-hydroxytryptamine) increased in the more susceptible populations, as infection progressed, but then decreased in the terminal stages of the disease. The correlation between the levels of skin serotonin and disease progression suggests that serotonin may be part of an immune response against *Bd*.

Serotonin is well studied as a neurotransmitter, but is also known to have antimicrobial, antifungal, and immunosuppressive effects. The amino acid tryptophan (Trp) is synthesized into 5-hydroxytryptophan, and then into serotonin. Oxidized Trp can become kynurenine (Kyn), a structural analog of serotonin, which has been shown to suppress amphibian lymphocytes *in vitro*. Frogs synthesize serotonin from exogenous Trp (frog are unable to produce Trp), while *Bd* produces Trp and Kyn, but does not make serotonin. Finally, some fungi have developed antioxidant defences against the reactive oxygen species from the host immune system, by using a process known as protein glutathionylation. Chapter 1 considered the role of glutathione in the ability of *Bd* to evade host immune defences.

The objective of Chapter 2 was to examine the role of serotonin in the pathogenesis of *Bd*, by determining the effects of serotonin and structural analaogues on *Bd* and lymphocytes *in vitro*. Growth assays were performed by treating *Bd* with serotonin and various structural analogues. The results showed that *Bd* growth decreased with increasing concentrations of serotonin (minimum inhibitory concentration was between 1.5 mM and 2 mM). The structural analogues Kyn and Trp did not reduce sporangial growth, suggesting that only the conversion of Trp into serotonin within frog skin may inhibit *Bd* growth. A structure-activity relationship analysis showed that the terminal primary amine (NH_2) and 5-membered ring of serotonin were important for inhibiting sporangial growth. Human Jurkat lymphocytes were grown as a proxy for amphibian lymphocytes in serotonin and Kyn, and a viability assay was performed. Human Jurkat lymphocytes from a patient are immortalized human T cell lymphocytes obtained from the blood of a boy with T cell leukemia (Schneider et al., 1977).

Serotonin and Kyn both inhibited human Jurkat lymphocyte growth. The host serotonin production may increase in susceptible frogs due to pathogen excretion of Trp, but may decrease in moribund frogs with reduced host metabolic activity. This study suggests that serotonin may have both antifungal and immunosuppressive effects.

Frog skin contains mucous and granular glands that contain biogenic amines that could affect immunity to *Bd*. Therefore, the presence and amount of granular gland serotonin was compared between four amphibian species (common eastern froglets (*Crinia signifera*), southern corroboree frogs (*Pseudophryne corroboree*), Baw Baw frogs (*Philoria frosti*), and cane toads (*Rhinella marina*)) that differ in resistance to chytridiomycosis. Hence, the objectives of Chapter 3 were to determine the location and amount of serotonin in frog and toad skin. Dorsal and ventral skin sites were compared in all species. Additional factors such as age were examined in the cane toads and infection status in the southern corroboree frogs, and a serotonin treatment (0.44 g/L serotonin bath) was administered in common eastern froglets.

Immunohistochemistry (IHC) was used to visualise the location of serotonin within frog and toad skin and high-performance liquid chromatography (HPLC) was used to quantify the amount within the skin. The location of the skin (dorsal versus ventral) did not have an effect on the amount of serotonin detected except in the southern corroboree frogs which had greater levels in dorsal skin. Serotonin was detected in all age classes in the cane toads. Overall, more susceptible species had less serotonin in their skin, suggesting that serotonin's antifungal effects may inhibit *Bd* in some hosts. This contrasts with previous work (Chapter 2) on alpine tree frogs where the more resistant population had lower serotonin levels when infected. Chapter 4 aimed to understand how serotonin inhibits *Bd* growth. *Bd* was grown with various concentrations of serotonin and luminescent assays were used to determine both the overall protease activity and the caspase-3/7 activity as a measure of apoptosis. The protease assay showed that total *Bd* zoospore protease activity decreased with increased concentrations of serotonin, possibly due to reduced cellular metabolic activity. Similarly, *Bd* was grown with various concentrations of serotonin, and RT-PCR was performed on three highly expressed protease genes. Semi-quantitative analysis showed that there was no difference in gene expression in highly expressed protease genes (aspartyl protease (ASP), serine peptidase (S41), and fungalysin metallopeptidase (M36)) in untreated and serotonin treated *Bd*. Real time quantification of expression of a wider array of genes expression would allow detection of more and smaller differences in gene expression between groups. After

performing a caspase assay, we found a decrease in caspase (apoptosis-mediator) activity with increased concentrations of serotonin. Therefore, *Bd* is not likely to be undergoing increased apoptosis in the presence of serotonin.

To determine if serotonin inhibited the Trp biosynthetic pathways, *Bd* zoospores were grown and supplemented with Trp and Kyn. Results showed that treatment of Trp and Kyn does not restore growth of *Bd* with increasing concentrations of serotonin. *Bd* was grown with various concentrations of serotonin, and RT-PCR was performed to determine the expression of the Trp synthase gene. Trp gene expression in *Bd* showed no differences between untreated and serotonin treated *Bd*. Kyn is believed to cause immunosuppression of lymphocytes via apoptosis, hence, a caspase assay was performed to detect if apoptosis occurred in *Bd* treated with serotonin. The results showed that caspase activity decreased with increased concentrations of serotonin further supporting a disruption of metabolic activity by serotonin. This could also be the mechanism of *Bd* cell death. The viability results suggest that serotonin becomes progressively more fungicidal to *Bd* as concentrations of serotonin increase. The effect of serotonin at high concentrations may not correspond to an impact on virulence *in vivo*.

Many pathogens use glutathione (GSH) to protect against host reactive oxygen species that are part of immune defences. Therefore, the objective of Chapter 5 was to determine the role of glutathione in *Bd*'s ability to evade host immune defenses. *Bd* was treated with i. a sublethal dosage of hydrogen peroxide (H_2O_2) as a proxy for host reactive oxygen species, ii. serotonin which in inhibits *in vitro Bd* growth *in vitro*, and iii. buthionine sulfoximine (BSO), which inhibits the rate-limiting enzyme in glutathione synthesis. The *Bd* cultures were analyzed via 1-D and 2-D Western blot, and 2-D gel electrophoresis and mass spectrometry to identify differences in glutathionylated proteins. The Western blots showed that oxidative stress had an effect on numerous glutathionylated proteins in *Bd*. Glutathionylated enolase was identified in the untreated control, but the enzyme was not glutathionylated at a sublethal concentration of H_2O_2. α-Enolase is found in many species of fungi and plays a part in the glycolytic pathway. This glutathionylated pattern and cellular abundance suggests a role for enolase as a glutathione reservoir. Therefore, enolase may provide reducing agents for *Bd* as a protective mechanism against host oxidative stress. For serotonin, the results showed that *Bd* does not appear to use protein glutathionylation to protect proteins from the inhibitory

effects of serotonin. Finally, There was no observable difference in glutathionylation of proteins in the untreated control and BSO control.

Lastly, Chapter 6 discusses the overall conclusions of this thesis, gaps in knowledge, and suggestions for future areas of investigation. In summary, serotonin is a bioactive compound with diverse functional effects, and further work is needed to determine its likely context specific roles in the pathogenesis of chytridiomycosis. Additional studies on glutathione stores may provide insight into *Bd*'s mechanisms of resistance against host immune defences, while identification of glutathionylated proteins may help characterise virulence factors in *Bd*. Given the apparent lack of a cellular immune response from amphibians to *Bd*, further work to understand metabolic immune responses and their role in resistance to chytridiomycosis are warranted even though as shown here, they can be complicated and require substantial effort to understand.

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Statement of Access ii

Declaration iii

Copyright iv

Acknowledgements v

Statement of the Contribution of Others vi

Abstract vii

Publications in Support of this Thesis xi

Conferences and Presentations xi

CHAPTER 1 | General Introduction 1

1.1 | Pathogenic fungi 1

1.2 | Chytridiomycosis 2

1.2.1 | Global amphibian declines 2

1.2.2 | Pathology and clinical signs 6

1.2.3 | Origin of *Bd* 6

1.2.4 | Taxonomy and life-cycle of *Bd* 7

1.2.5 | Lineages of *Bd* 9

1.2.6 | Ecology 9

1.2.7 | Transmission and spread of *Bd* 10

1.2.8 | Detection and treatment 11

1.3 | *Bd* virulence factors 12

1.3.1 | Proteins 12

1.3.2 | Metabolites 13

1.3.3 | Growth 13

1.4 | Antioxidants 13

1.5 | Amphibian immune response 14

1.5.1 | Adaptive immunity and innate immunity 14

1.5.2 | Serotonin 15

1.6 | Objectives 16

CHAPTER 2 | Susceptibility of frogs to chytridiomycosis correlates with increased levels of immunomodulatory serotonin in the skin 19

Co-author consent to include jointly authored papers in the thesis 20

2.1 | INTRODUCTION 22

2.2 | METHODS 24

2.2.1 | Study subjects, exposure experiment, and sampling 24

2.2.2 | Growth of *Bd* by liquid culture 24

2.2.3 | Metabolite isolation from skin tissue and *Bd,* and analysis by GC-MS and UPLC qTOF 25

2.2.4 | Immunohistochemistry 26

2.2.5 | Granular gland fullness 26

2.2.6 | *Bd* growth assays 27

2.2.7 | Supplementation experiments with Trp and Kyn 28

2.2.8 | Jurkat growth assays 28

2.2.9 | MTT cell proliferation assay 29

2.3 | RESULTS 29

2.3.1 | Serotonin levels change over the course of infection 29

2.3.2 | Location of serotonin in skin and changes in levels with infection status 33

2.3.3 | Serotonin inhibits *in vitro* growth of *Bd* sporangia 33

2.3.4 | Serotonin inhibits *in vitro* growth of Jurkat lymphocytes 39

2.4 | DISCUSSION 39

2.4.1 | Serotonin levels over the course of infection 39

2.4.2 | Localization of serotonin in frog skin 39

2.4.3 | The effects of serotonin *in vitro* 39

2.4.4 | Immunosuppressive effects of serotonin 40

2.5 | Conclusions 41

CHAPTER 3 | Characterisation of serotonin in amphibian skin 59

3.1 | INTRODUCTION 62

3.2 | METHODS 63

3.2.1 | Tissue collection 63

3.2.2 | Diagnosis of *Bd* by qPCR 66

3.2.3 | Immunohistochemistry (IHC) 67
3.2.4 | High-performance liquid chromatography (HPLC) 67
3.2.5 | Mass Spectrometry (MS) 68
3.2.6 | Quantification of granular gland abundance 68
3.3 | RESULTS 69
3.3.1 | Baw Baw frogs 69
3.3.2 | Cane toads 69
3.3.3 | Common eastern froglets 69
3.3.4 | Southern corroboree frogs 70
3.4 | DISCUSSION 85
CHAPTER 4 | Mechanisms of *Bd* inhibition by serotonin 96
4.1 | INTRODUCTION 98
4.2 | METHODS 99
4.2.1 | Growth of *Bd* in liquid culture 99
4.2.2 | RNA extraction and purification 100
4.2.3 | Complementary DNA (cDNA) synthesis 100
4.2.4 | Reverse transcription polymerase chain reaction (RT-PCR) 100
4.2.5 | Semi-quantitative transcript analysis 101
4.2.6 | Protease Assay 101
4.2.7 | Caspase assay 101
4.2.8 | Viability stain 102
4.3 | RESULTS 102
4.3.1 | Effect of serotonin on Trp synthase gene expression 102
4.3.2 | Decrease in protease and caspase activity 102
4.3.3 | Effect of serotonin on protease gene expression 102
4.3.4 | *Bd* viability stain 103
4.4 | DISCUSSION 109
CHAPTER 5 | Protein glutathionylation in *Bd* 114
5.1 | INTRODUCTION 117
5.2 | METHODS 121

5.2.1 Inhibitory dose of hydrogen peroxide 121
5.2.2 | *Bd* culture and treatments 121
5.2.3 | Protein extraction 124
5.2.4 | PAGE and Western Blot 124
5.2.5 | 2-D gel electrophoresis 125
5.2.6 | Bioinformatic Analysis 125
5.3 | RESULTS 126
5.3.1 | Decrease in overall protein glutathionylation with peroxide treatment 126
5.3.2 | Protein glutathionylation of enolase 127
5.4 | DISCUSSION 135
CHAPTER 6 | General Discussion 138
6.1 | Introduction 138
6.2 | Serotonin 138
6.2.1 | Growth assays 139
6.2.2 | Immunosuppressive effects 140
6.2.3 | Localisation and quantification 141
6.2.4 | The effect of serotonin on protease activity 144
6.2.5 | Future areas of investigation 145
6.3 | Protein Glutathionylation 145
6.4 | Conclusions 146
APPENDIX 1| James Cook University ethics approval for animal-based research or teaching. 149
APPENDIX 2 | Chapter 2 Publication 150

TABLE 1.1 Australian amphibians in decline, extinct or threatened due to *Bd* and their listing under the Commonwealth Environment Protection and Biodiversity Conservation Act 1999 (EPBC Act) (Skerratt et al., 2016, Scheele et al., 2017). 4
TABLE S2.1 Experimental design with the number of frogs from each population and treatment group (*Bd* infected or uninfected control) sampled at each time point post-exposure. 48
TABLE S2.2 Qualitative description of *Bd* treated with a range of serotonin concentrations in HEPES buffer. 49
TABLE S2.3 Genes in the tryptophan biosynthesis pathway from *Saccharomyces cerevisiae*, that are homologs found in *Batrachochytrium dendrobatidis*. 57
TABLE S2.4 Genes involved in tryptophan metabolism from *Saccharomyces cerevisiae*, that are homologs found in *Batrachochytrium dendrobatidis*. 58
TABLE 3.1 Statistical test of fixed effects for location and infection status, in the amount of serotonin produced in dorsal and ventral skin of southern corroboree frogs (*Pseudophryne corroboree*). 80
TABLE 3.2 Average concentration of serotonin in dorsal and ventral frog and toad skin, via high performance liquid chromatography (HPLC). 81
TABLE 3.3 Proportion of frogs and toads staining positive for serotonin via immunohistochemistry (IHC). 82
TABLE 3.4 Average concentration of serotonin (mg) ± standard error of the mean, in three replicates of the common eastern froglet (*Crinia signifera*) and the southern corroboree frog (*Pseudophryne corroboree*) based on mass spectrometry analysis, at m/z 224 and m/z 160. 84
TABLE S3.1 Concentration of serotonin in the skin of cane toads (*Rhinella marina*) per gram of skin tissue. ND = Not Detected. 89
TABLE S3.2 Concentration of serotonin in the skin of common eastern froglet (*Crinia signifera)* per gram of skin tissue. 90
TABLE S3.3 Concentration of serotonin in the skin of corroboree frogs (*Pseudophryne corroboree*) per 8 mm diameter skin punch. 91
TABLE 4.1 DNA primers used for the transcript analysis. 104
TABLE 5.1 Treatment concentrations and *Batrachochytrium dendrobatidis* growth times. 123
TABLE 5.3 Top 10 proteins identified from the *Batrachochytrium dendrobatidis* no treatment control, based on highest sum PEP score. 131

TABLE 5.4 Top 10 proteins identified from *Batrachochytrium dendrobatidis* in the high H_2O_2 for 24 h treatment, based on highest sum PEP score.....132

FIGURE 2.1 Skin Serotonin Levels over the Course of Infection in Three Populations of *L. v. alpina.*.....31
FIGURE 2.2 Infection Intensity and Concentration [Relative Units] of Serotonin within *L. v. alpina* skin.....32
FIGURE 2.3 Immunohistochemistry of ventral *Pseudophryne corroborree* skin with Serotonin Antibody.....34
FIGURE 2.5 Effect of Supplementation of Cultures.....36
FIGURE 2.6 Effect of Serotonin and Kynurenine (Kyn) on Jurkat Cells.....37
FIGURE 2.7 Average absorbance at 492 nm of *Bd* treated with serotonin.....38
FIGURE S2.1 The Product Ion from MS/MS Fragmentation of the Protonated Serotonin Extracted from Frog Skin.....43
FIGURE S2.2 Growth of *Bd* zoospores as measured by optical density using a spectrophotometer.....44
FIGURE S2.3 Representative Images of *Bd* Grown in Different Concentrations of Serotonin. 45
FIGURE S2.4 Tryptophan (Trp) Biosynthesis Pathway. 46
FIGURE S2.5 Tryptophan (Trp) Metabolism Pathways. 47
FIGURE 3.1 Representative size classes of cane toads (*Rhinella marina*) used for age comparison.....65
FIGURE 3.2 Immunohistochemistry staining for serotonin in the ventral skin of a representative Baw Baw frogs (*Philoria frosti*) (n = 6).....71
FIGURE 3.3 Immunohistochemistry staining for serotonin in the ventral skin of a representative cane toad (*Rhinella marina*) (n = 37). 72
FIGURE 3.4 Percentage of cane toads (*Rhinella marina*) that had granular gland staining for serotonin by immunohistochemistry (IHC), by age class and skin location.....73
FIGURE 3.5 High-performance liquid chromatography (HPLC) chromatograms. 74
FIGURE 3.6 Immunohistochemistry staining for serotonin in the ventral skin of a representative common eastern froglet (*Crinia signifera*) (n = 8).....75
FIGURE 3.7 Immunohistochemistry staining for serotonin in the ventral skin of a representative southern corroboree frog (*Pseudophryne corroboree*) (n = 15).....76
FIGURE 3.8 Representative high-performance liquid chromatography (HPLC) chromatogram. 77

FIGURE 3.9 Skin serotonin concentration of dorsal and ventral skin in infected and uninfected southern corroboree frogs (*Pseudophryne corroboree*).....78
FIGURE 3.10 Serotonin concentration vs. zoospore load in dorsal (green) and ventral (blue) skin from southern corroboree frogs (*Pseudophryne corroboree*). 79
FIGURE 3.11 Full scan mass spectrometry profiles of serotonin and tryptophan standards, and common eastern froglet (*Crinia signifera*) and corroboree frog (*Pseudophryne corroboree*) skin samples.....83
FIGURE S3.1 Mass spectrometry product ion scan for serotonin. 92
FIGURE S3.2 Mass spectrometry product ion scan for tryptophan. 93
FIGURE S3.3 Mass spectrometry product ion scan for serotonin in three replicates of common eastern froglet (*Crinia signifera*). 94
FIGURE S3.4 Mass spectrometry product ion scan for serotonin in three replicates of southern corroboree frog (*Pseudophryne corroboree*). 95
FIGURE 4.1 Gene expression of tryptophan synthase alpha-chain (TrpA) in *Bd* treated with and without 0.3125 mM serotonin.....105
FIGURE 4.2 Gene expression bands on an agarose gel for *Bd* treated with 0 mM and 0.3125 mM of serotonin.....106
FIGURE 4.3 Serotonin decreases both A. protease and B. caspase activity in *Bd in vitro*..107
FIGURE 4.4 Viability of *Batrachochytrium dendrobatidis* sporangia with increasing serotonin concentrations. 108
FIGURE 5.1 Oxidation of cysteine residues and glutathionylated protein formation, from Lushchak (2012). 120
FIGURE 5.2 Sporangia growth assay with hydrogen peroxide (H_2O_2) shows a reduction in *Batrachochytrium dendrobatidis* (*Bd*) sporangia size. 128
FIGURE 5.3 A. Polyacrylamide gel (PAGE) and B. chemiluminescence image of polyvinylidene fluoride (PVDF) membrane from Western blot analysis, for *Bd* treated with peroxide, serotonin, and buthionine sulfoximine (BSO). 129
FIGURE 5.4 Coomassie stained PAGE gels and Western blot analysis of control and 0.15 mM H_2O_2 24 h treatment samples.....130
FIGURE 5.5 Amino acid alignment of enolase proteins. 133
FIGURE 5.6 Three-dimensional model of enolase found in *Batrachochytrium dendrobatidis*. 134
FIGURE 6.1 Overall thesis findings.....148

CHAPTER 1 | General Introduction

1.1 | Pathogenic fungi

As of 2019, 25% of the world's plant and animal species were threated with extinction (IPBES, 2019a). The loss of biodiversity (the variety of living organisms) is largely due to anthropogenic causes, including overexploitation (Maxwell et al., 2016), habitat loss (Brooks et al., 2002), climate change (Thomas et al., 2004), toxicity of the environment (De Laender et al., 2014), introduction of invasive species (Simberloff, 2014), and pathogen pollution (Cunningham et al., 2003). Pathogens have greatly influenced the dynamics of wild animal populations, through emerging infectious diseases and introduced pathogens from captive-bred animals. For example, *ranavirus* has been transmitted into the wild by farmed American bullfrogs (*Lithobates catesbeianus*) that escaped into the environment (Ruggeri et al., 2019, Both et al., 2011). Changes in human and domestic animal migrations and trade of products and food are major means of transmitting pathogens globally.

Pathogenic fungi have caused population declines in a wide variety of animal taxa and environments; *Pseudogymnoascus destructans*. causes white-nose syndrome and has caused significant decline in bats (Gargas et al., 2009), *Batrochochytrium dendrobatidis* (*Bd*) causes chytridiomycosis and widespread declines and extinction of amphibian species (Berger et al., 1998a), *Fusarium solani* results in hatching failure in loggerhead sea turtles (Sarmiento-Ramirez et al., 2010), *Aspergillus sydowii* causes sea-fan aspergillosis in soft coral species (Kim and Harvell, 2004), and *Nosema* spp. cause colony collapse disorder and population declines in bees (Kim and Harvell, 2004).

Many emerging fungi have a broad host range that helps them spread widely and persist in the environment (Fisher et al., 2012). For exampe, the fungus *Bd* infects over 700 species of amphibians (Olson and Ronnenberg, 2014), while the fungus responsible for sudden oak death and ramorum blight, *Phytophthora ramorum*, infects over 100 plant species (Grunwald et al., 2008). Other strategies that pathogenic fungi use to persist and disperse in the environment include having saprobic life-stages or producing long-lived resistant spores (Fisher et al., 2012).

Some fungal pathogens are highly virulent and can cause mass mortality, leading to population declines or extinction. Virulence factors are mechanisms that increase disease severity, and include the production of molecules that improve infectivity or host immune system evasion. Pathogenicity is the ability to cause disease, whereas virulence is the degree

of pathogencity. A well-studied example is *Aspergillus fumigatus* which causes liver damage and produces aflatoxins with immunosuppressive effects on macrophage function in humans (Williams et al., 2004, Neldon-Ortiz and Qureshi, 1991). Fungal virulence can also vary among host species. In the globally distributed fungus *Bd*, virulence can vary amongst strains and amphibian hosts (Dang et al., 2017). In Brazil, *Bd* lineages have hyrbridized (Schloegel et al., 2012) and have high virulence in native host species (Greenspan et al., 2018).

1.2 | Chytridiomycosis

1.2.1 | Global amphibian declines

The pathogenic fungus *Bd* causes the disease chytridiomycosis and is a major cause of the global decline and extinction of amphibians (Skerratt et al., 2007). Recently, the Intergovernmental Science-Policy Platform on Biodiversity and Ecosystem Services (IPBES) reported that greater than 40% of global amphibian species are threatened with extinction (IPBES, 2019b). Infection with *Bd* is listed as notifiable by the World Organisation for Animal Health (OIE) (OIE, 2019). The fungus is found in frogs and toads (order Anura), salamanders and newts (order Caudata), and caecilians (order Gymnophiona). *Bd* is an aquatic fungi that infects the keratinized epidermis of amphibians (Berger et al., 1998a). The fungus is able to spread quickly in some locations, and has spread over much of South America in 20 years (Lips et al., 2008).

Since the 1980's, frog populations in Australia and globally have declined dramatically due to the spread of *Bd* (Skerratt et al., 2007). Six species of frog are extinct in Australia, seven are endangered (Table 1.1) and 22 others have declined substantially due to chytriomycosis (Skerratt et al., 2016). Australia has unique native fauna due to a long history of evolutionary isolation, and is thus susceptible to introduced parasites (Abbott, 2006). Chytridiomycosis has been reported in all Australian states and the Australian Captial Territory, but has not been found in the Northern Territory (Skerratt et al., 2008).

Chytridiomycosis occurs in a broad range of Australian climates. Spatial models of environmental suitability have predicted that *Bd* will persist in cool and wet areas of Australia, with the central region being unsuitable (Murray et al., 2011). *Bd* has likely reached its range limit in Australia, though models predict that areas where *Bd* has not been detected may be suitable, such Cape York, Queensland and southwest Tasmania (Murray et al., 2011). Other regions where no *Bd* sampling has occurred are predicted to be suitable for *Bd* (uplands in far north Queensland and Brigalow Belt, the western slopes of the Great

Dividing Range in New South Wales, the south-west central tablelands in New South Wales, and in South Australia in the regions surrounding the Mount Gambier and Mount Lofty Ranges) (Murray et al., 2011).

Recovery programs for Australian amphibian species affected by *Bd* have been developed. There are active recovery program for endangered corroboreee frogs (*Pseudophryne corroboree* and *Pseudophryne pengilleyi*). In Kosciuzko National Park, approximately 50 southern corroboree frogs remain in their original habitat. In New South Wales, the Australian Capital Territory, and Victoria, captive assurance colonies are held to allow eggs and tadpoles to be released in their former range. Captive breeding, reintroduction, and monitoring programs in Victoria for endangered spotted tree frogs (*Litoria spenceri*) and critically endangered Baw Baw frogs (*Philoria frosti*) are also in place (Skerratt et al., 2016, Hollis, 2011, NPWS, 2001).

There is no proven method to control chytridiomycosis in wild populations. It is important to understand how some frogs are able to resist *Bd* so that managers can use this knowledge to better protect threatened species. This chapter is a literature review that aims to provide a background on the current research concerning *Bd* and fungal virulence

TABLE 1.1 Australian amphibians in decline, extinct or threatened due to *Bd* and their listing under the Commonwealth Environment Protection and Biodiversity Conservation Act 1999 (EPBC Act) (Skerratt et al., 2016, Scheele et al., 2017).

Common Name	**Species**	**Region**	**EPBC Act Listing Status**
Armoured mist frog	*Litoria lorica*	Carbine Tablelands, northeast Queensland (JCU, 2008)	Critically Endangered
Baw baw frog	*Philoria frosti*	Baw Baw Plateau, Victoria (Hollis, 1995)	Critically Endangered
Kroombit tinkerfrog	*Taudactylus pleione*	Kroombit Tops, Queensland (Clarke et al., 1999)	Critically Endangered
Mountain mistfrog	*Litoria nyakalensis*	Montane, northeastern Queensland (Richards, 1993)	Critically Endangered
Northern corroboree frog	*Pseudophryne pengilleyi*	Brindabella Ranges in Australian Capital Territory and Bogong Mountains/Fiery Range in New South Wales (Osborne, 1989)	Critically Endangered
Northern gastric brooding frog	*Rheobatrachus vitellinus*	Rainforest, mideastern Queensland (Covacevich and McDonald, 1993)	Extinct (1985)
Northern tinkerfrog	*Taudactylus rheophilus*	Montane, northern Queensland (Hero et al., 1998)	Endangered
Peppered tree frog	*Litoria piperata*	Montane wet forest stream, New England Tablelands in New South Wales	Endangered

Sharp-snouted day frog	*Taudactylus acutirostris*	Montane, northern Queensland (McDonald, 1992)	Extinct (1997)
Southern corroboree frog	*Pseudophryne corroboree*	Jagungal Wilderness area, New South Wales (Hunter, 2013)	Critically Endangered
Southern day frog	*Taudactylus diurnus*	Sub-coastal ranges, southeastern Queensland (Hines et al., 1999)	Extinct (1979)
Southern gastric-brooding frog	*Rheobatrachus silus*	Montane, southeastern Queensland (Hines et al., 1999)	Extinct (1981)
Spotted tree frog	*Litoria spenceri*	Montane, Victoria and New South Wales (Gillespie and Hollis, 1996)	Endangered
Tasmanian tree frog	*Litoria burrowsae*	Tasmania	Not Listed

1.2.2 | Pathology and clinical signs

In Australia, chytridiomycosis is listed as a notifiable disease under Australia's National List of Reportable Diseases of Aquatic Animals. It is also listed as notifiable by the World Organisation for Animal Health (OIE) in the Aquatic Animal Health Code. Anurans (frogs and toads), urodeles (newts and salamanders), and caecilians are all susceptible to *Bd* infection (Gower et al., 2013). Declines and extinctions of amphibians in Central-America (Lips et al., 2006, Cheng et al., 2011), North America (Rachowicz et al., 2006), Australia (Berger et al., 1998a), and southern Europe (Bosch and Martinez-Solano, 2006) were caused by *Bd* infections. *Bd* has been found to co-infect amphibians with other pathogens, such as *Ranavirus* (Kik et al., 2012, Whitfield et al., 2013), *Chlamydia pneumonia* (Reed et al., 2000), *Aeromonas hydrophila* (Hill et al., 2010), and *Mycobaterium spp* (Hill et al., 2010).

Bd causes chytridiomycosis by infecting amphibian skin, causing epidermal hyperplasia (the increase of cells in tissue), hyperkeratosis (the thickening of the outer layer of the skin), and hindering cutaneous ion (sodium and potassium) transport, which ultimately results in death via cardiac arrest (Campbell et al., 2012, Voyles et al., 2009). Amphibian skin is important for osmoregulation (regulation of osmotic pressure of body fluid) and for O_2/CO_2 exchange (Heatwole, 1994).

The skin on the ventral abdomen, particularly the pelvic patch, which is a highly vascularized skin area, feet, and toes are common sites of infection in frogs and toads (Berger et al., 2005b, Puschendorf and Bolanos, 2006). In salamanders, the pelvic region, fore and hind limbs, and the central side of the tail are common infection sites (Van Rooij et al., 2011). Common signs of chytridiomycosis are excessive skin shedding, erythema, anorexia, skin discoloration, lethargy, abnormal posture, and poor swimming capabilities may reduce the amphibian's ability to forage, thereby reflecting a reduced body size (Hanlon et al., 2015). In metamorphosed amphibians, the signs vary from sudden death without obvious disease to significant skin disorder. A clinical sign of chytridiomycosis in anuran larvae is the depigmentation of mouthparts (Rachowicz and Vredenburg, 2004). In green tree frogs (*Litoria caerulea*) skin ulcerations occur in severely infected individuals, though uncommon in other species (Berger et al., 2005b). Tail autotomy (separation of the tail from the body) occurs in bolitoglossine salamanders infected with *Bd* (Pasmans et al., 2004).

1.2.3 | Origin of *Bd*

Two hypotheses were proposed to explain the origin of *Bd*: the novel pathogen hypothesis and the emerging endemic hypothesis. The novel pathogen hypothesis describes a

pathogen that has recently spread into a new geographic area, encountering naïve hosts that are susceptible to infection (Rachowicz et al., 2005, Skerratt et al., 2007). The novel pathogen hypothesis was suggested because of *Bd*'s irregular global distribution, wave-like global epidemics, and the existence of amphibian vectors (carriers) (Lips et al., 2006, Kolby et al., 2014, Garner et al., 2006). Alternatively, the emerging endemic hypothesis suggested that a pathogen increases in incidence due to environmental change (Rachowicz et al., 2005). This was supported by *Bd*'s presence in amphibian populations before the beginning of declines and because declines from chytridiomycosis are associated with changes in the environment, such as temperature (Weldon et al., 2004, Bosch et al., 2007).

Several studies have investigated the geographic origin of *Bd*. It is hypothesized that *Bd* has spread anthropogenically into naïve amphibian populations (Berger et al., 1999a). In the Americas, *Bd* appears to have caused a wave of population declines starting in the 1970s in Mexico, moving through Central America to Panama (Cheng et al., 2011). There is low genetic diversity in *Bd* strains across geographic locations with low genetic variation in isolates from North America, Africa, and Australia (Morehouse et al., 2003). Proposed origins included southern Africa, shown by an archival African clawed frogs (*Xenopus laevis*) from 1938 that tested positive for *Bd* (Weldon et al., 2004, Soto-Azat et al., 2010), North America due to genetic similarity with European strains (James et al., 2009), Asia from an archival sample collected in a Japanese giant salamander (*Andrias japonicas*) from 1902 (Goka et al., 2009, Bai et al., 2012), and Brazil from an archived sample from 1894 (Rodriguez et al., 2014). Recently, O'Hanlon et al. (2018) used whole-genome sequencing and found that *Bd* originated in the Korean peninsula in the early 20th century, from the ancestral population 'BdASIA-1'.

1.2.4 | Taxonomy and life-cycle of *Bd*

Bd was named for a blue poison dart frog (*Dendrobates azureus*) from which it was originally isolated (Longcore et al., 1999). *Bd* is a basal fungus within the "lower fungi" phylum Chytridiomycota, order *Rhizophydiales*, family *incertae sedis* (Longcore et al., 1999, Fisher et al., 2009b). Fungi in this phylum have flagellated, motile spores (zoospores). Chytrid fungi are generally saprobic and are found in moist soil and fresh water or parasitic to plants, algae or invertebrates (Longcore et al., 1999). Three species within Chytridiomycota infect vertebrates; *B. dendrobatidis* and *Batrochochytrium salamandrivorans* (*Bsal*) (infects salamanders) within the order Rhizophydiales, and *Ichthyochytrium vulgare* (infects freshwater fish). The closest relative to *Bd* and *Bsal* is *Homolaphlyctis polyrhiza*, which is a

non-pathogenic saprobic fungus. It is estimated that *Bd* and *Bsal* diverged from each other approximately 67.3 million years ago (Martel et al., 2014).

Bd likely reproduces asexually (Morehouse et al., 2003). Although rare, sexual recombination may also occur in *Bd*, as recombination occurs with some lineages of *Bd* (Morgan et al., 2007). The *Bd* life-cycle is divided into two major stages: the substrate-independent and the substrate-dependent stages (Rosenblum et al., 2008). The substrate-independent stage consists of motile, waterborne zoospores. The zoospores encyst on keratinized amphibian skin to invade the epidermis, their preferred substrate (Longcore et al., 1999). During this stage, the *Bd* flagellum is absorbed and the cell wall is formed (Berger et al., 2005a). Adhesion of *Bd* to amphibian skin occurs within 2-4 h after zoospore exposure (Van Rooij et al., 2012). Zoospores mature and cluster at the foci of infection. The zoospore cyst germinates and then develops a germ tube that enters the host epidermis. A new sporangia develops from the tip of the germ tube (Van Rooij et al., 2012, Greenspan et al., 2012b). The sporangia then grow and multiply within epidermal cells located in the stratum corneum and stratum granulosum. The sporangia are transported from the deeper skin to the skin surface by epidermal cells as they differentiate and turnover. Once the sporangium reaches maturity, it releases motile zoospores into the environment via discharge tubes that poke through to the skin surface (Berger et al., 2005a).

The life-cycle of *Bd* in culture is similar to that observed *in vivo*. In culture, *Bd* matures from zoospore to zoosporangium in approximately 4 to 5 days at 22ºC (Berger et al., 2005a). Keratin is not an essential ingredient for *in vitro* growth of *Bd*, though *Bd* can grow on substances that contain keratin, such as autoclaved snake skin, 1% keratin agar, frog skin agar, feathers and geese feet (Longcore et al., 1999, Piotrowski et al., 2004, Garmyn et al., 2012). In culture, the rates of growth and development vary depending on nutrient media. *Bd* can grow *in vitro* in mixtures of varying concentrations of peptonized milk, tryptone, gelatin hydrolysate, lactose, glucose, asparagine, yeast extract, malt extract, peptone, sucrose, maltose, sorbitol, and glycerol (Longcore et al., 1999, Piotrowski et al., 2004, Symonds et al., 2008).

It is suggested that *Bd* uses chemotaxis to find its way to keratinous areas of the host. *Bd* chemotaxis has been observed in studies with toe scales of geese and commercially available keratin (Garmyn et al., 2012). *Bd* may also be attracted to the mucous layer of amphibians, as reported for bacterial pathogens (Garmyn et al., 2012). In contrast, the

mucosome (micro-ecosystem of the mucus) may reduce the infection load on skin, by acting as a defence barrier (Van Rooij et al., 2012).

1.2.5 | Lineages of *Bd*

Phylogenetic studies have shown that there are different lineages of *Bd*. Many fungi can undergo horizontal gene transfer, hybridization or genetic recombination (Richards et al., 2011). This could lead to the creation of pathogenic lineages (Stukenbrock and McDonald, 2008) or increased virulence (Mallet, 2007).The global panzootic lineage (GPL) is the most widespread strain of *Bd* (Schloegel et al., 2012). Two clades of GPL exist: GPL-1 and GPL-2 (Schloegel et al., 2012). The GPL-1 lineage is commonly found in North America and Europe (Schloegel et al., 2012, Piovia-Scott et al., 2014). GPL-2 is found mostly in Central America and Australia (Lips et al., 2006, Picco and Collins, 2007, Schloegel et al., 2012).

GPL is highly pathogenic and causes mortality more quickly than other strains (Fisher et al., 2009b, Piovia-Scott et al., 2014). GPL contains additional copy numbers of chromosomal segments compared to the same chromosomes in isolates from other lineages (Piovia-Scott et al., 2014). Geographically distinct lineages have also been found from Japan (Goka et al., 2009), South Africa (Farrer et al., 2011), Switzerland (Farrer et al., 2011), Brazil (Schloegel et al., 2012) and Korea (Bataille et al., 2013). An isolate from Brazil showed the genetic signature of both an endemic Brazilian lineage and a globally distributed lineage (Schloegel et al., 2012).

1.2.6 | Ecology

Bd prefers cooler temperatures, between 4°C and 25°C (Piotrowski et al., 2004). Declines mostly occur in aquatic and riparian (near rivers and streams) amphibians in high elevations, with cool stream temperatures. Temperature may be important to amphibian immunity, because disease susceptibility may differ seasonally (Andre et al., 2008). A study of three *Bd* isolates from diverse latitudes in eastern Australia showed that each isolate had different responses to temperature (Stevenson et al., 2013). Each isolate had different thermal maxima for growth, zoospore production, and activity in relation to temperature that correlated to their original habitat (Stevenson et al., 2013). Some frogs show lower probability of infection if their body temperatures are greater than the thermal optima for *Bd* (Rowley and Alford, 2013). When *Bd* was grown *in vitro* in temperatures simulating frog basking temperatures, *Bd* growth was reduced (Daskin et al., 2011). The differences in thermal optima for growth is likely why the distribution of *Bd* is restricted to cooler environments. *Bd* poorly tolerates desiccation (Johnson et al., 2003), which may signify why

it is not found in arid environments. One study has shown that *Bd* infected Peron's tree frogs (*Litoria peronii*) exposed to sodium chloride (NaCl) had higher survival rates than those that were not (Stockwell et al., 2012).Warm, saline areas may be natural refuges for amphibians from *Bd* (Heard et al., 2014), as solutions of 5% NaCl are lethal to *Bd* (Johnson et al., 2003).

Macroinvertebrate community structure and algae may also influence the occurrence of *Bd* in the aquatic environment (Strauss and Smith, 2013). The microcrustacean zooplankton, water fleas (*Daphnia*), consume chytrid fungus spores and reduce infection risk (Searle et al., 2013). In addition, green algae may release secondary metabolites that harm *Bd* (Searle et al., 2013, Wolfe and Rice, 1979).

1.2.7 | Transmission and spread of *Bd*

Infection dynamics vary between host species with implications for transmission. The African clawed frog (*Xenopus laevis*) (Rollins-Smith et al., 2009) the North American bullfrog (*Lithobates catesbeianus*) (Greenspan et al., 2012a, Miaud et al., 2016), and the common eastern froglet (*Crinia signifera*) in Australia (Scheele et al., 2016), are believed to serve as *Bd* reservoirs. The North American bullfrogs are traded mostly within the US, and to South America, China, and Europe for food consumption (Herrel and van der Meijden, 2014, Fisher and Garner, 2007). The African clawed frog is traded mostly for scientific research (Weldon et al., 2004). Bullfrogs were introduced into Europe to create populations that could be sold for frog legs (Ficetola et al., 2008). They are traded internationally in large numbers and have a high prevalence of *Bd* (Fisher and Garner, 2007, Bai et al., 2010). Bullfrogs have higher zoospore loads compared to amphibians native to Colorado in the United States of America, and may contribute to maintaining *Bd* in the non-native species within the community (Peterson and McKenzie, 2014), though heavy infections can cause mortality in bullfrogs (Gervasi et al., 2013).

Bd is transmitted among hosts through either the motile zoospores or direct contact with other infected amphibians (Longcore et al., 1999, Rowley and Alford, 2007). Shedding of *Bd* zoospores by hosts into water may create environmental reservoirs of the fungus (Johnson and Speare, 2003, Johnson and Speare, 2005). Under sterile conditions, *Bd* can survive in moist soil and water from weeks to months (Johnson and Speare, 2003, Johnson and Speare, 2005).

Non-amphibian carriers of *Bd* have also been suggested. Crayfish tissues were positive by PCR in the wild (McMahon et al., 2013). Exposure experiments resulted in crayfish mortality, and *Bd* could be transmitted to amphibians that were co-housed

(McMahon et al., 2013). *Bd* DNA has been found on the skin of Panamanian lizards and snakes though it is unclear if *Bd* grows on reptile skin (Kilburn et al., 2011).The toenails of waterfowl may also be infected with *Bd* (Garmyn et al., 2012). Under laboratory conditions, *Bd* can also infect and induce mortality in zebrafish larvae (Liew et al., 2017) and nematode (*Caenorhabditis elegans*) (Shapard et al., 2012). This suggests that there are potentially non-amphibian reservoirs of *Bd*.

The amphibian trade is a major contributor to the dispersal of *Bd* globally, through the intentional release of imported frogs (Kolby, 2014, Kolby et al., 2014, Fisher and Garner, 2007). *Bd* was suspected to be present in Madagascar, when three frog species (*Heterixalus alboguttatus*, *Heterixalus betsileo*, and *Scaphiophryne spinosa*) imported from Madagascar into the United States of America, were positive for *Bd* (Kolby, 2014). *Bd* pathogen pollution from the amphibian trade may occur if infected wastewater is not disposed of correctly.

In the wild, disease risk depends on the complexity of the amphibian community. An increase in host and non-host species richness will reduce the impact of *Bd* prevalence and intensity, by affecting the rate of contact and transmission, though species with long-lived tadpole stages may enhance the persistance of infection within the community (Briggs et al., 2010). Increased population recruitment may allow populations to recover from declines caused by *Bd*. High population densities likely cause an increase in *Bd* infection intensity and continuous reinfection of infected amphibians (Briggs et al., 2010).

Species vary greatly in susceptibility. A recent study involved infecting 20 species and showed the frogs tested (from the *Ranidae*) had generally lower infection intensities and mortality than the toads (*Bufonidae*) (Gervasi et al., 2017). One isolate was used in this study (Gervasi et al., 2017), and different *Bd* isolates may have different effects. However, cane toads are a resistant species and adults are rarely infected (Brannelly et al., 2018a). The host life stage can also influence *Bd* transmission. As tadpoles do not usually die from infection, they can be intra-species reservoirs (Rachowicz and Vredenburg, 2004).

1.2.8 | Detection and treatment

Bd can be detected by histology, quantitative polymerase chain reaction (qPCR) via skin swabs and an IgM monoclonal antibody (mAb), 5C4 (Berger et al., 1999b, Hyatt et al., 2007, Dillon et al., 2017). The monoclonal antibody binds to a surface glycoprotein antigen on zoospores and zoosporangia (Dillon et al., 2017). Chytridiomycosis can be diagnosed in metamorphosed amphibians by observing sporangia in the keratinized skin layers. Irregular thickening of the outermost keratinized layers of the epidermis, erosion of the stratum

corneum, and increased tissue growth in the stratum spinosum is associated with infection. *Bd* does not disseminate into the deeper layers of skin or internal organs (Pessier, 2008).

Immunization against *Bd* has not been shown to be effective (Stice and Briggs, 2010). Probiotic treatment against *Bd* infection has been suggested, using skin bacterial flora that secrete antifungal compounds (Bletz et al., 2013, Woodhams et al., 2020). Effects of inhibitory skin bacteria vary depending on the *Bd* strain (Antwis et al., 2015). A range of antifungal drugs developed for humans can clear *Bd* infections, such as itraconazole and terbinafine. Itraconazole is commonly used, but is toxic to some species (Brannelly, 2014). Itraconazole inhibits the synthesis of ergosterol, which is part of the fungal cell membrane (Marichal et al., 1999).

1.3 | *Bd* virulence factors

Bd virulence factors are only just beginning to be identified. *Bd* is suspected to use a variety of mechanisms, such as proteins and metabolites to evade the host immune system. Attenuation can occur in *Bd* successively passaged on culture media, leading to weakened infectivity and pathogenicity which can be partially restored by passaging through an amphibian host (Brem et al., 2013).

1.3.1 | Proteins

The ability of *Bd* to invade the epidermis and evade the host immune system appears partially due to its secreted proteins, which have a variety of functions. Proteins produced by *Bd* disrupt skin intercellular junctions, causing apoptosis (programmed cell death) (Brutyn et al., 2012). Numerous proteases were identified from zoospore supernatants, suggesting a role for these enzymes in *Bd* infection. Proteolytic activity and elastin degradation may assist *Bd* to colonize the epidermis of their amphibian hosts (Moss et al., 2010). There are relatively few studies that have identified the function of *Bd* proteins (Symonds et al., 2008, Fisher et al., 2009a, Brutyn et al., 2012, Rosenblum et al., 2012, Thekkiniath et al., 2013, Thekkiniath et al., 2015). *Bd* has been shown to secrete a protease that can cleave some amphibian antimicrobial peptides (AMP's) (Thekkiniath et al., 2013). Other proteins, such as elastolytic protease and subtilisin-like serine protease (Moss et al., 2010, Thekkiniath et al., 2013), and genes for fungalysin metallopeptidase (involved in tissue repair) (Rosenblum et al., 2008) were also identified. Unique gene families for *Bd*, especially proteases, have been found by comparing *Bd* genomes to closely related genomes of other fungi (Joneson et al., 2011).

Non-protease proteins have also been identified in *Bd*; adhesins (that may assist adhesion to the epidermal surface) (Rosenblum et al., 2012) and virulence effectors (proteins

that aid in the virulence of the pathogen) such as crinkler proteins and serine peptidases, have been identified (Sun et al., 2011). Genes from the carbohydrate-binding module family 18 (CBM18), which are associated with protecting fungal pathogens from the host, were found in *Bd* (Liu and Stajich, 2015, Abramyan and Stajich, 2012). More research is needed that focuses on identifying *Bd* adhesin and their receptors on the host surface.

1.3.2 | Metabolites

Bd can also release metabolic products that may inhibit host immunity in the skin. *In vitro*, secretions from cultured *Bd* sporangia inhibit the proliferation of lymphocytes via apoptosis (Fites et al., 2013, Fites et al., 2014). *In vivo*, the metabolites inhibit the localised skin immune response (Fites et al., 2014, Piovia-Scott et al., 2014).Several immunosuppressive molecules have been identified from *Bd* supernatants, including methylthioadenosine (MTA), kynurenine (Kyn) and spermidine (Rollins-Smith et al., 2015, Rollins-Smith et al., 2019). MTA is a naturally occurring product of metabolism (Albers, 2009) and Kyn is a product of tryptophan metabolism. Kyn is important to immunity as it plays a role in T cell development (Mezrich et al., 2010). Kyn activates the aryl hydrocarbon receptor (AHR), which effects T cell differentiation in mice cells (Mezrich et al., 2010). Amphibian lymphocytes have been shown to be inhibited *in vitro* by Kyn (Rollins-Smith et al., 2015).

1.3.3 | Growth

An additional virulence factor of *Bd* may be growth rate. Growth rates vary between strains (Voyles et al., 2014). Lower temperatures result in slower population growth rates but longer periods of zoospore activity (Voyles et al., 2012, Stevenson et al., 2013). This could result in higher zoospore encounter rates by amphibians. Hence, virulence of *Bd* is a function of temperature.

1.4 | Antioxidants

Pathogens can face oxidative stress by the host immune system. Some fungi have developed antioxidant defences against the reactive oxygen species of the host immune system via glutathionylation. Glutathione is a tripeptide composed of three amino-acids (γ-L-glutamyl-L-cysteinyl-glycine), and functions as an antioxidant in eukaryotic cells (Sies, 1999). Glutathione can either be in its reduced (GSH) form or oxidized as glutathione disulfide (GSSG) which contains two glutathione molecules joined by a disulfide bond. GSSG is the main mechanism of glutathionylation; GSH is the active antioxidant, and GSSG must be reduced by enzymes, such as glutathione reductase. Protein cysteines connect to the

cysteine of glutathione by a disulfide bond. In some species of pathogenic fungi, disrupting biosynthesis of glutathione can lead to lower levels of virulence, as seen in the yeast *Candida albicans* (Yadav et al., 2011). Previous high performance liquid chromatography (HPLC) analysis of *Bd* extracts confirmed the presence of glutathione (A. Roberts, unpublished data). Glutathione likely provides an evolutionary advantage in *Bd*, as glutathione metabolism genes are thought to have arisen in *Bd* from bacteria via horizontal gene transfer from bacteria led to the transferal of genes responsible for glutathione metabolism (Sun et al., 2016).

1.5 | Amphibian immune response

Amphibians employ both adaptive and innate immunity to combat *Bd* infection. Although *Bd* is a highly virulent pathogen, some species of frogs are able to overcome infection (Davidson et al., 2003, Briggs et al., 2005). Mechanisms for resistance to *Bd* include thermoregulatory behaviour to increase body temperature, innate variations in immunity and presence of inhibitory bacterial flora on skin (Scheele et al., 2017). However, a robust adaptive immune response to *Bd* has not been observed in amphibians by any methods (Grogan et al., 2018c). For example, on histology, there is a marked absence of cellular inflammatory response to foci of infection in all species (Ellison et al., 2014b). This suggests resistance may be related to non-cellular factors.

1.5.1 | Adaptive immunity and innate immunity

Reinfection experiments with three frog species (Cuban tree frog: *Osteopilu septentrionalis*, oak toads: *Bufo quercicus*, Booroolong frog: *Litoria booroolongensis*) showed that although some immune responses to subsequent infections appeared to be stimulated, mortality rates remained high and the potential for developing a vaccination may be low (Cashins et al., 2013, McMahon et al., 2014, Berger et al., 2016).

However, amphibians resistant to *Bd* were shown to have conserved amino acids in the binding pockets of the antigen binding groove of the major histocompatibility complex (MHC) (Bataille et al., 2015). As part of the adaptive immune system, MHC class II is an antigen binding molecule located on antigen-presenting cells, enabling pathogen resistance in the host. Changes in amino acids in the deep pockets of the antigen binding grooves may affect MHC structure, and thereby change MHC's antigen binding affinity (Jones et al., 2006). In a study of MHC genotypes, MHC heterozygosity was related to survival when amphibians were experimentally infected with *Bd* (Savage and Zamudio, 2011). These studies suggest that there is some recognition of *Bd* antigens by the adaptive immune system

and that some resistance is conferred. However, it is possible that these findings have been confounded by another unknown immune mechanism that is non-functionally linked to MHC.

Currently, only the African clawed frog (*X. laevis*) has exhibited antibodies for *Bd*. After injection with heat-killed zoospores, *Bd* specific IgM, IgX and IgY antibodies were found in the skin (Ramsey et al., 2010). Immunization attempts have been made via subcutaneous or intraperitoneal injections of formalin or heat-killed *Bd*, but have not resulted in protective immunity (Stice and Briggs, 2010, Rollins-Smith et al., 2009).

Innate immune defences protect amphibian skin from pathogens in general. Macrophages such as Kupffer cells (Corsaro et al., 2000), complement via pathogen lysis (Lambris et al., 1995), natural killer cells (Horton et al., 2003), and antimicrobial peptides (AMP's) from frog skin glands occur in amphibian skin as a first line of defence against pathogens (Rollins-Smith et al., 2002).

AMP's and metabolites from bacteria can work synergistically to inhibit growth of *Bd* (Myers et al., 2012). Temporin family peptides, which are associated with some antibacterial activity and antifungal activity, were found to be active against *Bd* (Rollins-Smith et al., 2003). Secondary metabolites secreted by symbiotic bacteria on amphibian skin are known to defend against infection. The bacteria *Janthinobacterium lividum*, *Lysobacter gummosus* and *Pseudomonas fluorescens* secrete several metabolites found to inhibit *Bd* growth in vitro (i.e. 2,4-diacetylphloroglucinol, indol-3-carboxaldehyde and violacein) (Brucker et al., 2008, Myers et al., 2012, Lam et al., 2010).

1.5.2 | Serotonin

Little is known about metabolites that can defend the host against *Bd* infection. Recent work suggests a role for serotonin (5-hydroxytryptamine) in disease progression in *Bd* infected skin. A recent study using broad scale metabolomics found skin serotonin levels increased over the course of an experimental infection in susceptible frogs, more than in resistant frogs (Claytor et al., 2019, Grogan, 2014). However, the functional significance of this correlation was not determined.

Serotonin is highly conserved amongst vertebrate groups and has been well studied as a neurotransmitter, but also has other diverse biological activities including a role in vasoconstriction and platelet aggregation. Serotonin has been shown to have effects on other fungi, such as inhibiting sporulation of *Stagonospora nodorum* and *Pyrenophora tritici-repentis* in wheat (Du Fall and Solomon, 2013), and affecting hyphal growth and cell

membrane integrity in *Aspergillus* spp. (Perkhofer et al., 2007). Selective serotonin reuptake inhibitors (SSRIs), which increase available serotonin, were shown to have fungicidal effects *in vitro* on *Aspergillus terreus* and *Candida parapsilosis* (Lass-Florl et al., 2001). Serotonin decreases serine proteinase secretion in *C. albicans* (Mayr et al., 2005).

In humans, serotonin also has been shown to have a variety of effects on immune cells. Monocytes (Dürk et al., 2005) and lymphocytes (Iken et al., 1995) are stimulated by serotonin, which influences the secretion of cytokines. Neutrophils can be recruited (Duerschmied et al., 2013) and T-cells can be activated by serotonin (Nordlind et al., 1992). However, Kyn, a structural analog to serotonin, suppresses amphibian lymphocytes (Rollins-Smith et al., 2015).

Serotonin has previously been detected in the glands of frog skin (Roseghini et al., 1988, Şengezer-İnceli et al., 2004, Yoshie et al., 1985). Frog skin is made predominately of two types of dermal gland; the mucous and granular (poison) glands. Serotonin was detected in both mucus and poison glands of marsh frogs (*Rana ridibunda*) (Şengezer-İnceli et al., 2004), and in the poison glands of Northern leopard frogs (*Lithobates pipiens*) and African clawed frogs (*Xenopus laevis*) (Bennett et al., 1981). Immunohistochemistry revealed the occurrence of serotonin within frog dermal glands (Yoshie et al., 1985). Serotonin may regulate secretion in mucus glands and trigger secretory material in the poison glands of the marsh frog skin (Şengezer-İnceli et al., 2004). The mechanistic link between serotonin and increased susceptibility to *Bd* has not been elucidated. One possibility is via affecting the permeability of skin, since serotonin stimulates sodium transport and decreases the permeability of chloride (Dalton, 1979).

Serotonin is produced from the amino acid L-tryptophan (Trp) and is one of three main host neurotransmitters. Trp is converted to 5-hydroxytryptophan by the enzyme tryptophan 5-monooxygenase, and then converted to serotonin by 5HTP decarboxylase. Amphibians cannot produce Trp, and instead must acquire this amino acid from exogenous sources, such as diet. *Bd* produces Trp and Kyn, but does not produce serotonin (Rollins-Smith et al., 2015).

1.6 | Objectives

Host resistance to *Bd* varies between species and populations. The mechanisms of resistance to *Bd* are unknown, and negligible cellular inflammation is observed in the skin. Acquired immunity has had little effect on protecting amphibians from infection. Skin antimicrobial peptides and inhibitory bacterial skin flora have been shown to be part of a

frog's innate immunity, though little is known of the effects of host metabolites. Previous work by a former Ph.D. student in our laboratory, Dr. Laura Grogan, investigated survival in populations of vulnerable alpine tree frogs (*Litoria verreauxii alpina*) infected with *Bd* (Grogan et al., 2018b). In this work, a metabolomics approach was used to compare expression levels of metabolites excreted from infected frog skin. Serotonin levels increased in the skin of more susceptible frogs as infection progressed (Grogan, 2014). The correlation between the levels of skin serotonin and disease progression may mean that serotonin has a role in disease outcomes. This role could benefit the frog, such as having antifungal effects that are an immune defence against *Bd*. Alternatively, increased serotonin could result in more severe disease due to its immunosuppressive effects. Expanding upon L. Grogan's results regarding serotonin, is the first of two main objectives of this thesis:

- **1) To determine the role of serotonin in the pathogenesis of *Bd***

Within this broad objective there are five aims. My first aim relates to the antifungal and immunomodulatory functions of serotonin and is:

- To determine the effects of serotonin and structural analogues on growth of the *Bd* and human Jurkat lymphocytes *in vitro*

Serotonin is correlated with infection progression in frog skin, and different species and life stages of frogs have varying susceptibility to *Bd*. Serotonin is known to occur within frog skin mucous and granular glands. Therefore, to characterise the presence and amount of granular gland serotonin in some Australian frogs, my next aims are:

- To determine the location and amount of serotonin in the frog and toad skin
- To determine how serotonin levels in the skin are affected by different factors (species, location on the skin, age, infection status, treatment with serotonin)

Due to the complexity of serotonin activity in the host, it is important to understand the mechanism of *Bd* inhibition by serotonin. *Bd* produces Trp and Kyn, therefore, if serotonin inhibits the Trp biosynthetic pathways, we expect that serotonin would reduce Trp gene expression in *Bd*. *Bd* proteases are important for parasitism in frog skin, and therefore if serotonin inhibits *Bd* protease expression, the total protease activity and expression of key protease genes would decrease when *Bd* is treated with serotonin. Kyn has a similar structure to serotonin and causes immunosupression of lymphocytes, possibly via apoptosis mechanisms. Therefore, we hypothesize that serotonin (i) interrupts *Bd* Trp biosynthesis and

metabolism, (ii) inhibits *Bd* protease activity essential for growth, and (iii) causes apoptosis of *Bd*. The aims are:

- To determine if serotonin reduces Trp and protease gene expression in *Bd*
- To determine if serotonin causes apoptosis in *Bd*

Regarding the second objective of my thesis, it is unknown how *Bd* survives in the host skin. Many pathogens use glutathione to protect against oxidative stress from the host immune system. *Bd* produces glutathione, though it is unknown if glutathione will protect its proteins via protein S-glutathionylation. Therefore, the second objective of this thesis is:

- **2) To determine if glutathione is involved in *Bd*'s ability to evade host immune defenses**

Few proteins have been functionally characterized in *Bd*. It is possible that glutathionylated proteins are involved in *Bd* virulence. The sole aim within this objective is:

- To determine if oxidative stress affects protein glutathionylation patterns in *Bd* using sublethal H_2O_2
- To explore the function of glutathionylated proteins

In vitro cultures were treated with 1) hydrogen peroxide (H_2O_2) as a proxy for reactive oxygen species (ROS) stress, 2) serotonin, which inhibits *Bd* growth *in vitro*, and 3) buthionine sulfoximine (BSO), which inhibits glutamylcysteine synthetase (γ-GCS), the rate-limiting enzyme in glutathione synthesis. Western blotting, 2D gel electrophoresis, and mass spectrometry were used to identify differentially glutathionylated proteins.

This thesis is written as a series of connected manuscripts or chapters. Chapter 2 has been published and chapters 3, 4, and 5 are in preparation for publication. Chapter 2 investigates the effects of serotonin and structural analogues on *Bd* and lymphocytes *in vitro*. Chapter 3 characterizes serotonin in frog and toad skin, to determine where serotoin is found in the skin, and how much is present under various conditions. Chapter 4 investigates the mechanisms of *Bd* inhibition by serotonin, by focusing on the effect serotonin has on gene expression and protease activity, since proteases are important for parasitism in frog skin by causing epidermal degradation during infection. Chapter 5 investigates the role of glutathione in protecting *Bd* from host immune defences, by determining if glutathionylated proteins are imporant to *Bd* virulence. Chapter 6 summarizes the results of the research, discusses their significance and implications, and explores possible future research avenues.

CHAPTER 2 | Susceptibility of frogs to chytridiomycosis correlates with increased levels of immunomodulatory serotonin in the skin

OVERVIEW

Previous work by Laura F. Grogan found that serotonin levels increased in more susceptible populations of alpine tree frogs (*Litoria verreauxii alpina*) as *Batrachochytrium dendrobatidis* (*Bd*) infection progressed. However, the importance of this correlation was not determined. As other studies have found that serotonin has antifungal effects, it is possible that serotonin may be accumulated by the host as an immune defense against *Bd*. Alternatively, serotonin modulates immune responses in humans, and its structural analogue, kynurenine, has been shown to suppress amphibian lymphocytes. Hence, it may be contributing to the susceptibility of amphibians to *Bd*. The aim of this chapter is:

- To determine the effects of serotonin and structural analogues on growth of *Bd* and human Jurkat lymphocytes *in vitro*

This chapter is a copy of the published manuscript. Some data in this chapter is from Laura F. Grogan's PhD thesis (Figures 2.1, 2.2, and S2.1; Table S2.1). The clinical experiment and collected samples were performed by Laura Grogan. Joel Gummer performed the metabolite extractions and GC-MS analyses.

Grogan, L.F. (2014) *Understanding host and environmental factors in the immunology and epidemiology of chytridiomycosis in anuran populations in Australia*. PhD thesis, James Cook University.

Co-author consent to include jointly authored papers in the thesis

Thesis title: Characterization of *Batrachochytrium dendrobatidis* virulence factors

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Nature and extent of the intellectual input of each author, including the candidate:

This manuscript was submitted to the journal *Cellular Microbiology*. Laura Grogan, Lee Skerratt, Lee Berger, and Alexandra Roberts designed the research. Laura Grogan performed the clinical experiment and collected samples. Joel Gummer performed the metabolite extractions and GC-MS analysis. Sieara Claytor, Rebecca Webb, and Alexandra Roberts performed the growth assays and the supplementation experiment. Sieara Claytor performed the immunohistochemistry. Sieara Claytor and Laura Brannelly measured the granular gland fullness. Sieara Claytor and Alexandra Roberts performed the growth and cell proliferation assays. Sieara Claytor, Alexandra Roberts, Laura Grogan, and Joel Gummer performed the statistical analyses and wrote the paper. All authors reviewed the manuscript.

I confirm the candidate's contribution to this paper and consent to the inclusion of the paper in this thesis.

Name: Joel Gummer Signature:

Name: Laura Grogan Signature:

Name: Lee Skerratt Signature:

Name: Rebecca Webb Signature:

Name: Laura Brannelly Signature:

Name: Lee Berger Signature:

Name: Alexandra Roberts Signature:

The following is a modified version of the published manuscript (Appendix 2):

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Susceptibility of frogs to chytridiomycosis correlates with increased levels of immunomodulatory serotonin in the skin

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ABSTRACT

Chytridiomycosis, caused by the fungus *Batrachochytrium dendrobatidis* (*Bd*), is a skin disease responsible for the global decline of amphibians. Frog species and populations can vary in susceptibility, but this phenomenon remains poorly understood. Here, we investigated serotonin in the skin of infected and uninfected frogs. In more susceptible frog populations, skin serotonin rose with increasing infection intensity, but decreased in later stages of the disease. The more resistant population maintained a basal level of skin serotonin. Serotonin inhibited both *Bd* sporangial growth and Jurkat lymphocyte proliferation *in vitro*. However, serotonin accumulates in skin granular glands, and this compartmentalisation may prevent inhibition of Bd growth *in vivo*. We suggest that skin serotonin increases in susceptible frogs due to pathogen excretion of precursor tryptophan, but that resistant frogs are able to control the levels of serotonin. Overall, the immunosuppressive effects of serotonin may contribute to the susceptibility of frogs to chytridiomycosis.

2.1 | INTRODUCTION

The fungus *Batrachochytrium dendrobatidis* (*Bd*) causes the disease chytridiomycosis, which has caused the global decline and extinction of numerous amphibian species (Skerratt et al., 2007). Fungal sporangia grow within amphibian epidermal skin cells, and cause hyperplasia, hyperkeratosis, and inhibition of cutaneous ion transport, leading to cardiac arrest (Voyles et al., 2009). Species vary greatly in their susceptibility to *Bd*, ranging from 100% mortality to those that can clear infection, while others can be resistant reservoirs (Brannelly et al., 2018b, Miaud et al., 2016, Rollins-Smith et al., 2009, Scheele et al., 2016, Berger et al., 2016). Recent studies have shown population level differences in susceptibility within species (Bataille et al., 2015). Metabolomic analysis of skin from infected and uninfected alpine tree frogs *Litoria verreauxii alpina* (listed as vulnerable in Australia) from these populations revealed a correlation between serotonin (5'-hydroxytryptamine) identified by gas chromatography-mass spectrometry and the progression of *Bd* infection (Grogan et al., 2018d). Understanding variation in susceptibility is necessary to improve the survival of vulnerable species.

Differential susceptibility (mortality after infection) might correspond to the ability of the host to develop a robust immune response to *Bd* (Grogan et al., 2016, Bataille et al., 2016). Pathogen-induced suppression of lymphocytes is believed to reduce the effectiveness

of this response (Ellison et al., 2014a, Fites et al., 2013, Young et al., 2014). The immunosuppressive effect of *Bd* is likely due to the secretion of various metabolites, including kynurenine (Kyn), methylthioadenosine (MTA) and spermidine (a biogenic amine), which cause lymphocyte apoptosis *in vitro* (Rollins-Smith et al., 2015, Rollins-Smith et al., 2019). Kynurenine and its amino acid precursor tryptophan (Trp) were detected in *Bd* supernatants, with Trp being one of the most abundantly excreted molecules (Rollins-Smith et al., 2015). MTA is a produced from the methionine salvage pathway (Albers, 2009). The fate of pathogen-excreted metabolites, such as Trp, has not been explored *in vivo*.

Frogs must obtain Trp from their diet as they are unable to biosynthesise Trp. Frogs can metabolize Trp into serotonin, 5'-hydroxytryptophan, tryptamine, and 5'-hydroxy 3'-indoleacetic acid (5'HIAA). In contrast, although *Bd* produces Trp (Rollins-Smith et al., 2015), the pathogen lacks the enzymes to metabolise it into serotonin (Fig S5).

Serotonin occurs within the skin of many vertebrates, including frogs and fish (Fasulo et al., 1993), and has been suggested to have antifungal activities against *Candida albicans* and fungal plant pathogens (Du Fall and Solomon, 2013, Lass-Flörl et al., 2003, Mayr et al., 2005). The role of serotonin in immune function is complex, with conflicting functions proposed in the existing literature (Herr et al., 2017). Serotonin can be synthesised by mast cells and T-cells, is secreted by platelets during inflammation, and can be taken up by macrophages, dendritic cells, and B-cells (Ahern, 2011, Herr et al., 2017). The immunomodulatory functions of serotonin include: controlling cytokine secretion by macrophages (Sternberg et al., 1986), inducing proliferation of T-cells (Leon-Ponte et al., 2007), and promoting apoptosis of Burkitt lymphoma cells (Serafeim et al., 2002).

There is a correlation between the levels of *L. v. alpina* skin serotonin and disease progression (Grogan et al., 2018d), however, the mechanism of serotonin's effect on the host-pathogen interaction remains unknown. In this study, we sought to investigate the role of serotonin in frog immunity and its effect on the pathogen, *Bd*. We hypothesized that frogs use serotonin as an immune defence against *Bd*. Our objectives were (i) to determine serotonin levels over the course of *Bd* infection and correlate this to pathogen load and host susceptibility, (ii) to determine the location and abundance of serotonin in frog skin, (iii) to determine the effects of serotonin and structural analogues on *Bd* and lymphocytes *in vitro*.

2.2 | METHODS

2.2.1 | Study subjects, exposure experiment, and sampling

Study populations were sourced by D. Hunter in accordance with Scientific Licence number S12848 (Grogan et al., 2018a, Grogan et al., 2018d, Grogan et al., 2018b). Source populations originated in Kosciuszko National Park, New South Wales, Australia, from three sites; Kiandra (Population K), Eucumbene (Population E), and Grey Mare (Population G). Experimental procedures followed approved guidelines under permits issued by James Cook University (A1408) and Taronga Conservation Society (4c/01/10). Adult *L. v. alpina* (n = 44) were inoculated with *Bd* and a further 15 frogs were mock inoculated with dilute salts solution (negative control group) (Bataille et al., 2015). Animals were sampled throughout the course of infection in a random block design (see Table S2.1 for details). Thirty-six animals were euthanized during the subclinical phase of infection at 4, 14, and 18 d post exposure (n = 12 at each time point). Fifteen control animals were also sampled (n = 5 at each time point). The remaining eight exposed frogs were sampled from 28 d post exposure when they began to exhibit clinical signs of chytridiomycosis and were hence in the final stages of disease. Frogs were swabbed to quantify *Bd* infection intensity via qPCR, and examined and measured before being euthanized for tissue sample collection (Grogan et al., 2018d). Tissues were collected from ventral abdominal skin, the common site of *Bd* infection (Berger et al., 1998b, Berger et al., 2005a), and were immediately transferred to 500 µL of 100% methanol (Fisher Scientific) and stored at -80ºC. This study was part of a larger project investigating the effect of *Bd* on host immune responses (Grogan et al., 2018b).

2.2.2 | Growth of *Bd* by liquid culture

Bd (isolate AbercrombieR-Lbooroolongensis-09-LB1) were cultured at 20 °C on three mTGhL agar plates (8 g L^{-1} tryptone, 2 g L^{-1} gelatin hydrolysate, 4 g L^{-1} lactose, 10 g L^{-1} agar, with the addition of 200 mg L^{-1} penicillin-G and 200-500 mg L^{-1} streptomycin sulfate) before flooding plates with 10 mL dilute salts solution (1.0 mM KH_2PO_4, 0.2 mM $CaCl_2.H_2O$, 0.1 mM $MgCl_2.2H_2O$) for 20 min to harvest the sporangia and zoospores. The suspension was then centrifuged at 2,800 x *g* for 10 min in a refrigerated Eppendorf Centrifuge 5702 (Eppendorf, USA), the supernatant was removed, and the pellet resuspended in a 2 mL cryotube with 1 mL methanol before storage at -80 ºC.

2.2.3 | Metabolite isolation from skin tissue and *Bd,* and analysis by GC-MS and UPLC qTOF

Skin metabolites were extracted as previously described (Grogan et al., 2018d), however the metabolite extracts were analysed by 10 µL injection into a UPLCqTOF-MS, and without first drying the extracts. Metabolites from cultured *Bd* were isolated for analysis by GC-MS. *Bd* metabolites were extracted from sporangia (in 1 mL methanol) by sonication for five min at ambient temperature, followed by vigorous mixing by vortex to further disrupt the cells. The suspension was then transferred to a 2 mL microcentrifuge tube and 300 µL water added before a further round of cell disruption, as already described. Cell debris were collected by centrifuge at 16,100 *g* for 10 min at 4 °C, the supernatant transferred to a fresh tube and 65 µL of 10 µg.mL^{-1} $^{13}C_6$-sorbitol standard added. The *Bd* metabolite extracts were dried in preparation for GC-MS analysis as previously described (Gummer *et al*., 2013), by first concentrating the extract in an Eppendorf Concentrator Plus vacuum concentrator, before then submersion in liquid nitrogen and lyophilising to dryness in a Labconco Freezone 2.5 Plus depressurized with a JLT-10 JAVAC high vacuum pump.

For GC-MS analysis, metabolites were derivatised with *N*-Methyl-*N*-(trimethylsilyl) trifluoroacetamide (MSTFA, Sigma-Aldrich). Analysis was using a Shimadzu QP2010 Ultra GC-MS (Kyoto, Japan) as described in (Grogan et al., 2018d). The identity of serotonin and tryptophan were assigned by chromatographic retention time and mass-spectral match, by comparison to an authentic metabolite standard. Supportive data for the identification of serotonin was by accurate mass measurement using a Waters Synapt G2S quadrupole time-of-flight mass spectrometer operating in positive electrospray ionisation mode and coupled to a Waters Acquity liquid chromagraphy system (UPLC qTOF/MS). Chromatography was conducted using a Kinetex 1.7 µm HILIC column (Phenomenex Inc.). The mobile phases consisted of A; 7.5 mM ammonium formate and B; 90:8:2, acetonitrile:isopropanol:water (7.5 mM ammonium formate). The mobile phase flow of 500 µL min^{-1} was equilibrated and held at 99% B for 2.5 min before a linear gradient to 95% B over 12.5 min. The ion source was operated with a capillary voltage of 3.0 kV, desolvation gas flow of 1000 L h^{-1} and at a temperature of 150 °C. MS data were collected in continuum mode, at a mass range of m/z 100 – 1,000, with a scan time of 0.5 s and operating at a resolution of 38,000. Mass accuracy was maintained by infusion of leucine encephalin reference. Analyses were conducted using SPSS Statistics Version 22 (IBM).

General linear models (univariate) were fitted to serotonin levels measured in skin between days 4 to 14 post-exposure to better understand likely determinants including treatment effects (infected versus control), pathogen burden (infection load as measured in zoospore equivalents), population of origin and duration of infection (days of the experiment). One-way analysis of variance and non-parametric Kruskal-Wallis tests were conducted to compare serotonin levels on different days.

2.2.4 | Immunohistochemistry

To determine the location of serotonin in *Bd* infected and *Bd* uninfected frog skin, immunohistochemistry (IHC) was performed. Skin tissue from the original, 8-month old *L. v. alpina* used in the exposure experiment was not viable for immunohistochemistry, and as additional animals were not available from this endangered species we changed to an alternative alpine species. We were able to collect fresh skin samples from 3 year old Southern Corroboree frogs (*Pseudophyrne corroboree*) that were euthanized in an unrelated infection experiment. The inoculation and husbandry of *P. corroboree* followed (Brannelly et al., 2015). Skin sections (5 μm thick) were cut from *Bd* infected and *Bd* uninfected *P. corroboree* and embedded in paraffin blocks. Tissue collection followed approved guidelines under James Cook University permit (A2171). Slides were deparaffinised and rehydrated with xylene and ethanol, and rinsed with 1x phosphate-buffered saline (PBS). The slides were incubated overnight at 4 °C with the mouse monoclonal anti-serotonin primary antibody (ThermoFisher Scientific Cat# MA5-12111, RRID: AB_10982728) at a 1:20 dilution, in Tris hydrochloride (Tris-HCL) buffer with 1% bovine serum albumin (BSA). Slides were again rinsed with 1x PBS, and incubated overnight with rabbit anti-mouse IgG secondary antibody, HRP (ThermoFisher Scientific Cat#: PA 1-28568, RRID: AB_10983403) at a 1:1,500 dilution in Tris-HCL buffer 1% BSA. After rinsing with 1x PBS, antibody detection was performed by using 20 μL 3,3'-diaminobenzidine substrate-chromagen (DAB+ Chromagen) in 1 mL DAB+ Substrate Buffer (Dako EnVision+ System-HRP (DAB)) for approximately 10 min. Slides were rinsed with distilled water, counterstained with haematoxylin, dehydrated with ethanol and xylene, and mounted.

2.2.5 | Granular gland fullness

Granular gland fullness was measured in dorsal and ventral skin from 10 infected and 10 uninfected moribund *P. corroboree*. Tissue was prepared as previously described in the section above. Slides were stained with haematoxylin and eosin, dehydrated with ethanol and

xylene, and mounted with mounting medium. Photos of granular glands were taken with a microscope, at 20x magnification. Photos of glands were analysed for size and fullness, following Brannelly L. (unpublished data) using ImageJ (Schneider et al., 2012). The protocol for measuring gland fullness consisted of fixing the photo white balance, outlining the gland, adjusting photo saturation and brightness, analysing the area within the outlined gland, and calculating the proportion full. The proportion of gland filled was measured for five granular glands in dorsal and ventral tissues. The proportion filled was not normally distributed, and therefore arcsine transformed. A linear mixed model was performed in SPSS v. 25 where the arcsine transformed proportion filled was the dependent variable, fixed effects were the location of granular glands (dorsal or ventral), infection status (exposed or unexposed), and the interaction of location and status.

2.2.6 | *Bd* growth assays

The *Bd* isolate "Ethel creek-Lnannotis-2013-LB" (isolated from a Waterfall frog, *Litoria nannotis*, in 2013 from northern Queensland, Australia) was grown on sterile tryptone/gelatin hydrolysate/lactose (TGhL) plates using established methods (Longcore et al., 1999). Plates were flooded with TGhL media to release zoospores, which were syringe filtered to remove sporangia using a 10 μm isopore filter (Millipore). Chemicals were obtained from the Sigma-Aldrich Corporation. The chemicals tested were serotonin and the structural analogues tryptamine, histamine, 5-hydroxyindole-3-acetic acid, Trp, 5'-hydroxy L-tryptophan, and Kyn, as well as other biogenic amines ornithine, putrescine, spermidine, and spermine at final assay concentrations of 0.156 mM, 0.312 mM, 0.625 mM, 1.25 mM, 2.5 mM, 5 mM, and 10 mM.

Bd zoospores (100 μL, concentration between 2.5 x 10^5 and 1.0 x 10^6) were inoculated in a 96 well plate with an equal volume of the chemical of interest in 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) pH 7.4. Sterile deionised water and 20 mM HEPES negative controls were included. Sporangial size was measured at 3-4 d post inoculation, to capture the maximal mature sporangial size of each culture. For each concentration of serotonin a photograph was taken, and the 10 largest sporangia were selected per well and the sporangial diameter was measured, using a light microscope at 20X magnification (Fisher et al., 2009a) .

Bd from isolate "Ethel creek-Lnannotis-2013-LB" was grown on a 96 well sterile plate with 20 mM HEPES and serotonin at concentrations of 0.156 mM, 0.312 mM, 0.625

mM, 1.25 mM, 2.5 mM, 5 mM, and 10 mM. Absorbance at 492 nm was measured on a plate reader, and was averaged.

To confirm reproducibility of results, inhibitory experiments were repeated using the isolate "AbercrombieR-Lbooroolongensis-09-LB1", isolated from the Booroolong frog (*Litoria booroolongensis*), from New South Wales, Australia. One-way analysis of variance was performed in SPSS v.22 using with the sporangia size data obtained from the inhibitory assay. This was used to test the effect of concentration for each chemical.

The minimum inhibitory concentration (MIC) of *Bd* with serotonin was quantified using the isolate "AbercrombieR-Lbooroolongensis-09-LB1". We defined MIC as the lowest concentration that prevented *Bd* from completing its life cycle (release of zoospores from sporangia) (Martel et al., 2011). We observed *Bd* at 0.5 mM, 1.0 mM, 1.25 mM, 1.5 mM and 2 mM concentrations of serotonin for two weeks and noted when sporangia encysted, whether zoospores formed inside sporangia, and when motile zoospores were released (Table S2.2).

2.2.7 | Supplementation experiments with Trp and Kyn

To determine whether serotonin inhibits the Trp or Kyn biosynthetic pathways, a supplementation experiment was performed. A 96 well plate was inoculated with 100 µL of *Bd* from the "Ethel creek-Lnannotis-2013-LB" isolate, as above. Fifty microliters of either 10 mM Trp (in 20 mM HEPES), 10 mM Kyn (in 20 mM HEPES), or 20 mM HEPES buffer was added with increasing concentrations of serotonin (0 mM, 0.16 mM, 0.3125 mM, 0.625 mM, 1.25 mM, and 2.5 mM final concentration in 20 mM HEPES buffer). Each condition was tested in triplicate. After day 5, the 10 largest sporangia were measured in each well, using a light microscope at 20x magnification (Fisher et al., 2009a).

2.2.8 | Human Jurkat growth assays

Huma Jurkat T cells (a commercially available human lymphocyte cell line) were used as a proxy for frog T cells, due to the difficulty of extracting T cells from frogs (Piovia-Scott et al., 2014). Human Jurkat T cells were warmed at 37 °C for approximately two min in a water bath. Warm RPMI medium (ThermoFisher Scientific) supplemented with 10% fetal bovine serum (FBS), 100 IU/mL penicillin, and 100 µg/mL of streptomycin, was added to the cells. Cells were centrifuged at 200 x *g* for five min at 23 °C. Supernatant was removed, and cells were resuspended in fresh media. Twenty-five cm^2 flasks were inoculated with inoculum, and to a final volume of 10 mL with media. Cells were incubated at 37 °C in 5% CO_2 - 95% air and passaged 1:10 with fresh media when 80% confluence was reached

(approximately every 3 d). Ninety µL of human Jurkat T cells were inoculated on a 96 well plate in triplicate, with either 10 µL of serotonin suspended in 20 mM HEPES (final concentrations of 0.156 mM, 0.312 mM, 0.625 mM, 1.25 mM, 2.5 mM, 5 mM, and 10 mM), 10 µL L-Kyn suspended in 20 mM HEPES (final concentrations of 0.625 mM, 1.25 mM, 2.5 mM), or 10 µL of 25 µg/mL etoposide. Cells were grown for 2 d at 37 °C in 5% CO_2 - 95% air.

2.2.9 | MTT cell proliferation assay

To determine the amount of T cell proliferation when inoculated with varying concentrations of serotonin, a thiazolyl blue tetrazolium bromide (MTT) cell proliferation assay was performed. In a 96 well plate, 100 µL of MTT (500 µg/mL) was added to each human Jurkat T cell sample and incubated for four h at 37 °C in 5% CO_2-95% air. The plate was spun for 30 min at 4,000 x *g*. Supernatant was removed and 100 µL of dimethyl sulfoxide (DMSO) was added to each well. The plate was incubated for 10 min at 37 °C in 5% CO_2 - 95% air. Absorbance was read in a plate reader at 570 nm after a two-min shake.

2.3 | RESULTS

2.3.1 | Serotonin levels change over the course of infection

Firstly, we confirmed the identity of serotonin in the skin of *Bd*-infected frogs (Grogan et al., 2018b, Grogan et al., 2018d) using accurate-mass mass spectrometry (Ultra-Performance Liquid Chromatography-quadrupole-Time of Flight-mass spectrometry; UPLC-qTOF-MS) with comparison to a metabolite reference standard (Figure S2.1a).

The full model including all explanatory variables, treatment effect (infected versus control), population of origin, pathogen burden (infection load), duration of infection (days of the experiment), and their interactions was significant $p < 0.001$ and had an $R^2 = 0.60$. However, of the explanatory variables only infection load was significant, $p < 0.001$. It positively covaried with serotonin levels. When infection load was excluded from the model then only days of the experiment significantly covaried, $p = 0.01$, but $R^2 = 0.31$. Step-wise elimination of non-significant variables and their interactions led to infection load and population of origin as being the only significant explanatory variables, $p < 0.001$ and $p = 0.04$, The estimated marginal means for serotonin levels among frogs from differing populations of origin differed between population E (long exposed), serotonin = 0.021^a, and those from population K (longer surviving in infection experiments than G and E, long exposed) and population G (naïve), which were similar, serotonin = 0.011^a. Covariates[a]

appearing in the model were evaluated at infection load = 6011.85 zoospore equivalents. R^2 = 0.59. The lack of fit test for the model was significant p = 0.01. This was because the residuals for serotonin levels from the model increased as serotonin levels increased. This likely reflects activation of more biochemical pathways and feedback loops as the infection progresses disrupting the linear relationship. The fit of the model was improved by taking the square root of serotonin levels, p = 0.4. However, R^2 = 0.54, infection load and population of origin remained significant explanatory variables, p < 0.001 and p = 0.01, respectively.

Our results demonstrated that there were two types of serotonin responses to *Bd* fungal infection. Serotonin concentrations increased over the course of infection in the more susceptible (higher mortality rates) Populations E and G, demonstrating a 4.8-fold increase in skin serotonin in the first 14 days (Figures 2.1a and 2.1b). In contrast, the more resistant (longer surviving) Population K, had no significant change in serotonin levels over the course of the infection (R^2 = 0.0526, Figure 2.1c). We found a positive correlation between subclinical serotonin levels and pathogen burden in the susceptible populations for the first 14 days of infection (population E, R^2 = 0.4584; population G, R^2 = 0.6139, Figure 2.2b). The 'baseline' or mean concentration of serotonin for control animals was 0.0091 relative units. The linear models could not be fitted if day 28, when frogs developed clinical signs, was included. This was because serotonin levels decreased dramatically, by three orders of magnitude in conjunction with severe disease and frogs becoming moribund. One-way analysis of variance showed that serotonin levels differed among days post-exposure for infected frogs, p = 0.028. A non-parametric Kruskal-Wallis test was also significant, p = 0.001. Bonferroni post hoc tests suggested that the main difference occurred between days 14 and 28, p = 0.039.

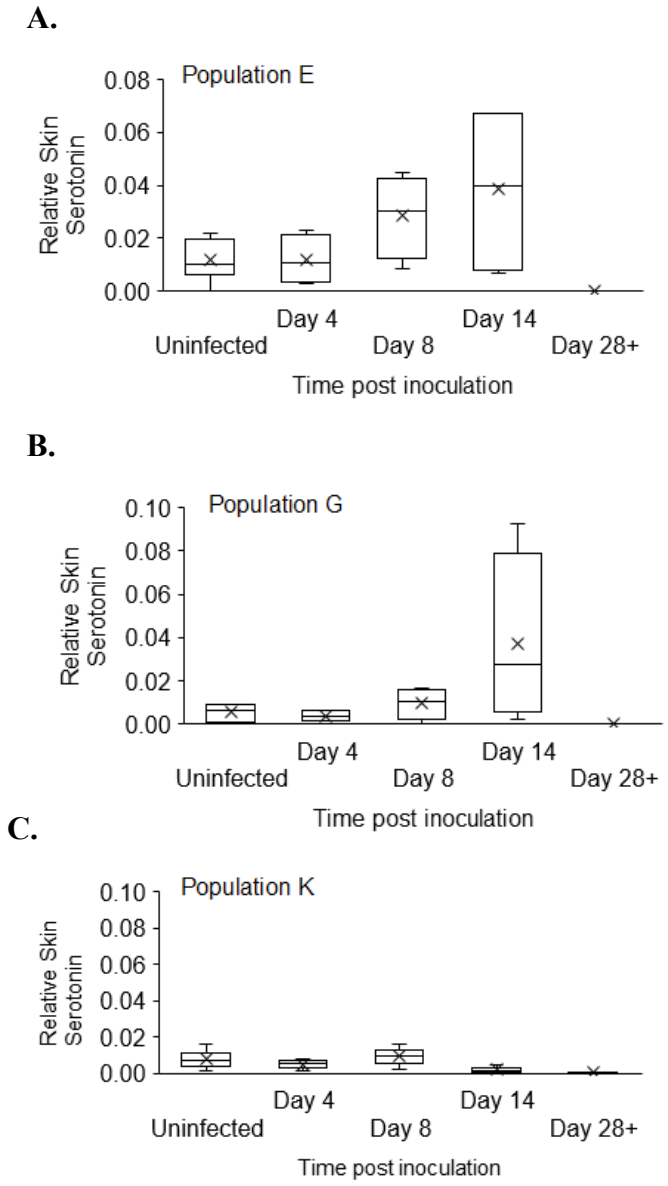
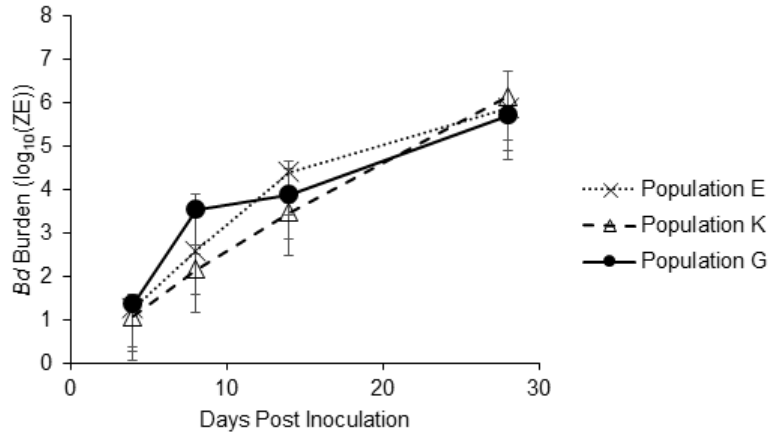


FIGURE 2.1 Skin Serotonin Levels over the Course of Infection in Three Populations of *L. v. alpina.*

X's represents the mean. A-B. Average serotonin concentrations increase over the course of infection in populations Eucumbene (Population E) and Grey Mare (Population G), prior to falling dramatically in moribund animals (Day 28+). C. Average serotonin concentrations remain low throughout the duration of infection in the Kiandra (Population K) population. Serotonin concentrations are relative to the normalised peak intensities in the dataset. Each box and whiskers plot represents the median and the interquartile range.

A.



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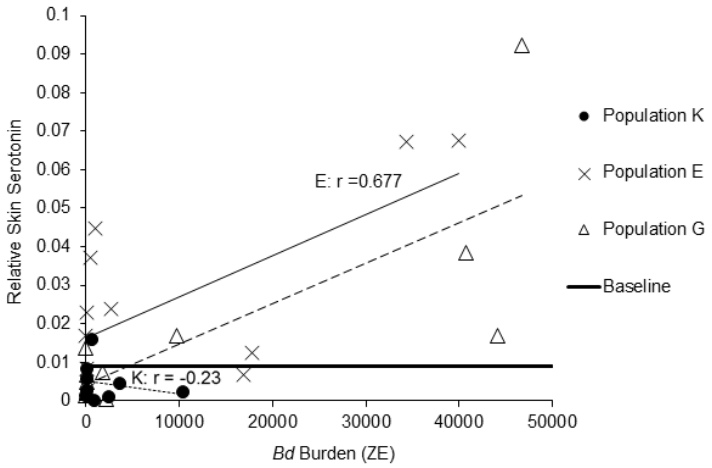

FIGURE 2.2 Infection Intensity and Concentration [Relative Units] of Serotonin within *L. v. alpina* skin.

A. *Bd* burden over the course of infection. B. Serotonin concentrations correlate positively with infection intensity in Populations E and G, but not in Population K. Baseline represents the mean concentration of serotonin for control animals.

2.3.2 | Location of serotonin in skin and changes in levels with infection status

Immunohistochemistry showed that serotonin in the skin was concentrated in granular skin glands and the linear mixed model showed that dorsal glands had more serotonin than ventral ($F_{1, 156} = 4.365$, $p = 0.041$). There was an accumulation of serotonin in uninfected control frogs at day 35 (Figure 2.3a), but no serotonin was visible in the frogs moribund with chytridiomycosis ($F_{1, 156} = 10.422$, $p = 0.002$) (day 35, Figure 2.3b). There was no significant interaction between granular gland location and infection status for serotonin ($F_{1, 156} = 0.031$, $p = 0.861$).

2.3.3 | Serotonin inhibits *in vitro* growth of *Bd* sporangia

Serotonin inhibited the growth of *Bd in vitro* (Figure 2.4a) and decreased the sporangial size (Figures 2.4a and 2.5). The average absorbance of Bd cultures also decreased with increasing concentrations of serotonin (Figure 2.7). Serotonin caused a significant reduction in sporangial size at concentrations equal to and above 0.3125 mM ($p < 0.002$). At 0.3125 mM and 1.25 mM serotonin, sporangial size was decreased by 30% and 60%, respectively. The minimum inhibitory concentration (where *Bd* was unable to release zoospores to complete its life cycle) of serotonin that inhibited the development of *Bd* sporangia was 1.5-2 mM. Similar results were observed for both strains of *Bd* tested, where both strains were from the phylogenetically-related global panzootic lineage (O'Hanlon et al., 2018).

In structure-activity relationship assays, serotonin structural analogues were tested for their ability to inhibit *Bd* growth (Figure 2.4). We tested *Bd*-produced analogues including Trp (Figure S2.1b) and Kyn; associated frog metabolites such as tryptamine, 5-hydroxy 3-indole acetic acid (5'-HIAA), and 5-hydroxytryptophan; as well as structurally similar histamine (produced from histidine) (Fig S2.5). Frog metabolites histamine, tryptamine, and 5'-HIAA inhibited *Bd* sporangial growth, while 5-hydroxytryptophan did not. The greatest decrease in sporangial size occurred with tryptamine, which had a similar effect on growth to serotonin (Figure 2.4a-4b). The endogenous *Bd*-produced metabolite analogues, Trp and Kyn did not affect growth. The addition of either 10 mM Trp or Kyn did not restore *Bd* growth in the presence of increasing serotonin concentrations (Figure 2.5a). Other biogenic amines were also tested as potential inhibitory agents of *Bd*. Putrescine, spermidine, and spermine (which are metabolically related to *Bd* immunosuppressant MTA) inhibited sporangial growth, while ornithine did not (Figure 2.4c).

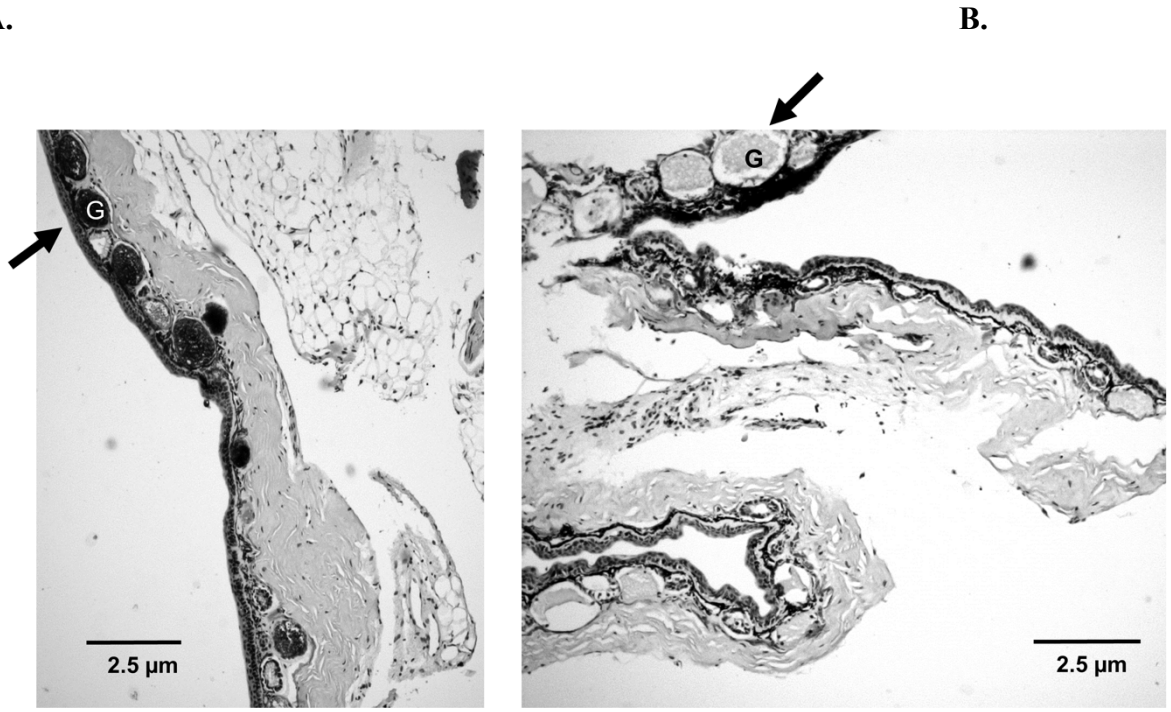

FIGURE 2.3 Immunohistochemistry of ventral *Pseudophryne corroborree* skin with Serotonin Antibody.

A. In uninfected control skin (day 35), serotonin accumulates within the granular glands (G). B. In infected frogs showing clinical signs of chytridiomycosis (day 35), serotonin is noticeably absent from granular glands. The arrows in both panels are pointing to granular glands. The chromagen staining is prominent in the granular glands in uninfected animals (panel a, i.e. the dark staining inside the glands), and absent from the granular glands in infected animals (panel b).

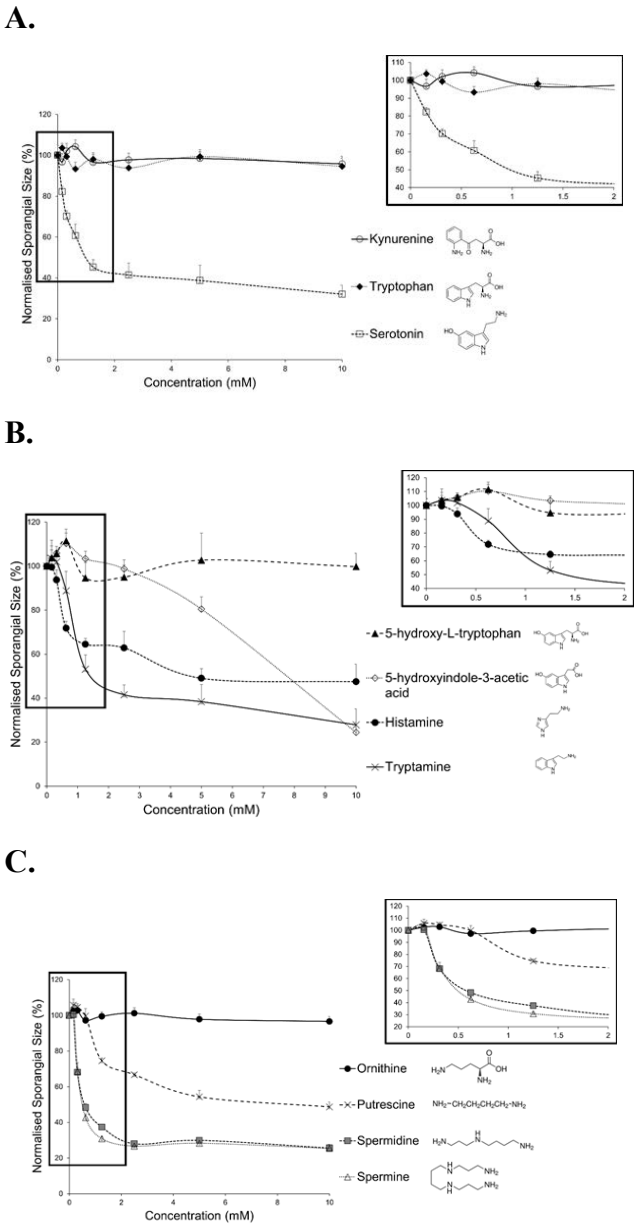

FIGURE 2.4 Sporangial Growth Assays with Serotonin and Structural Analogues Show that an Ethylamine Attached to a Basic Group Reduces *Bd* Sporangia Size. Bolded square corresponds to the inset that shows the data from concentrations of 0-2 mM. A. Serotonin (produced in frog skin) reduces sporangial growth, while the analogues kynurenine (Kyn) and tryptophan (Trp) (produced by *Bd*) do not affect fungal growth. B. Inhibition of sporangial growth occurs with histamine and tryptamine, which possess both the terminal primary amine and pyrrole ring of serotonin. At high concentrations (5-10 mM), 5-hydroxyindole-3-acetic acid inhibits growth, while 5-hydroxy-L-tryptophan does not have any effect. C. Other biogenic amines that contain an aliphatic primary amine, such as spermine, spermidine and putrescine inhibit growth. However, this effect is negated if the ethylamine is attached to an acidic group, as in ornithine.

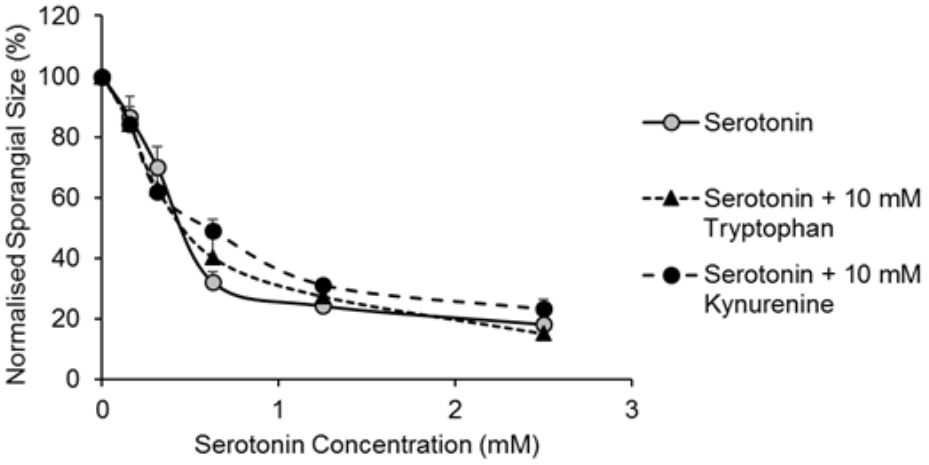

FIGURE 2.5 Effect of Supplementation of Cultures.

Treatment with 10 mM tryptophan (Trp) or 10 mM kynurenine (Kyn) does not restore the growth of *Bd* with increasing concentrations of serotonin.

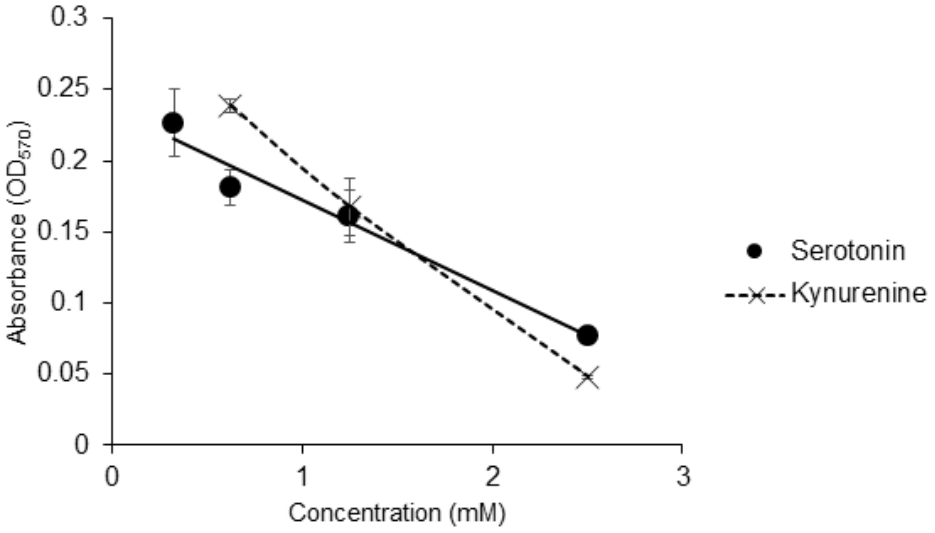

FIGURE 2.6 Effect of Serotonin and Kynurenine (Kyn) on human Jurkat Cells.
A trend line is shown for the serotonin data points. High absorbance serotonin inhibited human Jurkat lymphocytes at concentrations over 500 μM, similar to the effect for Kyn.

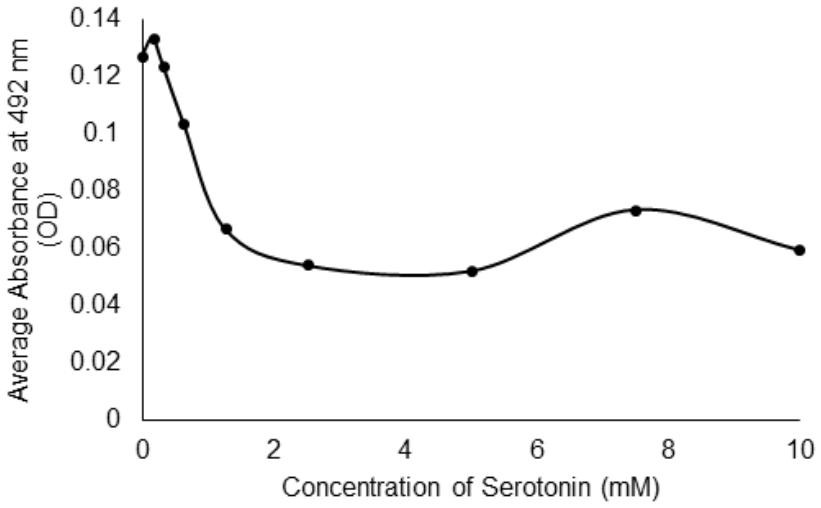

FIGURE 2.7 Average absorbance at 492 nm of *Bd* treated with serotonin.

The average absorbance of *Bd* decreased with increased concentrations of serotonin.

2.3.4 | Serotonin inhibits *in vitro* growth of human Jurkat lymphocytes

The human Jurkat lymphocyte cell line was used as a proxy to test the effect of serotonin on amphibian lymphocytes, as in previous studies (Piovia-Scott et al., 2014). Serotonin inhibited human Jurkat lymphocytes at concentrations over 0.5 mM (Fig. 6), similar to the immunosuppressive effects of Kyn (Rollins-Smith et al., 2015).

2.4 | DISCUSSION

2.4.1 | Serotonin levels over the course of infection

We used host skin metabolomics to assess whether survival rates (Bataille et al., 2015) correlated with skin serotonin levels. In populations with higher susceptibility to chytridiomycosis (Populations E and G), skin serotonin levels increased during the subclinical phase of infection, and were correlated to pathogen burden. However, in moribund frogs, serotonin levels dramatically decreased to basal pre-infection levels. In the more resistant Population K, serotonin levels remained at basal levels throughout the course of infection and were not correlated with infection burden.

2.4.2 | Localization of serotonin in frog skin

Immunohistochemistry indicated that serotonin is localised in granular glands within the frog skin, as shown previously in Marsh frogs (*Rana ridibunda*) (Şengezer-İnceli et al., 2004). The lack of serotonin antibody staining in moribund frogs corresponds to the reduction of serotonin to sub-basal levels in our metabolomic time-course study. Serotonin is expressed in the sweat and sebaceous glands of psoriasis lesions in humans (Huang et al., 2004), and might be excreted by epidermal mast cells to stimulate keratinocyte proliferation (Maurer et al., 1997). Hence, it is possible that the hyperkeratosis observed with chytridiomycosis (Berger et al., 2005a) is mediated by increased levels of skin serotonin during the subclinical phase of the disease.

2.4.3 | The effects of serotonin *in vitro*

Although Trp is a metabolic precursor to serotonin, Trp did not inhibit *Bd* growth *in vitro*. To provide insight into the effect of Trp metabolism to serotonin on *Bd*, we undertook structure-activity relationship assays to determine which structural motifs within serotonin were involved in inhibition of cultured *Bd*. These results indicate that the terminal primary amine and the pyrrole ring in serotonin inhibits normal *Bd* growth, as histamine (which only possesses these motifs) had a similar effect on *Bd* as serotonin. Furthermore, 5-hydroxyindoleacetic acid (5'-HIAA), which does not contain a terminal primary amine, only

inhibited sporangia at high concentrations. Given these results, conversion of Trp into tryptamine within the skin of frogs might also inhibit growth of *Bd*, whereas biotransformation to 5'-HIAA and the similar 5'-hydroxytryptophan are unlikely to affect *Bd.* Interestingly, high levels of 5'-HIAA were identified in the skin of moribund frogs (Grogan et al., 2018d), indicating another important fate of *Bd*-secreted Trp in the skin of infected frogs. Other unrelated biogenic amines also inhibited *Bd* growth, including putrescine, spermidine and spermine, which are biosynthetic metabolites of *Bd*-excreted MTA (Rollins-Smith et al., 2015) . As spermidine affects cell proliferation (Kihara and Snell, 1957) and controls the membrane potential of potassium, sodium, and calcium ATPases, future work should consider the effect of these biogenic amines on the susceptibility of frogs to chytridiomycosis.

We hypothesised that serotonin might inhibit *Bd* by mimicking metabolites within the Trp/Kyn biosynthetic pathway. Trp analogues inhibited Kyn production (a Trp metabolite) and hyphae development in *Candida albicans* (Bozza et al., 2005). However, supplementation with 10 mM Kyn or Trp in our study did not restore growth in the presence of increasing serotonin concentrations. Therefore, despite the structural similarities, serotonin does not seem to inhibit Trp or Kyn biosynthetic pathways in *Bd*. It is therefore unlikely that serotonin is mimicking Trp or Kyn precursors to competitively inhibit upstream metabolic enzymes. Despite the inhibition of *Bd* by serotonin *in vitro*, increasing levels of serotonin do not protect the frogs from *Bd in vivo*. It is possible that localization of serotonin in glands results in compartmentalization away from the pathogen.

Finally, Trp is an essential amino acid that frogs obtain from their diet. It is possible that increased skin serotonin during infection is due to a specific host response or due to increased Trp excreted from the rising burden of *Bd* (Priya et al., 2014, Rollins-Smith et al., 2015). As *Bd* has been shown to alter amphibian skin microbial communities (Jani and Briggs, 2018), Trp from other skin microbes may also contribute, but the relative abundance of *Bd* likely renders these sources negligible. As *Bd*-infected frogs lose appetite in late stages of disease, this may be an explanation for the final drop in serotonin.

2.4.4 | Immunosuppressive effects of serotonin

Serotonin inhibited the proliferation of human Jurkat lymphocytes with a similar effect to the previously reported Kyn produced by *Bd* (Rollins-Smith et al., 2015). *Bd* cannot produce serotonin, only its precursor Trp. Frogs, on the other hand, do not possess the necessary biochemistry for the *de novo* synthesis of Trp (Figure S2.4), so the serotonin

precursor must be introduced exogenously (Table S2.3). As expected, serotonin was not detected from *Bd* cultures, but the precursor metabolites Trp (Figure S2.1b) and serine were identified (Grogan et al., 2018d), consistent with previous reports (Rollins-Smith et al., 2015). Therefore, it is possible that the high levels of Trp secreted by *Bd* are metabolised into serotonin in the infected host, increasing host susceptibility to *Bd* due to immunosuppression. The immunomodulatory role of serotonin as seen in our study has been described in other organisms. Low concentrations of serotonin (50 μM) cause apoptosis and suppression of DNA synthesis in Burkitt lymphoma cells (Serafeim et al., 2002). Serotonin has been shown to be involved in host defence against other fungal pathogens (Du Fall and Solomon, 2013, Lass-Flörl et al., 2003, Mayr et al., 2005). Despite serotonin inhibiting *Bd* growth *in vitro*, there was no correlation of serotonin to increased resistance of frogs to chytridiomycosis. In this host-pathogen interaction, it seems that the effect of elevated skin serotonin is more detrimental to the frog. Overall, these results indicate that increased skin serotonin in susceptible frogs is a response to infection and high levels of pathogen-secreted Trp. However, the ability of more resistant populations to maintain low serotonin levels, despite increasing pathogen burden, could be linked to survival. Our study has brought new insight to the role of serotonin in *Bd* infection. Further investigation is needed to confirm serotonin as detrimental to the host during *Bd* infection, and to understand other effects of this bioactive molecule on pathogenesis. With the overall aim of improving outcomes for frogs with chytridiomycosis, future work could test therapeutics that alter skin serotonin levels to assess survival rates in infected frogs.

2.5 | Conclusions

The contribution of frog metabolites to immune defence against the deadly amphibian fungus *Bd* is not well understood. Here, we found a correlation between serotonin levels and pathogen burden, which suggests that serotonin may be produced by frog skin in response to *Bd* infection. Despite inhibiting the fungus *in vitro*, the immunosuppressive effects of serotonin likely contribute to the increased susceptibility of frogs to chytridiomycosis. This study improves the understanding of the role of metabolites in the host response to *Bd*, and provides a potential avenue for improving frog survival.

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CONFLICT OF INTEREST

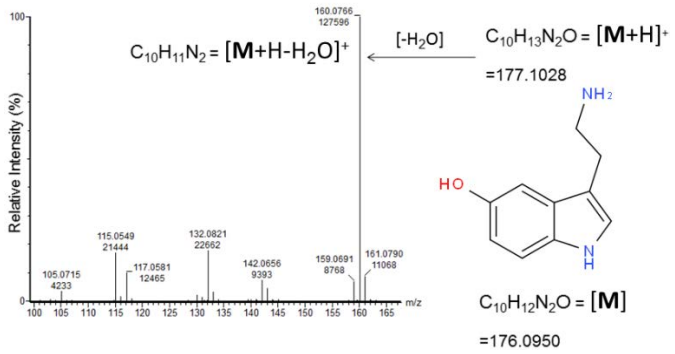
The authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS

L.F.G, L.F.S., L.B., and A.A.R. designed research. L.F.G. performed the clinical experiment and collected samples. J.P.A.G. performed metabolite extractions and GC-MS analyses. S.C.C., R.J.W., and A.A.R. performed the growth assays and the supplementation experiment. S.C.C. performed the immunohistochemistry. S.C.C. and L.A.B. measured the granular gland fullness. S.C.C. and A.A.R. performed the growth and cell proliferation assays. S.C.C., A.A.R., L.F.G. and J.P.A.G. performed the statistical analyses and wrote the paper. All authors reviewed the manuscript.

SUPPLEMENTAL INFORMATION

A.



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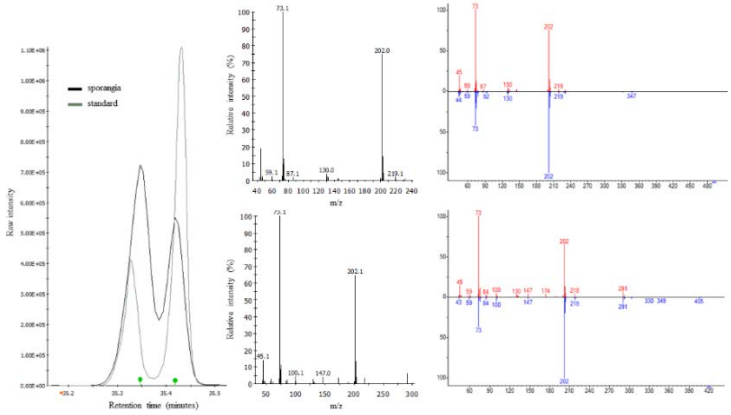

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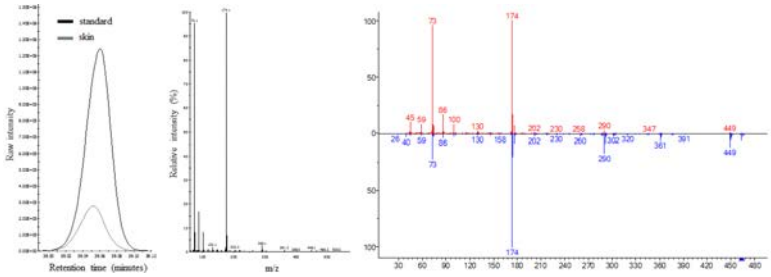

FIGURE S2.1 The Product Ion from MS/MS Fragmentation of the Protonated Serotonin Extracted from Frog Skin.

Fragmentation of the pseudo-molecular ion produces the dominant observed product ion, m/z 160 (<5 ppm; Massbank). A. relative intensity vs. retention time of serotonin, B. serotonin and Trp from sporangia compared to standard control, the graph on the left are from high-performance liquid chromatography and the right is from mass spectrometry and C. serotonin and Trp from skin compared to standard control, the graph on the left are from high-performance liquid chromatography and the right is from mass spectrometry.

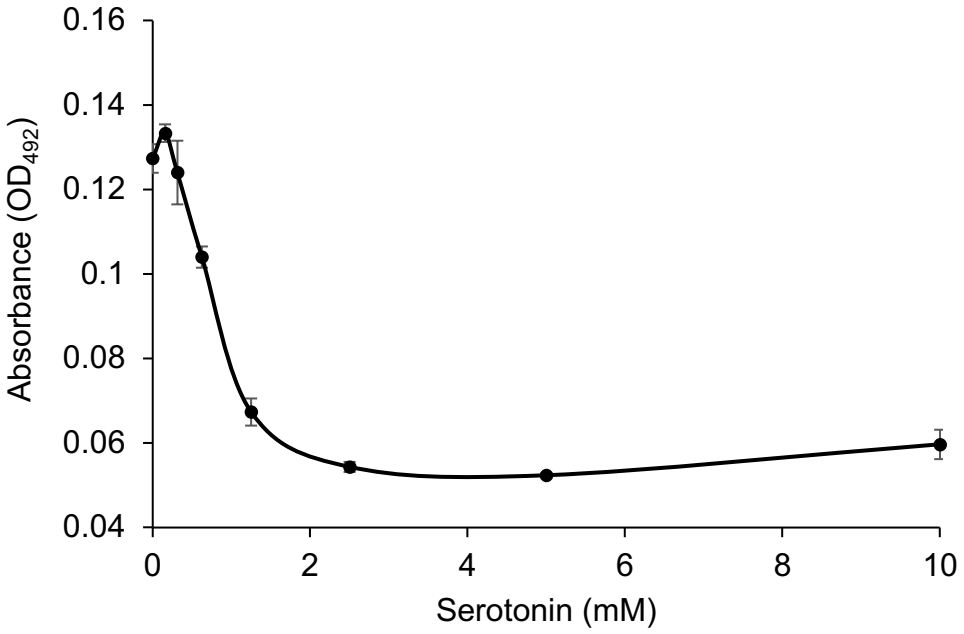

FIGURE S2.2 Growth of *Bd* zoospores as measured by optical density using a spectrophotometer.

Zoospores treated with 0-10 mM serotonin, measured by absorbance at 492 nm. Higher absorbance values coincide with more *Bd* growth

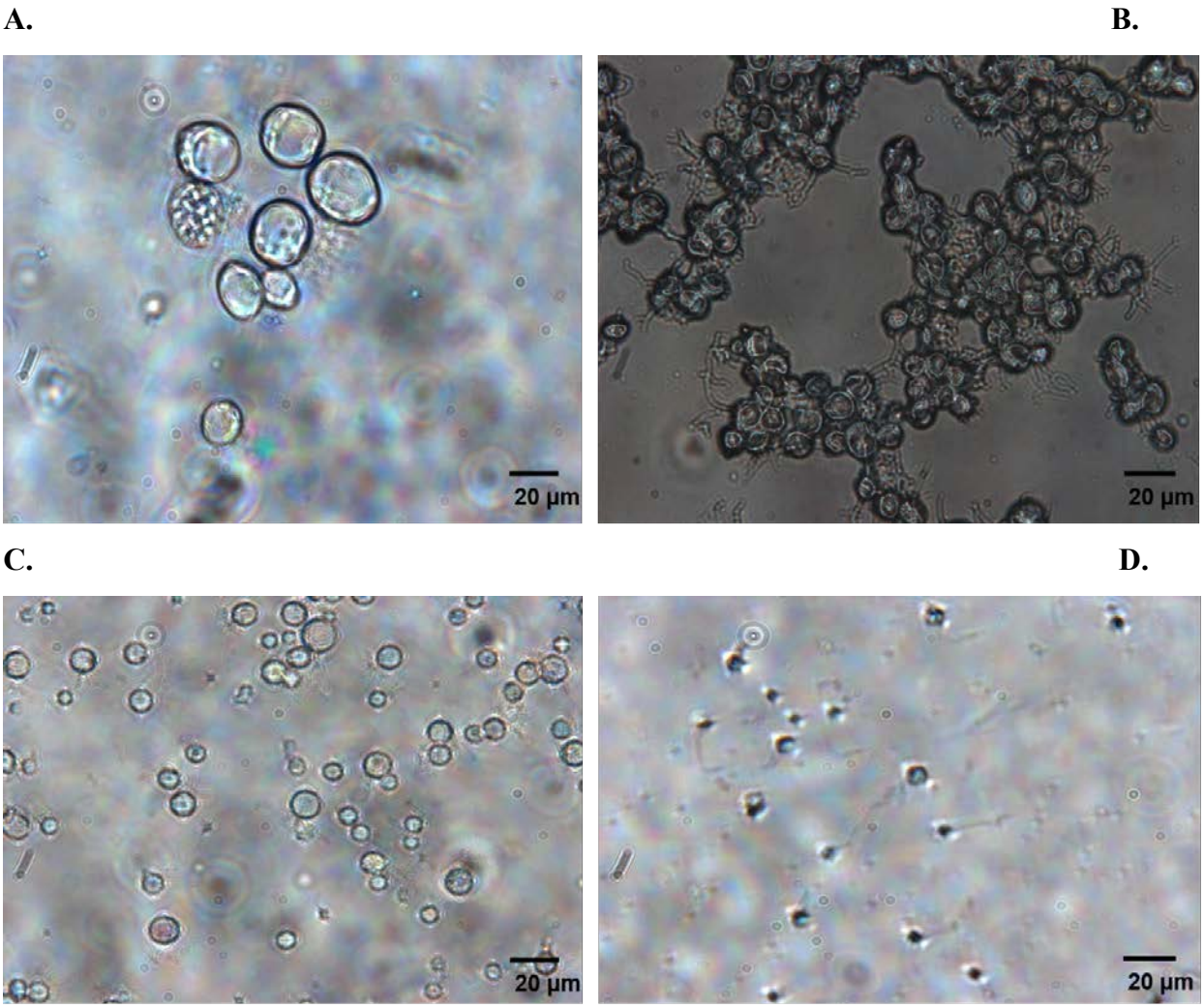

FIGURE S2.3 Representative Images of *Bd* Grown in Different Concentrations of Serotonin. A. Control - 0 mM, B. 0.625 mM, C. 2.5 mM, D. 10 mM.

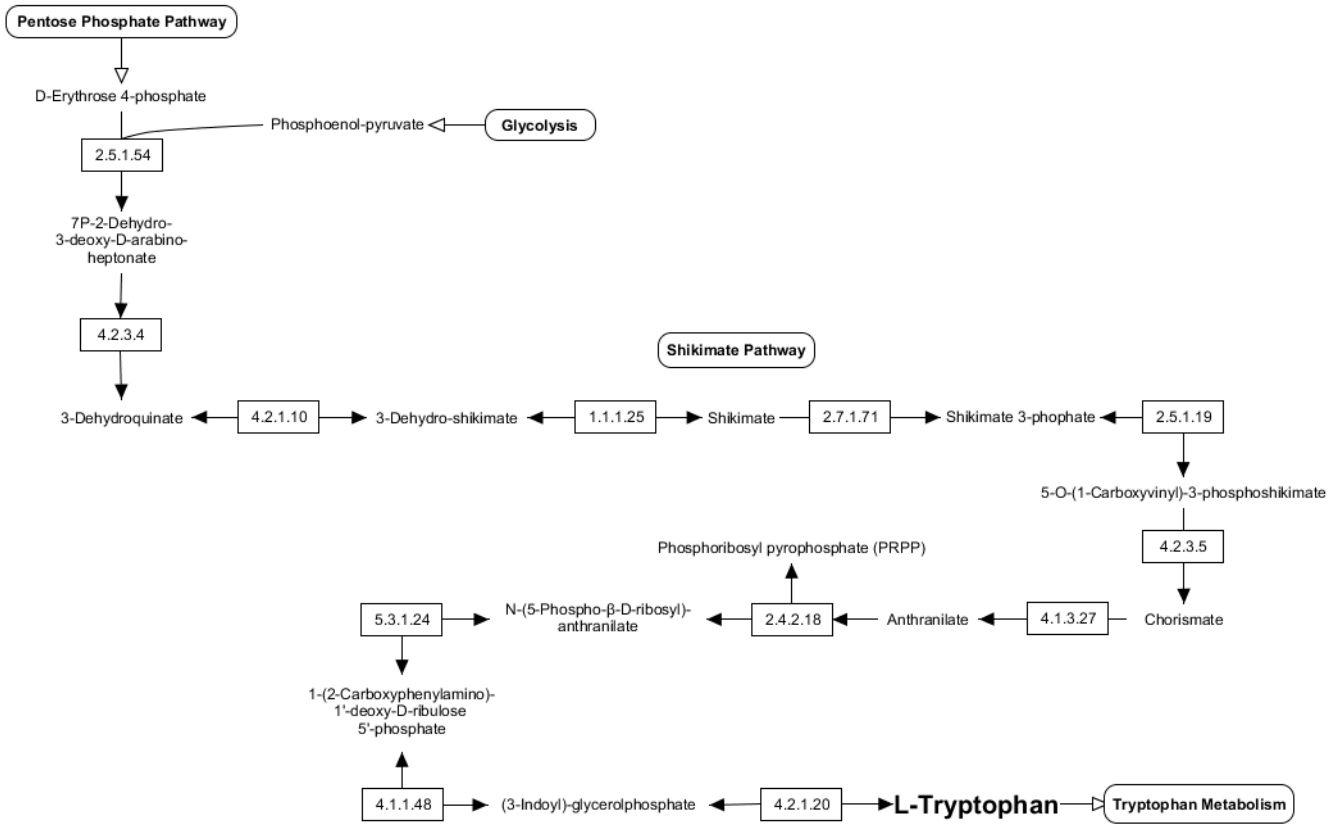

FIGURE S2.4 Tryptophan (Trp) Biosynthesis Pathway.

Numbers represent are the identification number of the protein in the Kyoto Encyclopedia for Genes and Genomes (KEGG) software. White boxes represent genes found in *Saccharomyces cerevisiae*, which are homologous in *Batrachochytrium dendrobatidis*. The biosynthetic pathway for *S. cerevisiae* has been well established, and useful for comparison to *B. dendrobatidis*. The Trp biosynthesis pathway is not found in the frog model organism *Xenopus tropicalis*.

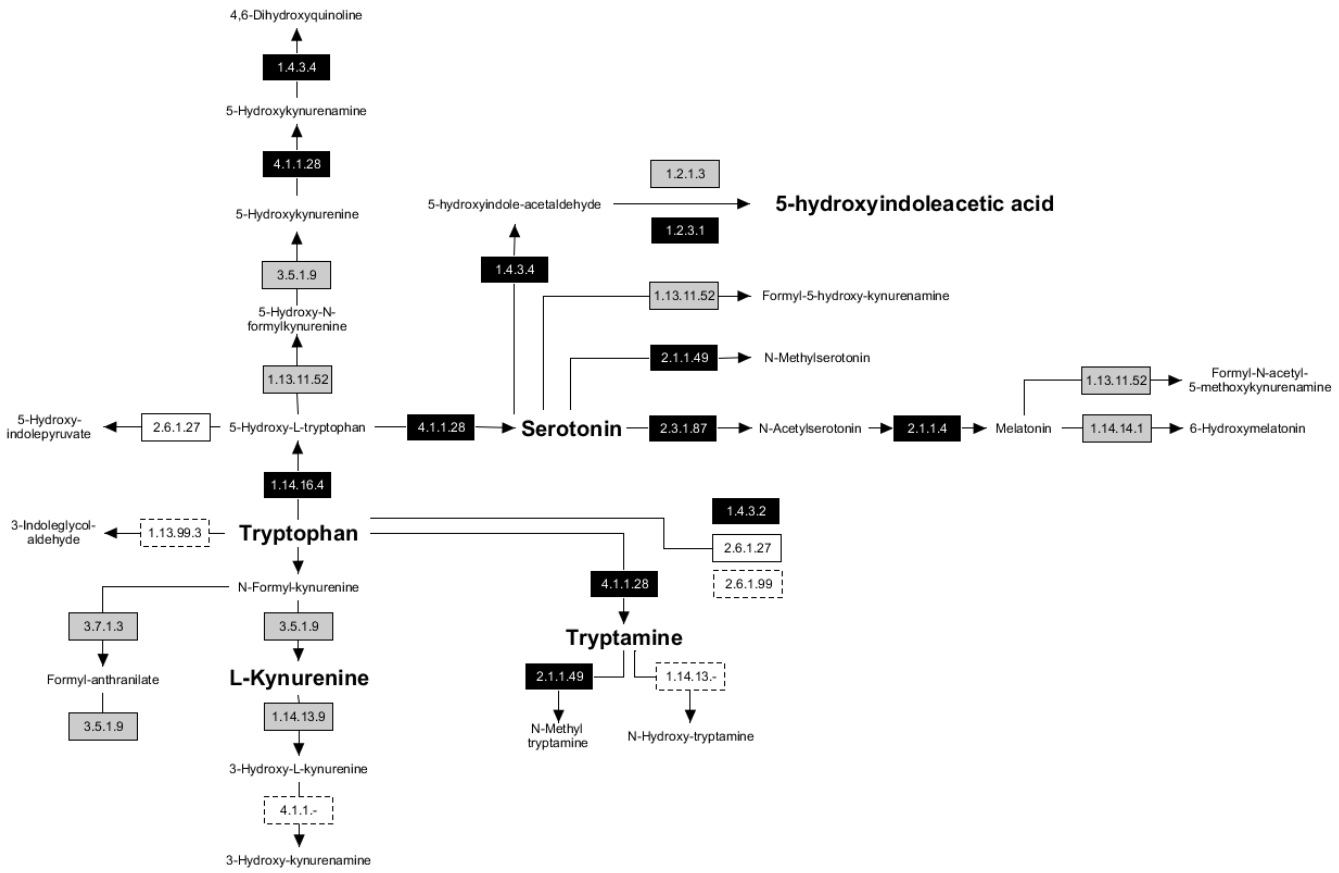

FIGURE S2.5 Tryptophan (Trp) Metabolism Pathways.

White boxes represent genes in *Saccharomyces cerevisiae*, which are homologs in *Batrachochytrium dendrobatidis,* black boxes represent genes found in the frog model organism *Xenopus tropicalis*, gray boxes are for genes found in both *S. cerevisiae* and *X. tropicalis*, dashed boxes are genes found in neither. The serotonin biosynthesis pathway is not found in the pathogen.

TABLE S2.1 Experimental design with the number of frogs from each population and treatment group (*Bd* infected or uninfected control) sampled at each time point post-exposure.

Populations	Day 4 Subclinical		Day 8 Subclinical		Day 14 Subclinical		Day 28+ Moribund	Total Number	
	Infected	Control	Infected	Control	Infected	Control	Infected	Infected	Control
Population G	4	1	4	1	4	1	4	16	3
Population E	4	2	4	2	4	2	2	14	6
Population K	4	2	4	2	4	2	2	14	6

TABLE S2.2 Qualitative description of *Bd* treated with a range of serotonin concentrations in HEPES buffer.

+ = yes, - = no.

Day	Observation	Concentration of serotonin (mM)				
		0.5	1.0	1.25	1.5	2.0
1	Encysted	+	+	+	+	+
	Zoospores inside sporangia	-	-	-	-	-
	Motile zoospores	+	-	+	+	-
	Notes	Developing sporangia	Developing sporangia	Developing sporangia	Developing sporangia	Developing sporangia
2	Encysted					
	Zoospores inside sporangia	-	-	-	-	-

	Motile zoospores	-	-	-	-	-
	Notes	Medium developing sporangia	Medium developing sporangia	Medium developing sporangia	Mostly small sporangia, some medium sporangia	Mostly small sporangia, some medium sporangia
3	Encysted					
	Zoospores inside sporangia	+	+	+	+	-
	Motile zoospores	-	-	-	-	-
	Notes	Large sporangia	Medium sporangia	Small-medium sporangia	Small sporangia	Small sporangia
4	Encysted					
	Zoospores inside sporangia	+	+	+	-	-

	Motile zoospores	+	-	-	-	-
	Notes	Large sporangia	Large and medium sporangia	Medium sporangia	Medium sporangia	Some medium sporangia
5	Encysted	+				
	Zoospores inside sporangia	+	+	+	+	+
	Motile zoospores	+	+	+	-	-
	Notes	Large sporangia, small encysted sporangia, many motile zoospores	Large and medium sporangia	Large and medium sporangia, with large vacuoles	Medium and small sporangia, with large vacuoles	Medium and small sporangia
6	Encysted					
	Zoospores inside sporangia	+	+	+	+	+

	Motile zoospores	+	+	+	+	-
	Notes	Large sporangia, many motile zoospores	Large sporangia	Large and medium sporangia	Large and medium sporangia with large vacuoles	Medium and small sporangia with vacuoles
7	Encysted					
	Zoospores inside sporangia	+	+	+	+	+
	Motile zoospores	+	+	+	+	-
	Notes	Large sporangia, many motile zoospores	Large sporangia	Large and medium sporangia	Large and medium sporangia	Medium and small sporangia
8	Encysted					

	Zoospores inside sporangia	+	+	+	+	+
	Motile zoospores	+	+	+	+	-
	Notes	Small to large sporangia	Large and medium sporangia	Large and medium sporangia	Large and small sporangia	Small to large sporangia
9	Encysted					
	Zoospores inside sporangia	+	+	+	+	+
	Motile zoospores	+	+	+	+	-
	Notes	Large sporangia	Large and small sporangia	Large and small sporangia	Large and small sporangia	Large and small sporangia
10	Encysted					

	Zoospores inside sporangia	+	+	+	+	+
	Motile zoospores	+	+	+	+	-
	Notes	Large and medium sporangia	Large and medium sporangia	Large and medium sporangia	Small to large sporangia	Small to large sporangia
11	Encysted	+	+	+	+	+
	Zoospores inside sporangia	+	+	+	+	+
	Motile zoospores	+	+	-	-	-
	Notes	Large and medium sporangia, many encysted zoospores	Large and medium sporangia, many encysted zoospores	Large and medium sporangia, some small developing sporangia	Large sporangia	Large sporangia
12	Encysted	+	+	+	+	+

	Zoospores inside sporangia	+	+	+	+	+
	Motile zoospores	+	+	-	-	-
	Notes	Small to large sporangia	Small to large sporangia	Small to large sporangia	Small to large sporangia	Small to large sporangia
13	Encysted	+	+	+	+	+
	Zoospores inside sporangia	+	+	+	+	+
	Motile zoospores	+	+	+	-	-
	Notes	Small to large sporangia	Small to large sporangia	Small to large sporangia	Small to large sporangia	Small to large sporangia
14	Encysted	+	+	+	+	+

Zoospores inside sporangia	+	+	+	+	+
Motile zoospores	-	-	-	-	-
Notes	Small to large sporangia	Small to large sporangia	Small to large sporangia	Small to large sporangia	Small to large sporangia

TABLE S2.3 Genes in the tryptophan biosynthesis pathway from *Saccharomyces cerevisiae*, that are homologs found in *Batrachochytrium dendrobatidis*.

Enzyme	Gene Name	GenBank® Accession Number
1.1.1.25	shikimate dehydrogenase	XP_006681594.1
2.4.2.18	anthranilate phosphoribosyltransferase	OAJ40366.1
2.5.1.19	3-phosphoshikimate 1-carboxyvinyltransferase	OAJ43256.1
2.5.1.54	3-deoxy-7-phosphoheptulonate synthase	OAJ40577.1
2.7.1.71	shikimate kinase	OAJ44380.1
4.1.1.48	indole-3-glycerol-phosphate synthase	OAJ41374.1
4.1.3.27	anthranilate synthase	OAJ41375.1
4.2.1.10	3-dehydroquinate dehydratase I	-
4.2.1.20	tryptophan synthase alpha chain	XP_006680001.1
4.2.3.4	3-dehydroquinate synthase	XP_006681594.1
4.2.3.5	chorismate synthase	OAJ32760.1
5.3.1.24	anthranilate synthase	OAJ41375.1

TABLE S2.4 Genes involved in tryptophan metabolism from *Saccharomyces cerevisiae*, that are homologs found in *Batrachochytrium dendrobatidis*.

Enzyme	Gene Name	GenBank® Accession Number
1.13.11.52	indoleamine 2,3-dioxygenase	BAO42882.1
1.14.13.9	kynurenine 3-monooxygenase	XP_006681171
1.14.14.1	unspecific monooxygenase	-
1.2.1.3	aldehyde dehydrogenase	XP_006683530
2.6.1.27	tryptophan transaminase	-
3.5.1.9	arylformamidase	-
3.7.1.3	kynureninase	OAJ42861.1

CHAPTER 3 | Characterisation of serotonin in amphibian skin

OVERVIEW

Chapter 2 showed that serotonin levels in skin are correlated with the progression of infection of *Batrachochytrium dendrobatidis* in alpine tree frogs (*Litoria verreauxii alpina*). To characterise the occurrence of serotonin in amphibian skin, we opportunistically collected dorsal and ventral skin tissue from Baw Baw frogs (*Philoria frosti*), cane toads (*Rhinella marina*), common eastern froglets (*Crinia signifera*), and southern corroboree frogs (*Pseudophryne corroboree*). As these animals were obtained opportunistically from separate unrelated studies, I was unable to use consistent group sizes or to examine all factors of interest in each species. The objectives of this chapter were:

- To determine the location and amount of serotonin in frog and toad skin
- To determine how serotonin levels in skin are affected by different factors (species, body site, age, infection status, and treatment with serotonin)

The following text is a copy of the manuscript in preparation, prior to its submission.

Identification and quantification of serotonin in frog and toad skin

Claytor, S.C. [1], Llewelyn, V.K. [2,3], Boughton, B.[4,5], Burns, T.[6], de Lourdes Frias Alvarez, P. [1], Webb, R. J. [1], Robertson, S.[2], Brannelly, L.A. [7], Driscoll, D.[6] , Mendez, D. [1], Skerratt, L.F. [7], Roberts, A.A. [1], and Berger, L[7].

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KEYWORDS | serotonin, 5-hydroxytryptomine, *Batrachochytrium dendrobatidis*, *Crinia signifera*, *Philoria frosti*, *Pseudophryne corroboree*, *Rhinella marina*

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ABSTRACT

The fungus *Batrachochytrium dendrobatidis* (*Bd*) causes the disease chytridiomycosis and is responsible for the global decline and extinction of amphibians. A previous study found that skin serotonin levels increase in the skin of susceptible alpine tree frogs (*Litoria verreauxii alpina*) as *Bd* infection progresses. To further characterise the occurrence of serotonin and determine whether this observation is a consistent across amphibian species,

we collected dorsal and ventral skin tissue from four Australian frog species with differing susceptibility to chytridiomycosis: Baw Baw frogs (*Philoria frosti*), cane toads (*Rhinella marina*), common eastern froglets (*Crinia signifera*), and southern corroboree frogs (*Pseudophryne corroboree*). We used immunohistochemistry to identify the location of serotonin within the skin and high-performance liquid chromatography to quantify the amount of serotonin. We also assessed if serotonin levels in skin are affected by various factors (species, body site, age, infection status, and treatment with serotonin). Overall, we found that species known to be more susceptible to *Bd* had less serotonin in their skin, in contrast to that observed in alpine tree frogs. Serotonin was detected in the granular glands of all species tested except the Baw Baw frogs. Dorsal skin in southern corroboree frogs had higher serotonin levels than ventral skin. However, in contrast to previous work we did not observe an effect of infection status or the interaction of location and infection status. Age and infection status did not have an effect on the amount of serotonin detected in the other species. Skin serotonin levels due to *Bd* pathogenesis may differ among frog species.

3.1 | INTRODUCTION

The fungus *Batrachochytrium dendrobatidis* (*Bd*) causes the disease chytridiomycosis and is responsible for the global decline and extinction of amphibians (Skerratt et al., 2007). Susceptibility varies among species and individuals, and older frogs tend to be more resistant (Berger et al., 2016, Brannelly et al., 2018a), but mechanisms for this variation remains unclear (Grogan et al 2018b). Sporangia of *Bd* grow within amphibian epidermal cells on ventral skin surfaces and there is limited to no inflammatory cell response observed histologically. Frogs with chytridiomycosis may have eroded skin surfaces, with fewer epidermal cell layers (Berger et al., 2005b).

A pervious experiment found that the amount of serotonin (5-hydroxytryptamine) in the skin of susceptible alpine tree frogs (*Litoria verreauxii alpina*) increased as disease progressed (Grogan et al., 2018b). Additionally, alpine tree frogs from a more resistant (lower mortality rate) population had lower levels of skin serotonin, suggesting that serotonin may play a role in *Bd* pathogenesis (Claytor et al 2019). Serotonin is well-known as a neurotransmitter (Dicke et al., 1997) with a variety of other biological functions (Van Nueten et al., 1985), such as regulatory cardiovascular (Kaumann and Levy, 2006, Singh, 2004) and digestive (Tecott, 2007) functions in humans. Although serotonin has fungicidal effects on *Bd* cultures *in vitro*, it also possesses lymphocyte inhibitory activity, supporting a role in the increased susceptibility of some *L. v. alpina* populations to *Bd* (Claytor et al., 2019).

Previous studies have quantified the amount of serotonin within amphibian skin (Şengezer-İnceli et al., 2004, Seki et al., 1995, Seki et al., 1989, Bennett et al., 1981). In the marsh frog (*Rana ridibunda*) found in Europe, serotonin was detected in the mucous and poison glands using immunohistochemistry (IHC) (Şengezer-İnceli et al., 2004). In the *Bd* resistant African clawed frog (*Xenopus laevis*) (Rollins-Smith et al., 2009), the amount of serotonin in the skin tissue was quantified by high-performance liquid chromatography (HPLC), and the amount was shown to increase with developmental stage; less than 1 ng/mg of serotonin in tadpole wet tissue, which increased to 63.2 ± 3.14 ng/mg of wet tissue in metamorphs, and 203 ± 17.1 ng/mg of wet tissue in adults (Seki et al., 1989).

The composition of frog skin can vary between species (Toledo and Jared, 1995, Brown et al., 1981) and between skin sites (Yorio and Bentley, 1977). Amphibian skin contains granular glands, which produce noxious or toxic secretions that can be used as a defence against predators (Toledo and Jared, 1995). These glands contain serotonin and other alkaloids, proteins, and anti-microbial peptides (AMP's) used for antimicrobial defence

(Woodhams et al., 2007), nutrient storage, defence against predators, chemosensory communication, and reproduction (Roseghini et al., 1986, Wang et al., 2014, Vanable, 1964). In the western clawed frog (*Xenopus tropicalis*), granular glands are found uniformly distributed both dorsally and ventrally, which may be due to their aquatic lifestyles, and used as a defence from predators attacking from all directions in the aquatic environment (Le Quang Trong, 1975). In the narrow-mouthed toad (*Gastrophryne carolinesnsis*), granular glands are evenly distributed throughout their body, which is believed to prevent attack from ants, which are their prey species (Garton and Mushinsky, 1979). The crab-eating frog (*Rana cancrivora*), did not have typical granular distribution (Seki et al., 1995).

There are no studies that have quantified serotonin in skin of Australian frogs, many of which have undergone declines due to *Bd* (Scheele et al., 2019). Due to the suggested involvement of serotonin in amphibian susceptibility to *Bd*, we investigated serotonin in native Australian frogs in 1) two *Bd* susceptible, critically endangered frog species, (the southern corroboree frog (*Pseudophryne corroboree*) and the Baw Baw frog (*Philoria frosti*)) (Hollis, 2004), 2) one *Bd* tolerant frog species, the common eastern froglet (*Crinia signifera*)) which is a reservoir of *Bd*, as they persist with high infection prevalence and intensity without disease sign (Brannelly et al., 2018b); and 3) the non-native invasive and *Bd* resistant cane toad (*Rhinella marina*), native to South America (Brannelly et al., 2018a).

In this study, we aimed to: 1) determine the location and amount of serotonin in frog and toad skin and correlate its occurrence with various host factors and 2) better understand its role in the pathogenesis of chytridiomycosis. We collected dorsal and ventral skin tissue from all species, which were obtained opportunistically from various studies. We used IHC and HPLC to determine how serotonin levels were affected by species, skin location (in all species), age (in cane toads), a bath treatment with serotonin (in common eastern froglets), and infection status (in corroboree frogs and common eastern froglets).

3.2 | METHODS

3.2.1 | Tissue collection

Frogs were opportunistically sampled (available due to being used in previous studies). Frogs and toads from 4 species (3 wild-caught, 1 captive bred) were euthanised with 0.2% buffered tricaine methane sulfonate (MS-222) baths (Brannelly et al., 2016). Approximately 1-4, 10 mm^3 or 8 mm diameter punches of skin tissue were dissected from the

midline dorsum and ventrum, weighed and stored in 70% ethanol for IHC, and 100% methanol for quantification by HPLC.

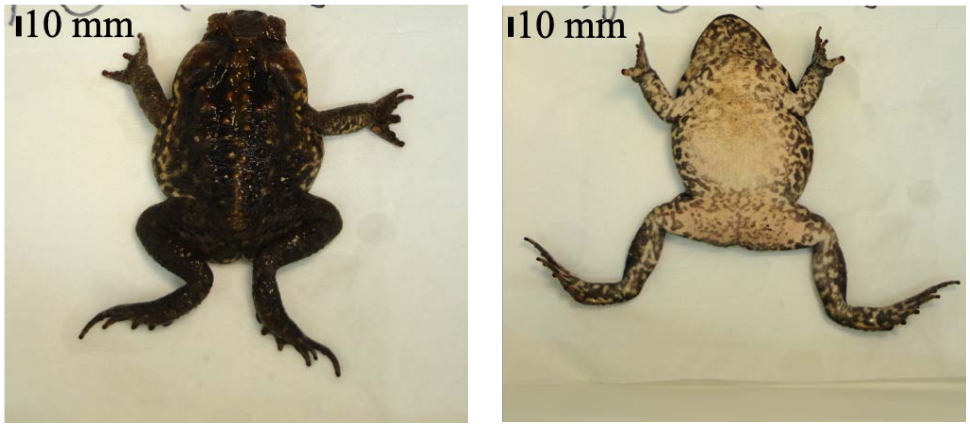
3.2.1.1 | Baw Baw frogs

Six uninfected adult Baw Baw frogs were obtained from the Baw Baw plateau in the central highlands of Victoria, Australia, and held in captivity (Deakin University Animal Ethics permit 10008298). Dorsal and ventral skin tissue samples were collected from the euthanised individuals. The skin tissue of all 6 individuals was analysed via IHC, and 4 individuals' samples were analysed via HPLC.

3.2.1.2 | Cane toads

Thirty-seven uninfected cane toads were collected from Townsville (a *Bd* negative zone), Queensland, Australia (James Cook University Animal Ethics permits A2503 and A2551) and euthanised (Figure 3.1). Each toad was assigned to age classes based on snout-venter length (SVL). Adults were 90 to 150 mm (n = 22), juveniles 40 to 80 mm (n = 13), and metamorphs were less than 40 mm (n = 2) (Figure 3.1). IHC analysis was performed on the dorsal and ventral skin samples of all thirty-seven individuals. Dorsal and ventral skin tissue from seven individuals of different age classes were used for HPLC analysis (2 adults, 3 juveniles, and 2 metamorphs).

A.



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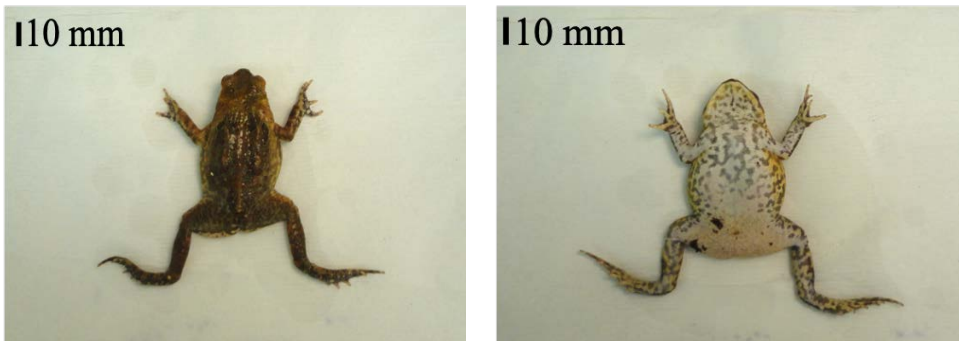


C.

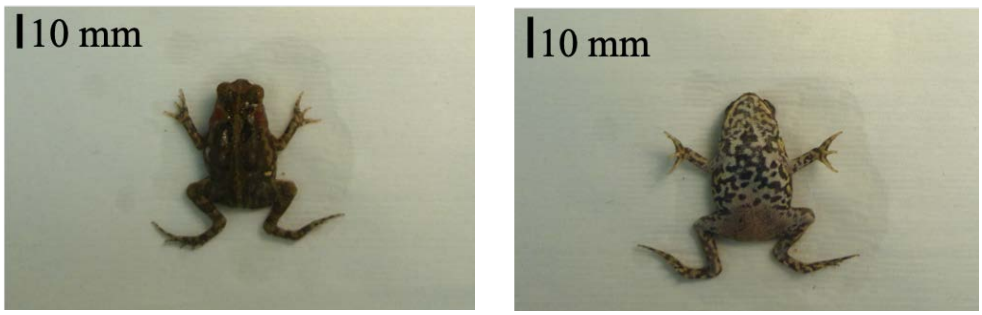


FIGURE 3.1 Representative size classes of cane toads (*Rhinella marina*) used for age comparison.

Left: dorsal view, right: ventral view. A. adult, snout-venter length (SVL) was 125 mm, B. juvenile, SVL was 66 mm, and C. metamorph, SVL was 37 mm.

3.2.1.3 | Common eastern froglets

Eleven frogs were collected from the wild in Kosciuszko, Brindabella, and Namadgi National Parks, and Micalong State Forest, in southeastern Australia and held in captivity during previous studies (collected in November 2015) (Scheele et al., 2016). They were tested for *Bd* by qPCR. Ten of the 11 frogs were positive for *Bd* by qPCR before the serotonin treatment.

To test if serotonin levels could be manipulated, four frogs were bathed in 20 mL of water, as treatment controls, and seven frogs were bathed in 0.2 mM (44 mg/L) serotonin in 20 mL baths, for one hour (h) daily, for 5 days (James Cook University Animal Ethics permit: A2503). Previous work with *C. signifera* showed that one h daily baths do not cause distress to the animals (Roberts et al., 2018). After the final treatment, frogs were euthanised, rinsed with reverse osmosis water, and skin was collected. Dorsal and ventral skin from 8 individuals was used for IHC analysis (3 no treatment control, and 5 treated with serotonin). The dorsal and ventral skin from five individuals was used for HPLC analysis (2 no treatment control, and 3 treated with serotonin).

3.2.1.4 | Southern corroboree frogs

Fifteen captive-bred southern corroboree frogs were obtained from a previous experiment (James Cook University Animal Ethics permit # A2374), in which 8 of the individuals were inoculated with *Bd* and 7 were uninfected controls, following established methods (Brannelly et al., 2016). Zoospore loads were quantified via qPCR. The infected frogs were euthanised after showing clinical signs of chytridiomycosis. Dorsal and ventral skin from all 15 individuals was used for IHC analysis. The dorsal and ventral skin from nine individuals was used for HPLC analysis (5 infected and 4 uninfected).

3.2.2 | Diagnosis of *Bd* by qPCR

Skin swabs were obtained by gently rubbing and rotating a cotton swab across the skin surface, with 5 strokes on the dorsum, 5 on each side, 5 on the venter, and 5 on each foot. To test for *Bd*, quantitative polymerase chain reaction (qPCR) was performed as per standardised methods (Hyatt et al 2007). *Bd* was extracted from the swabs by adding PrepMan™ Ultra Sample Preparation Reagent (ThermoFisher Scientific), then bead beating twice for 1 min, heating at 100 °C for 10 min, and removing supernatant. The qPCR used Bioline 2x Master Mix, 50 μM primers (primer 1: 5'-CCTTGATATAATACAGTGTGCCAATGTC-3'; primer 2: 5'-

AGCCAAGAGATCCGTTGTCAAA-3'), 100 μM TaqMan probe, 0.109 g/mL bovine serum albumin (BSA), and ultra-pure water.

3.2.3 | Immunohistochemistry (IHC)

To determine the location of serotonin in the skin, IHC was performed on dorsal and ventral skin tissue from all species. Skin samples were embedded into paraffin blocks and sectioned 5 μm thick. Slides were deparaffinised and rehydrated with xylene and ethanol, and then rinsed with 1x phosphate-buffered saline (PBS) pH 7.2 containing 10.5 mM potassium phosphate monobasic, 1.6 M sodium chloride, and 29.6 mM sodium phosphate dibasic (Gibco®, ThermoFisher Scientific). Slides were incubated overnight at 4 °C with the mouse monoclonal anti-serotonin primary antibody (ThermoFisher Scientific Cat# MA5-12111, RRID: AB_10982728) at a 1:20 dilution, in 0.5 M Tris hydrochloride (Tris-HCl) buffer with 1% bovine serum albumin (BSA). Slides were rinsed with 1x PBS and incubated overnight with rabbit anti-mouse IgG secondary antibody, HRP (ThermoFisher Scientific Cat#: PA 1-28568, RRID: AB_10983403) at a 1:1,500 dilution in Tris-HCL buffer 1% BSA. Slides were rinsed with 1x PBS, and antibody detection was performed with 3,3'-diaminobenzidine substrate-chromagen (DAB+ Chromagen) in DAB+ Substrate Buffer (Dako EnVision+ System-HRP (DAB)). Slides were rinsed with distilled water, counterstained with haematoxylin, dehydrated with ethanol and xylene, and mounted with mounting medium. Negative controls were tissue sections that were not treated with the primary serotonin antibody. Positive controls were skin sections from one individual corroboree frog that stained for serotonin.

3.2.4 | High-performance liquid chromatography (HPLC)

HPLC was used to quantify the amount of serotonin in both dorsal and ventral skin, of all individuals. The skin tissue was bead-beated 9 times for 20 s, and vacuum dried until all liquid was removed. The samples were suspended in 212 μL of 50% methanol and spun at 16,000 x *g* for 5 min. The supernatant was removed and placed into a new microcentrifuge tube for derivatisation with 250 μL of 2 mg/mL dabsyl chloride (DABS) and 188 μL of bicarbonate (pH 8.95). The tubes were then heated at 67 °C for 10 min. Contents of the tubes were syringe filtered with a 0.2 μm Captiva Premium syringe filter, regenerated cellulose (RC) membrane (Agilent, product 5190-5108) for analysis by HPLC. Five serotonin standards (1 mg/mL, 0.5 mg/mL, 0.25 mg/mL, 0.125 mg/mL, and 0.0625 mg/mL) were derivatised following the aforementioned methods.

Derivatised skin extracts were injected onto an Agilent Poroshell 120 EC-C18 4 μm, 46 x 150 mm column, on an Agilent high performance liquid chromatography (HPLC) instrument. All solvents were HPLC grade (ThermoFisher Scientific). The initial buffer composition was 60% Buffer A (45 mM sodium acetate, pH 4.9, 10% acetonitrile) and 40% Buffer B (100% acetonitrile) for 2 min, followed by a stepwise gradient of 55% Buffer B at 40 min, and 95% Buffer B at 42 min, followed by column equilibration, following previously established methods (Chen et al., 2003).

3.2.5 | Mass Spectrometry (MS)

Three replicates of de-salted dabsylated samples and standards were dissolved in 0.5 mL acetonitrile and ultrapure water. Mass spectrometry (MS) was performed on an Agilent 1200 Series LC coupled to an Agilent Triple Quad 6410 LC/MS. An Agilent Eclipse XDB-C18; 2.1 x 50 mm column, maintained at 30 °C, was used with an injection volume of 1 μL, and flow rate of 300 μL/min. The buffer composition was Buffer A (ultrapure water, 0.1% formic acid) and Buffer B (acetonitrile, 0.1% formic acid). The gradient programme was 0-1 min with 1% Buffer B, 1-12 min linear gradient of 1-100% Buffer B, 12-14 min of 100% Buffer B, and 14-19 min of 1% Buffer B. ESI-MS was run in positive ion mode, and collision energy was optimized and set to 20V.

MS was used to confirm the presence of serotonin in the samples analysed by HPLC following previously published methods (Chen et al., 2003). Derivatised skin extracts, and derivatised serotonin and tryptophan (structurally similar to serotonin) standards were de-salted using a SUPELCO Supelclean™ LC-18 SPE tube (Sigma Aldrich). Desalting consisted of nitrogen drying the sample, resuspending it in 1 mL of ultra-pure water, filtering through the SUPELCO Supelclean™ LC-18 SPE tube, nitrogen drying the sample, resuspending in 1 mL of acetonitrile, and nitrogen drying the sample. To confirm that serotonin was eluted from the column, de-salted dabsylated samples and standards were dissolved in 0.5mL acetonitrile and ultra-pure water, and 10 μL of sample was injected onto a Poroshell 120 EC-C18 column, 4 μm 46 x 150 mm. All solvents were MS grade. The initial buffer composition was 85% Buffer A (deionised water, 0.1% formic acid) and 15% Buffer B (acetonitrile, 0.1% formic acid) for 3 min, followed by a stepwise gradient to 85% Buffer B at 63 min and 70 min, and15% Buffer B at 74 and 80 min.

3.2.6 | Quantification of granular gland abundance

We used corroboree frogs to estimate the abundance of skin glands in this species, and to determine if this parameter influenced serotonin levels. The number of granular glands

in a field of view at 20x magnification was counted for each individual that showed staining for serotonin, via IHC. A linear mixed model was used in SPSS statistics software (IBM Corp.) to determine the significance of location, infection status, and the interaction between the two.

3.3 | RESULTS

3.3.1 | Baw Baw frogs

For the HPLC, the limit of detection for serotonin was 50 µg/µL. Serotonin was not detected in the granular skin glands nor the rest of the skin structures of the Baw Baw frog samples after IHC staining (Figure 3.2). Serotonin was not detected in dorsal nor ventral skin via HPLC (Table S3.1).

3.3.2 | Cane toads

Serotonin was detected in the skin of all age classes of cane toad (*R. marina*) via IHC (Figure 3.3). Serotonin was detected in the dorsal skin of 83.7% (31/37) of individuals and 86.4% (32/37) for ventral skin samples (Figure 3.4). Of the adults, 86.3% (19/22) of the dorsal and ventral skin stained for serotonin (n = 22). For juveniles, 92% (12/13) of the dorsal and ventral skin stained for serotonin (n = 13). For the metamorphs, none of the dorsal skin samples stained by IHC, and 50% of the ventral skin samples (1/2) stained.

Serotonin was detected via HPLC in two out of two adults and two out of three juvenile cane toads (Figure 3.5, Table S3.2). The adults had a dorsal skin serotonin concentration of 254.9122 µg/g and 14,087 µg/g of tissue, and a ventral skin concentration of 22.2635 µg/g and 489.0516 µg/g of tissue (n = 2). The juvenile had a dorsal skin concentration of 840.3690 µg/g and 120.9276 µg/g, while the ventral skin concentrations were below the limit of detection (n = 3).

3.3.3 | Common eastern froglets

Ten of the 11 frogs were positive for *Bd* by qPCR before the serotonin treatment, and 9 frogs were positive after treatment. There was no significant difference in zoospore loads before and after treatment ($F_{1,21}$ = 3,551, p = 0.073). Serotonin was visible by IHC in all dorsal and 87.5% (7/8) of the ventral granular glands in the common eastern froglets (*C. signifera*). There were no differences in serotonin concentration between animals treated with a serotonin or control bath as determined by IHC (Figure 3.6). HPLC detected serotonin in the skin of one out of five individuals, with a concentration of 137.5431 µg/g of tissue (n = 5). No toxic effect of the serotonin baths was observed in the common eastern froglets.

3.3.4 | Southern corroboree frogs

The IHC staining showed that serotonin was present in some dorsal and ventral skin granular glands, in infected and uninfected southern corroboree frogs (*P. corroboree*) (Figure 3.7). In a field of view (x20), serotonin was detected in a mean of 3.94 glands in dorsal skin samples and 4.00 glands in ventral skin samples. The average number of granular glands was a mean of 3.25 for dorsal skin, and 4.10 in ventral skin in uninfected frogs; and a mean of 4.63 in dorsal skin, and 3.83 in ventral skin in infected frogs.

Based on the HPLC analysis, dorsal skin had more serotonin than ventral skin samples, in both infected and uninfected individuals (Figure 3.8, Table 3.1). The location of the skin (dorsal vs. ventral) had a significant effect on serotonin levels ($F_{1,12} = 6.101$, $p = 0.03$) (Table 3.1), while infection status ($F_{1,12} = 0.269$, $p = 0.614$), and the interaction of location and infection status ($F_{1,12} = 1.847$, $p = 0.199$) did not (Figure 3.10, Table 3.1).

3.3.5 | Mass Spectrometry

Serotonin was detected in the skin via HPLC and MS in the same cane toad individual (n=1), mass spectrometry was able to detect lower concentrations of serotonin (Figure 3.11, Table 3.4). In the common eastern froglet, no serotonin was detected in the skin via HPLC, but was detected by mass spectrometry (n = 1) (Figure 3.11, Table 3.4).

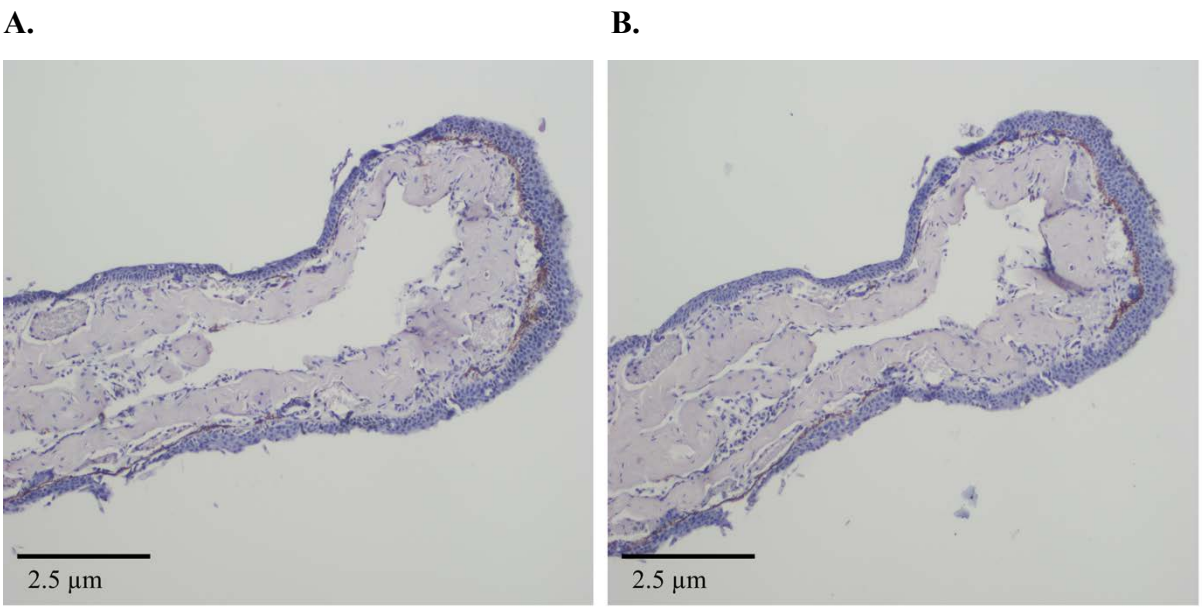


FIGURE 3.2 Immunohistochemistry staining for serotonin in the ventral skin of a representative Baw Baw frogs (*Philoria frosti*) (n = 6).
Serotonin was not detected in this species. A. control without mouse monoclonal anti-serotonin primary antibody, B. with mouse monoclonal anti-serotonin primary antibody (1:40 dilution). Dark brown staining at the base of epidermis in all samples is due to the normal presence of melanin pigment at this location.

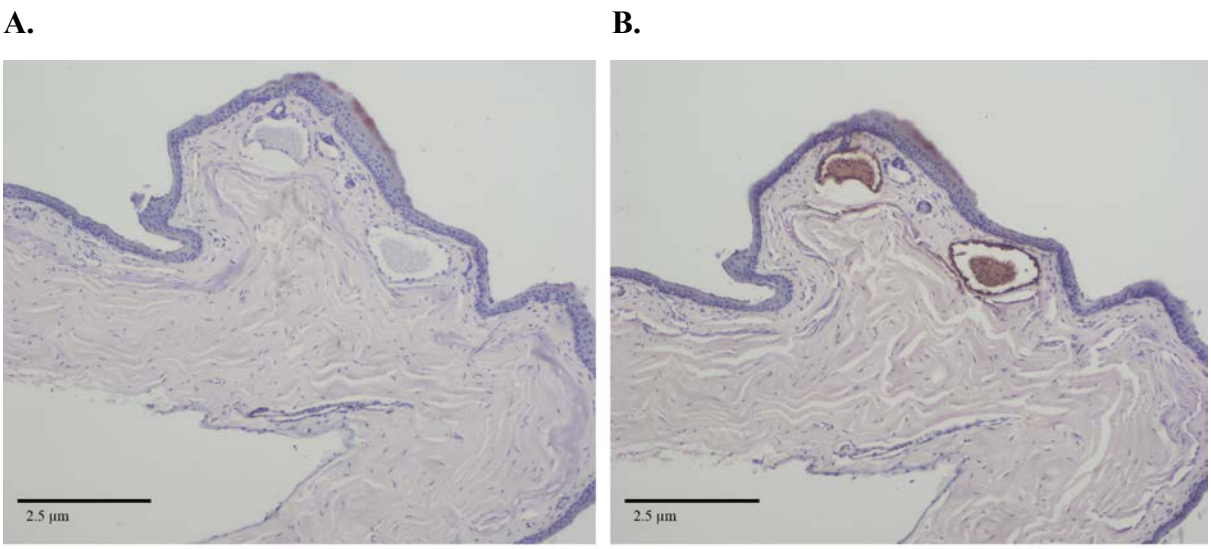


FIGURE 3.3 Immunohistochemistry staining for serotonin in the ventral skin of a representative cane toad (*Rhinella marina*) (n = 37).

A. control mouse monoclonal anti-serotonin primary antibody, B. with mouse monoclonal anti-serotonin primary antibody (1:40 dilution), DAB+ chromagen staining for serotonin in granular glands shown in dark brown.

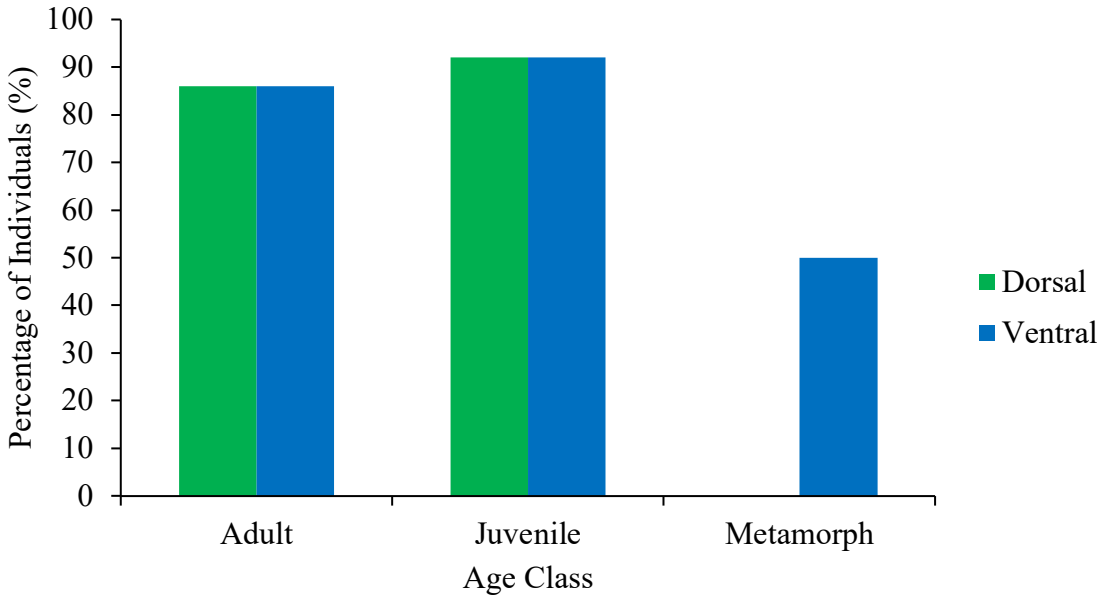
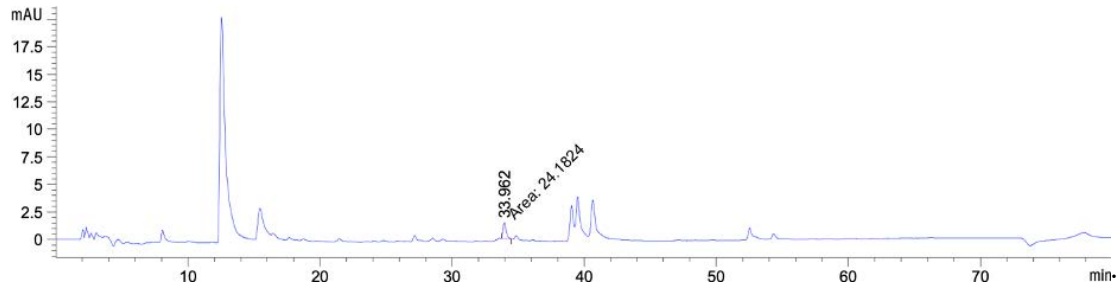


FIGURE 3.4 Percentage of cane toads (*Rhinella marina*) that had granular gland staining for serotonin by immunohistochemistry (IHC), by age class and skin location.
Adult: n = 22, juvenile: n = 13, metamorph: n = 2.

A.



B.

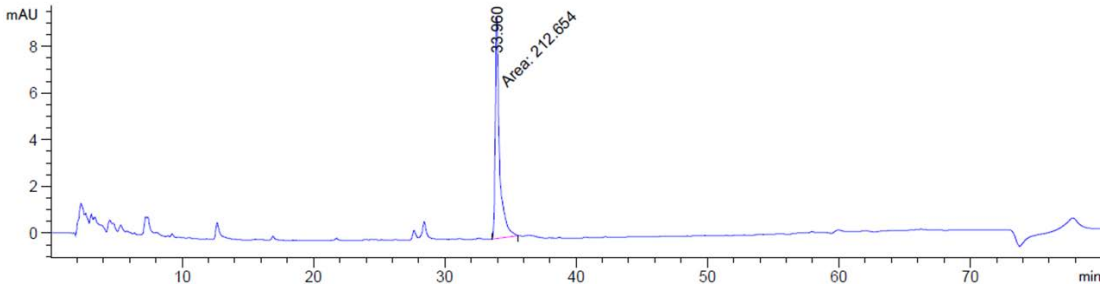


FIGURE 3.5 High-performance liquid chromatography (HPLC) chromatograms. A. representative cane toad (*Rhinella marina*) at 436 nm, serotonin retention time 33.962 min, area of 24.1824, B. 0.5 mg/mL serotonin control at 436 nm, serotonin retention time 33.960 min, area 212.654. Concentration of serotonin in the skin tissue is 840.3690 µg/g

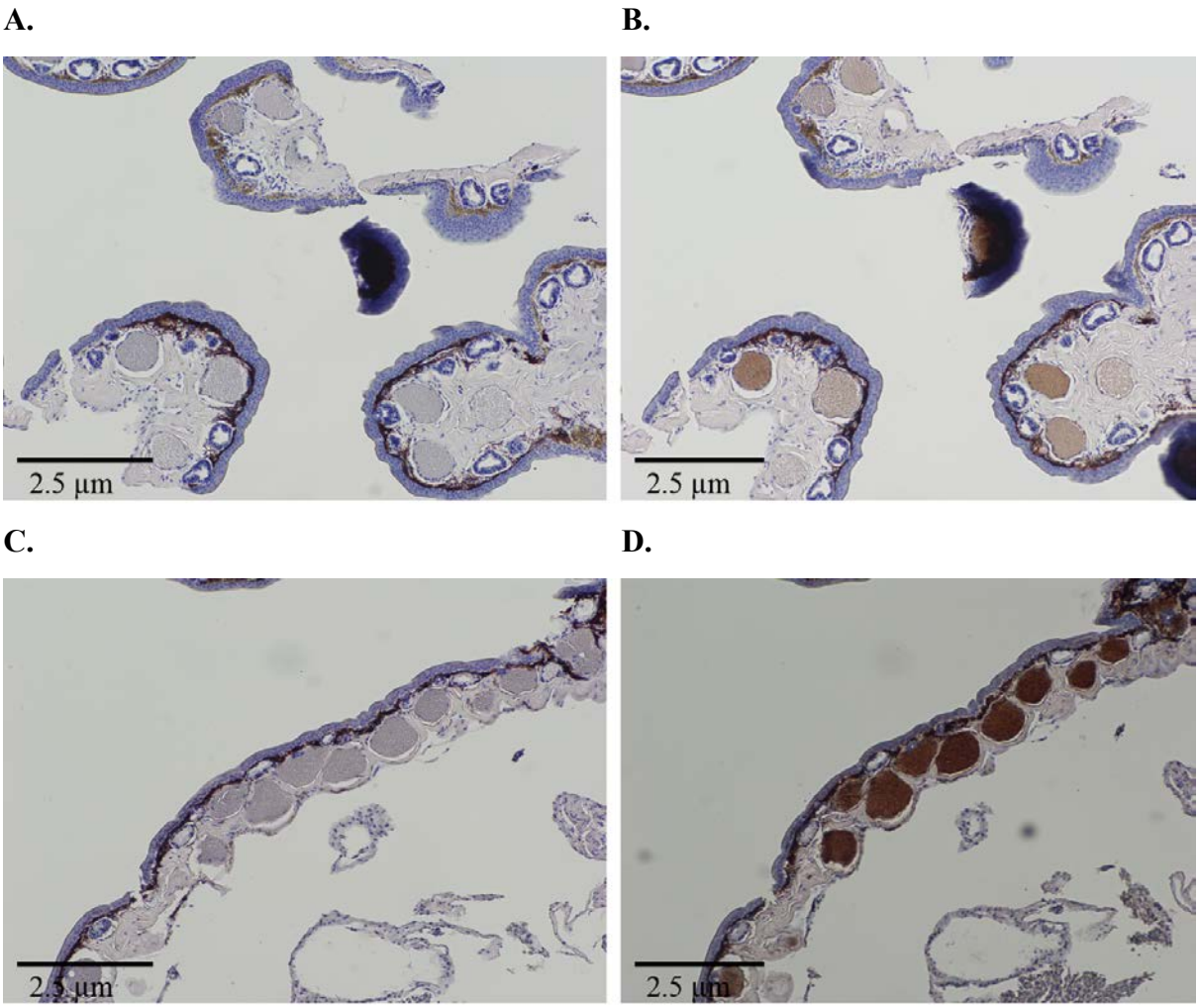


FIGURE 3.6 Immunohistochemistry staining for serotonin in the ventral skin of a representative common eastern froglet (*Crinia signifera*) (n = 8).
A-B. control bath treatment without serotonin, C-D. 0.044 g/L serotonin bath treatment. A & C. immunohistochemistry (IHC) control without mouse monoclonal anti-serotonin primary antibody, B & D. with mouse monoclonal anti-serotonin primary antibody (1:40 dilution), DAB+ chromagen staining for serotonin in granular glands shown in dark brown. Dark brown staining at the base of epidermis in all samples is due to the occurrence of normal melanin pigment.

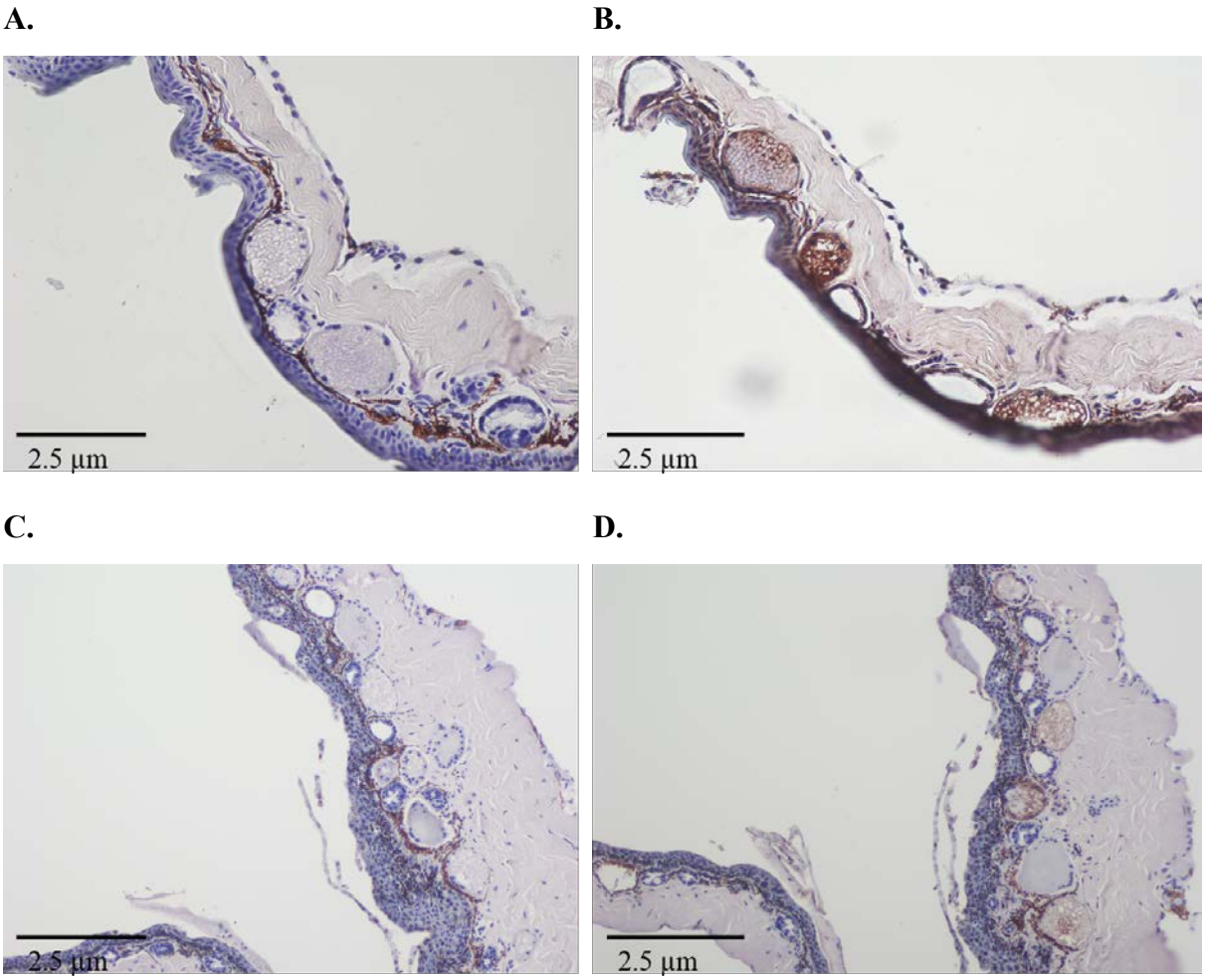
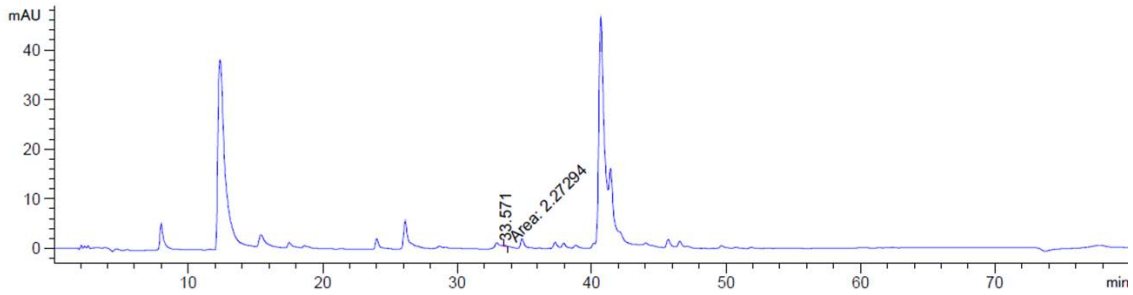


FIGURE 3.7 Immunohistochemistry staining for serotonin in the ventral skin of a representative southern corroboree frog (*Pseudophryne corroboree*) (n = 15).
A-B. *Bd* negative C-D. *Bd* positive (27 days of infection). A & C. without mouse monoclonal anti-serotonin primary antibody, B & D. with mouse monoclonal anti-serotonin primary antibody (1:40 dilution), DAB+ chromagen staining for serotonin in granular glands shown in dark brown.

A.



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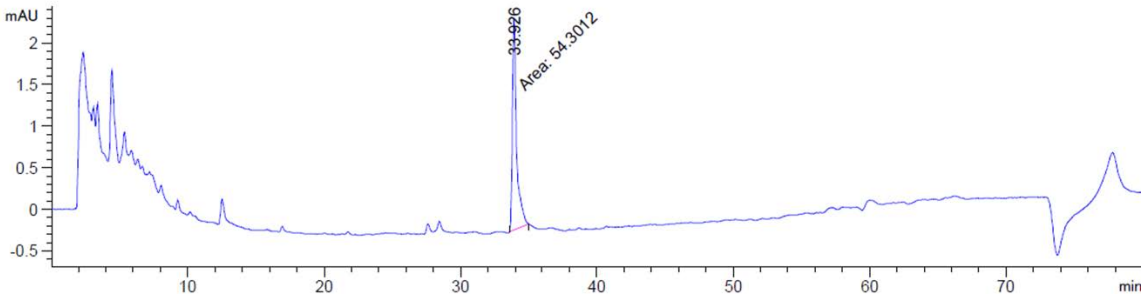

FIGURE 3.8 Representative high-performance liquid chromatography (HPLC) chromatogram.

A. southern corroboree frog (*Pseudophryne corroboree*), at 436 nm, serotonin retention time 33.571 min, area of 2.27294, B. 0.5 mg/mL serotonin control at 436 nm, serotonin retention time 33.926 min, area 54.3012.

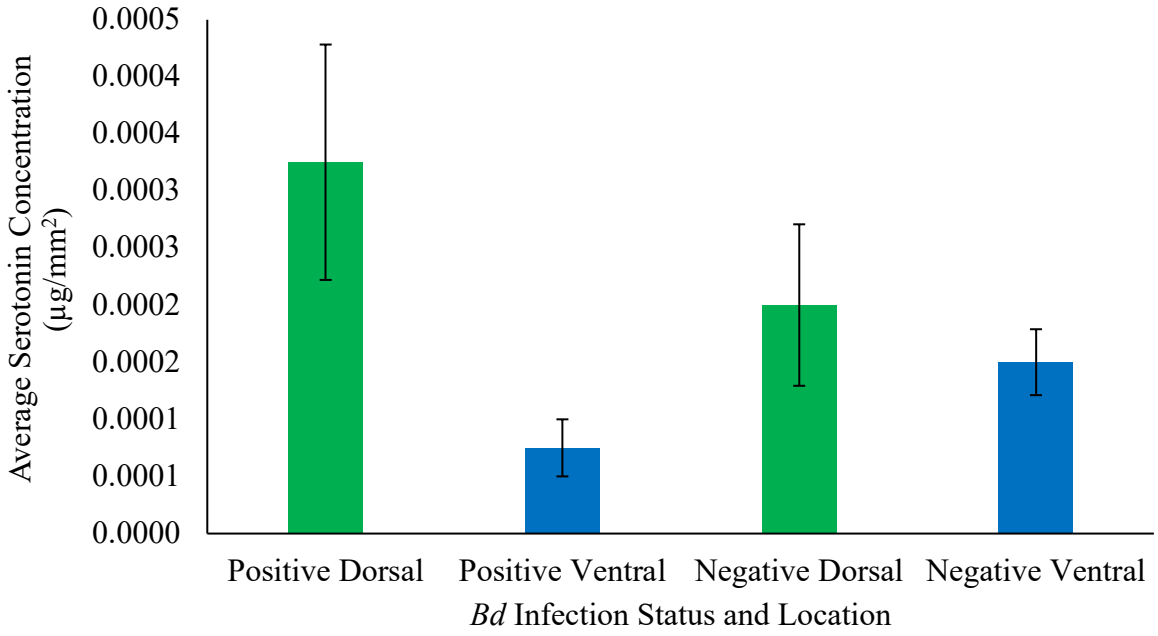

FIGURE 3.9 Skin serotonin concentration of dorsal and ventral skin in infected and uninfected southern corroboree frogs (*Pseudophryne corroboree*).
Error bars are standard error of the mean (SEM).

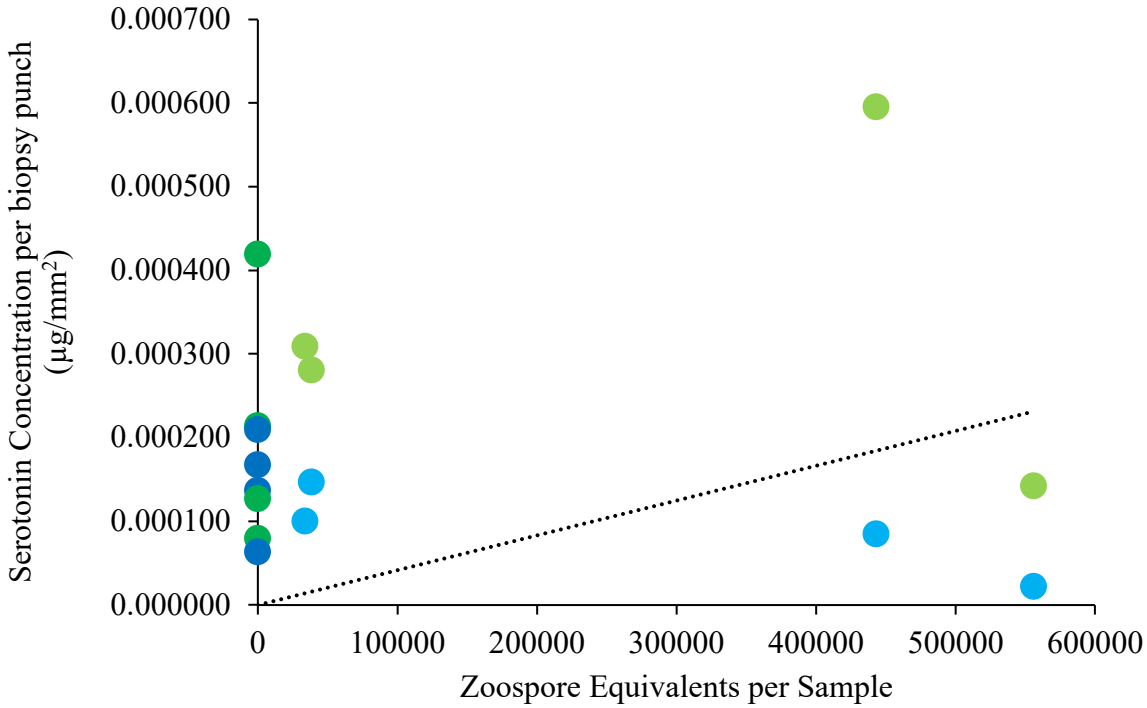

FIGURE 3.10 Serotonin concentration vs. zoospore load in dorsal (green) and ventral (blue) skin from southern corroboree frogs (*Pseudophryne corroboree*).

TABLE 3.1 Statistical test of fixed effects for location and infection status, in the amount of serotonin produced in dorsal and ventral skin of southern corroboree frogs (*Pseudophryne corroboree*).

Source	*Numerator df*	*Denominator df*	*F*	*Sig.*
Intercept	1	12	36.974	0.000
Location	1	12	6.077	0.030
Bd infection status	1	12	0.268	0.614
*Location * infection status*	1	12	1.834	0.201

[1]Dependent variable: Serotonin concentration per punch (μg/mm^2).

TABLE 3.2 Average concentration of serotonin in dorsal and ventral frog and toad skin, via high performance liquid chromatography (HPLC).

Common Name	Species	Number of individuals	Average Concentration of Serotonin	
			Dorsal	Ventral
Baw Baw frog	*Philoria frosti*	**4**	ND	ND
Cane toad	*Rhinella marina*	**7**	3,826.9226 µg/g	255.6576 µg/g
	Adult	2	7,171.3969 µg/g	255.6576 µg/g
	Juvenile	3	480.6483 µg/g	ND
	Metamorph	2	ND	ND
Common eastern froglet	*Crinia signifera*	**5**	137.5431 µg/g	
	No treatment	2	ND	
	0.044 mg/mL serotonin treatment	3	137.5431 µg/g	
Southern corroboree frog	*Pseudophryne corroboree*	**9**	0.0003 µg/mm^2	0.0001 µg/mm^2
	Infected	5	0.0003 µg/mm^2	0.0001 µg/mm^2
	Uninfected	4	0.0002 µg/mm^2	0.0002 µg/mm^2

ND: Not detected

TABLE 3.3 Proportion of frogs and toads staining positive for serotonin via immunohistochemistry (IHC).

			Proportion of individuals staining positive for serotonin in granular glands (%)	
Common Name	Species	Number of Individuals	Dorsal	Ventral
Baw Baw frog	*Philoria frosti*	**6**	0	0
Cane toad	*Rhinella marina*	**37**	83.8	86.5
	Adult	22	86.4	86.4
	Juvenile	13	92.3	92.3
	Metamorph	2	0	50.0
Common eastern froglet	*Crinia signifera*	**8**	100.0	87.5
	No treatment	3	100.0	100.0
	0.044 mg/mL serotonin treatment	5	100.0	80.0
Southern corroboree frog	*Pseudophryne corroboree*	**15**	26.7	26.7
	Infected	8	6.7	6.7
	Uninfected	7	20.0	20.0

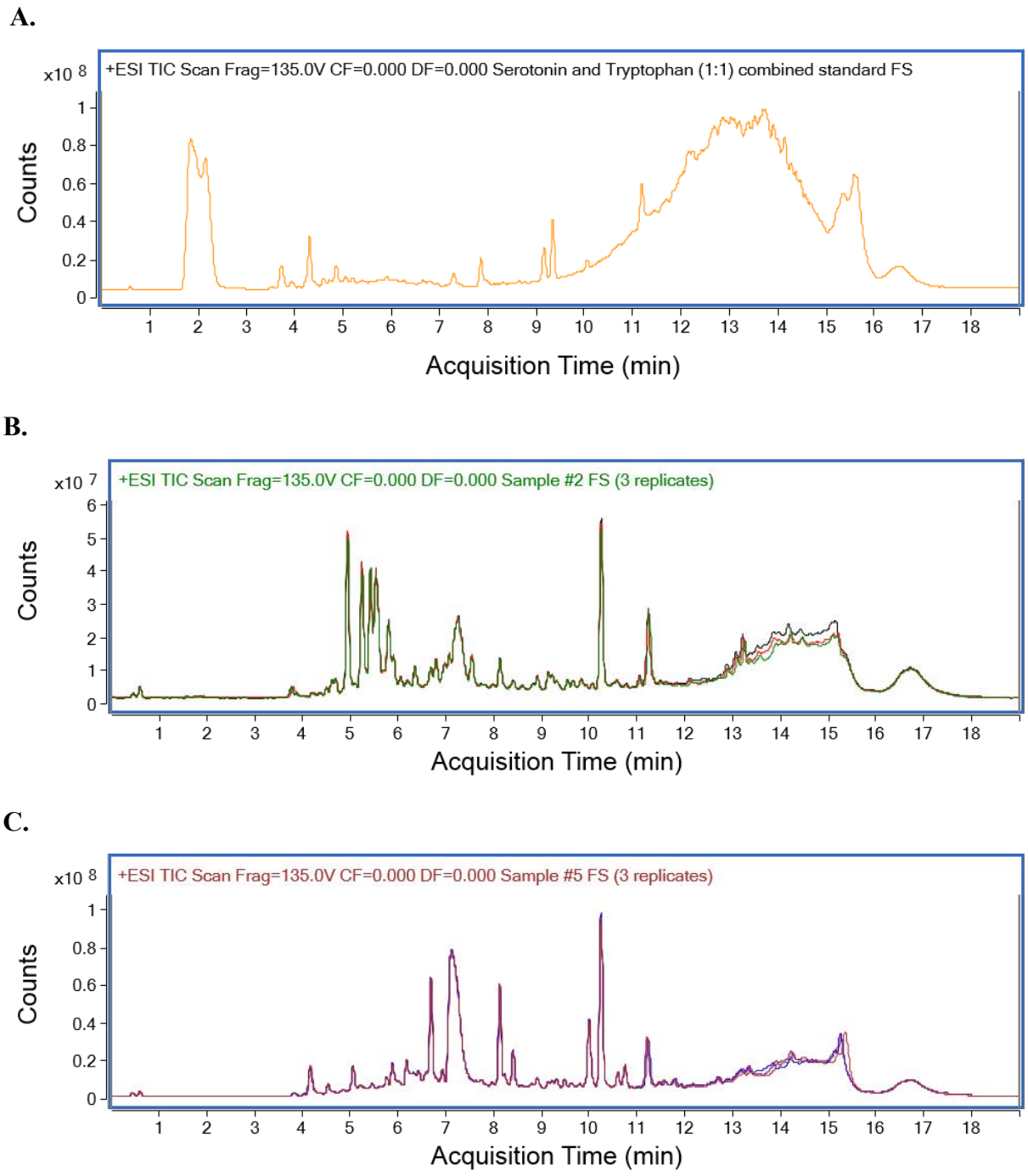

FIGURE 3.11 Full scan mass spectrometry profiles of serotonin and tryptophan standards, and common eastern froglet (*Crinia signifera*) and corroboree frog (*Pseudophryne corroboree*) skin samples.

A. 1:1 mix of de-salted derivatised serotonin and tryptophan standards, B. de-salted derivatised common eastern froglet skin sample, C. de-salted derivatised Corroboree frog skin sample. Serotonin acquisition time 9.1 min, tryptophan acquisition time 9.3 min.

TABLE 3.4 Average concentration of serotonin (mg) ± standard error of the mean, in three replicates of the common eastern froglet (*Crinia signifera*) and the southern corroboree frog (*Pseudophryne corroboree*) based on mass spectrometry analysis, at m/z 224 and m/z 160.

Sample	Concentration at m/z 224 (mg)	Concentration at m/z 160 (mg)
Common eastern froglet	0.0573 ± 0.0047	0.0508 ± 0.0033
Southern corroboree frog	0.0370 ± 0.0024	0.0361 ± 0.0024

3.4 | DISCUSSION

In this study, we aimed to determine the location and amount of serotonin in frog and toad skin, to correlate its occurrence with various host factors (skin location, age, infection status, and serotonin bath treatment) and further understand its potential role in *Bd* pathogenesis. Previous studies have found that skin serotonin levels increase in the skin of susceptible alpine tree frogs (*Litoria verreauxii alpina*) as *Bd* infection progresses (Grogan et al., 2018b). We found that the more susceptible species (Baw Baw frogs and metamorph cane toads, unlike adult cane toads) had less serotonin in their skin than more susceptible species, as measured by both IHC and HPLC (Table 3.2 and 3.3). This result contrasts with the pattern found in Grogan et al. (2018b). Baw Baw frogs are highly susceptible to *Bd* (Hollis, 2004) and are listed as critically endangered in Australia according to the Environment Protection and Biodiversity Conservation (EPBC) Act. The Baw Baw frogs showed no serotonin in IHC nor HPLC skin analyses for any individual. The serotonin may not be present in the glands and more diffusely distributed within the skin, though HPLC suggests that it is not present at detectable concentrations within the skin tissue in this species. This may be due to serotonin being in concentrations lower than the limit of detection for either method. Since the Baw Baw frogs were not infected with *Bd*, the low levels of skin serotonin may be characteristic of this species, which could relate to their susceptibility to *Bd*. Southern corroboree frogs are susceptible to *Bd* infection (Kosch et al., 2017). Serotonin was detected in *Bd* infected and uninfected corroboree frogs via HPLC analysis, but not always via IHC. Cane toad metamorphs are highly susceptible to *Bd* infection, while adults are resistant (Brannelly et al., 2018a). However, serotonin was detected consistently in all ages of cane toads by IHC but only in adults and one juvenile via HPLC. Serotonin treated and untreated common eastern froglets also stained consistently for serotonin via IHC, but levels were not detectable by HPLC. These frogs are tolerant to infection and therefore fit the pattern seen here of higher serotonin levels in less susceptible species; however, this result is confounded as 9 of the 11 froglets were infected with *Bd* before the treatment.

HPLC appeared to be more sensitive in detecting serotonin in corroboree frogs but less sensitive in common eastern froglets. Serotonin inhibits both *Bd* growth and lymphocytes in culture (Claytor et al., 2019), but overall it appeared to be detrimental to controlling chytridiomycosis in alpine tree frogs, possibly due to its immunosuppressive effects (Claytor et al., 2019). Our treatment of common eastern froglets aimed to test whether serotonin levels could be manipulated through supplementation, which would be useful in

assessing its role in pathogenesis. However, we did not observe an increase of serotonin after treatment as measured by IHC, and levels were undetectable via HPLC. Llewelyn et al. (2020) found that percutaneous absorption in cane toads *in vivo* was less than that predicted *in vitro* skin permeability experiments. The serotonin from the treatments may have been absorbed and metabolized by the frogs, leaving levels lower than the limit of detection for the HPLC. The dosage of 0.044 g/L proved to be non-lethal. Most of the froglets tested positive for low levels of *Bd* at the start of treatment and all except one remained positive and burdens were unchanged, suggesting this dosage did not impact infections. This may be due to the dosage not being large enough to effectively inhibit *Bd* infection. Further studies should aim to identify non-lethal dosage of serotonin that effectively inhibits *Bd* infectivity in amphibians. Also, determining the effect of selective serotonin reuptake inhibitors (SSRIs) is an area for further investigation. SSRIs block the reuptake of serotonin and it would be useful to determine their effect on chytridiomycosis.

Dorsal skin contained higher amounts of serotonin than ventral skin in southern corroboree frogs. The quantification of skin glands in corroboree frogs revealed no difference in the number of granular glands in the dorsal or ventral skin, showing that the difference in serotonin levels in this species was not confounded by gland abundance and likely reflects a difference in gland composition. However, in some species, there are more granular glands dorsally, such as adult green tree frogs (*Litoria caerulea*) (Berger et al 2005). Analysis of the number of granular glands in dorsal and ventral skin of the Baw Baw frogs, common eastern froglets, and cane toads should be performed, to determine if there is a difference between species and if this determines serotonin levels in skin. Though the amount of serotonin in the southern corroboree frogs was not due to the number of glands, there may be significant associations amongst other species.

Infection status was not associated with serotonin in corroboree frogs, which is in contrast to the previous study on alpine tree frogs, where skin serotonin rose with increasing infection intensity in more *Bd* susceptible frog populations (Claytor et al., 2019). Species specific differences may contribute to the conflicting results in our study. Other limitations to the study making comparisons across species difficult are the differences between the study animals (wild vs captive bred, infected vs uninfected, serotonin treatment vs no treatment).

There are no studies in the literature that have determined the amount of serotonin in amphibian skin. Our study found that the average concentration of serotonin in uninfected frog and toad skin varies between species and ranges from 0 μg/g to 7.171 μg/g in dorsal

skin, and 0 μg/g to 0.26 mg/g in ventral skin. In humans, serotonin was found to be 113.18 ± 13.34 ng/mL (Slominski et al., 2020). Serotonin may not have been detected in skin structures other than the glandular glands, due to the concentration in the frog or toad skin being below the limit of detection. Alternatively, there may be little serotonin in frog skin. The majority of serotonin is synthesized in the intestine and travels into the bloodstream, where it is then transported to the tissues (Palego et al., 2016). In amphibians, serotonin is stored and not synthesized in granular glands (Daly, 1995). Future work should sample more frog species to determine a general value of skin serotonin in frogs.

In summary, the location of the skin (dorsal or ventral) did not have an effect on the amount of serotonin detected in all species except corroboree frogs. Serotonin was detected in all age classes in cane toads. There was no correlation between infection status and the amount of serotonin. Overall, more susceptible species had less serotonin in their skin. This suggests that serotonin's antifungal effects may be inhibiting *Bd*, but this conclusion contrasts with previous work on alpine tree frogs where the more resistant population of alpine tree frogs had lower levels. Serotonin is a bioactive compound with diverse functional effects, and further work is needed to determine its role in the pathogenesis of chytridiomycosis. Future work should test potential therapeutics that alter skin serotonin levels to determine their effect on intensity of infection, and rates of survival in infected frogs and the suitability of these drugs as a treatment against *Bd*.

ACKNOWLEDGEMENTS

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

AUTHOR CONTRIBUTIONS

S.C.C., A.A.R., L.B., L.F.S., and D.M. designed the research. S.C.C., V.K.L., S.R. collected and provided skin tissue for cane toads. T.B. and D. D. collected and provided skin tissue for the Baw Baw frogs. P.L.F.A. and R.J.W. collected and provided the skin tissue for

the southern corroboree frogs. S.C.C. and L.B. performed the serotonin baths. S.C.C. performed the experiments. S.C.C., A.A.R., V.K.L., and B.B. performed calculations. S.C.C. and L.A.B. performed statistical analysis. S.C.C. and S.R. performed the nitrogen drying. All authors reviewed the manuscript.

SUPPLEMENTAL INFORMATION

TABLE S3.1 Concentration of serotonin in the skin of cane toads (*Rhinella marina*) per gram of skin tissue. ND = Not Detected.

Individual	Location	Size class	Concentration of serotonin per gram (μg/g)
1	Dorsal	Adult	254.9122
1	Ventral	Adult	22.2635
2	Dorsal	Adult	14,087.8817
2	Ventral	Adult	489.0516
3	Dorsal	Juvenile	840.3690
3	Ventral	Juvenile	ND
4	Dorsal	Juvenile	120.9276
4	Ventral	Juvenile	ND
5	Dorsal	Juvenile	ND
5	Ventral	Juvenile	ND
6	Dorsal	Metamorph	ND
6	Ventral	Metamorph	ND
7	Dorsal	Metamorph	ND
7	Ventral	Metamorph	ND

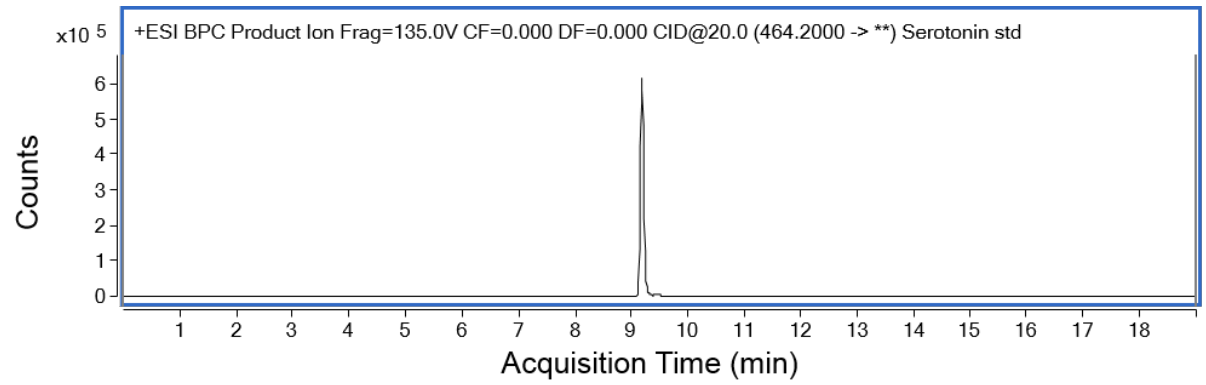
TABLE S3.2 Concentration of serotonin in the skin of common eastern froglet (*Crinia signifera)* per gram of skin tissue.

Individual	Location	Treatment	Concentration of serotonin per gram (μg/g)
1	Dorsal and Ventral	No treatment	ND
2	Dorsal and Ventral	No treatment	ND
3	Dorsal and Ventral	0.44 g/L serotonin	ND
4	Dorsal and Ventral	0.44 g/L serotonin	ND
5	Dorsal and Ventral	0.44 g/L serotonin	137.5431

TABLE S3.3 Concentration of serotonin in the skin of corroboree frogs (*Pseudophryne corroboree*) per 8 mm diameter skin punch.

Individual	Location	*Bd* Infection Status	Concentration of serotonin ($\mu g/mm^2$)
1	Dorsal	Infected	0.0001
1	Ventral	Infected	0.0000
2	Dorsal	Infected	0.0001
2	Ventral	Infected	0.0002
3	Dorsal	Infected	0.0003
3	Ventral	Infected	0.0001
4	Dorsal	Infected	0.0001
4	Ventral	Infected	0.0001
5	Dorsal	Uninfected	0.0003
5	Ventral	Uninfected	0.0001
6	Dorsal	Uninfected	0.0002
6	Ventral	Uninfected	0.0002
7	Dorsal	Uninfected	0.0006
7	Ventral	Uninfected	0.0001
8	Dorsal	Uninfected	0.0004
8	Ventral	Uninfected	0.0001

A.



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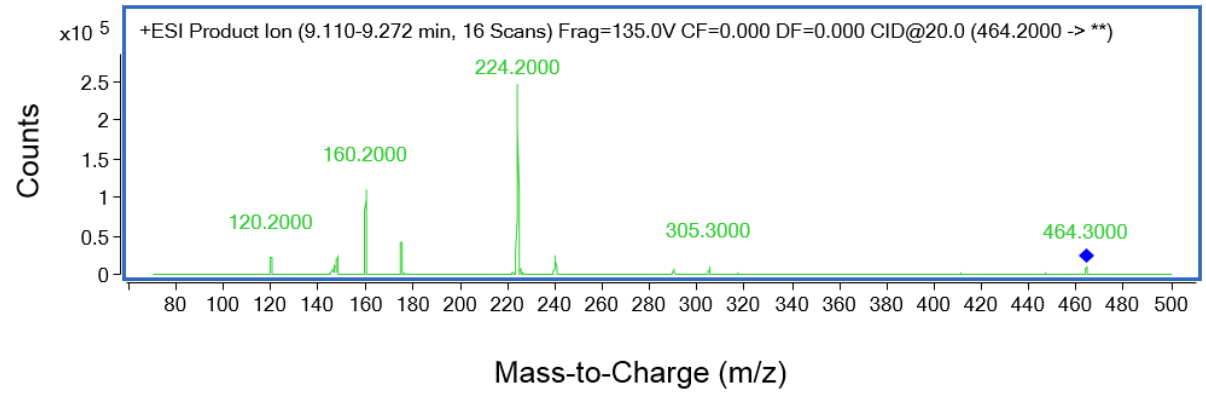
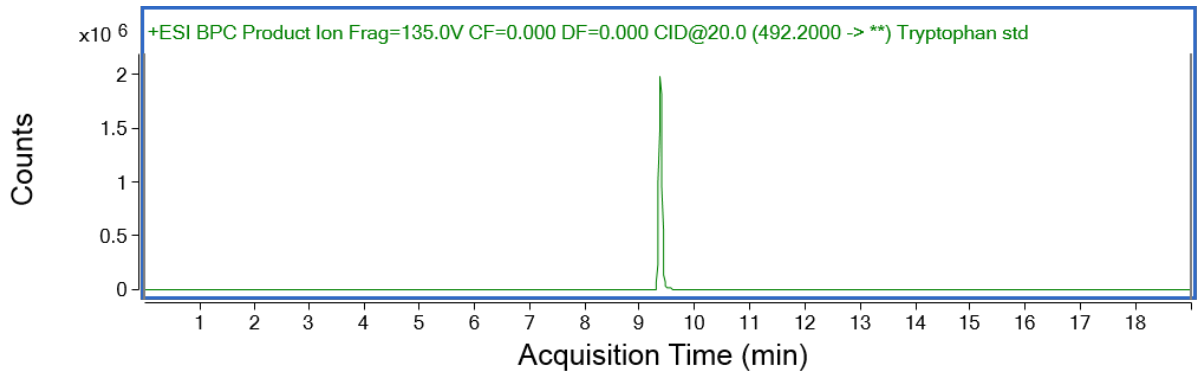


FIGURE S3.1 Mass spectrometry product ion scan for serotonin.

A. Acquisition time, B. mass-to-charge.

A.



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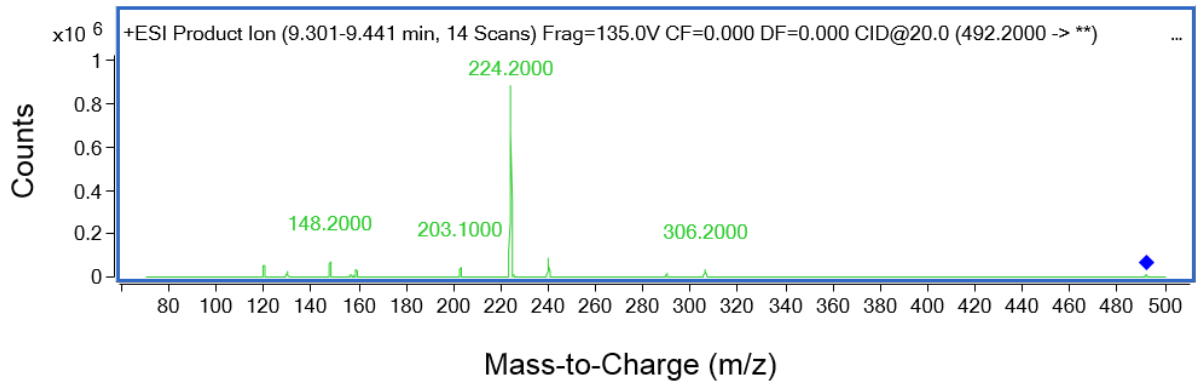

FIGURE S3.2 Mass spectrometry product ion scan for tryptophan.

A. Acquisition time, B. mass-to-charge.

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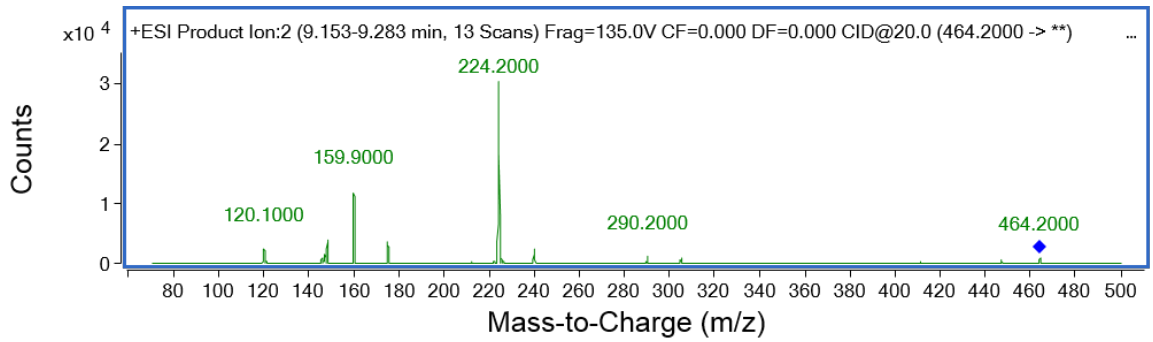

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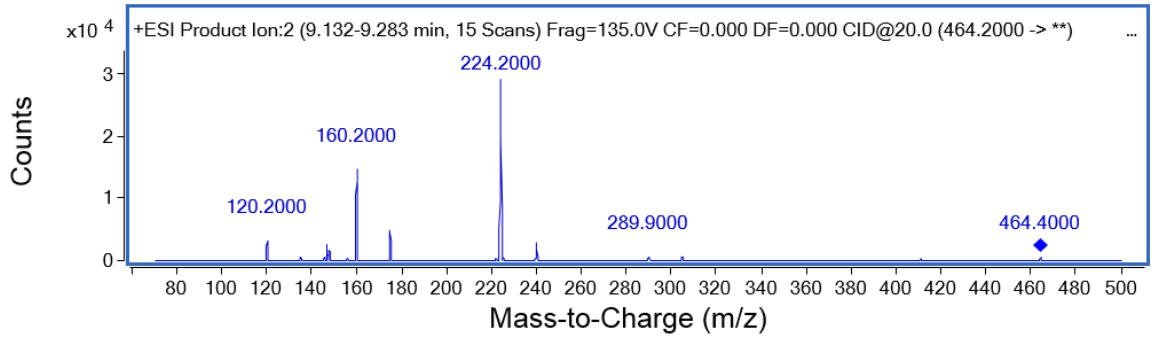

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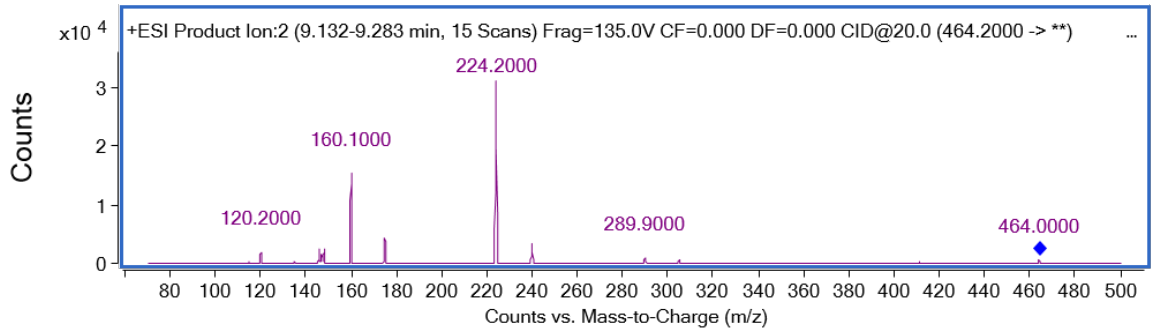

FIGURE S3.3 Mass spectrometry product ion scan for serotonin in three replicates of common eastern froglet (*Crinia signifera*).

A-D. Mass-to-charge

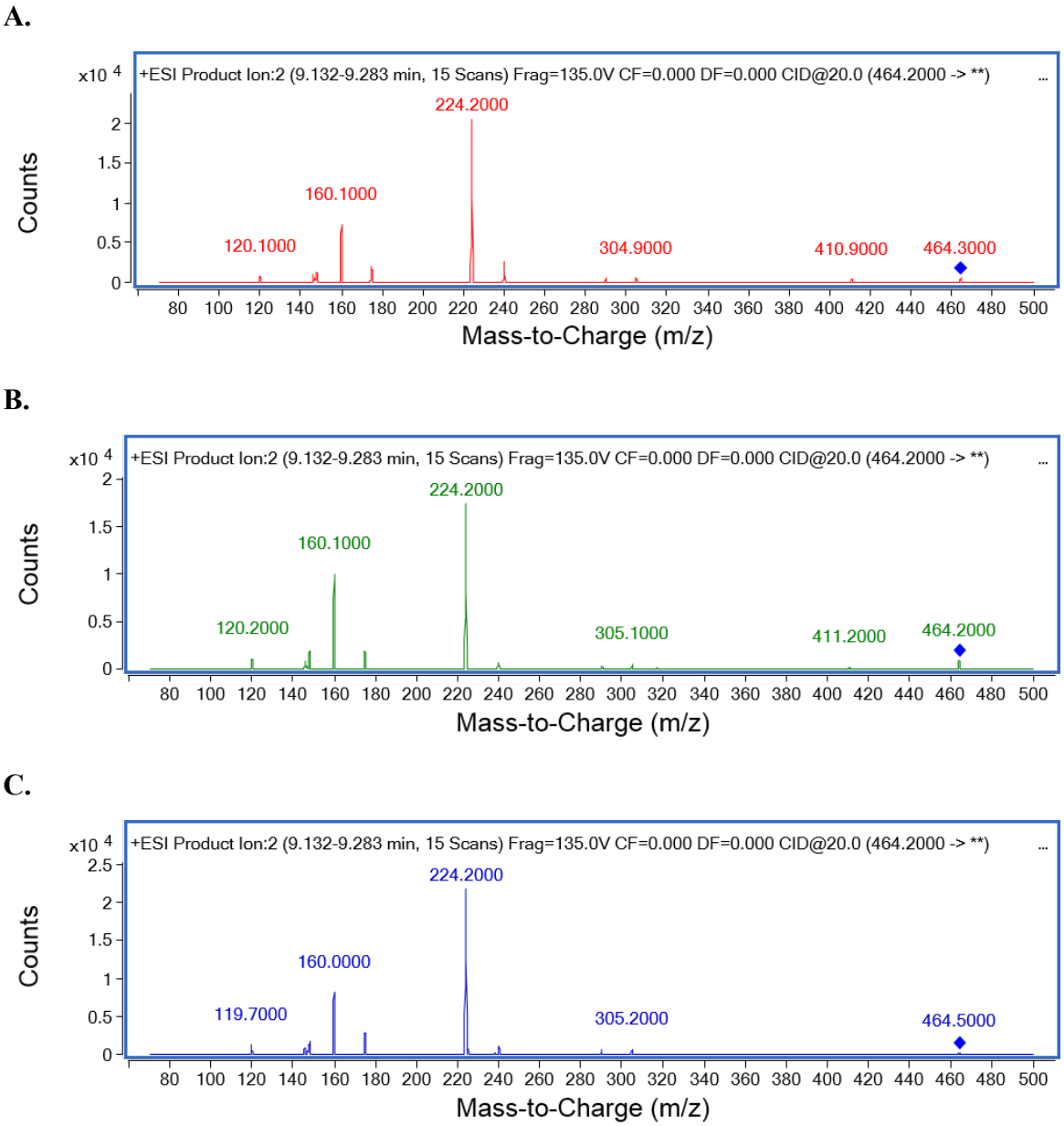

FIGURE S3.4 Mass spectrometry product ion scan for serotonin in three replicates of southern corroboree frog (*Pseudophryne corroboree*).

A-D. Mass-to-charge

CHAPTER 4 | Mechanisms of *Bd* inhibition by serotonin

OVERVIEW

The objective of this chapter is to understand how serotonin inhibits *Batrachochytrium dendrobatidis* (*Bd*) growth. The function of serotonin in amphibians is complex, and the mechanism of *Bd* inhibition by serotonin is unknown. Chapter 2 showed that serotonin and structural analogues inhibit growth of *Bd in vitro*, but that *Bd* metabolites tryptophan (Trp) and kynurenine (Kyn) do not restore *Bd* growth in the presence of serotonin. Therefore, despite the similarity in structures, the mechanism of *Bd* inhibition by serotonin is not likely to be via disruption of the Trp or Kyn metabolic pathways. To confirm this result, we used transcript analysis to quantify Trp biosynthesis gene expression in the presence of serotonin. We also explored other potential mechanisms of *Bd* inhibition by serotonin, including disruption of *Bd* protease activity and promotion of caspase-mediated cell death in *Bd*.

The following text is a copy of the manuscript in preparation, prior to submission.

Decrease in protease and caspase activity in *Batrachochytrium dendrobatidis* treated with serotonin

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KEYWORDS

Batrachochytrium dendrobatidis, chytridiomycosis, serotonin, 5-hydroxytryptamine, tryptophan, aspartyl protease, serine peptidase, fungalysin metallopeptidase

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ABSTRACT

The fungus *Batrachochytrium dendrobatidis* (*Bd*) causes the disease chytridiomycosis and is responsible for the global decline and extinction of amphibians. Previous studies have found that serotonin inhibits *Bd* growth *in vitro*, but the mechanism of inhibition has not been elucidated. Here, we investigated the role of serotonin in *Bd* by assessing the impact on tryptophan (serotonin precursor) and protease gene expression, protease activity, and caspase activity. We found a decrease in total protease and caspase activity in *Bd* with increasing concentrations of serotonin. The decrease in protease activity and apoptosis may be due to a lack of metabolic activity of *Bd* at higher concentrations of serotonin. Transcript analysis showed no statistical difference in tryptophan, aspartyl protease (ASP), serine peptidase (S41), and metallopeptidase (M36) gene expression when treated with serotonin. This study provides more insight into the mechanisms of serotonin inhibition of *Bd* and how serotonin may affect host/*Bd* interactions.

4.1 | INTRODUCTION

The pathogenic fungus *Batrachochytrium dendrobatidis* (*Bd*) causes the disease chytridiomycosis and is the major cause of global amphibian declines (Skerratt et al., 2007). The neurotransmitter serotonin (5-hydroxytryptamine) has antifungal properties against some fungi (Lass-Flörl et al., 2003, Mayr et al., 2005), including inhibition of *Bd in vitro* (Claytor et al., 2019). Serotonin is produced in the skin glands of frogs, but despite increased levels of serotonin during *Bd* infection, frogs were more susceptible to this fungal pathogen (Claytor et al., 2019). The role of serotonin in the frog-*Bd* interaction is not fully understood, but in addition to its inhibitory effects on *Bd*, serotonin was shown to inhibit lymphocytes *in vitro* (Claytor et al., 2019). This dichotomy requires more study if we are to resolve the role of serotonin in the frog-*Bd* interaction. Here, we further examine the ways that serotonin could inhibit *Bd*.

The serotonin analogues tryptophan (Trp) and kynurenine (Kyn) form part of the Trp synthesis pathway which is vital for *Bd* growth. We hypothesized that excess serotonin may provide a feedback loop and inhibit Trp synthesis. However, supplementation with Trp and Kyn does not restore *Bd* growth in the presence of serotonin (Claytor et al., 2019). This observation suggests that serotonin does no inhibit Trp synthesis pathways in *Bd*, and further studies are required to elucidate the function of serotonin.

In *Candida*, serotonin inhibits fungal proteases and hyphal elongation at concentrations above 0.46 mM (Mayr et al., 2005), however, it is not known whether the same phenomenon occurs in *Bd*. Proteases and peptidases cleave the peptide bonds of proteins, breaking them into peptides or free amino acids. Proteases are classified by their catalytic mechanism and active sites, and include aspartyl-, cysteine-, metallo-, serine-, threonine-, and other proteases. Numerous metallo-, serine-, and aspartyl- proteases are upregulated in *Bd* when grown in media supplemented with pulverised amphibian skin compared with tryptone broth alone (Rosenblum et al., 2012). *Bd* proteases are likely to play an important role in infection and pathogenicity, as zoospores secrete proteases that disrupt epidermal intercellular junctions in anuran skin (Brutyn et al., 2012). Similarly, aspartyl proteases aid in fungal invasion of mice host tissue by *Aspergillus fumigatus* (Lee and Kolattukudy, 1995), while fungalysin metallopeptidases (M36 family) are involved in keratin degradation in the human dermatophytes *Trichophyton rubrum*, *Trichophyton mentagrophytes*, and *Microsporum canis* (Jousson et al., 2004). Therefore, in this study, we explore the effect of serotonin on proteases of *Bd*.

Apoptosis is a form of programmed cell death (Lockshin and Williams, 1965, Kerr et al., 1972) that occurs in most living organisms, including fungi (Ramsdale, 2006). There are two major routes of apoptosis, the extrinsic and intrinsic pathways (Elmore, 2007), both of which cause the cleavage of the effector caspase-3. Extrinsic pathways occur when an extracellular ligand activates death receptors on the cell membrane. Fungi only use the intrinsic pathway for apoptosis (Shlezinger et al., 2012), which occurs when there is cell damage or during a specific stage of development. Kynurenine, which is structurally similar to serotonin, causes immunosuppression of lymphocytes, possibly through an apoptosis mechanism (Rollins-Smith et al., 2015). Given serotonin, which is secreted by amphibians and structurally similar to Kyn, may cause apoptosis in lymphocytes, it may cause apoptosis in *Bd*. Therefore, the effect of serotonin on *Bd* caspase activity was investigated as a proxy for apoptosis.

In summary, the aim of this study was to elucidate the function of serotonin in *Bd* inhibition by testing the effects of serotonin on protease and caspase activity. Gene expression of Trp, and M36, S41, and ASP proteases was also examined to verify that serotonin does not inhibit the Trp synthesis pathway and whether these specific proteases are downregulated by serotonin. This study provides further understanding of how serotonin may inhibit *Bd* growth through its effects on the ability of *Bd* to break down proteins, on inducing the apoptotic pathway in *Bd* and on its feedback on the Trp synthesis pathway essential for *Bd* growth.

4.2 | METHODS

4.2.1 | Growth of *Bd* in liquid culture

The *Bd* isolate "Ethel creek-Lnannotis-2013-LB" (isolated from a Waterfall frog, *Litoria nannotis*, in 2013 from northern Queensland, Australia) was grown on sterile tryptone/gelatin hydrolysate/lactose (TGhL) plates using established methods (Longcore et al., 1999). Plates were flooded with TGhL media to release zoospores, which were syringe filtered to remove sporangia using a 10 µm isopore filter (Millipore). Chemicals were obtained from the Sigma-Aldrich Corporation. Serotonin was added at a final assay concentration of 0.3125 mM to a total volume of 30 mL and the culture with zoospore concentration approximately 2.5 x 10^5, was incubated for 6 d. A no serotonin control was included. Cultures were harvested at 8,000 x *g* for 5 min, the supernatant was removed, and pellets were stored in RNAlater™ (Sigma-Aldrich) at -80 °C until extraction.

4.2.2 | RNA extraction and purification

RNeasy® mini kit (QIAGEN®) was used to extract RNA from *Bd* cells, following the manufacturer's protocol for yeast. The quality and concentration of each RNA sample was measured on a NanoDrop™ ND-1000 spectrophotometer.

Milli-Q® water was added to the RNA samples, to a total volume of 500 µL. Then, 500 µL of phenol:chloroform:isoamyl alcohol (125:24:1, pH 4.5) was added to the sample. Tubes were vortexed for approximately 1 min and spun at 16,000 x *g* in a microcentrifuge for 5 min. The upper, aqueous layer was transferred into a new microcentrifuge tube. One volume of chloroform was added to the sample, vortexed for 1 min, and centrifuged for 5 min at16,000 x *g*. The upper, aqueous layer was transferred to a new microcentrifuge tube. Then, 0.1 volume of 3 M sodium acetate (pH 5.2) and 1 volume cold isopropanol was added to the tube. Tubes were mixed by aspiration and placed on ice for 1 h, then spun at 16,000 x *g* in microcentrifuge for 10 min. The supernatant was discarded, and the pellet was washed with 1 mL of cold 70% ethanol and spun for 5 min in microcentrifuge at 16,000 x *g*. Supernatant was removed and spun again at 16,000 x *g* for 30 s and remaining liquid removed. Pellets were air dried for 5 min and RNA samples were resuspended in 50 µL of nuclease-free water.

4.2.3 | Complementary DNA (cDNA) synthesis

A ProtoScript® II First Strand cDNA Synthesis Kit was used to synthesise cDNA. In a RNase-free microfuge tube, 6 µL RNA and 2 µL anchored oligo-dT [$d(T)_{23}VN$] primer (New England BioLabs) was added to each sample. Tubes were mixed, spun, and then denatured for 5 min at 65 °C in a Veriti™ 96-Well thermal cycler. Samples were immediately cooled on ice for approximately 1 min, spun, and 10 µL ProtoScript® II Reaction Mix (2X) and 2 µL ProtoScript® II Enzyme Mix (10X) (New England BioLabs) were added to each tube, then mixed and spun at 16,000 x *g*. Samples were incubated at 42 °C for 1 h, 80 °C for 5 min, and then cooled to 4 °C, in the thermal cycler.

4.2.4 | Reverse transcription polymerase chain reaction (RT-PCR)

DNA primers were designed using Primer-BLAST (NCBI) software (Ye et al., 2012). Primers for metallo-peptidase 'M36' (BATDEDRAFT_1502), serine-peptidase 'S41' (BATDEDRAFT_25462), and aspartyl-protease 'ASP' (BATDEDRAFT_90236) were designed based on the *Bd* genomes JAM81 (NCBI reference sequence NW_006281099.1) and JEL423 (GenBank® assembly accession GCA_000149865.1) in GenBank®. These three protease genes were selected as representatives of the three major protease families with the greatest change in differential expression when *Bd* was grown on pulverised frog skin

(Rosenblum et al., 2012) (Table 4.1). 'TrpA' was designed based on the tryptophan synthase alpha-chain gene found in GenBank® (Accession #: XP_006680001.1), which catalyses the penultimate step Trp biosynthesis. Reverse transcription PCR was performed using 1 µL of *Bd* cDNA, 10 mM dNTPs, 10 µM forward and reverse primers (Merck), 5.0 U Taq DNA polymerase (New England BioLabs®), and 10X ThermoPol® buffer (New England BioLabs®) in a Veriti™ 96-Well thermal-cycler. The thermal-cycler settings were: denaturation at 95 °C for 30 s, 30 cycles of 95 °C for 30 s, 55 °C for 30 s, 68 °C for 40 s, and extension at 68 °C for 5 min.

4.2.5 | Semi-quantitative transcript analysis

RT-PCR products were run in triplicate replicates with a GeneRuler 1 kb Plus DNA ladder (Thermo Scientific) on a 1% tris/acetate/ethylenediaminetetraacetic acid (EDTA) (TAE) agarose gel at 95 volts for 1.5 h. The band intensity for each band was measured using ImageJ 1.52a software (Schneider et al., 2012, The University of Queensland, 2017). The band intensities were then averaged for the replicates of each sample. A Mann-Whitney test was performed to determine if there was a difference in band intensity between the no treatment control and 0.3125 mM serotonin treated *Bd*.

4.2.6 | Protease Assay

An EnzChek™ Peptidase/Protease Assay Kit (Invitrogen™) was used to perform the protease assay, following the manufacturer's protocol. Three replicates of 25 µL *Bd* (grown in TGhL broth for 6 d in a clear Thermo Scientific™ 96-Well, Nunclon Delta surface microplate) and 12.5 µL of 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer was added to a white Greiner 384 microplate (Greiner Bio-One GmbH). Next, 12.5 µL of either water or serotonin at 0.156 mM or 0.3125 mM was added to wells and incubated for 6 d at 21 °C. Then, 50 µL of EnzChek™ peptidase/protease substrate working solution was added to each well. The microplate was incubated at room temperature for 1 h, protected from light. Fluorescence was measured at 485 nm on a POLARstar® Omega microplate reader spectrophotometer. Relative fluorescence values were standardized by subtracting the fluorescence values from wells without *Bd*, containing TGHL broth and HEPES buffer. Serotonin concentration vs. fluorescence was plotted to determine relative protease activity.

4.2.7 | Caspase assay

A Caspase-Glo® 3/7 Assay (Promega) was used to measure the activity of caspase-3 and caspase-7, activated during the extrinsic and intrinsic apoptosis pathway, in *Bd* with

varying serotonin concentrations. Three replicates of 25 µL *Bd* (grown in TGhL broth for 6 d) and 12.5 µL of 20 mM HEPES buffer was added to a white Greiner 384 microplate. Next, 12.5 µL of either water or serotonin at 0.156 mM, 0.3125 mM, 0.625 mM, 1.25 mM, 2.5 mM, 5 mM, or 10 mM was added to each well and incubated for 6 d at 21 °C. 50 µL of Caspase-Glo® 3/7 Reagent was added to each well, and the plate was incubated at room temperature for up to 1 h. Luminescence was measured on a microplate reader. Luminescence values were standardized by subtracting the luminescence values from wells without *Bd*.

4.2.8 | Viability stain

Bd from isolate "Ethel creek-Lnannotis-2013-LB" was grown on a 24-well sterile plate with 20 mM HEPES and serotonin at concentrations of 0.156 mM, 0.312 mM, 0.625 mM, 1.25 mM, 2.5 mM, 5 mM, and 10 mM. *Bd* was incubated for 4 d at 21 °C, and then stained with 0.01 mg/mL methylene blue for 2 min, following established methods (Webb et al., 2019). Liquid was removed, attached cells were rinsed with deionised water, and photos were taken of each well by an inverted microscope at 20x magnification, counting 3 replicate field views. Viable sporangia remained unstained, while non-viable sporangia stained dark blue.

4.3 | RESULTS

4.3.1 | Effect of serotonin on Trp synthase gene expression

Semi-quantitative comparison of gene expression for tryptophan synthase showed no statistical difference in gene expression in *Bd* grown in untreated and serotonin-treated *Bd* (treatment mean = 6,856.092, 25th percentile – 75th percentile = 5,022.874 – 8,537.317, Z = -1.964, p = 0.100).

4.3.2 | Decrease in protease and caspase activity

The total protease activity decreased with concentrations over 0.156 mM serotonin (Figure 3.2). At 2.5 mM there was a 5.8-fold decrease in relative protease activity. There was also a decrease in caspase activity with concentrations over 0.156 mM serotonin (Figure 4.2). At 0.156 mM there was a 1.5-fold decrease in relative caspase activity.

4.3.3 | Effect of serotonin on protease gene expression

Semi-quantitative analysis of gene expression of the proteases ASP, S41, and M36 was performed for *Bd* grown with and without serotonin. There was no significant difference between the control and the 0.3125 mM serotonin-treated *Bd* for any of the genes. (units = band intensity, ASP mean = 13,862.508, 25th percentile – 75th percentile = 13,076.772 –

14,321.354, Z = -0.655, p = 0.700; S41 mean = 12,379.129, 25th percentile – 75th percentile =11,830.706 – 12,872.585, Z = -1.964, p = 0.100; M36 gene mean = 12,118.323, 25th percentile – 75th percentile = 9,079.116 – 14,874.490, Z = -1.964, p = 0.100).

4.3.4 | *Bd* viability stain

There were no viable sporangia observed at serotonin concentrations ≥ 5 mM. Serotonin did not impact *Bd* viability below 5 mM.

TABLE 4.1 DNA primers used for the transcript analysis.

Primer	Sequence
TrpA_F	5’ AAAACGACCTGCGTTTGTAG 3’
TrpA_R	5’ CTGGAAACACCGAATCCAAC 3’
ASP_F	5’ TGGTGGAAACCCCGATCAAG 3’
ASP_R	5’ TGGTGAGTCTACCCATGCAAC 3’
S41_F	5’ TGAAGGTGCTGGTGGATGTC 3’
S41_R	5’ ACGTTTCCAATCCCTCGTGT 3’
M36_F	5’ GTTCGACGGTGAGGCTAGAG 3’
M36_R	5’ AGCCCACACTTCACCAACAA 3’

Note: ‘F’, forward primer, ‘R’, reverse primer, ‘TrpA’, tryptophan synthase subunit alpha gene, ‘ASP’ represents the aspartyl protease gene, ‘S41’ represents the peptidase S41 gene, and ‘M36’ represents the metallopeptidase M36 gene. Primers for genes were designed based on the *Bd* genomes JAM81 (NCBI reference sequence NW_006281099.1) and JEL423 (GenBank® assembly accession GCA_000149865.1).

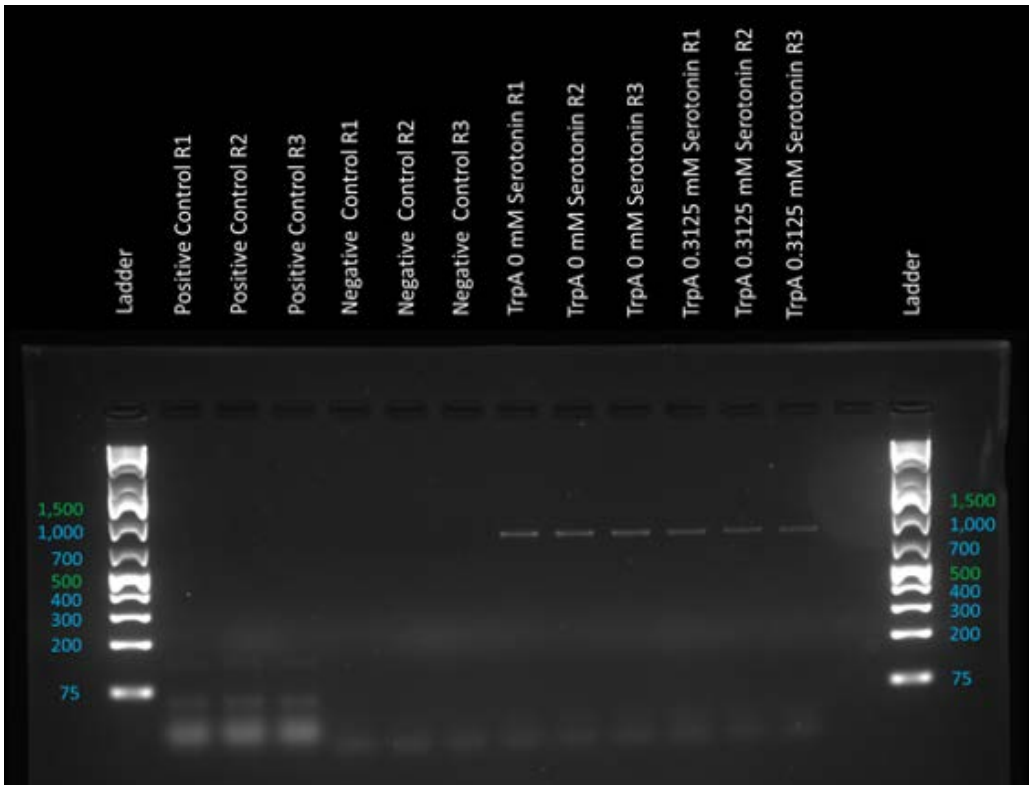

FIGURE 4.1 Gene expression of tryptophan synthase alpha-chain (TrpA) in *Bd* treated with and without 0.3125 mM serotonin.

Triplicate RT-PCR samples were run on 2% agarose gel. The positive control primers (BOB5 and BOB6) were from Boyle et al. (2004) targeting *Bd* 18S and 28S rDNA, and the ladder was a GeneRuler 1 kb Plus DNA ladder (Thermo Fisher Scientific) with the bp size as indicated. There was no statistically significant difference between the no serotonin control *Bd* and 0.325 mM serotonin treated *Bd*, according to the ImageJ band intensity analysis.

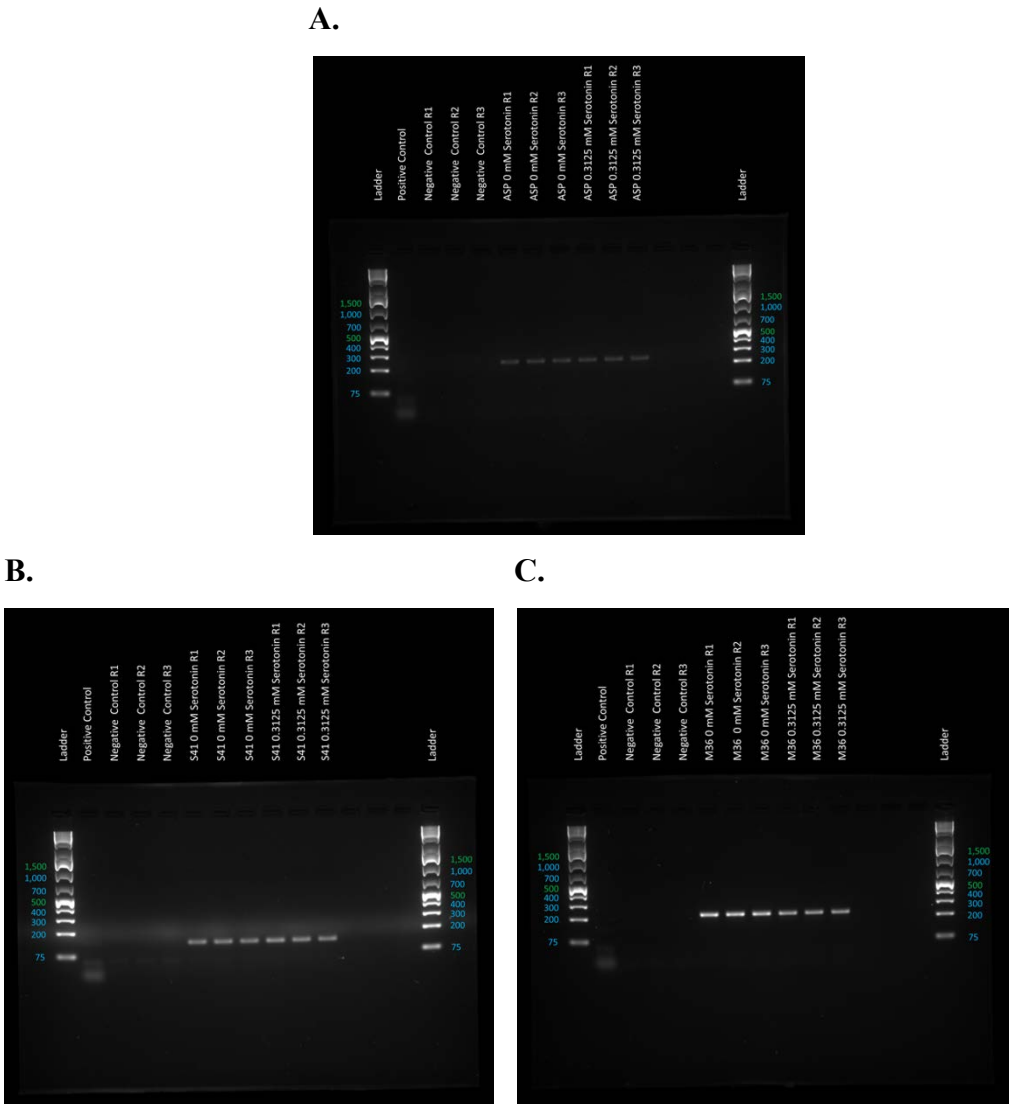

FIGURE 4.2 Gene expression bands on an agarose gel for *Bd* treated with 0 mM and 0.3125 mM of serotonin.

A. aspartyl protease (ASP), B. serine peptidase (S41), and C. fungalysin metallopeptidase (M36) gene expression. Triplicate RT-PCR samples were run on 2% agarose gel. The positive control primers (BOB 5 and BOB 6) were from (Boyle et al., 2004) targeting *Bd* 18S and 28S rDNA, and the ladder was a GeneRuler 1 kb Plus DNA ladder (Thermo Fisher Scientific) with the bp size as indicated. There was no statistically significant difference between the no serotonin control *Bd* and 0.325 mM serotonin treated *Bd* for any gene, according to the ImageJ band intensity analysis.

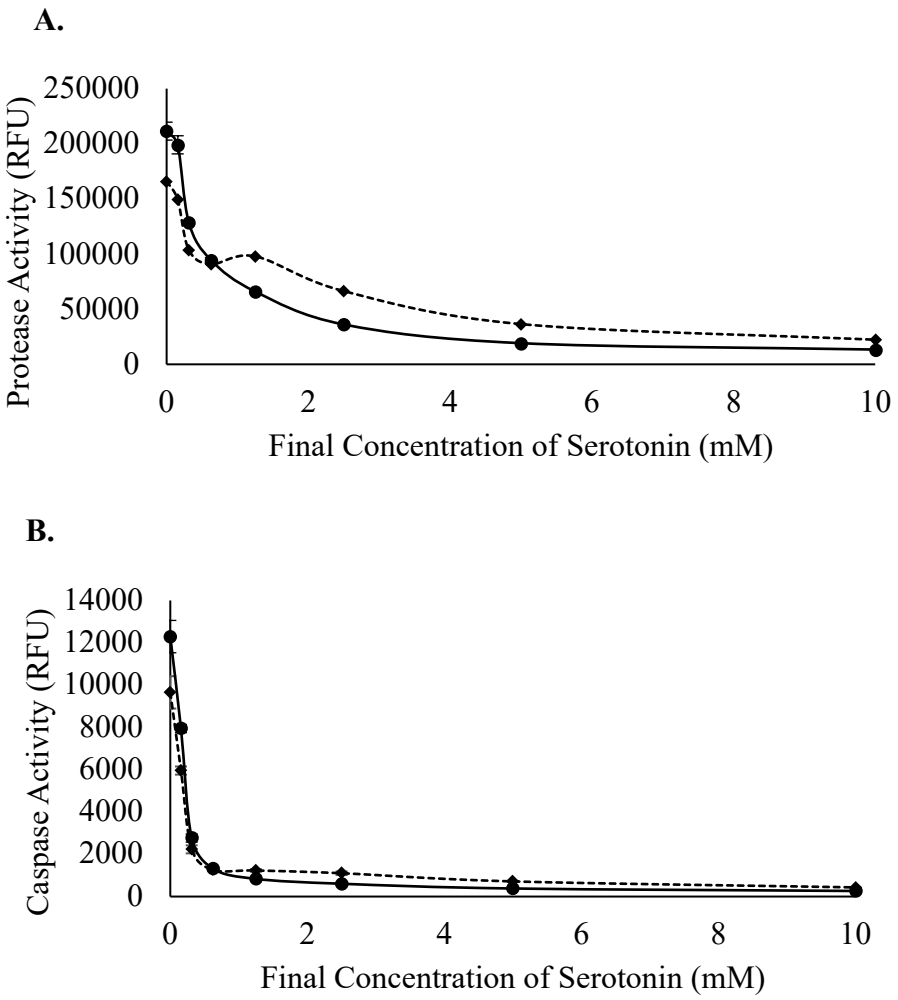

FIGURE 4.3 Serotonin decreases both A. protease and B. caspase activity in *Bd in vitro*. Raw luminescence (dashed line) and luminescence normalized (data divided by absorbance) to *Bd* growth (absorbance at 492 nm) (solid line).

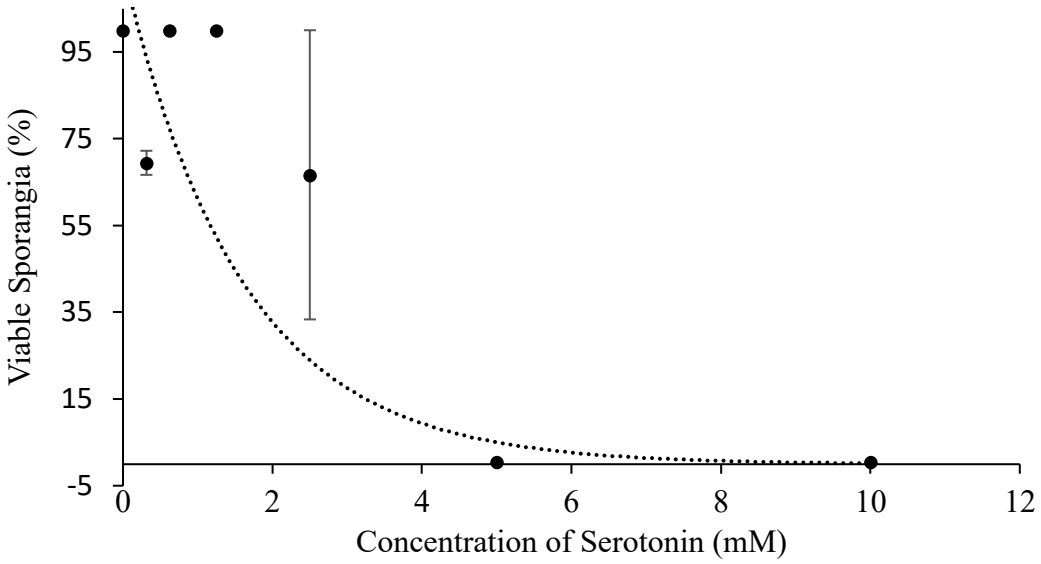

FIGURE 4.4 Viability of *Batrachochytrium dendrobatidis* sporangia with increasing serotonin concentrations.

Viability was determined with a methylene blue assay. Error bars are the standard error of the mean, for two biological replicates.

4.4 | DISCUSSION

Previous studies found a correlation between the levels of serotonin in disease progression in the skin of alpine tree frogs (*Litoria verreauxii alpina*) (Grogan et al., 2018b). To better understand the effect serotonin has on *Bd* protease expression, we first characterized the gene expression of tryptophan in *Bd* treated with serotonin. Tryptophan (Trp) is a metabolic precursor to serotonin. *Bd* produces Trp but lacks the enzymes necessary to metabolise it into serotonin (Rollins-Smith et al., 2015). In contrast, frogs biosynthesize exogenous Trp into serotonin. We found no statistical difference in protease gene expression of the tryptophan alpha subunit gene in *Bd* treated with serotonin. Therefore, serotonin likely does not influence the expression of the tryptophan synthesis gene, which supports our previous studies where supplementation with Trp was unable to reverse the inhibition of *Bd* by serotonin *in vitro*. Trp from other skin microbes may also contribute, but the abundance of *Bd* in infected frogs makes the contributions from other microbes negligible. *Bd*-infected frogs lose appetite as disease progresses and therefore, the Trp is likely provided from *Bd*. A limitation to our gene expression study was that we tested the effect of a concentration of serotonin (0.3125 mM), which is below the minimum inhibitory concentration for *Bd in vitro* (Claytor et al., 2019). Further work should investigate gene expression at levels of serotonin closer to biological levels found in amphibian skin. The average concentration of serotonin in uninfected frog and toad skin ranges from 0 μg/g to 7.171 μg/g in dorsal skin, and 0 μg/g to 0.26 μg/g in ventral skin (Chapter 3). The variation in concentration of serotonin within the skin is location (dorsal vs. ventral) and species specific (Chapter 3). Due to species specific differences in skin levels of serotonin (Claytor et al. unpublished data), a more pronounced effect of serotonin on gene expression of Trp and protease genes may be seen in species with higher concentrations of serotonin.

We hypothesized that serotonin would inhibit *Bd* protease activity as a mechanism for growth restriction (Mayr et al., 2005). *Candida albicans* virulence properties such as hyphal elongation, phospholipase activity, and secreted aspartyl proteinases (Saps) activity was affected at concentrations between 3.7 – 14.7 mM, 1.8 – 14.7 mM, and 1.8-7.3 mM of serotonin *in vitro* (Mayr et al., 2005). *In vivo* concentrations of serotonin in frogs may differ from the concentrations used in our *in vitro* study. An individual human blood platelet carries 178 μM of serotonin (Lee et al., 2020), and concentrations of serotonin in frog skin vary depending on species and life stage (Claytor et al. unpublished data). As *Bd* produces numerous uncharacterised proteases, we assessed the total protease activity, by performing a

protease assay. We found that there was an overall decrease in protease activity with increasing concentrations of serotonin. However, our study found that there was no statistical difference in ASP, S41, or M36 gene expression, and therefore serotonin likely does not influence the expression of the selected protease genes. Quantification of protease gene expression with qRT-PCR would allow for a more robust comparison of gene expression between various concentrations of serotonin. The lack of statistically different changes in gene expression of protease genes, but an overall decrease in protease activity suggests that serotonin may inhibit the activity of some or many of the other 100+ protease genes not analysed in our study. In addition, the effect of serotonin on protease gene expression may be more apparent at higher concentrations, as observed in *Candida* above 0.46 mM (Mayr et al., 2005). Since we normalised the protease activity to *Bd* growth, this reduced activity is not likely due to smaller cell size alone. However, there may be an effect of serotonin on reducing the overall metabolic activity in smaller *Bd* cells rather than a specific effect on protease activity, which could be confirmed by an MTT cell proliferation assay, to measure cellular growth rates.

We found a decrease in caspase (apoptosis-mediator) activity with increased concentrations of serotonin. Therefore, *Bd* is not likely to be undergoing increased apoptosis in the presence of serotonin. Although serotonin inhibits the growth of *Bd*, the inhibition may not correlate with cell death, and may merely be disruption of metabolic activity as per the effect on protease activity. Our viability assay showed that *Bd* sporangia were viable in 2.5 mM of serotonin. The zoospores do not develop into sporangia at 5 mM and 10 mM of serotonin, and the encysted zoospores were non-viable at these concentrations. In Chapter 2, serotonin was shown to be fungistatic to *Bd in vitro* at serotonin concentrations between 1.5 – 2 mM. The results in this chapter suggest that serotonin becomes progressively more fungicidal to *B*d as concentrations of serotonin increase towards 5 mM and fungicidal at concentrations greater than 5 mM. While there is an effect of serotonin at high concentrations observed on *Bd*, the effect may not correspond to an impact on virulence *in vivo*. Interestingly, the selective serotonin reuptake inhibitors (SSRIs) fluoxetine, paroxetine, and sertraline cause death in *Candida* species by damaging their plasma and mitochondrial membranes, which activates their apoptotic signalling pathways (Costa Silva et al., 2017). Future work should investigate the role of SSRIs in *Bd* apoptosis.

Future work should investigate the effect of serotonin on the expression of other potential *Bd* virulence genes. Adhesin genes (Rosenblum et al., 2012), crinklers and crinkler-

like effectors (CRNs) genes (Sun et al., 2011, Rosenblum et al., 2012), and triglyceride lipase genes (Joneson et al., 2011, Rosenblum et al., 2012), all have been shown to play a role in *Bd* virulence. Understanding the role of serotonin on virulence gene expression may aid in determining why *Bd* virulence varies among amphibian species (Dang et al., 2017).

The mechanisms by which serotonin act on *Bd* are not yet unknown. Our results improve on the limited understanding of the role of serotonin on the host/*Bd* interaction. *Bd* is responsible for the declines and extinction of many species of amphibians globally. Therefore, understanding the mechanisms of serotonin inhibition of *Bd* may lead to a treatment for chytridiomycosis and aid in the development of new antifungal treatments.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

AUTHOR CONTRIBUTIONS

A.A.R., L.B., and L.F.S. designed the research. S.C.C. performed the experiments. S.C.C., A.A.R., L.B., L.F.S., and D.M. analysed the data, and also wrote and reviewed the manuscript.

TABLE S4.1. Concentrations of serotonin used in *Bd* assays

Concentration (mM)		
EnzChek™ Peptidase/Protease Assay Kit (Invitrogen)	Caspase-Glo® 3/7 Assay (Promega)	Growth (Absorbance)
0	0	0
0.156	0.156	0.156
0.3125	0.3125	0.3125
0.625	0.625	0.625
1.25	1.25	1.25
2.5	2.5	2.5
5	5	5
-	7.5	-
10	10	10

CHAPTER 5 | Protein glutathionylation in *Bd*

OVERVIEW

Little is known of the function of proteins expressed by *Bd,* which remain a largely unexplored component of the host-pathogen interaction. To protect against oxidative stress from the host immune system, some fungi use the process of protein S-glutathionylation. To explore if this process is employed by *Bd*, protein S-glutathionylation was investigated *in vitro*. I treated *in vitro* cultures with 1) hydrogen peroxide (H_2O_2) as a proxy for reactive oxygen species (ROS) stress, 2) serotonin, which inhibits *Bd* growth *in vitro* (Chapter 2), and 3) buthionine sulfoximine (BSO), which inhibits glutamylcysteine synthetase (γ-GCS), the rate-limiting enzyme in glutathione synthesis. The aims of this chapter are:

- To determine if oxidative stress affects protein glutathionylation patterns in *Bd* using sublethal H_2O_2, serotonin, and BSO
- To explore the function of glutathionylated proteins

The following text is a copy of the manuscript in preparation, prior to submission.

Protein glutathionylation in *Batrachochytrium dendrobatidis*

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KEYWORDS

Batrachochytrium dendrobatidis, glutathione, protein S-glutathionylation, enolase

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ABSTRACT

The pathogenic fungus *Batrachochytrium dendrobatidis* (*Bd*) has caused the global decline and extinction of amphibians worldwide. *Bd* proteins remain a largely unexplored component of the host-pathogen interaction. Some fungi use protein S-glutathionylation, to protect active site cysteines from oxidative stress. Here, we treat *Bd* with sublethal levels of hydrogen peroxide (H_2O_2) as a proxy for reactive oxygen species (ROS) stress produced by hosts to attack pathogens, serotonin, which inhibits *Bd* growth *in vitro*, and buthionine sulfoximine (BSO), which inhibits glutamylcysteine synthetase (γ-GCS), the rate-limiting enzyme in glutathione synthesis, to investigate the role of protein glutathionylation in oxidative stress protection. Western blot with anti-glutathione antibodies showed reduced glutathionylation of proteins in the 55 kDa region when treated with 0.015 and 0.15 mM H_2O_2. Baseline levels of glutathionylation recovered after 24 h for the 0.015 mM treatment, while the reduced glutathionylation pattern continued at 24 h with 0.15 mM H_2O_2. We used 2D gel

electrophoresis and mass spectroscopy to identify differentially glutathionylated proteins in the 0.15 mM H_2O_2 treatment. A protein with homology to the glycolytic enolase enzyme was found to be constitutively glutathionylated in the untreated control but not in the H_2O_2 treatment sample. Also, *Bd* does not appear to use glutathionylation to protect proteins from the inhibitory effects of serotonin. Lastly, there was no observable difference in glutathionylation of proteins in the untreated BSO treated samples. This study suggests that *Bd* does not use S-glutathionylation to protect the glycolytic pathway from oxidative stress *in vitro*, but that enolase may be a reservoir for glutathione stores.

5.1 | INTRODUCTION

The pathogenic fungus *Batrachochytrium dendrobatidis* (*Bd*) is responsible for the global decline and extinction of amphibians. The mechanisms that enable *Bd* to decimate amphibian populations are a complex combination of environmental factors, host susceptibility, and pathogen virulence. Research has focused on identifying potential virulence factors such as mechanisms for evading the host immune system. Candidate virulence genes have been identified through proteome- and transcriptome-wide comparisons between strains of varying virulence, *Bd* life stages, and growth on different substrates. For example, a role in virulence has been implicated for HSP70, 26S protease regulatory subunit 6A-B, and proteasome subunit beta type 3, as they are differentially expressed between high and low virulence strains of *Bd* (Fisher et al., 2009a). In the common toad (*Bufo bufo*), HSP70 allows for a heat-induced increase in peroxide resistance, which may help in antioxidant defence (Miller et al., 2000). When *Bd* is exposed to thyroid hormone, which is involved in control of amphibian metamorphosis, 263 proteins were differentially expressed (Thekkiniath et al., 2015). There was an increase in proteases and crinkler-like effector proteins, which could be important for invasion of keratinised frog skin (Moss et al., 2010). Brutyn et al. (2012) found that zoospore supernatants were able to disrupt intercellular junctions in frog skin, and that the extracellular components included proteins such as proteases, biofilm-associated proteins, and carotenoid ester lipase. Extracellular lipases are virulence factors in pathogenic *Candida* species (Trofa et al., 2009), and may play a role in nutrient acquisition, host adhesion, hydrolysis, and evasion of host defence systems in *Bd* (Brutyn et al., 2012). Other putative virulence proteins have been identified based on transcriptomic data from *Bd* growth on amphibian skin (Rosenblum et al., 2008, Rosenblum et al., 2012): including metallo-, serine-, and aspartyl- proteases, which are upregulated when *Bd* is grown on pulverised toad skin compared to laboratory media. Only a few candidate virulence proteins have been functionally characterised in *Bd*, due to limited molecular biology tools developed in this species (Rosenblum et al., 2011). These virulence factors include a subtilisin-like serine protease that cleaves frog skin antimicrobial peptides (Thekkiniath et al., 2013) and partial purification of an elastolytic enzyme found in infected frog skin (Moss et al., 2010).

An important aspect of pathogenicity is the ability to evade the host immune response. Host immune cells, such as macrophages and neutrophils, can release reactive oxygen species (ROS) to eliminate invasive microorganisms (‘respiratory burst’ or ‘oxidative

burst') (Franchini et al., 2013, Christoffersson and Phillipson, 2018). ROS are highly reactive radical and non-radicals (Aung-Htut et al., 2012) that cause damage to various cellular macromolecules, such as proteins, lipids, polysaccharides, nucleic acids (Lushchak, 2014, Breitenbach et al., 2015). ROS have been used as a host defence against pathogenic fungi, such as *Candida albicans*, *Cryptococcus neoformans*, and *Aspergillus fumigatus* (Missall et al., 2004, Philippe et al., 2003).

Some fungi have developed antioxidant defences against host ROS. *Cryptococcus neoformans* uses the enzyme S-nitrosoglutathione (GSNO) reductase to detoxify $NO^{\bullet -}$ related molecules, which contributes to its virulence (de Jesús-Berríos et al., 2003). Another mechanism of oxidative stress protection is via the antioxidant glutathione (GSH), a tripeptide composed of three amino-acids (γ-L-glutamyl-L-cysteinyl-glycine). GSH is found in most eukaryotes and some prokaryotes in concentrations of up to 10 mM (Penninckx, 2002). Glutathione can either be in its reduced (GSH) form or oxidized as glutathione disulfide (GSSG), which can be reduced back to GSH by glutathione disulfide reductase. GSH can protect cells from oxidation (Mehdi and Penninckx, 1997), and also contribute to detoxification of xenobiotics, and aid in the resistance to heavy metals (eg. cadmium in *Schizosaccharomyces pombe* (Al-Lahham et al., 1999) and arsenite in *Saccharomyces cervisiae*) (Ghosh et al., 1999). In some species of pathogenic fungi, disrupting biosynthesis of glutathione can lead to lower levels of virulence, as seen in the pathogenic yeast *Candida albicans* in a mouse model (Yadav et al. 2011).

GSH can also be used in a reversible mechanism to protect proteins via protein S-glutathionylation of cysteinyl residues through an oxidised sulfenic acid intermediate. This glutathionylation protects the protein from damage by over-oxidation to the sulfinic acid and irreversible sulfonic acid forms (Sies 1999) (Figure 5.1). When the oxidative stressor is removed, proteins are de-glutathionylated via glutaredoxin (Grx) enzymes, which restores protein functionality without resynthesis (Dalle-Donne et al., 2009). For example, the filamentous form of *C. albicans* uses protein S-glutathionylation to protect the enzyme isocitrate lyase, which is part of the glyoxylate cycle (Gergondey et al., 2016). Glutathionylation can also be used as a redox-switch to alter the function of proteins. For example, in the rat carbonic anhydrase isozyme III (CAIII), glutathionylation can both activate or block activity of the protein depending on which protein cysteine is glutathionylated (Cabiscol and Levine, 1996).

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Bd, like many other fungi, produces GSH (A. Roberts, unpublished data), but its role in oxidative stress protection in *Bd* has not been elucidated. Sun et al. (2016) identified GSH as a potential virulence factor in *Bd*, as GSH genes responsible for glutathione metabolism were likely horizontally transferred from bacteria as an evolutionary advantage. Here, we treated *Bd* with sublethal levels of hydrogen peroxide as a proxy for host ROS, and buthionine sulfoximine (BSO) which inhibits the rate limiting enzyme in GSH biosynthesis, to determine its effect on protein glutathionylation patterns. We also observed the effect of serotonin on protein glutathionylation, to gain insight into the mechanism of *Bd* inhibition by serotonin.

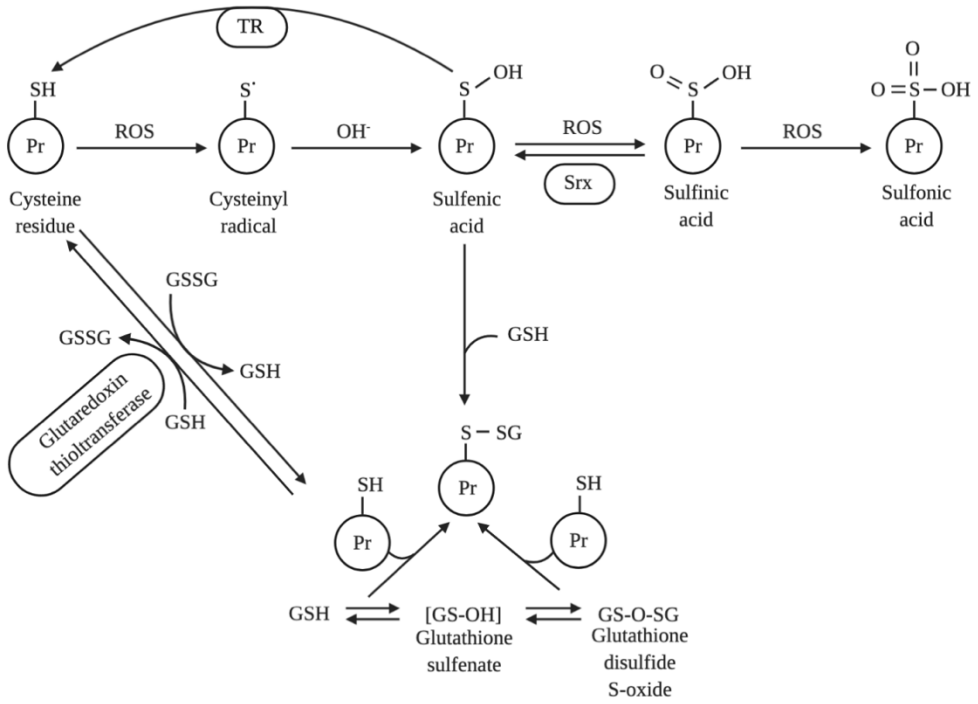

FIGURE 5.1 Oxidation of cysteine residues and glutathionylated protein formation, from Lushchak (2012).

Pr = protein, TR = thioredoxin, Srx = sulfiredoxin, GSH = glutathione, GSSG = glutathione disulfide, ROS = reactive oxygen species

5.2 | METHODS

5.2.1 Inhibitory dose of hydrogen peroxide

Bd was treated with six concentrations of hydrogen peroxide (H_2O_2) to determine the inhibitory dose. The *Bd* isolate "Ethel creek-Lnannotis-2013-LB" (isolated from a Waterfall frog (*Litoria nannotis*), in 2013 from northern Queensland, Australia) was grown on tryptone/gelatin hydrolysate/lactose (TGhL) agar. After 4 d, petri dishes were flooded with 1 mL of TGhL broth and zoospores were syringe filtered to remove sporangia using a 10 µm isopore filter (Millipore). Five hundred microlitres of zoospores approximately (1.3×10^5 zoospores/mL) and 500 µL of H_2O_2 (for final concentrations of 0 mM, 0.0125 mM, 0.025 mM, 0.05 mM, 0.1 mM, and 0.2 mM) was added to a 24-well plate in triplicate and incubated at 21°C for 4 d. Each well was assessed by microscopy for growth, zoospore motility, and encystation for the next 7 days. The experiment was repeated, zoospores (approximately 1.0×10^5 zoospores/mL) from the *Bd* isolate "Ethel creek-Lnannotis-2013-LB" were inoculated in a 96-well plate. On the third day of incubation, a photograph was taken for each concentration of H_2O_2. The 10 largest sporangia were selected per image and the sporangial diameter was measured, using a light microscope at 20x magnification (Fisher et al., 2009a).

5.2.2 | *Bd* culture and treatments

Bd from isolate "Ethel creek-Lnannotis-2013-LB" was grown in TGhL broth for 4 d in tissue culture flasks (Greiner), and 3 mL was used to inoculate cultures for protein extraction. Cultures were treated with serotonin or H_2O_2 and grown for various times as indicated in Table 5.1. Control cultures included 20 mM buthionine sulfoximine (BSO), which inhibits the rate limiting enzyme in GSH biosynthesis (added on day 1) and a no treatment control culture. The concentrations of H_2O_2 and serotonin were below the minimum inhibitory concentration (MIC) for *Bd* (Claytor et al., 2019). After treatment, cells were harvested by centrifugation at 16,000 x *g* for 10 min at 4 °C and the supernatant was removed. Pellets were then suspended in 3 mL of buffer containing protease inhibitor cocktails 15 µL P8215 and 300 µL P2714 (Sigma-Aldrich), 1 mM phenylmethane sulfonyl fluoride (PMSF), and 0.5 M ethylenediaminetetraacetic acid (EDTA). Pellets were then frozen in liquid nitrogen and freeze-dried. Protease inhibitor cocktail P8215 (Sigma-Aldrich) contained 4-(2-aminoethyl)benzenesulfonyl fluoride (AEBSF), pepstatin A, E-64, and 1,10-phenanthroline, which inhibits serine, cysteine, aspartic, and metalloproteases. Protease

inhibitor cocktail P2714 (Sigma-Aldrich) contained AEBSF, aprotinin, bestatin, E-64, EDTA, and leupeptin, which inhibits serine, cysteine, and metalloproteases.

TABLE 5.1 Treatment concentrations and *Batrachochytrium dendrobatidis* growth times.

Treatment Concentration	Treatment Added	Exposure Time
No treatment control	Day 4	1 h
No treatment control	Day 5	24 h
0.015 mM H_2O_2 ('low')	Day 4	1 h
0.15 mM H_2O_2 ('high')	Day 5	24 h
0.625 mM serotonin	Day 1	4 d
100 μM buthionine sulfoximine (BSO)	Day 1	4 d

5.2.3 | Protein extraction

Freeze-dried pellets were suspended in a lysis buffer containing: 0.5 M iodoacetamide (IAM), 0.5 M EDTA, 1 M HEPES, 0.967 M thiourea, 8 M urea, and 5 µL of P8215 and 100 µL of P2714 protease inhibitor cocktails (Sigma-Aldrich). Samples were sonicated 3 times on a Branson Digital Sonifier® at 10% amplitude, for 20 s, with 0.5 s pulses, resting on ice between cycles. Samples were spun at 16,000 x *g* at 4 °C for 15 min. Supernatant was pipetted into a new tube and the pellet was discarded. A Bradford assay (Merck) was used to determine protein concentration, using bovine serum albumin (BSA) as the protein standard.

5.2.4 | PAGE and Western Blot

A Western blot was performed to determine whether serotonin or H_2O_2 altered the glutathionylation profile of *Bd* proteins. To each protein sample, 0.25 M N-ethylmaleimide (NEM) was added. Polyacrylamide gel electrophoresis (PAGE) was performed with two Mini-PROTEAN® TGX 4-20% precast gels (Bio-Rad). Gels were run in Tris-glycine-SDS buffer (Biorad), in a Mini-PROTEAN® tetra cell (Bio-Rad) at 100 V for 1.5 h. One gel was stained with Instant Blue™ Ultra Fast Protein Stain (Merck) and a PageRuler™ prestained 10 - 180 kDa protein ladder (Thermo Fisher).

A Immobilon-P 0.45 µm pore (Merck), polyvinylidene difluoride (PVDF) transfer membrane was prepared by soaking in 100% methanol for 3 seconds and then washed with PBST (phosphate buffered saline (PBS) and 0.1% Tween 20). The second PAGE gel was transferred to the PVDF transfer membrane in a 20% methanol, 25 mM Tris, 192 mM glycine transfer buffer, for 1 h at 100 V. The PVDF membrane was washed three times (10 min each) in a solution of PBST. The membrane was blocked on a shaker overnight at 4°C, in a solution of 0.25M NEM, 5% powdered skim milk, and PBST. A 1:200 dilution of the primary glutathione antibody (D8) mouse monoclonal IgG_{2a} (Santa Cruz Biotechnology, sc-52399) in PBST was incubated on the membrane overnight on a shaker at 4°C. The membrane was washed another 3 times (10 min each) in PBST. A 1:2,000 dilution of the secondary antibody goat anti-mouse IgG-HRP (Santa Cruz Biotechnology, sc-2005) in PBST was incubated on the membrane overnight on a shaker at 4°C. Another three washes (10 min each) with PBST and 1 wash with PBS (10 min) was performed on the membrane. For chemiluminescent detection, 1 mL of SuperSignal™ West Femto Maximum Sensitivity Substrate (Thermo Scientific) was pipetted onto the membrane and incubated for 5 min. Chemiluminescence was imaged on a G:Box system (SYNGENE) using the Genesys® software.

5.2.5 | 2-D gel electrophoresis

To separate proteins for mass spectroscopy analysis, 2-dimensional gel electrophoresis (2D GE) was performed on samples from *Bd* treated with 0.015 mM hydrogen peroxide for 24 h, and a no peroxide control. These samples were selected as they had differential glutathionylation patterns in the one-dimensional Western blot. A 2D Clean-Up Kit (GE Healthcare) was used to prepare samples for 2D GE, following the manufacturer's protocol. Samples were re-suspended in rehydration buffer containing 8 M urea, 2% CHAPS (3-((3-cholamidopropyl) dimethylammonio)-1-propanesufonate), 2% Biol-lyte® 40% 3/10 ampholytes (Bio-Rad), 0.025 M NEM, ultrapure water, and trace amount of bromophenol blue. Thirty micrograms of each *Bd* sample and an SDS Page Standard (Bio-Rad), were loaded onto separate wells within a 11 cm rehydration/equilibration tray (two replicates for each sample). A 11 cm ReadyStrip™ pH 4-7 IPG strip was placed on top of each sample and standard. Mineral oil (2 mL) was applied on top of the strips to prevent evaporation, and then left to rehydrate overnight at room temperature. Each IPG strip was transferred onto the focusing tray, covered with 2 mL mineral oil, and placed into the Bio-Rad PROTEAN® IEF cell. The electrophoresis was run at 250 V for 20 min in a linear ramp, then 8,000 V for 2.5 h in a linear ramp, and then 8,000 V for 20,000 V-h in a rapid ramp. IPG strips were equilibrated for 10 min sequentially in 4 mL of each equilibrium buffer. Buffer 1: 6 M urea, 2% sodium dodecyl sulfate (SDS), 0.375 M Tris-HCL (pH 8.8), 20% glycerol, and ultra-pure water; Buffer 2: 6 M urea, 2% SDS, 0.375 M Tris-HCL (pH 8.8), 20% glycerol, 0.5 g of iodoacetamide, and ultra-pure water. Each IPG strip was added to the well of a Criterion™ TGX™, 4-15%, IPG+1 precast gel, and covered with overlay agarose solution containing 0.025 M NEM. The gels were electrophoresed in 1x Tris/glycine/SDS buffer at 200 V for 65 min. Then one each replicate sample and control gel were then stained with Coomassie Brilliant Blue and imaged on a G:Box gel doc system (SYNGENE) in the Genesys® software. A Western blot was performed on the second replicate sample and control gel to locate the differentially glutathionylated proteins (see Western blot methods).

Protein spots that showed differential glutathione labelling were excised from the 2D-PAGE gel and sent to the Australian Proteome Analysis Facility (Macquarie University), for identification via mass spectrometry analysis.

5.2.6 | Bioinformatic Analysis

Conserved domain analysis was performed to determine the location of the S-glutathionylated cysteine within the tertiary structure of the enolase protein. The full-length

Bd enolase protein sequence was obtained from GenBank® (OAJ38561.1) and analysed in BLASTp (Altschul et al., 1997), to identify conserved metal binding sites, substrate binding pockets, and dimer interfaces. Clustal Omega (Madeira et al., 2019) was used to align the *Bd* sequence to enolases from *Batrachochytrium salmandriovrans* (*Bsal*)(GenBank® Accession OON05269.1), *Candida albicans* (GenBank® Accession AAA71939.1), and *Saccharomyces cerevisiae* (GenBank® Accession AAA71939.1), *Homo sapiens* (GenBank® Accession CAA47179.1), and two amphibian species *Xenopus laevis* (GenBank® Accession NP001079392.1) and *Xenopus tropicalis* (GenBank® Accession NP989144.1).

A three-dimensional (3-D) model of the enolase protein was constructed. The sequence of *Bd* (GenBank® OAJ38561.1) was converted into a 3-D model file in the Phyre2 Protein Fold Recognition Server (Kelley et al., 2015), using default settings. The model was then compared to 'human enolase 1' (PDB archive #: 3B97) in PDBeFold (Krissinel and Henrick, 2004) using default settings. The superimposed file was then visualized in Jmol version 14.30.2 software (Hanson and Lu, 2017).

5.3 | RESULTS

5.3.1 | Decrease in overall protein glutathionylation with peroxide treatment

The inhibitory concentration of H_2O_2 for *Bd* was 0.2 mM, with fungal growth occurring at 0.1 mM. Zoospore motility was absent at 0.2 mM but was observed from day 3 onwards at lower concentrations. The diameter of *Bd* sporangia decreased with increasing concentrations of H_2O_2, with a large decline at 0.325 mM of H_2O_2. The samples in the 1D PAGE of the H_2O_2 and control protein samples showed similar overall protein expression patterns, although there were slightly darker bands in the low (0.015 mM) H_2O_2 (24 h) and 100 μM BSO treatments suggesting some proteins were at greater abundance. In the 1D Western blot, many of the glutathionylated proteins were in the 55 kDa region (indicated by an asterisk in Figure 5.3). Compared to the untreated control, there was a decrease in glutathionylation of 55 kDa region proteins in the low peroxide treatment at 1 h, the high peroxide (0.15 mM) treatment at 1 and 24 h and the serotonin treatment. The control glutathionylation pattern was similar to the low H_2O_2 at 24 h, and BSO treatment. When exposed to 0.15 mM H_2O_2, the amount of protein glutathionylation decreased and remained so over 24 h of exposure.

We used 2D gel electrophoresis to identify glutathionylated proteins in *Bd*, in a no treatment control (24 h) and high H_2O_2 treatment (24 h) (Figure 5.3). There were more

Coomassie stained proteins in the no treatment control (24h) than the high H_2O_2 (24 h) treatment, despite attempts at even loading of samples. For the 2D Western blot, most of the glutathionylated proteins were also between 20-80 kDa, with pI greater than 5. There was a differential glutathionylation pattern between peroxide treated and control samples, with an overall decrease in glutathionylated proteins in the high H_2O_2 (24 h) treatment compared to the no treatment control (24 h) (Figure 5.3).

5.3.2 | Protein glutathionylation of enolase

Two protein spots were cut out of the 2D PAGE no treatment control (protein 1) and high H_2O_2 24 h treatment (protein 6) PAGE gels. Mass spectrometry identified protein 1 as a mixture of mostly cytosol AP domain-containing protein (52.74 kDa) and glutathionylated enolase (46.9 kDa). No glutathionylated protein was found in the high H_2O_2 treatment (24 h) sample (protein 6). The most abundant protein in the sample was non-glutathionylated enolase (46.9 kDa).

Mass spectrometry of excised protein 1 identified the location of the glutathionylated cysteine at position 339 of the enolase protein (GenBank® Accession OAJ38561.1) (Figure 5.6). By comparing the *Bd* enolase sequence to conserved domains from functionally characterised enolases, the putative residues in the metal binding site, substrate binding pocket, and dimer interface were identified (Figure 5.5). This alignment shows the glutathionylated *Bd* Cys339 correlating to a highly conserved Cys in many diverse species, including the related *B. salamandrivorans*, white nose syndrome pathogen *Pseudogymnoascus destructans*, frogs *Xenopus laevis* and *Xenopus tropicalis*, and *Homo sapiens* (Figure 5.5). On analysis of the human enolase crystal structure, the corresponding cysteine residue (Cys366) is not located within a conserved functional domain of the protein, but is found on a beta sheet adjacent to the metal binding residue (Figure 5.6).

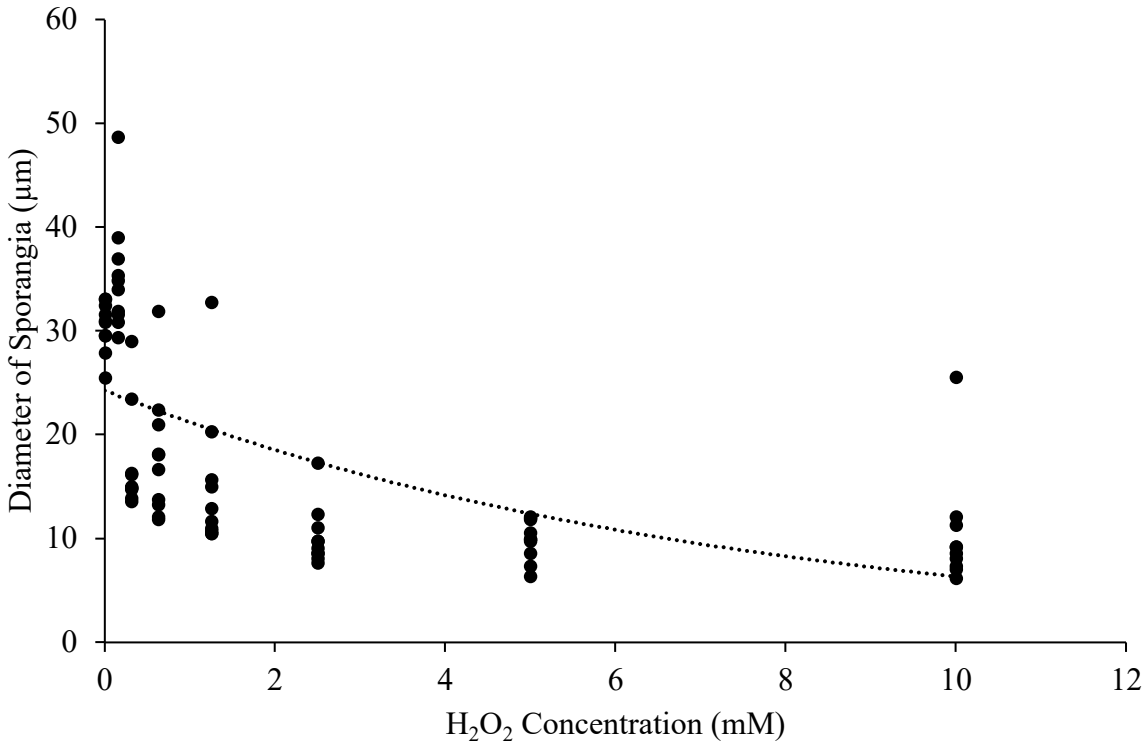

FIGURE 5.2 Sporangia growth assay with hydrogen peroxide (H_2O_2) shows a reduction in *Batrachochytrium dendrobatidis* (*Bd*) sporangia size.

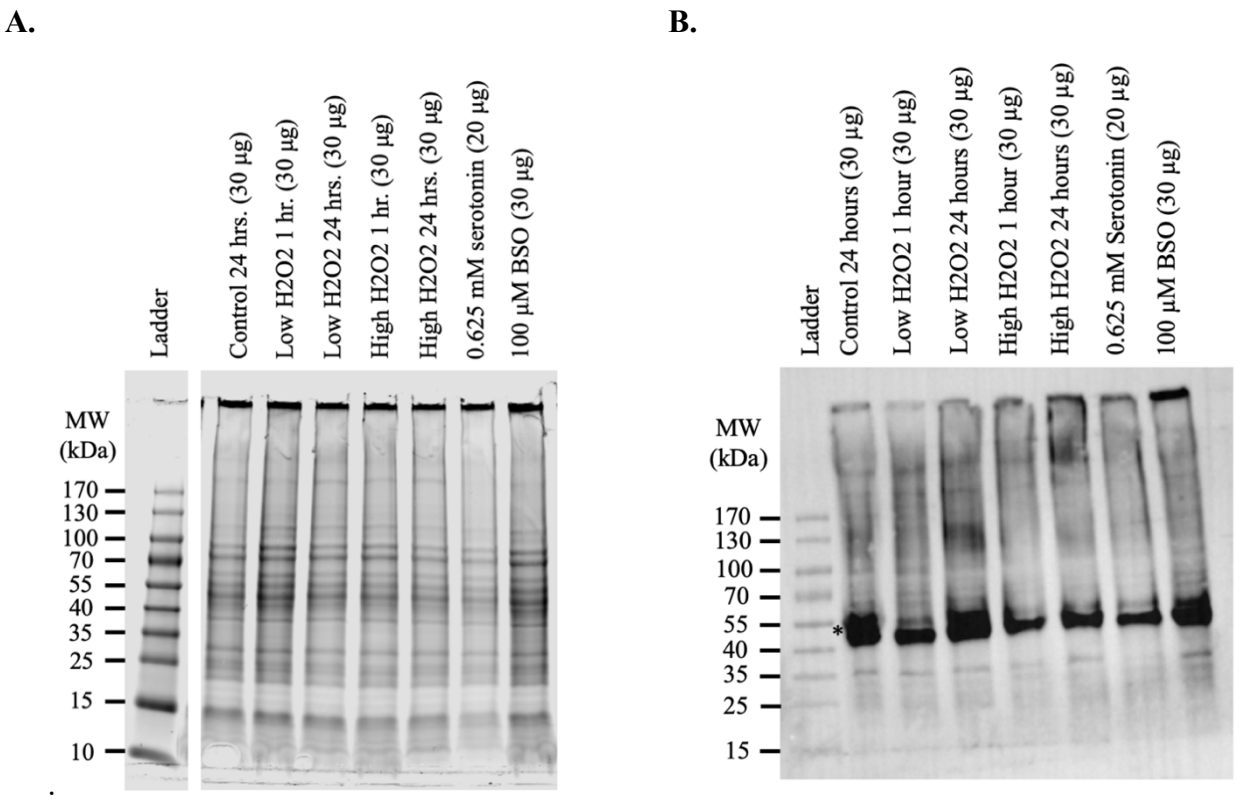

FIGURE 5.3 A. Polyacrylamide gel (PAGE) and B. chemiluminescence image of polyvinylidene fluoride (PVDF) membrane from Western blot analysis, for *Bd* treated with peroxide, serotonin, and buthionine sulfoximine (BSO).

* Indicates glutathionylated proteins in the 55 kDa region; 20 µg and 30 µg represent the amount of total protein loaded into the well; low H_2O_2 is 0.015mM of H_2O_2, and high H_2O_2 is 0.15 mM of H_2O_2.

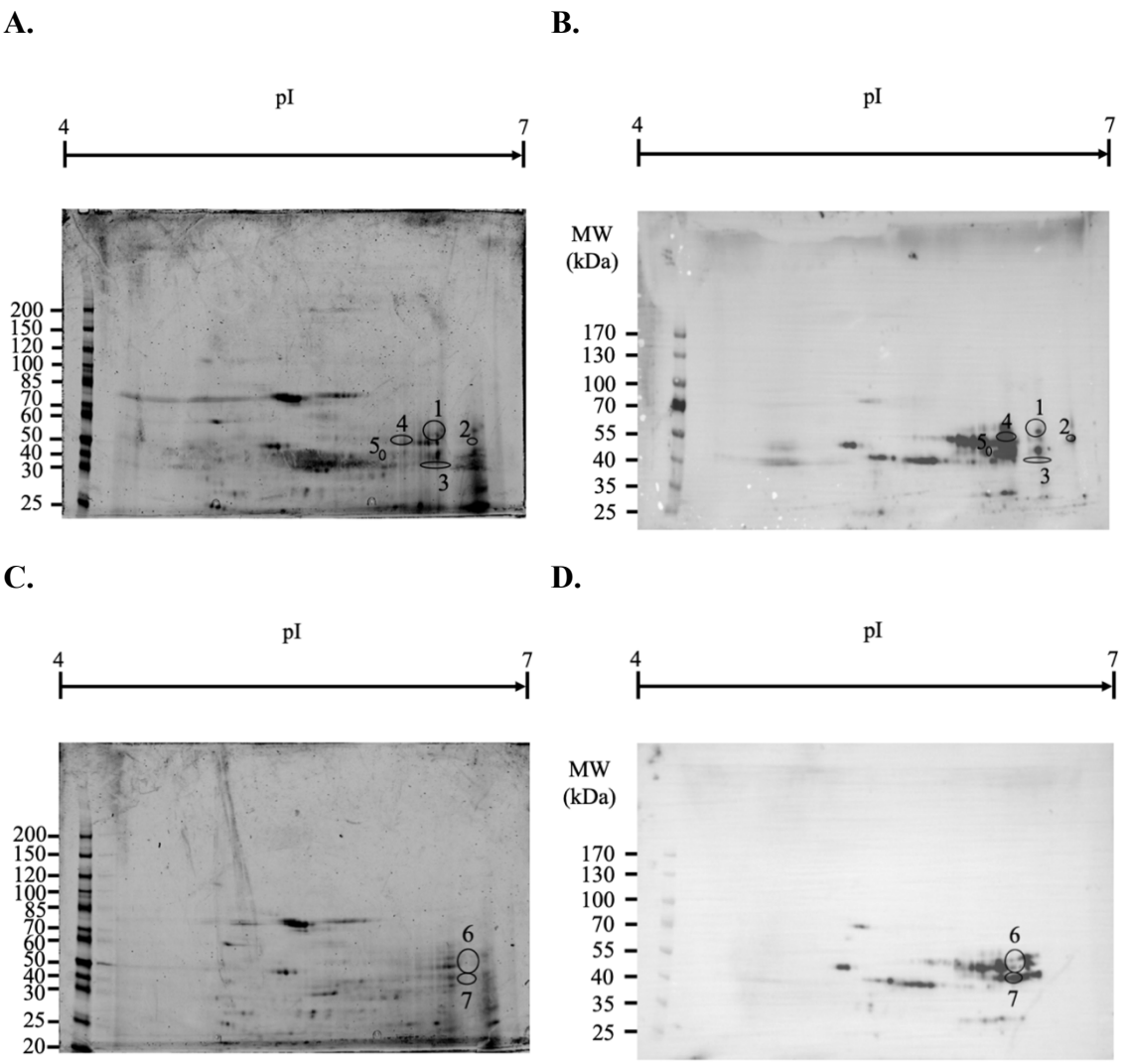

FIGURE 5.4 Coomassie stained PAGE gels and Western blot analysis of control and 0.15 mM H_2O_2 24 h treatment samples.

A. Control Coomassie stained PAGE gel, B. control Western blot, C. 0.15 mM H_2O_2 Coomassie stained PAGE gel, D. 0.15 mM H_2O_2 Western blot. Circles and numbers represent proteins that were differentially glutathionylated between the two samples. Proteins number 1 and 6 were sent for identification via mass spectrometry.

TABLE 5.3 Top 10 proteins identified from the *Batrachochytrium dendrobatidis* no treatment control, based on highest sum PEP score.

PEP = Posterior Error Probability

Protein Name	Accession	Sum PEP Score	Molecular Weight (kDa)	Number of Proteins	Protein Coverage	Modifications
Cytosol_AP domain-containing protein	F4P386	129.7247	52.7400	21	70.5765	
Enolase	A0A177WGS1	78.1884	46.8620	20	68.8940	Glutathionylated
Fumarate hydratase, mitochondrial	A0A177WVH1	38.6957	56.3500	12	41.1538	
ATP synthase subunit beta	A0A177WR09	29.1384	54.0350	11	28.0079	
Aldedh domain-containing protein	F4NUJ3	19.0568	53.9800	10	24.7505	
Dihydrolipoyl dehydrogenase	F4NY10	56.0630	53.7230	10	35.3516	
Uncharacterized protein	F4NSA1	14.4776	71.8070	9	18.7879	
Aldedh domain-containing protein	A0A177WBH6	21.5284	58.6310	9	29.7445	
Stress-70 protein, mitochondrial	A0A177WFS4	15.2208	73.3410	9	14.0325	
Uncharacterized protein	F4P0W3	21.6113	177.7240	9	9.1374	

TABLE 5.4 Top 10 proteins identified from *Batrachochytrium dendrobatidis* in the high H_2O_2 for 24 h treatment, based on highest sum PEP score.

PEP = Posterior Error Probability

Protein Name	Accession	Sum PEP Score	Molecular Weight (kDa)	Number of Proteins	Protein Coverage
Enolase	A0A177WGS1	14.7305	46.8620	12	33.4101
Uncharacterized protein	F4NYX0	4.4692	42.0060	5	14.0625
Cytosol_AP domain-containing protein	F4P386	2.7970	52.7400	3	7.7535
Fumarate hydratase, mitochondrial	A0A177WVH1	4.9464	56.3500	1	2.8846
Thioredoxin domain-containing protein	F4PFM4	0.6340	25.5510	1	4.7414
DPPIV_N domain-containing protein	F4P242	0.3092	129.1810	1	0.9418
Uncharacterized protein	F4P6E4	0.6278	76.2160	1	1.0204
Lipoyl-binding domain-containing protein	A0A177W9V3	0.9427	50.5750	1	4.1841
Uncharacterized protein	F4NSR5	0.2940	280.6020	1	0.2457
S-adenosylmethionine synthase	F4NV90	2.1277	42.2620	1	3.8961

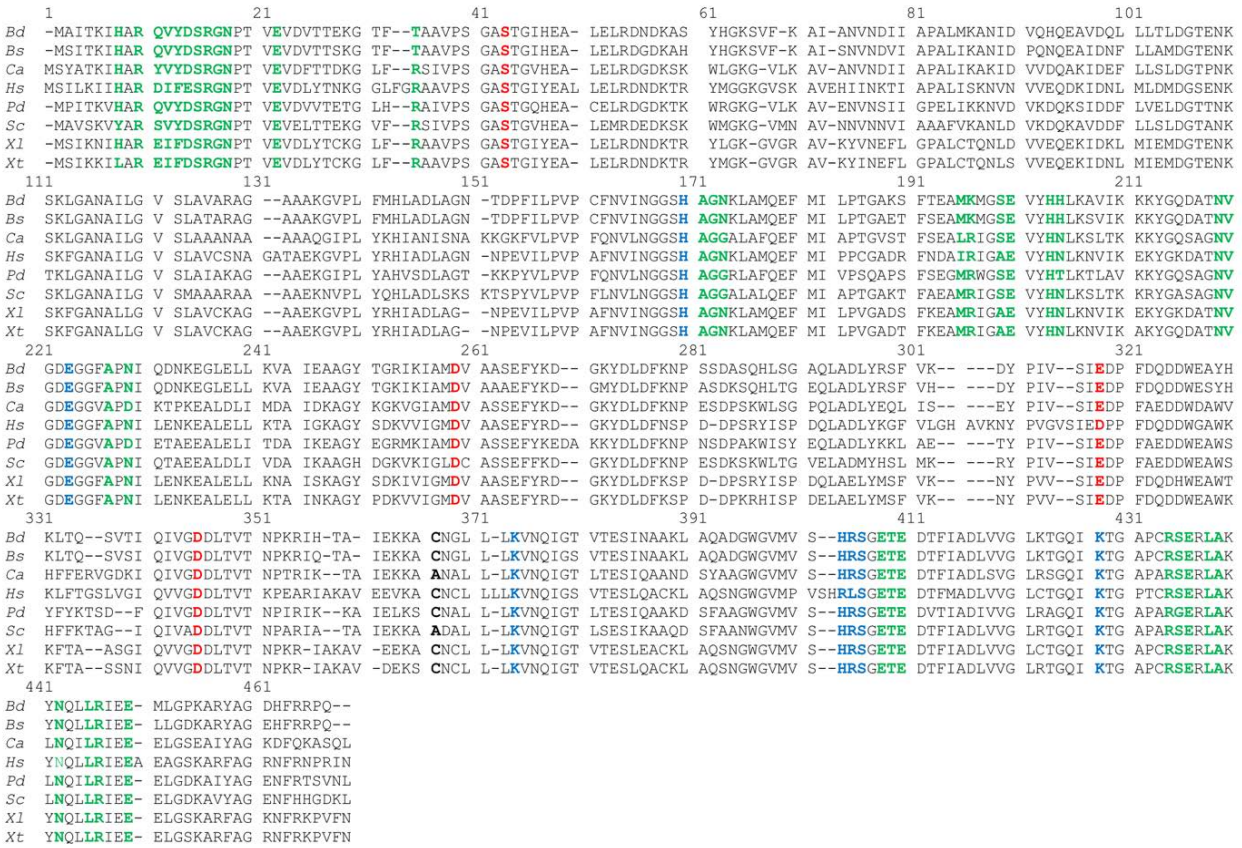

FIGURE 5.5 Amino acid alignment of enolase proteins.

Bd = *Batrachochytrium dendrobatidis* (GenBank® Accession OAJ38561.1), *Bs* = *Batrachochytrium salmandrivorans* (GenBank® Accession OON05269.1), *Ca* = *Candida albicans* (GenBank® Accession AAA71939.1), *Hs* = *Homo sapiens* (GenBank® Accession CAA47179.1), *Pd* = *Pseudogymnoascus destructans* (GenBank® Accession ELR04711.1), *Sa* = *Saccharomyces cerevisiae* (GenBank® Accession AAA71939.1), *Xl* = *Xenopus laevis* (GenBank® Accession NP001079392.1), and *Xt* = *Xenopus tropicalis* (GenBank® Accession NP989144.1). Conserved domains: red represents the conserved metal binding site, blue represents the substrate binding pocket, and green represents the dimer interface. Bolded in black is the location of the cysteine found in *Bd*.

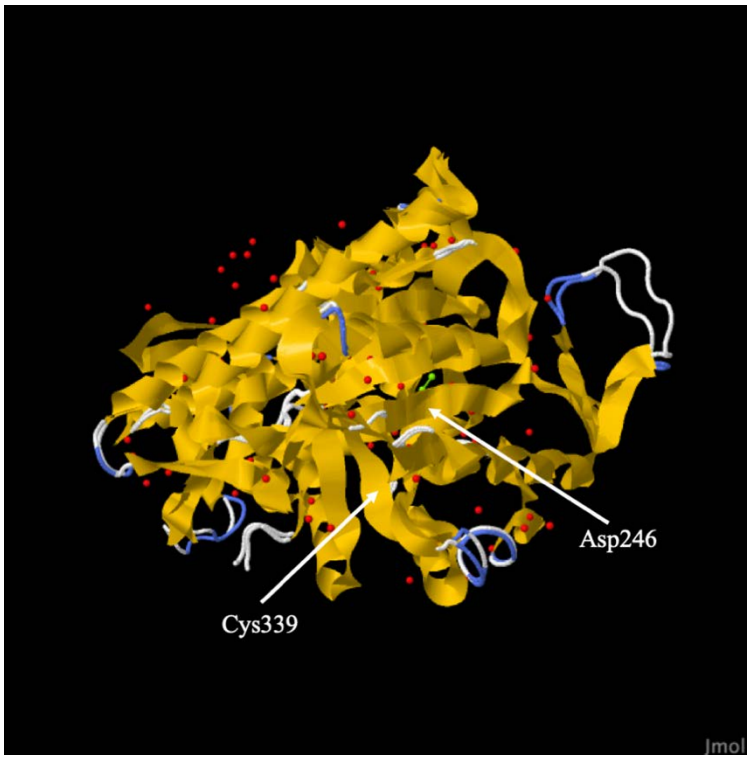

FIGURE 5.6 Three-dimensional model of enolase found in *Batrachochytrium dendrobatidis*. Amino acid numbering corresponds with UniProt A0A177WGS1; Amino acids found in the metal binding conserved domain: Cys339 and Asp246. Cys is not in the active site.

5.4 | DISCUSSION

We used H_2O_2 as a proxy for host ROS, to investigate whether *Bd* uses protein glutathionylation to protect itself from the host immune system. The most marked changes in glutathionylation pattern were in proteins around 55 kDa when treated with 0.015 and 0.15 mM H_2O_2. The baseline levels of glutathionylation recovered after 24 h for the 0.015 mM treatment, while the reduced glutathionylation pattern continued at 24 h with 0.15 mM H_2O_2. Surprisingly, there were less glutathionylated proteins in the high H_2O_2 (24 h) treatment than the no treatment control, which suggests that oxidative stress reduces glutathionylation of these proteins of interest. Mass spectrometry identified one of the glutathionylated proteins in the no treatment control as the glycolytic enolase enzyme. In the corresponding high H_2O_2 sample (protein 6) enolase was not glutathionylated.

The function of *Bd* enolase has not been studied, but in many fungi metalloenzyme α-enolase (2-phospho-D-glycerate hydrolase) is a glycolytic enzyme that catalyses the conversion of 2-phosphoglycerate to phosphoenol pyruvate. The analysis of the conserved domains (metal binding site, substrate binding pocket, and dimer interface) showed that the glutathionylated cysteine identified from mass spectrometry was not in the active site, though the cysteine is conserved in many species. The reduced glutathionylation of *Bd* enolase under peroxide stress suggests that the pathogen does not protect the glycolytic pathway from ROS, in contrast to other published studies in other organisms. The enolase in human T cell blasts contains multiple cysteine residues that are constitutively glutathionylated, with an increase in glutathionylation under peroxide stress (Fratelli et al., 2002). *In vitro* activity studies suggested that increased glutathionylation of enolase corresponded to a reduction in catalytic function, although the specific cysteine residues involved were not identified. Similarly, in Alzheimer's disease oxidative stress increases glutathionylation of α-enolase protein, resulting in reduced enzyme activity in the inferior parietal lobule (Newman et al., 2007).

A reduction in *Bd* enolase glutathionylation under peroxide stress suggests that the pathogen may increase metabolism and growth via glycolysis to overcome oxidative stress. However, our results suggested that growth is not significantly altered with 0.15 mM H_2O_2, and that the modified Cys339 residue is not within the active site of the enzyme. *In vitro* activity assays are required to confirm whether glutathionylation of Cys339 affects glycolysis in *Bd*. An alternate hypothesis is that since enolase is a highly abundant cellular protein that is constitutively glutathionylated, it serves as a GSH reservoir (Fratelli et al., 2002). The reduction in glutathionylated enolase during peroxide exposure may thus correspond to the

increased requirement for free GSH as a reducing agent under oxidative conditions. Analysis of total cellular GSH and oxidised glutathione (GSSG) levels under peroxide stress would enable investigation of the GSH reservoir for *Bd*.

Enolase has also been detected on the cell surface of many fungal species (Funk et al., 2016). In *Candida albicans*, enolase is an immunogenic antigen during infection, and causes host damage by binding to plasminogen and fibrin (Jong et al., 2003, Angiolella et al., 1996). Deletion of the α-enolase gene in *C. albicans* reduces its growth rate and affects drug sensitivity, the formation of mycelium and virulence (Ko et al., 2013). In bacteria, such as *Staphylococcus aureus*, enolase is a receptor for host proteins and plays a role in immune evasion and tissue invasion (Mölkänen et al., 2002). It is possible that enolase plays a similar role in *Bd* virulence, the function of which may be altered by glutathionylation. Immunohistochemistry could be used to localise the expression of *Bd* enolase *in situ* to determine whether it functions as a cell surface virulence factor.

Future studies could also identify the other differentially glutathionylated proteins (highlighted in Figure 5.3), to better understand oxidative stress in the host-pathogen response. Additionally, this study focused primarily on intracellular *Bd* proteins. Many of the candidate *Bd* virulence proteins associated have been identified from *Bd* secretions (Brutyn et al., 2012) (Ribas et al., 2009, Rosenblum et al., 2009), and therefore glutathionylation patterns of secreted proteins warrants investigation. A more comprehensive proteomic analysis could identify the complete "glutathiome" of all glutathionylated proteins in *Bd*, using techniques such as BODIPY labelled mass spectrometry (Chi et al., 2013).

We used 100 μM buthionine sulfoximine (BSO) as a positive control in this study. BSO inhibits a rate limiting enzyme in glutathione synthesis to deplete GSH levels. However, there was no observable difference in glutathionylation of proteins in the untreated control and BSO control. The concentrations of BSO used in this study were established for mammalian cells and may not have been high enough to deplete GSH levels or alter protein glutathionylation in *Bd*. Future work could monitor the levels of reduced cellular GSH and oxidised GSSG with BSO to determine the appropriate concentration for use in *Bd*.

In this study, we also investigated the glutathionylation of *Bd* proteins after treatment with serotonin. Previous studies have shown that serotonin has an inhibitory effect on *Bd in vitro* (Claytor et al., 2019). The 1D Western blot showed reduced glutathionylation of proteins at the 55 kDa size, similar to that observed for high H_2O_2. Thus, *Bd* does not appear to use increased glutathionylation to protect proteins from the inhibitory effects of serotonin.

Overall, our study showed that *Bd* proteins are glutathionylated, including constitutive glutathionylation of enolase. *Bd* does not use S-glutathionylation to protect the glycolytic pathway from oxidative stress *in vitro*, but enolase may be a reservoir for glutathione stores. Further analysis should confirm the function of more differentially glutathionylated proteins between various oxidative treatments for a more robust understanding of factors that enable *Bd* to evade its host immune system.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

AUTHOR CONTRIBUTIONS

S.C.C., A.A.R., L.B., and L.F.S. designed the research. S.C.C. performed the experiments. The Australian Proteome Analysis Facility (APAF), in Macquarie University, performed mass spectrometry protein identification. All authors analysed the results and reviewed the manuscript.

CHAPTER 6 | General Discussion

6.1 | Introduction

This chapter discusses the key findings of this thesis and addresses knowledge gaps in the pathogenesis of *Batrachochytrium dendrobatidis* (*Bd*) infection, focussing on understanding the role of selected metabolites and antioxidants. There is a lack of visible cellular immune response in the skin of infected frogs, which suggests that non-cellular immune mechanisms may be important, including metabolites that may interfere with pathogenesis and virulence of *Bd*. Insight into the function of serotonin (5-hydroxytryptamine) in *Bd* pathogenesis and host response during infection could aid in the improvement of frog survival, through selective breeding of resistant frogs and in the targeted development of antifungal treatments. Characterisation of glutathionylated proteins from *Bd* provides a platform for future functional studies on virulence factors and potential drug targets.

6.2 | Serotonin

Previous studies on amphibian bioactive compounds in *Bd* resistance have focused on antimicrobial peptides (AMPs). These molecules are secreted by amphibian skin glands and inhibit the growth of *Bd in vitro* by disrupting the membrane of the *Bd* zoospores (Rollins-Smith and Conlon, 2005, Rollins-Smith et al., 2011, Myers et al., 2012, Rollins-Smith et al., 2006, Rollins-Smith, 2009, Ramsey et al., 2010). In a non-targeted metabolomic study of the endangered alpine tree frog (*Litoria verreauxii alpina*), levels of the metabolite serotonin increased in the skin of frogs from the more *Bd*-susceptible populations as infection progressed (Grogan et al., 2018b, Grogan et al., 2018d). Serotonin levels remained low in frogs from a more resistant population. Based on this interesting correlation, my first aim was to determine the role of serotonin in the skin, and to determine its implications for host immunity and *Bd* infection.

The potential functions of serotonin are complex. In humans serotonin plays a role in maintaining homeostasis and healing of tissue (Duerschmied and Bode, 2009), and has regenerative functions in the liver (Lesurtel et al., 2006), and fibroproliferative functions in the liver and lung (Sadri et al., 2016, Welsh et al., 2004). Serotonin has immunomodulatory functions and affects the secretion of cytokines, by stimulating monocytes (Dürk et al., 2005) and lymphocytes (Iken et al., 1995). In the human gastrointestinal tract, serotonin reduces virulence gene expression of enterohaemorrhagic *Escherichia coli* and *Citrobacter rodentium*, by inactivating the transcriptional factor that controls for the virulence genes

(Kumar et al., 2020). Excess serotonin can cause serotonin syndrome in humans, which presents clinical signs such as changes in mental status, agitation, myoclonus (muscle jerking), hyperreflexia, diaphoresis (sweating), shivering, tremor, diarrhoea, and incoordination (Sternbach, 1991). Interestingly, some of these symptoms are similar to the signs of late stage chytridiomycosis in amphibians (convulsions and lack of righting reflex) and may be part of the biological response in addition to the known altered potassium and sodium levels in this disease. Future work should investigate blood levels of serotonin throughout infection and correlate the levels to clinical signs.

The results of this thesis showed the many complicated and sometimes contradictory roles of serotonin in *Bd* pathogenesis. Frogs are able to produce serotonin but not the serotonin precursor tryptophan (Trp), and therefore must obtain Trp exogenously. Chapter 2 showed that serotonin inhibited *Bd* growth *in vitro*, though Trp did not. Supplementation with Trp did not restore the growth of *Bd* in the presence of serotonin (Chapter 2). Serotonin also inhibited human Jurkat lymphocytes *in vitro* (Chapter 2). Immunohistochemistry enabled clear visualisation of serotonin in the dermal granular glands of three of the four frog species examined (Chapter 3). Generally, more susceptible species had less detectable levels of serotonin in the skin (Chapter 3), which contrasts with previous studies in alpine tree frogs (*L. v. alpina*), where the more resistant populations had lower levels of skin serotonin (Grogan et al., 2018d). Chapter 4 showed that there was a reduction in overall protease activity of *Bd* with increasing concentrations of serotonin. In *Bd* treated with serotonin, there was no significant difference in protease gene of TrpA (tryptophan synthase) and selected protease genes (Chapter 4). Overall, the results of this thesis suggest that rather than being a host response to *Bd*, the variation in serotonin levels may be correlated with *Bd* virulence mediated through the production of Trp. Future work is needed to determine if the increase in serotonin is stress response to *Bd* infection which may result in greater synthesis or retention of serotonin in the skin.

6.2.1 | Growth assays

My first aim was to study the effect of serotonin on *Bd* growth. *In vitro* sensitivity trials showed that serotonin has a dose dependent inhibitory effect on *Bd* growth in culture (Chapter 2, Figure 6.1). I then used a structure-activity relationship assay to determine which structural motifs of serotonin are involved in the inhibition. A comparison of the inhibitory effect of serotonin and structural analogues showed that the terminal primary amine and the pyrrole ring were important to *Bd* inhibition. Frogs are able to produce serotonin from the

metabolic precursor Trp however, frogs must obtain Trp exogenously. *Bd* has been shown to secrete Trp (Rollins-Smith et al., 2015), and oxidised metabolite kynurenine (Kyn), and as expected, neither molecule inhibited *Bd in vitro*.

To examine possible mechanisms of this inhibition, I tested whether serotonin acts by mimicking Trp or Kyn precursors to competitively inhibit upstream metabolic enzymes. However, neither Trp or Kyn supplementation restored the growth of *Bd* in the presence of serotonin. Therefore, serotonin does not likely inhibit the Trp or Kyn biosynthetic pathways. *Bd* also produces other biogenic amines, such as putrescine and spermidine, the latter showing immunosuppressive effects on amphibian lymphocytes (Rollins-Smith et al., 2019). I tested the effect of related biogenic amines on *Bd* growth. Interestingly, spermine, putrescine, and spermidine inhibited growth of *Bd*, despite the latter two being secreted by *Bd* in culture. Further studies should investigate the effect of spermidine *in vivo*, to examine if the antifungal or immunosuppressive effects are more physiologically relevant for determining disease outcomes.

There was no statistically significant difference in protease gene expression of the TrpA (tryptophan synthase) in *Bd* treated with serotonin. Frogs can only synthesise serotonin via exogenous tryptophan, and *Bd* does not secrete serotonin. Therefore, serotonin found in host skin may come from exogenous Trp produced by *Bd*. A limitation to the gene expression study was that only one sublethal concentration of serotonin was compared to the no treatment control. Further studies should investigate the effect of higher concentrations of serotonin on tryptophan gene expression. Analysis of caspase activity showed that there was no increase in apoptosis with increased concentrations of serotonin.

6.2.2 | Immunosuppressive effects

As Kyn, a structural analogue of serotonin, is a suspected immunosuppressant that inhibits amphibian and human Jurkat lymphocytes (Rollins-Smith et al., 2015), I investigated whether serotonin also inhibits lymphocytes (Chapter 2). I found that serotonin inhibited human Jurkat lymphocyte proliferation *in vitro* (Figure 6.1). Although this effect of serotonin in a human cell line has not been confirmed with amphibian lymphocytes, other studies have validated the use of human Jurkat cells as model systems for amphibians (Piovia-Scott et al., 2014). The results suggest while the increase in serotonin observed in susceptible infected frogs may inhibit *Bd*, the potential for lymphocyte immunosuppression may increase susceptibility to *Bd* overall. Inhibition of the lymphocytes by serotonin may be a mechanism that allows *Bd* to suppress the host immune response and could explain the findings in

Grogan et al. (2018a, 2018c) where higher skin serotonin was linked to susceptible populations. Serotonin inhibited human Jurkat lymphocytes at a minimal inhibitory concentration of 500 µM and *Bd* sporangia at 1.5-2 mM (Claytor et al., 2019). Therefore, serotonin may affect host lymphocytes more than *Bd* sporangia, although future studies should confirm this result *in vivo*. Resistant and susceptible species show little inflammatory response to *Bd* infection, and therefore more work is needed to better understand the effect serotonin has on host lymphocytes.

The mechanisms of serotonin activity within frog skin during infection is complex, and largely unknown. It is unclear how serotonin affects lymphocytes *in vivo*. Immunohistochemistry (IHC) showed serotonin accumulated in the skin granular glands (Chapter 3), although it is likely also distributed throughout the skin at levels below the limit of detection by IHC. As *Bd* burden increases, it is likely that Trp secretion will increase, with a concomitant increase in the production of serotonin in infected frog skin. This proposed mechanism of serotonin could contribute to immune suppression observed in susceptible frogs with chytridiomycosis (Young et al., 2014, Rollins-Smith et al., 2019). Future work should aim to determine the origin of the serotonin within the granular glands, as melanocytes are believed to be the origin of serotonin in human skin (Johansson et al., 1998). *Bd* could be grown with labelled Trp precursors (e.g. chorismite or anthranilate) and then frogs could be infected with the *Bd*, to investigate the conversion of labelled Trp into serotonin in frogs. Alternatively, frogs could be treated with baths containing labelled Trp to investigate the conversion of Trp to serotonin.

6.2.3 | Localisation and quantification

To better understand the role of serotonin in *Bd* pathogenesis, Chapter 3 aimed to localise and quantify the amounts of serotonin within frog skin at different body sites across four species with different in susceptibility to *Bd*. The few previous studies that identified or quantified skin serotonin via IHC showed serotonin accumulation in granular glands in marsh frogs (*Rana ridibunda*) (Şengezer-İnceli et al., 2004), crab-eating frogs (*Rana cancrivora*) (Seki et al., 1995), African clawed frogs (*X. laevis*) (Seki et al., 1989, Bennett et al., 1981), and northern leopard frogs (*Rana pipiens*) (Bennett et al., 1981). No studies of Australian frogs have been performed, therefore, I used IHC to localise serotonin and high-performance liquid chromatography (HPLC) to quantify serotonin in skin. The composition of frog skin varies between species and location on the body (Llewelyn et al., 2018). Therefore, serotonin was identified and quantified in four opportunistically collected species: Baw Baw frogs

(*Philoria frosti*), cane toads (*Rhinella marina*), common eastern froglets (*Crinia signifera*), and southern corroboree frogs (*Pseudophryne corroboree*), and from (i) dorsal and ventral skin (all species), (ii) different ages (cane toads), (iii) different *Bd* infection status (southern corroboree frogs and common eastern froglets), and (iv) with serotonin bath treatments (common eastern froglets).

Generally, the more susceptible species (Baw Baw frogs and metamorph cane toads) had less detectable amounts of serotonin in the skin. This contrasts with our previous studies in alpine tree frogs, where the more resistant populations had lower levels of skin serotonin (Grogan et al., 2018d). No serotonin was detected in the skin of the *Bd* susceptible Baw Baw frogs. In cane toads, metamorphs are susceptible to *Bd*, though the adults are resistant (Brannelly et al., 2018a). No serotonin was detected by HPLC in cane toad metamorphs and only one metamorph showed granular gland IHC staining for serotonin, whereas it was detected in adults and juvenile. The *Bd* susceptible southern corroboree frogs had detectable levels of serotonin in some, but not all *Bd* infected and uninfected individuals. This may be due to sample size and the small size of the frogs. There was no significant difference in serotonin between infected and uninfected individuals. These results show that serotonin levels in the skin vary among *Bd* susceptible species and between individuals within a population. Future work should aim to determine if the correlation between serotonin and infection progression (Grogan et al., 2018b) is present in other susceptible species. Base-line levels of serotonin should be determined among different species, to test for a robust link with resistance. A possible method would be to stimulate secretion of granular glands with norepinephrine injections which has been performed in frog antimicrobial peptide (AMP) research (Gammill et al., 2012), and then measure the amount of serotonin in the secretions. Other sensitive methods to quantify serotonin could be utilized, such as using liquid chromatography- mass spectrometry (LC-MS) or matrix assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF) which would allow for the identification of serotonin molecules *in situ*.

To see whether serotonin can be absorbed transdermally in frog skin, infected *Bd* tolerant common eastern froglets were bathed in 0.44 mg/mL serotonin. Serotonin was identified in dorsal and ventral granular glands of all individuals, via IHC. However, there was no increase in serotonin in the treated animals. Interestingly, skin serotonin could only be quantified by HPLC in one individual. The detection of serotonin in the skin may be affected by the method of detection, the sample size and/or the small size of the frogs. IHC seemed a

more effective method of detecting serotonin than HPLC and the amount of serotonin in the skin may be below the limit of detection by HPLC. Mass spectrometry (MS) was able to quantify serotonin in the skin of individuals that had levels undetectable by HPLC. Further work should use MS to quantify serotonin in the skin, given that it seems to be more sensitive. Also, further work should validate these techniques in more individuals and species.

Since the available eastern froglets were naturally infected, I included an assessment of the effect of serotonin on *Bd* burdens. The chosen serotonin dosage (0.44 mg/mL for 1 h) was non-lethal to common eastern froglets, however, this dose had no impact on *Bd* infection levels. Most individuals tested positive for low levels of *Bd* before treatment and all except one remained positive post-treatment with unchanged *Bd* burdens. Further work should aim to manipulate serotonin levels in various species of frogs, including infected alpine tree frogs where initial variations in serotonin were observed. Higher concentrations of serotonin could be trialled, but since there are no studies in the literature reporting dosages or toxicity (LD50) for serotonin in adult frogs, safety studies would be required. An alternate approach to alter serotonin levels *in vivo* could be to bathe frogs in the serotonin precursor, the amino acid Trp. Exogenous tryptophan from food is absorbed from the intestines, and travels into the bloodstream, where it is then transported to the tissues, muscles, and liver, and used for the synthesis of proteins (Palego et al., 2016). Trp was shown to be absorbed transdermally through mouse skin, but because amino acids have low permeability coefficients, Trp cannot be administered transdermally without penetration enhancers or iontophoretic treatment (Ruland and Kreuter, 1991). This suggests that future work should consider using penetration enhancers to enable Trp to be absorbed into frog skin. Presently, no studies were found that measured transdermal serotonin absorption in frogs. Further research should determine if serotonin or Trp can be absorbed transdermally in frogs at a non-lethal dosage.

A method to manipulate serotonin levels, such as with serotonin reuptake inhibitors, would enable a clearer understanding of its function. In the mammalian nervous system, SSRIs such as sertraline, inhibit reuptake of serotonin by presynaptic cells resulting in a sustained effect in the synaptic cleft (Åberg-Wistedt, 1989). It would be interesting to explore whether SSRIs are able to alter serotonin levels in the skin, and affect *Bd in vivo*, particularly as SSRIs have been shown to have antifungal effects against other species of fungi *in vitro* (Zhai et al., 2012, Lass-Flörl et al., 2003), and *in vivo* (Lass-Florl et al., 2001). However, it is likely that the antifungal effects of SSRIs occur via lipophilic cytotoxicity rather than specific

inhibition of the serotonin transporter (Young et al., 2003). Furthermore, the SSRIs fluoxetine and sertraline also cause developmental toxicity in frogs (Conners et al., 2009), thus they are not likely to be practical treatments against *Bd in vivo*. As higher serotonin levels may correspond to increased susceptibility to *Bd* in some frog species (Grogan et al., 2018b), a therapeutic approach may include inhibition of serotonin levels in the skin via chemicals means or via transgenic methods. Genetic manipulation has already been used in *X. laevis* by using CRISPR/Cas9, for example to test immune gene function (Banach et al., 2017). In a transgenic rat model, serotonin biosynthesis in the brain were manipulated by knocking out the tryptophan hydroxylase gene isoform (TRP-2), which is a rate limiting enzyme in the biosynthesis of serotonin (Matthes et al., 2019). Future work could aim to use genetic manipulation to knock down key genes in the biosynthesis of serotonin with frogs. However, serotonin has diverse functional effects, and further work is needed to determine its precise role in the pathogenesis of chytridiomycosis and as a potential treatment against *Bd* in frogs.

6.2.4 | The effect of serotonin on protease activity

Serotonin has been shown to decrease the protease activity of *C. albicans* (Lass-Flörl et al., 2003). Serotonin may decrease the virulence properties of *C. albicans in vitro*, by affecting hyphal extension, phospholipase activity, and the production of secreted aspartyl proteinases (Saps) (Mayr et al., 2005). As *Bd* secretes many proteases that are involved in pathogenicity (Rosenblum et al., 2008, Fisher et al., 2009a, Brutyn et al., 2012, Thekkiniath et al., 2013), the aim of Chapter 4 was to determine the effect of serotonin on overall protease activity and gene expression of three highly expressed fungal proteases: aspartyl protease (ASP), and serine peptidase (S41), and metallopeptidase (M36) (Rosenblum et al., 2012).

There was a reduction in overall *Bd* protease activity (normalised to growth) with increasing concentrations of serotonin. Serotonin may reduce the metabolic activity of *Bd* cells and future work should measure how serotonin affects *Bd* cellular growth rates. There was no statistically significant difference in ASP, S41, or M36 gene expression with serotonin, although quantification of gene expression with qRT-PCR would allow for a more comprehensive gene expression comparison. *Bd* expresses numerous proteases and a microarray approach may be more effective in identifying whether the expression of specific proteases is impacted by serotonin.

6.2.5 | Future areas of investigation

Another known action of serotonin that is relevant to chytridiomycosis is its effect on ion transport in frog skin (Kosik-Bogacka and Tyrakowski, 2006). Amphibian skin facilitates the exchange of electrolytes (Krogh, 1937, Ussing, 1949), water (Parsons and Mobin, 1991), and gases (Whitford and Hutchison, 1965). Death from chytridiomycosis occurs due inhibition of electrolyte transport (sodium and potassium) across the epidermis of frog skin leading to terminally low blood electrolyte levels and cardiac arrest (Voyles et al., 2009). Experiments found that serotonin inhibits the sodium ion current across skin in Edible frogs (*Rana esculenta Linnaeus*) (Kosik-Bogacka and Tyrakowski, 2006) and high concentrations of the 5-hydroxytryptamine creatinine sulphate complex (5-HTCS) inhibits the transport of sodium in Common frogs (*Rana temporaria*) (Dalton, 1977). It is possible that the increased susceptibility of frogs to chytridiomycosis is mediated through serotonin-associated electrolyte disturbances. Further work using Ussing chambers could investigate whether increased serotonin during *Bd* infection contributes to the disrupted ion transport in frog skin. Although serotonin has antifungal effects against *Bd* and anti-proliferative effects on lymphocytes *in vitro*, serotonin treatment *in vivo* may interfere with ion transport in frog skin. This is an important consideration for further development of therapeutics that manipulate serotonin levels as a treatment to fight *Bd* infection. Commonly used *Bd* antifungals such itraconazole can have toxic effects on frogs (Brannelly, 2014) and cause skin depigmentation in tadpoles (Garner et al., 2009). Therefore, investigating other potential therapeutics that are well tolerated by frogs is very desirable.

6.3 | Protein Glutathionylation

The exponential growth of *Bd* in susceptible hosts implies an ability of the pathogen to evade the host immune system. In addition to the production of immunosuppressants (Fites et al., 2013), it is likely that *Bd* uses other mechanisms, such as antioxidants, to defend against the host. The fungi *Saccharomyces cerevisiae* uses the antioxidant glutathione as an adaptive defence against reactive oxygen species (ROS) (Herrero et al., 2008). The process of protein S-glutathionylation protects protein cysteines against overoxidation by ROS by binding glutathione (GSH) to form a mixed disulfide with the protein cysteinyl group. We have shown that *Bd* produces glutathione as its major low molecular weight thiol. Therefore, Chapter 5 aimed (i) to determine if *Bd* uses protein S-glutathionylation to protect its proteins from oxidative stress, and (ii) to identify glutathionylated proteins in *Bd*.

Bd was grown under low and high sublethal concentrations of hydrogen peroxide (H_2O_2), as a proxy for reactive oxygen species of the host immune system. The most marked changes in glutathionylation pattern were in proteins around the 55 kDa region. There were less glutathionylated proteins in the high H_2O_2 (24 h) treatment than the no treatment control (Figure 6.1). This suggests, surprisingly, that oxidative stress reduces glutathionylation of these proteins. Mass spectrometry identified the glutathionylation of the glycolytic enolase enzyme in the control, but not the in H_2O_2 treated sample. This suggests that *Bd* may not protect the glycolytic pathway from oxidative stress *in vitro*.

Bd may overcome oxidative stress by increasing metabolism and growth via glycolysis. Since enolase is a highly abundant cellular protein that is constitutively glutathionylated, it probably serves as a GSH reservoir (Fratelli et al., 2002). Thus, the reduction of glutathionylated enolase with peroxide treatment may reflect the higher need for reducing agents in the oxidised cell. To confirm this, cellular GSH and GSSH levels should be correlated with glutathionylated enolase under conditions of peroxide stress. In the future, immunohistochemistry could also be used to localise *in situ* enolase expression and provide more insight into the function of enolase in *Bd*. In the human bacterial pathogen *Streptococcus pyrogenes*, enolase is excreted into the extracellular environment where it plays a role in the degradation of host tissues and immune evasion (Walker et al., 2005). In parasitic trypanosomatids, enolase is likely a virulence factor due to having multiple functions in sugar metabolism and as a plasminogen receptor (Avilán et al., 2011). Further research should aim to identify differentially glutathionylated proteins from various oxidative treatments (ex. superoxide anion ($O_2\cdot^-$) and hydroxyl radical (OH^-)). A comprehensive proteomic analysis could identify the complete *Bd* "glutathiome", to better understand how *Bd* is able to evade the host immune system and to identify new virulence factors as therapeutic targets.

6.4 | Conclusions

Overall, my research found that the metabolite serotonin plays a complex role in *Bd* infection and that the serotonin mediated susceptibility to *Bd* is likely a result of increased Trp production from the pathogen. As serotonin has antifungal and immunosuppressive effects, a method to manipulate levels in the host may elucidate its overall impact on disease outcomes in individuals, which has been difficult to clarify. *Bd* also likely uses enolase glutathionylation as a glutathione reservoir for times of oxidative stress. Future investigations

should look deeper into the role of metabolites in *Bd* infection dynamics, as this is an untapped source of possible therapeutic targets.

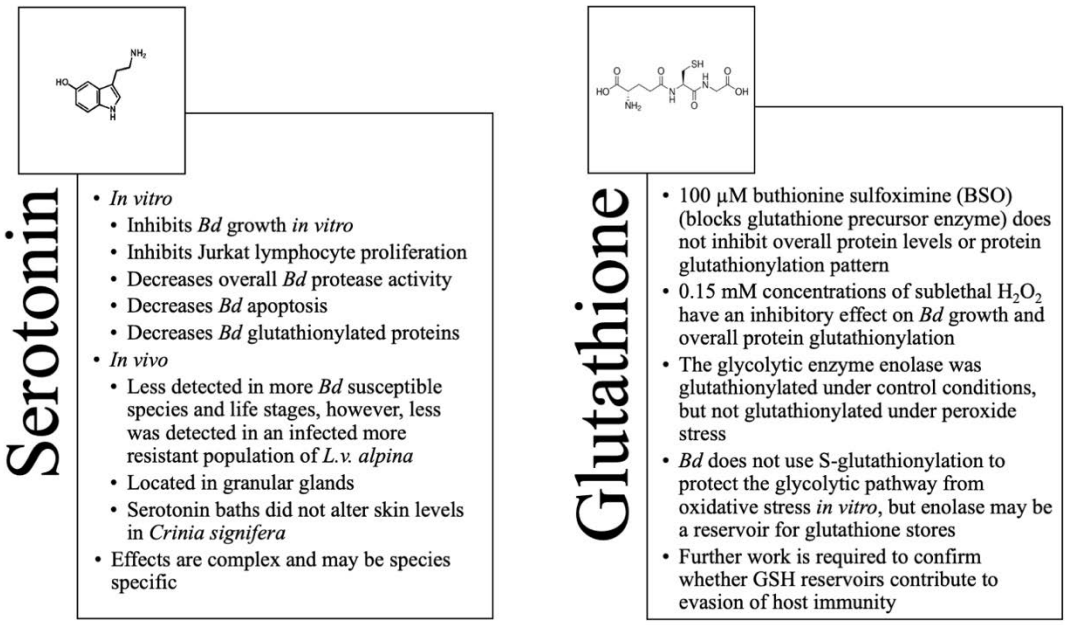

FIGURE 6.1 Overall thesis findings.

APPENDIX 1| James Cook University ethics approval for animal-based research or teaching.

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APPENDIX 2 | Chapter 2 Publication

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RESEARCH ARTICLE

WILEY

Susceptibility of frogs to chytridiomycosis correlates with increased levels of immunomodulatory serotonin in the skin

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Abstract

Chytridiomycosis, caused by the fungus *Batrachochytrium dendrobatidis* (*Bd*), is a skin disease responsible for the global decline of amphibians. Frog species and populations can vary in susceptibility, but this phenomenon remains poorly understood. Here, we investigated serotonin in the skin of infected and uninfected frogs. In more susceptible frog populations, skin serotonin rose with increasing infection intensity, but decreased in later stages of the disease. The more resistant population maintained a basal level of skin serotonin. Serotonin inhibited both *Bd* sporangial growth and Jurkat lymphocyte proliferation in vitro. However, serotonin accumulates in skin granular glands, and this compartmentalisation may prevent inhibition of *Bd* growth in vivo. We suggest that skin serotonin increases in susceptible frogs due to pathogen excretion of precursor tryptophan, but that resistant frogs are able to control the levels of serotonin. Overall, the immunosuppressive effects of serotonin may contribute to the susceptibility of frogs to chytridiomycosis.


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REFERENCES

ABBOTT, I. 2006. Mammalian faunal collapse in Western Australia, 1875-1925: the hypothesised role of epizootic disease and a conceptual model of its origin, introduction, transmission, and spread. *Australian Zoologist,* 33**,** 530-561.

ÅBERG-WISTEDT, A. 1989. The antidepressant effects of 5-HT uptake inhibitors. *British Journal of Psychiatry,* 155**,** 32-40.

ABRAMYAN, J. & STAJICH, J. E. 2012. Species-specific chitin-binding module 18 expansion in the amphibian pathogen *Batrachochytrium dendrobatidis*. *mBio,* 3**,** e00150-12.

AHERN, G. P. 2011. 5-HT and the immune system. *Current Opinion in Pharmacology,* 11**,** 29-33.

AL-LAHHAM, A., ROHDE, V., HEIM, P., LEUCHTER, R., VEECK, J., WUNDERLICH, C., WOLF, K. & ZIMMERMANN, M. 1999. Biosynthesis of phytochelatins in the fission yeast. phytochelatin synthesis: a second role for the glutathione synthetase gene of *Schizosaccharomyces pombe*. *Yeast,* 15**,** 385-396.

ALBERS, E. 2009. Metabolic characteristics and importance of the universal methionine salvage pathway recycling methionine from 5′-methylthioadenosine. *IUBMB Life,* 61**,** 1132-1142.

ALTSCHUL, S. F., MADDEN, T. L., SCHAFFER, A. A., ZHANG, J., ZHANG, Z., MILLER, W. & LIPMAN, D. J. 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res,* 25**,** 3389-402.

ANDRE, S. E., PARKER, J. & BRIGGS, C. J. 2008. Effect of temperature on host response to *Batrachochytrium dendrobatidis* infection in the mountain yellow-legged frog (*Rana muscosa*). *Journal of Wildlife Diseases,* 44**,** 716-20.

ANGIOLELLA, L., FACCHIN, M., STRINGARO, A., MARAS, B., SIMONETTI, N. & CASSONE, A. 1996. Identification of a glucan-associated enolase as a main cell wall

protein of *Candida albicans* and an indirect target of lipopeptide antimycotics. *The Journal of Infectious Diseases,* 173**,** 684-690.

ANTWIS, R. E., PREZIOSI, R. F., HARRISON, X. A. & GARNER, T. W. 2015. Amphibian symbiotic bacteria do not show a universal ability to inhibit growth of the global panzootic lineage of *Batrachochytrium dendrobatidis*. *Applied Environmental Microbiology,* 81**,** 3706-11.

AUNG-HTUT, M. T., AYER, A., BREITENBACH, M. & DAWES, I. W. 2012. Oxidative stresses and ageing. *Subcellular Biochemistry,* 57**,** 13-54.

AVILÁN, L., GUALDRÓN-LÓPEZ, M., QUIÑONES, W., GONZÁLEZ-GONZÁLEZ, L., HANNAERT, V., MICHELS, P. A. M. & CONCEPCIÓN, J.-L. 2011. Enolase: a key player in the metabolism and a probable virulence factor of trypanosomatid parasites-perspectives for its use as a therapeutic target. *Enzyme research,* 2011**,** 932549-932549.

BAI, C., GARNER, T. W. & LI, Y. 2010. First evidence of *Batrachochytrium dendrobatidis* in China: discovery of chytridiomycosis in introduced American bullfrogs and native amphibians in the Yunnan Province, China. *Ecohealth,* 7**,** 127-34.

BAI, C., LIU, X., FISHER, M. C., GARNER, T. W. J. & LI, Y. 2012. Global and endemic Asian lineages of the emerging pathogenic fungus *Batrachochytrium dendrobatidis* widely infect amphibians in China. *Diversity and Distributions,* 18**,** 307-318.

BANACH, M., EDHOLM, E.-S. & ROBERT, J. 2017. Exploring the functions of nonclassical MHC class Ib genes in Xenopus laevis by the CRISPR/Cas9 system. *Developmental Biology,* 426**,** 261-269.

BATAILLE, A., CASHINS, S. D., GROGAN, L., SKERRATT, L. F., HUNTER, D., MCFADDEN, M., SCHEELE, B., BRANNELLY, L. A., MACRIS, A., HARLOW, P. S., BELL, S., BERGER, L. & WALDMAN, B. 2015. Susceptibility of amphibians to chytridiomycosis is associated with MHC class II conformation. *Proceedings of the Royal Society B: Biological Sciences,* 282**,** 20143127.

BATAILLE, A., FONG, J. J., CHA, M., WOGAN, G. O. U., BAEK, H. J., LEE, H., MIN, M.-S. & WALDMAN, B. 2013. Genetic evidence for a high diversity and wide distribution of endemic strains of the pathogenic chytrid fungus *Batrachochytrium dendrobatidis* in wild Asian amphibians. *Molecular Ecology,* 22**,** 4196-4209.

BATAILLE, A., LEE-CRUZ, L., TRIPATHI, B., KIM, H. & WALDMAN, B. 2016. Microbiome Variation Across Amphibian Skin Regions: Implications for Chytridiomycosis Mitigation Efforts. *Microbial Ecology,* 71**,** 221-232.

BENNETT, G. W., BALLS, M., CLOTHIER, R. H., MARSDEN, C. A., ROBINSON, G. & WEMYSS-HOLDEN, G. D. 1981. Location and release of TRH and 5-HT from amphibian skin. *Cell Biology International Reports,* 5**,** 151-8.

BERGER, L., HYATT, A. D., SPEARE, R. & LONGCORE, J. E. 2005a. Life cycle stages of the amphibian chytrid *Batrachochytrium dendrobatidis*. *Diseases of Aquatic Organisms,* 68**,** 51-63.

BERGER, L., ROBERTS, A. A., VOYLES, J., LONGCORE, J. E., MURRAY, K. A. & SKERRATT, L. F. 2016. History and recent progress on chytridiomycosis in amphibians. *Fungal Ecology,* 19**,** 89-99.

BERGER, L., SPEARE, R., DASZAK, P., GREEN, D. E., CUNNINGHAM, A. A., GOGGIN, C. L., SLOCOMBE, R., RAGAN, M. A., HYATT, A. D., MCDONALD, K. R., HINES, H. B., LIPS, K. R., MARANTELLI, G. & PARKES, H. 1998a. Chytridiomycosis causes amphibian mortality associated with population declines in the rain forests of Australia and Central America. *Proceedings of the National Academy of Sciences,* 95**,** 9031-9036.

BERGER, L., SPEARE, R. & HYATT, A. 1999a. Chytrid fungi and amphibian declines: overview, implications and future directions. *Environment Australia: Declines and Disappearances of Australian Frogs.* Canberra.

BERGER, L., SPEARE, R. & KENT, A. 1999b. Diagnosis of chytridiomycosis in amphibians by histologic examination. World Wide Web file.

BERGER, L., SPEARE, R. & SKERRATT, L. F. 2005b. Distribution of *Batrachochytrium dendrobatidis* and pathology in the skin of green tree frogs *Litoria caerulea* with severe chytridiomycosis. *Diseases of Aquatic Organisms,* 68.

BERGER, L. B., SPEARE, R., DASZAK, P., GREEN, D. E., CUNNINGHAM, A. A., GOGGIN, C. L., SLOCOMBE, R., RAGAN, M. A., HYATT, A. D., MCDONALD, K. R., HINES, H., LIPS, K. R., MARANTELLI, G. & PARKES, H. 1998b. Chytridiomycosis causes amphibian mortality associated with population declines in the rain forests of Australia and Central America. *Proceedings of the National Academy of Sciences, USA,* 95**,** 6.

BLETZ, M. C., LOUDON, A. H., BECKER, M. H., BELL, S. C., WOODHAMS, D. C., MINBIOLE, K. P. C. & HARRIS, R. N. 2013. Mitigating amphibian chytridiomycosis with bioaugmentation: characteristics of effective probiotics and strategies for their selection and use. *Ecology Letters,* 16**,** 807-820.

BOSCH, J., CARRASCAL, L. M., DURÁN, L., WALKER, S. & FISHER, M. C. 2007. Climate change and outbreaks of amphibian chytridiomycosis in a montane area of Central Spain; is there a link? *Proceedings of the Royal Society B: Biological Sciences,* 274**,** 253-260.

BOSCH, J. & MARTINEZ-SOLANO, I. 2006. Chytrid fungus infection related to unusual mortalities of *Salamandra salamandra* and *Bufo bufo* in the Penalara Natural Park, Spain. *Oryx,* 40**,** 84-89.

BOTH, C., LINGNAU. R., SANTOS-JR, A., MADALOZZO, B., LIMA, L. P. & GRANT, T. 2011. Widespread occurrence of the American Bullfrog, *Lithobates catesbeianus* (Shaw, 1802) (Anura: Ranidae), in Brazil. *South American Journal of Herpetology,* 6**,** 127-134, 8.

BOYLE, D., OLSEN, V., MORGAN, J. & HYATT, A. 2004. Rapid quantitative detection of chytridiomycosis (Batrachochytrium dendrobatidis) in amphibian samples using real-time Taqman PCR assay. *Diseases of aquatic organisms,* 60 2**,** 141-8.

BOZZA, S., FALLARINO, F., PITZURRA, L., ZELANTE, T., MONTAGNOLI, C., BELLOCCHIO, S., MOSCI, P., VACCA, C., PUCCETTI, P. & ROMANI, L. 2005. A crucial role for tryptophan catabolism at the host *Candida albicans* interface. *Journal of Immunology,* 174**,** 2910-2918.

BRANNELLY, L. A. 2014. Reduced itraconazole concentration and durations are successful in treating *Batrachochytrium dendrobatidis* infection in amphibians. *Journal of Visualized Experiments : JoVE***,** 51166.

BRANNELLY, L. A., BERGER, L., MARRANTELLI, G. & SKERRATT, L. F. 2015. Low humidity is a failed treatment option for chytridiomycosis in the critically endangered southern corroboree frog. *Wildlife Research,* 42**,** 44-49.

BRANNELLY, L. A., MARTIN, G., LLEWELYN, J., SKERRATT, L. F. & BERGER, L. 2018a. Age- and size-dependent resistance to chytridiomycosis in the invasive cane toad *Rhinella marina*. *Diseases of Aquatic Organisms,* 131**,** 107-120.

BRANNELLY, L. A., WEBB, R., SKERRATT, L. F. & BERGER, L. 2016. Amphibians with infectious disease increase their reproductive effort: evidence for the terminal investment hypothesis. *Open Biology,* 6**,** 150251.

BRANNELLY, L. A., WEBB, R. J., HUNTER, D. A., CLEMANN, N., HOWARD, K., SKERRATT, L. F., BERGER, L. & SCHEELE, B. C. 2018b. Non-declining amphibians can be important reservoir hosts for amphibian chytrid fungus. *Animal Conservation,* 21**,** 91-101.

BREITENBACH, M., WEBER, M., RINNERTHALER, M., KARL, T. & BREITENBACH-KOLLER, L. 2015. Oxidative stress in fungi: its function in signal transduction, interaction with plant hosts, and lignocellulose degradation. *Biomolecules,* 5**,** 318-342.

BREM, F. M. R., PARRIS, M. J. & PADGETT-FLOHR, G. E. 2013. Re-isolating *Batrachochytrium dendrobatidis* from an amphibian host increases pathogenicity in a subsequent exposure. *PLOS ONE,* 8**,** e61260.

BRIGGS, C. J., KNAPP, R. A. & VREDENBURG, V. T. 2010. Enzootic and epizootic dynamics of the chytrid fungal pathogen of amphibians. *Proceedings of the National Academy of Sciences,* 107**,** 9695-9700.

BRIGGS, C. J., VREDENBURG, V. T., KNAPP, R. A. & RACHOWICZ, L. J. 2005. Investigating the population-level effects of chytridiomycosis: an emerging infectious disease of amphibians. *Ecology,* 86**,** 3149-3159.

BROOKS, T. M., MITTERMEIER, R. A., MITTERMEIER, C. G., DA FONSECA, G. A. B., RYLANDS, A. B., KONSTANT, W. R., FLICK, P., PILGRIM, J., OLDFIELD, S., MAGIN, G. & HILTON-TAYLOR, C. 2002. Habitat loss and extinction in the hotspots of biodiversity. *Conservation Biology,* 16**,** 909-923.

BROWN, D., GROSSO, A. & DE SOUSA, R. C. 1981. The amphibian epidermis: distribution of mitochondria-rich cells and the effect of oxytocin. *Journal of Cell Science,* 52**,** 197.

BRUCKER, R. M., HARRIS, R. N., SCHWANTES, C. R., GALLAHER, T. N., FLAHERTY, D. C., LAM, B. A. & MINBIOLE, K. P. 2008. Amphibian chemical defense: antifungal metabolites of the microsymbiont *Janthinobacterium lividum* on the salamander *Plethodon cinereus*. *Journal of Chemical Ecology,* 34**,** 1422-9.

BRUTYN, M., D'HERDE, K., DHAENENS, M., VAN ROOIJ, P., VERBRUGGHE, E., HYATT, A. D., CROUBELS, S., DEFORCE, D., DUCATELLE, R., HAESEBROUCK, F., MARTEL, A. & PASMANS, F. 2012. *Batrachochytrium dendrobatidis* zoospore secretions rapidly disturb intercellular junctions in frog skin. *Fungal Genetics & Biology,* 49**,** 830-7.

CABISCOL, E. & LEVINE, R. L. 1996. The phosphatase activity of carbonic anhydrase III is reversibly regulated by glutathiolation. *Proceedings of the National Academy of Sciences of the United States of America,* 93**,** 4170-4174.

CAMPBELL, C. R., VOYLES, J., COOK, D. I. & DINUDOM, A. 2012. Frog skin epithelium: electrolyte transport and chytridiomycosis. *International Journal of Biochemical Cellular Biology,* 44**,** 431-4.

CASHINS, S. D., GROGAN, L. F., MCFADDEN, M., HUNTER, D., HARLOW, P. S., BERGER, L. & SKERRATT, L. F. 2013. Prior infection does not improve survival against the amphibian disease chytridiomycosis. *PLOS ONE,* 8**,** e56747.

CHEN, Y.-H., SHIH, L.-L., LIOU, S.-E. & CHEN, C.-C. 2003. Analysis of dabsyl-Cl derivated amino acids by high performance liquid chromatography and tandem mass spectrometry. *Food Science and Technology Research,* 9**,** 276-282.

CHENG, T. L., ROVITO, S. M., WAKE, D. B. & VREDENBURG, V. T. 2011. Coincident mass extirpation of neotropical amphibians with the emergence of the infectious fungal pathogen *Batrachochytrium dendrobatidis*. *Proceedings of the National Academy of Sciences of the United States of America,* 108**,** 9502-9507.

CHI, B. K., ROBERTS, A. A., HUYEN, T. T. T., BÄSELL, K., BECHER, D., ALBRECHT, D., HAMILTON, C. J. & ANTELMANN, H. 2013. S-bacillithiolation protects conserved and essential proteins against hypochlorite stress in firmicutes bacteria. *Antioxidants & Redox Signaling,* 18**,** 1273-1295.

CHRISTOFFERSSON, G. & PHILLIPSON, M. 2018. The neutrophil: one cell on many missions or many cells with different agendas? *Cell Tissue Research,* 371**,** 415-423.

CLARKE, J. M., BORSBOOM, A. C., CUNNINGHAM, M. & HINES, H. 1999. The recovery process for the Kroombit tinkerfrog, *Taudactylus pleione*. *In:* BOYES, B. R. (ed.) *Rainforest Recovery for the New Millennium.* Sydney: WWF 1998 SE Qld Rainforest Recovery Conference.

CLAYTOR, S. C., GUMMER, J. P. A., GROGAN, L. F., SKERRATT, L. F., WEBB, R. J., BRANNELLY, L. A., BERGER, L. & ROBERTS, A. A. 2019. Susceptibility of frogs to chytridiomycosis correlates with increased levels of immunomodulatory serotonin in the skin. *Cellular Microbiology,* 0**,** e13089.

CONNERS, D. E., ROGERS, E. D., ARMBRUST, K. L., KWON, J. W. & BLACK, M. C. 2009. Growth and development of tadpoles (*Xenopus laevis*) exposed to selective serotonin reuptake inhibitors, fluoxetine and sertraline, throughout metamorphosis. *Environmental Toxicology & Chemistry,* 28**,** 2671-6.

CORSARO, C., SCALIA, M., LEOTTA, N., MONDIO, F. & SICHEL, G. 2000. Characterisation of Kupffer cells in some Amphibia. *Journal of Anatomy,* 196**,** 249-261.

COSTA SILVA, R. A., DA SILVA, C. R., DE ANDRADE NETO, J. B., DA SILVA, A. R., CAMPOS, R. S., SAMPAIO, L. S., DO NASCIMENTO, F. B. S. A., DA SILVA GASPAR, B., DA CRUZ FONSECA, S. G., JOSINO, M. A. A., GRANGEIRO, T. B., GASPAR, D. M., DE LUCENA, D. F., DE MORAES, M. O., CAVALCANTI, B. C. & NOBRE JÚNIOR, H. V. 2017. In vitro anti-Candida activity of selective serotonin reuptake inhibitors against fluconazole-resistant strains and their activity against biofilm-forming isolates. *Microbial Pathogenesis,* 107**,** 341-348.

COVACEVICH, J. A. & MCDONALD, K. R. 1993. Distribution and conservation of frogs and reptiles of Queensland rainforests. *Memoirs of the Queensland Museum,* 34**,** 189-199.

CUNNINGHAM, A. A., DASZAK, P. & RODRIGUEZ, J. P. 2003. Pathogen pollution: defining a parasitological threat to biodiversity conservation. *Journal of Parasitology,* 89**,** S78-S83.

DALLE-DONNE, I., ROSSI, R., COLOMBO, G., GIUSTARINI, D. & MILZANI, A. 2009. Protein S-glutathionylation: a regulatory device from bacteria to humans. *Trends in Biochemical Sciences,* 34**,** 85-96.

DALTON, T. 1977. The effect of 5-hydroxytryptamine creatinine sulphate on sodium transport across isolated frog skin. *Comparative Biochemistry & Physiology C,* 56**,** 41-7.

DALTON, T. 1979. Pharmacology of 5-hydroxytryptamine receptors in frog skin epithelium. *British Journal of Pharmacology,* 65**,** 579-585.

DALY, J. W. 1995. The chemistry of poisons in amphibian skin. *Proceedings of the National Academy of Sciences of the United States of America,* 92**,** 9-13.

DANG, T. D., SEARLE, C. L. & BLAUSTEIN, A. R. 2017. Virulence variation among strains of the emerging infectious fungus *Batrachochytrium dendrobatidis* (*Bd*) in multiple amphibian host species. *Diseases of Aquatic Organisms,* 124**,** 233-239.

DASKIN, J. H., ALFORD, R. A. & PUSCHENDORF, R. 2011. Short-term exposure to warm microhabitats could explain amphibian persistence with *Batrachochytrium dendrobatidis*. *PLOS ONE,* 6**,** e26215.

DAVIDSON, E. W., PARRIS, M., COLLINS, J. P., LONGCORE, J. E., PESSIER, A. P. & BRUNNER, J. 2003. Pathogenicity and transmission of chytridiomycosis in tiger salamanders (*Ambystoma tigrinum*). *Copeia***,** 601-607.

DE JESÚS-BERRÍOS, M., LIU, L., NUSSBAUM, J. C., COX, G. M., STAMLER, J. S. & HEITMAN, J. 2003. Enzymes that counteract nitrosative stress promote fungal virulence. *Current Biology,* 13**,** 1963-1968.

DE LAENDER, F., MELIAN, C. J., BINDLER, R., VAN DEN BRINK, P. J., DAAM, M., ROUSSEL, H., JUSELIUS, J., VERSCHUREN, D. & JANSSEN, C. R. 2014. The contribution of intra- and interspecific tolerance variability to biodiversity changes along toxicity gradients. *Ecology Letters,* 17**,** 72-81.

DICKE, U., WALLSTEIN, M. & ROTH, G. 1997. 5-HT-like immunoreactivity in the brains of plethodontid and salamandrid salamanders (*Hydromantes italicus*, *Hydromantes genei*, *Plethodon jordani*, *Desmognathus ochrophaeus*, *Pleurodeles waltl*): an immunohistochemical and biocytin double-labelling study. *Cell and Tissue Research,* 287**,** 513-523.

DILLON, M. J., BOWKETT, A. E., BUNGARD, M. J., BECKMAN, K. M., O'BRIEN, M. F., BATES, K., FISHER, M. C., STEVENS, J. R. & THORNTON, C. R. 2017. Tracking the amphibian pathogens *Batrachochytrium dendrobatidis* and *Batrachochytrium salamandrivorans* using a highly specific monoclonal antibody and lateral-flow technology. *Microbial Biotechnology,* 10**,** 381-394.

DU FALL, L. A. & SOLOMON, P. S. 2013. The necrotrophic effector SnToxA induces the synthesis of a novel phytoalexin in wheat. *New Phytology,* 200**,** 185-200.

DUERSCHMIED, D. & BODE, C. 2009. The role of serotonin in haemostasis. *Hamostaseologie,* 29**,** 356-359.

DUERSCHMIED, D., SUIDAN, G. L., DEMERS, M., HERR, N., CARBO, C., BRILL, A., CIFUNI, S. M., MAULER, M., CICKO, S., BADER, M., IDZKO, M., BODE, C. & WAGNER, D. D. 2013. Platelet serotonin promotes the recruitment of neutrophils to sites of acute inflammation in mice. *Blood,* 121**,** 1008-1015.

DÜRK, T., PANTHER, E., MÜLLER, T., SORICHTER, S., FERRARI, D., PIZZIRANI, C., DI VIRGILIO, F., MYRTEK, D., NORGAUER, J. & IDZKO, M. 2005. 5-Hydroxytryptamine modulates cytokine and chemokine production in LPS-primed human monocytes via stimulation of different 5-HTR subtypes. *International Immunology,* 17**,** 599-606.

ELLISON, A. R., SAVAGE, A. E., DIRENZO, G. V., LANGHAMMER, P., LIPS, K. R. & ZAMUDIO, K. R. 2014a. Fighting a losing battle: vigorous immune response countered by pathogen suppression of host defenses in the chytridiomycosis-susceptible frog *Atelopus zeteki*. *G3 (Bethesda),* 4**,** 1275-89.

ELLISON, A. R., TUNSTALL, T., DIRENZO, G. V., HUGHEY, M. C., REBOLLAR, E. A., BELDEN, L. K., HARRIS, R. N., IBÁÑEZ, R., LIPS, K. R. & ZAMUDIO, K. R. 2014b. More than skin deep: functional genomic basis for resistance to amphibian chytridiomycosis. *Genome biology and evolution,* 7**,** 286-298.

ELMORE, S. 2007. Apoptosis: a review of programmed cell death. *Toxicologic Pathology,* 35**,** 495-516.

FARRER, R. S., WEINERT, L. A., BIEBLY, J., GARNER, T. W., BALLOUX, F., CLARE, F., BOSCH, J., CUNNINGHAM, A. A., WELDON, C., DU PREEZ, L. H., ANDERSON, L., KOSAKOVSKY POND, S. L., SHAHAR-GOLAN, R., HENK, D. A. & FISHER, M. C. 2011. Multiple emergences of genetically diverse amphibian-infecting chytrids include a globalized hypervirulent recombinant lineage. *Proceedings of the National Academy of Sciences, USA,* 108**,** 5.

FASULO, S., TAGLIAFIERRO, G., CONTINI, A., AINIS, L., RICCA, M. B., YANAIHARA, N. & ZACCONE, G. 1993. Ectopic expression of bioactive peptides and serotonin in the sacciform gland cells of teleost skin. *Archives of Histology and Cytology,* 56**,** 117-25.

FICETOLA, G. F., BONIN, A. & MIAUD, C. 2008. Population genetics reveals origin and number of founders in a biological invasion. *Molecular Ecology,* 17**,** 773-82.

FISHER, M. C., BOSCH, J., YIN, Z., STEAD, D. A., WALKER, J., SELWAY, L., BROWN, A. J., WALKER, L. A., GOW, N. A., STAJICH, J. E. & GARNER, T. W. 2009a. Proteomic and phenotypic profiling of the amphibian pathogen *Batrachochytrium dendrobatidis* shows that genotype is linked to virulence. *Molecular Ecology,* 18**,** 415-29.

FISHER, M. C., GARNER, T. W. & WALKER, S. F. 2009b. Global emergence of *Batrachochytrium dendrobatidis* and amphibian chytridiomycosis in space, time, and host. *Annual Review of Microbiology,* 63**,** 291-310.

FISHER, M. C. & GARNER, T. W. J. 2007. The relationship between the emergence of *Batrachochytrium dendrobatidis*, the international trade in amphibians and introduced amphibian species. *Fungal Biology Reviews,* 21**,** 2-9.

FISHER, M. C., HENK, D. A., BRIGGS, C. J., BROWNSTEIN, J. S., MADOFF, L. C., MCCRAW, S. L. & GURR, S. J. 2012. Emerging fungal threats to animal, plant and ecosystem health. *Nature,* 484**,** 186.

FITES, J. S., RAMSEY, J. P., HOLDEN, W. M., COLLIER, S. P., SUTHERLAND, D. M., REINERT, L. K., GAYEK, A. S., DERMODY, T. S., AUNE, T. M., OSWALD-RICHTER, K. & ROLLINS-SMITH, L. A. 2013. The invasive chytrid fungus of amphibians paralyzes lymphocyte responses. *Science,* 342**,** 366-9.

FITES, J. S., REINERT, L. K., CHAPPELL, T. M. & ROLLINS-SMITH, L. A. 2014. Inhibition of local immune responses by the frog-killing fungus *Batrachochytrium dendrobatidis*. *Infection & Immunity,* 82**,** 4698-706.

FRANCHINI, A. M., HUNT, D., MELENDEZ, J. A. & DRAKE, J. R. 2013. FcgammaR-driven release of IL-6 by macrophages requires NOX2-dependent production of reactive oxygen species. *Journal of Biological Chemistry,* 288**,** 25098-108.

FRATELLI, M., DEMOL, H., PUYPE, M., CASAGRANDE, S., EBERINI, I., SALMONA, M., BONETTO, V., MENGOZZI, M., DUFFIEUX, F., MICLET, E., BACHI, A., VANDEKERCKHOVE, J., GIANAZZA, E. & GHEZZI, P. 2002. Identification by redox proteomics of glutathionylated proteins in oxidatively stressed human T lymphocytes. *Proceedings of the National Academy of Sciences of the United States of America,* 99**,** 3505-3510.

FUNK, J., SCHAARSCHMIDT, B., SLESIONA, S., HALLSTRÖM, T., HORN, U. & BROCK, M. 2016. The glycolytic enzyme enolase represents a plasminogen-binding protein on the surface of a wide variety of medically important fungal species. *International Journal of Medical Microbiology,* 306**,** 59-68.

GAMMILL, W. M., SCOTT FITES, J. & ROLLINS-SMITH, L. A. 2012. Norepinephrine depletion of antimicrobial peptides from the skin glands of Xenopus laevis. *Developmental & Comparative Immunology,* 37**,** 19-27.

GARGAS, A., TREST, M. T., CHRISTENSEN, M., VOLK, T. J. & BLEHERT, D. S. 2009. *Geomyces destructans* sp. nov. associated with bat white-nose syndrome. *Mycotaxon,* 108**,** 147-154.

GARMYN, A., VAN ROOIJ, P., PASMANS, F., HELLEBUYCK, T., VAN DEN BROECK, W., HAESEBROUCK, F. & MARTEL, A. 2012. Waterfowl: potential environmental reservoirs of the chytrid fungus *Batrachochytrium dendrobatidis*. *PLOS ONE,* 7**,** e35038.

GARNER, T. W., GARCIA, G., CARROLL, B. & FISHER, M. C. 2009. Using itraconazole to clear Batrachochytrium dendrobatidis infection, and subsequent depigmentation of Alytes muletensis tadpoles. *Dis Aquat Organ,* 83**,** 257-60.

GARNER, T. W. J., PERKINS, M. W., GOVINDARAJULU, P., SEGLIE, D., WALKER, S., CUNNINGHAM, A. A. & FISHER, M. C. 2006. The emerging amphibian pathogen *Batrachochytrium dendrobatidis* globally infects introduced populations of the North American bullfrog, *Rana catesbeiana*. *Biology Letters,* 2**,** 455-459.

GARTON, J. D. & MUSHINSKY, H. R. 1979. Integumentary toxicity and unpalatability as an antipredator mechanism in the narrow mouthed toad, *Gastrophryne carolinensis*. *Canadian Journal of Zoology,* 57**,** 1965-1973.

GERGONDEY, R., GARCIA, C., SERRE, V., CAMADRO, J. M. & AUCHÈRE, F. 2016. The adaptive metabolic response involves specific protein glutathionylation during the filamentation process in the pathogen *Candida albicans*. *Biochimica et Biophysica Acta (BBA) - Molecular Basis of Disease,* 1862**,** 1309-1323.

GERVASI, S. S., STEPHENS, P. R., HUA, J., SEARLE, C. L., XIE, G. Y., URBINA, J., OLSON, D. H., BANCROFT, B. A., WEIS, V., HAMMOND, J. I., RELYEA, R. A. & BLAUSTEIN, A. R. 2017. Linking ecology and epidemiology to understand predictors of multi-host responses to an emerging pathogen, the amphibian chytrid fungus. *PLOS ONE,* 12**,** e0167882.

GERVASI, S. S., URBINA, J., HUA, J., CHESTNUT, T., R, A. R. & A, R. B. 2013. Experimental evidence for American bullfrog (*Lithobates catesbeianus*) susceptibility to chytrid fungus (*Batrachochytrium dendrobatidis*). *Ecohealth,* 10**,** 166-71.

GHOSH, M., SHEN, J. & ROSEN, B. P. 1999. Pathways of As(III) detoxification in *Saccharomyces cerevisiae*. *Proceedings of the National Academy of Sciences of the United States of America,* 96**,** 5001-5006.

GILLESPIE, G. & HOLLIS, G. 1996. Distribution and habitat of the spotted tree-frog, *Litoria spenceri Dubois* (Anura : Hylidae), and an assessment of potential causes of population declines. *Wildlife Research,* 23**,** 49-75.

GOKA, K., YOKOYAMA, J., UNE, Y., KUROKI, T., SUZUKI, K., NAKAHARA, M., KOBAYASHI, A., INABA, S., MIZUTANI, T. & HYATT, A. D. 2009. Amphibian chytridiomycosis in Japan: distribution, haplotypes and possible route of entry into Japan. *Molecular Ecology,* 18**,** 4757-74.

GOWER, D. J., DOHERTY-BONE, T., LOADER, S. P., WILKINSON, M., KOUETE, M. T., TAPLEY, B., ORTON, F., DANIEL, O. Z., WYNNE, F., FLACH, E., MULLER, H., MENEGON, M., STEPHEN, I., BROWNE, R. K., FISHER, M. C., CUNNINGHAM, A. A. & GARNER, T. W. J. 2013. *Batrachochytrium dendrobatidis* infection and lethal chytridiomycosis in caecilian amphibians (Gymnophiona). *Ecohealth,* 10**,** 173-183.

GREENSPAN, S. E., CALHOUN, A. J., LONGCORE, J. E. & LEVY, M. G. 2012a. Transmission of *Batrachochytrium dendrobatidis* to wood frogs (*Lithobates sylvaticus*) via a bullfrog (*L. catesbeianus*) vector. *Journal of Wildlife Diseases,* 48**,** 575-82.

GREENSPAN, S. E., LAMBERTINI, C., CARVALHO, T., JAMES, T. Y., TOLEDO, L. F., HADDAD, C. F. B. & BECKER, C. G. 2018. Hybrids of amphibian chytrid show high virulence in native hosts. *Scientific Reports,* 8**,** 9600.

GREENSPAN, S. E., LONGCORE, J. E. & CALHOUN, A. J. 2012b. Host invasion by *Batrachochytrium dendrobatidis*: fungal and epidermal ultrastructure in model anurans. *Diseases of Aquatic Organisms,* 100**,** 201-10.

GROGAN, L. F. 2014. *Understanding host and environmental factors in the immunology and epidemiology of chytridiomycosis in anuran populations in Australia.* PhD, James Cook University.

GROGAN, L. F., CASHINS, S. D., SKERRATT, L. F., BERGER, L., MCFADDEN, M. S., HARLOW, P., HUNTER, D., A., SCHEELE, B. C. & MULVENNA, J. 2018a. Evolution of resistance to chytridiomycosis is associated with a robust early immune response. *Molecular Ecology,* 27**,** 919-934.

GROGAN, L. F., MULVENNA, J., GUMMER, J. P. A., SCHEELE, B. C., BERGER, L., CASHINS, S. D., MCFADDEN, M. S., HARLOW, P., HUNTER, D. A., TRENGOVE, R. D. & SKERRATT, L. F. 2018b. Survival, gene and metabolite responses of *Litoria verreauxii alpina* frogs to fungal disease chytridiomycosis. *Scientific Data,* 5**,** 180033.

GROGAN, L. F., PHILLOTT, A. D., SCHEELE, B. C., BERGER, L., CASHINS, S. D., BELL, S. C., PUSCHENDORF, R. & SKERRATT, L. F. 2016. Endemicity of chytridiomycosis features pathogen overdispersion. *Journal of Animal Ecology,* 85**,** 806-816.

GROGAN, L. F., ROBERT, J., BERGER, L., SKERRATT, L. F., SCHEELE, B. C., CASTLEY, J. G., NEWELL, D. A. & MCCALLUM, H. I. 2018c. Review of the amphibian immune response to chytridiomycosis, and future directions. *Frontiers in Immunology,* 9.

GROGAN, L. F., SKERRATT, L. F., BERGER, L., CASHINS, S. D., TRENGOVE, R. D. & GUMMER, J. P. A. 2018d. Chytridiomycosis causes catastrophic organism-wide metabolic dysregulation including profound failure of cellular energy pathways. *Scientific Reports,* 8**,** 8188.

GRUNWALD, N. J., GOSS, E. M. & PRESS, C. M. 2008. *Phytophthora ramorum*: a pathogen with a remarkably wide host range causing sudden oak death on oaks and ramorum blight on woody ornamentals. *Molecular Plant Pathology,* 9**,** 729-40.

HANLON, S. M., LYNCH, K. J., KERBY, J. & PARRIS, M. J. 2015. *Batrachochytrium dendrobatidis* exposure effects on foraging efficiencies and body size in anuran tadpoles. *Diseases of Aquatic Organisms,* 112**,** 237-42.

HANSON, R. M. & LU, X.-J. 2017. DSSR-enhanced visualization of nucleic acid structures in Jmol. *Nucleic Acids Research,* 45**,** W528-W533.

HEARD, G. W., SCROGGIE, M. P., CLEMANN, N. & RAMSEY, D. S. L. 2014. Wetland characteristics influence disease risk for a threatened amphibian. *Ecological Applications,* 24**,** 650-662.

HEATWOLE, H. 1994. *Amphibian biology,* Chipping Norton, NSW, Surrey Beatty & Sons.

HERO, J.-M., HINES, H. B., MEYER, E., MORRISON, C., STREATFEILD, C. & ROBERTS, L. 1998. New records of "declining" frogs in Queensland, Australia. *Froglog,* 29-Jan**,** 1-4.

HERR, N., BODE, C. & DUERSCHMIED, D. 2017. The Effects of Serotonin in Immune Cells. *Frontiers in Cardiovascular Medicine,* 4.

HERREL, A. & VAN DER MEIJDEN, A. 2014. An analysis of the live reptile and amphibian trade in the USA compared to the global trade in endangered species. *Herpetological Journal,* 24**,** 103-110.

HERRERO, E., ROS, J., BELLI, G. & CABISCOL, E. 2008. Redox control and oxidative stress in yeast cells. *Biochimica et Biophysica Acta,* 1780**,** 1217-35.

HILL, W. A., NEWMAN, S. J., CRAIG, L., CARTER, C., CZARRA, J. & BROWN, J. P. 2010. Diagnosis of *Aeromonas hydrophila*, Mycobacterium species, and

Batrachochytrium dendrobatidis in an African clawed frog (*Xenopus laevis*). *Journal of the American Associan of Laboratory Animal Science,* 49**,** 215-20.

HINES, H., MAHONY, M. & MCDONALD, K. 1999. An assessment of frog declines in wet subtropical Australia. Canberra.

HOLLIS, G. J. 1995. Reassessment of the distribution, abundance and habitat of the baw baw Frog *Philoria frosti Spence*r: preliminary findings. *Victorian Naturalist,* 112**,** 190-201.

HOLLIS, G. J. 2004. *Ecology and conservation biology of the baw baw frog Philoria frosti (Anura: Myobatrachidae): distribution, abundance, autoecology and demography.* Ph.D., University of Melbourne.

HOLLIS, G. J. 2011. *National recovery plan for the baw baw frog Philoria frosti* [Online]. Victoria: Department of Sustainability and Environment. [Accessed].

HORTON, T. L., STEWART, R., COHEN, N., RAU, L., RITCHIE, P., WATSON, M. D., ROBERT, J. & HORTON, J. D. 2003. Ontogeny of *Xenopus* NK cells in the absence of MHC class I antigens. *Developmental & Comparative Immunology,* 27**,** 715-726.

HUANG, J., LI, G., XIANG, J., YIN, D. & CHI, R. 2004. Immunohistochemical study of serotonin in lesions of psoriasis. *International Journal of Dermatology,* 43**,** 408-11.

HUNTER, D. 2013. Southern corroboree frog - species information sheet. Provided to the Department of Sustainability, Environment, Water, Population and Communities. NSW Office of Environment and Heritage.

HYATT, A. D., BOYLE, D. G., OLSEN, V., BOYLE, D. B., BERGER, L., OBENDORF, D., DALTON, A., KRIGER, K., HEROS, M., HINES, H., PHILLOTT, R., CAMPBELL, R., MARANTELLI, G., GLEASON, F. & COILING, A. 2007. Diagnostic assays and sampling protocols for the detection of *Batrachochytrium dendrobatidis*. *Diseases of Aquatic Organisms,* 73**,** 175-92.

IKEN, K., CHHENG, S., FARGIN, A., GOULET, A. C. & KOUASSI, E. 1995. Serotonin upregulates mitogen-stimulated B lymphocyte proliferation through 5-HT1A receptors. *Cellular Immunology,* 163**,** 1-9.

IPBES 2019a. Global assessment report on biodiversity and ecosystem services. Intergovernmental Science-Policy Platform on Biodiversity and Ecosystem Services (IPBES).

IPBES 2019b. Summary for policymakers of the global assessment report on biodiversity and ecosystem services of the Intergovernmental Science-Policy Platform on Biodiversity and Ecosystem Services. *In:* S. DÍAZ, J. S., E. S. BRONDIZIO E.S., H. T. NGO, M. GUÈZE, J. AGARD, A. ARNETH, P. BALVANERA, K. A. BRAUMAN, S. H. M. BUTCHART, K. M. A. CHAN, L. A. GARIBALDI, K. ICHII, J. LIU, S. M. SUBRAMANIAN, G. F. MIDGLEY, P. MILOSLAVICH, Z. MOLNÁR, D. OBURA, A. PFAFF, S. POLASKY, A. PURVIS, J. RAZZAQUE, B. REYERS, R. ROY CHOWDHURY, Y. J. SHIN, I. J. VISSEREN-HAMAKERS, K. J. WILLIS, AND C. N. ZAYAS (ed.). Bonn, Germany.

JAMES, T. Y., LITVINTSEVA, A. P., VILGALYS, R., MORGAN, J. A. T., TAYLOR, J. W., FISHER, M. C., BERGER, L., WELDON, C., DU PREEZ, L. & LONGCORE, J. E. 2009. Rapid global expansion of the fungal disease chytridiomycosis into declining and healthy amphibian Populations. *PLoS Pathogens,* 5**,** e1000458.

JANI, A. J. & BRIGGS, C. J. 2018. Host and Aquatic Environment Shape the Amphibian Skin Microbiome but Effects on Downstream Resistance to the Pathogen Batrachochytrium dendrobatidis Are Variable. *Frontiers in Microbiology,* 9.

JCU 2008. “Extinct" frog found thriving. James Cook University.

JOHANSSON, O., LIU, P.-Y., LIANG, Y., GANGI, S., BONDESSON, L., NORDLIND, K., OLSSON, M. J., LÖNTZ, W. & VERHOFSTAD, A. 1998. A Serotonin-like Immunoreactivity is Present in Human Cutaneous Melanocytes. *Journal of Investigative Dermatology,* 111**,** 1010-1014.

JOHNSON, M. L., BERGER, L., PHILIPS, L. & SPEARE, R. 2003. Fungicidal effects of chemical disinfectants, UV light, desiccation and heat on the amphibian chytrid *Batrachochytrium dendrobatidis*. *Diseases of Aquatic Organisms,* 57**,** 255-60.

JOHNSON, M. L. & SPEARE, R. 2003. Survival of *Batrachochytrium dendrobatidis* in water: quarantine and disease control implications. *Emerging Infectious Disease Journal,* 9**,** 922.

JOHNSON, M. L. & SPEARE, R. 2005. Possible modes of dissemination of the amphibian chytrid *Batrachochytrium dendrobatidis* in the environment. *Diseases of Aquatic Organisms,* 65**,** 181-6.

JONES, E. Y., FUGGER, L., STROMINGER, J. L. & SIEBOLD, C. 2006. MHC class II proteins and disease: a structural perspective. *Nature Reviews Immunology,* 6**,** 271-82.

JONESON, S., STAJICH, J. E., SHIU, S. H. & ROSENBLUM, E. B. 2011. Genomic transition to pathogenicity in chytrid fungi. *PLOS Pathogens*.

JONG, A. Y., CHEN, S. H. M., STINS, M. F., KIM, K. S., TUAN, T.-L. & HUANG, S.-H. 2003. Binding of *Candida albicans* enolase to plasmin(ogen) results in enhanced invasion of human brain microvascular endothelial cells. *Journal of Medical Microbiology,* 52**,** 615-622.

JOUSSON, O., LÉCHENNE, B., BONTEMS, O., CAPOCCIA, S., MIGNON, B., BARBLAN, J., QUADRONI, M. & MONOD, M. 2004. Multiplication of an ancestral gene encoding secreted fungalysin preceded species differentiation in the dermatophytes *Trichophyton* and *Microsporum*. *Microbiology,* 150**,** 301-310.

KAUMANN, A. J. & LEVY, F. O. 2006. 5-hydroxytryptamine receptors in the human cardiovascular system. *Pharmacology &Therapeutics,* 111**,** 674-706.

KELLEY, L. A., MEZULIS, S., YATES, C. M., WASS, M. N. & STERNBERG, M. J. E. 2015. The Phyre2 web portal for protein modeling, prediction and analysis. *Nature Protocols,* 10**,** 845-858.

KERR, J. F., WYLLIE, A. H. & CURRIE, A. R. 1972. Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics. *British Journal of Cancer,* 26**,** 239-257.

KIHARA, H. & SNELL, E. E. 1957. Spermine and related polyamines as growth stimulants for *Lactobacillus casei*. *Proceedings of the National Academy of Sciences of the United States of America,* 43**,** 867-871.

KIK, M., STEGE, M., BOONYARITTICHAIKIJ, R. & VAN ASTEN, A. 2012. Concurrent ranavirus and *Batrachochytrium dendrobatidis* infection in captive frogs (*Phyllobates* and *Dendrobates* species), the Netherlands, 2012: a first report. *Vet J,* 194**,** 247-9.

KILBURN, V. L., IBANEZ, R. & GREEN, D. M. 2011. Reptiles as potential vectors and hosts of the amphibian pathogen *Batrachochytrium dendrobatidis* in Panama. *Diseases of Aquatic Organisms,* 97**,** 127-34.

KIM, K. & HARVELL, C. D. 2004. The rise and fall of a six-year coral-fungal epizootic. *The American Naturalist,* 164**,** S52-S63.

KO, H.-C., HSIAO, T.-Y., CHEN, C.-T. & YANG, Y.-L. 2013. *Candida albicans* ENO1 null mutants exhibit altered drug susceptibility, hyphal formation, and virulence. *Journal of Microbiology,* 51**,** 345-351.

KOLBY, J. E. 2014. Presence of the amphibian chytrid fungus *Batrachochytrium dendrobatidis* in native amphibians exported from Madagascar. *PLOS ONE,* 9**,** e89660.

KOLBY, J. E., SMITH, K. M., BERGER, L., KARESH, W. B., PRESTON, A., PESSIER, A. P. & SKERRATT, L. F. 2014. First evidence of amphibian chytrid fungus *Batrachochytrium dendrobatidis* and ranavirus in Hong Kong amphibian trade. *PLoS ONE,* 9**,** e90750.

KOSCH, T. A., EIMES, J. A., DIDINGER, C., BRANNELLY, L. A., WALDMAN, B., BERGER, L. & SKERRATT, L. F. 2017. Characterization of MHC class IA in the endangered southern corroboree frog. *Immunogenetics,* 69**,** 165-174.

KOSIK-BOGACKA, D. I. & TYRAKOWSKI, T. 2006. The effect of serotonin on epinephrine modulation of transepithelial ion transport in isolated frog skin. *Folia Biologica (Krakow),* 54**,** 109-15.

KRISSINEL, E. & HENRICK, K. 2004. Secondary-structure matching (SSM), a new tool for fast protein structure alignment in three dimensions. *Acta Crystallographica Section D-Structural Biology,* 60**,** 2256-2268.

KROGH, A. 1937. Osmotic regulation in the frog (*R. esculenta*) by active absorption of chloride ions. *Skandinavisches Archiv Für Physiologie,* 76**,** 60-74.

KUMAR, A., RUSSELL, R. M., PIFER, R., MENEZES-GARCIA, Z., CUESTA, S., NARAYANAN, S., MACMILLAN, J. B. & SPERANDIO, V. 2020. The Serotonin Neurotransmitter Modulates Virulence of Enteric Pathogens. *Cell Host & Microbe,* 28**,** 41-53.e8.

LAM, B. A., WALKE, J. B., VREDENBURG, V. T. & HARRIS, R. N. 2010. Proportion of individuals with anti-*Batrachochytrium dendrobatidis* skin bacteria is associated with population persistence in the frog *Rana muscosa*. *Biological Conservation,* 143**,** 529-531.

LAMBRIS, J. D., PAPPAS, J., MAVROIDIS, M., WANG, Y., MANZONE, H., SCHWAGER, J., DU PASQUIER, L., SILIBOVSKY, R. & SWAGER, J. 1995. The third component of *Xenopus* complement: cDNA cloning, structural and functional analysis, and evidence for an alternate C3 transcript. *European Journal of Immunology,* 25**,** 572-8.

LASS-FLORL, C., DIERICH, M. P., FUCHS, D., SEMENITZ, E., JENEWEIN, I. & LEDOCHOWSKI, M. 2001. Antifungal properties of selective serotonin reuptake

inhibitors against *Aspergillus* species in vitro. *Journal of Antimicrobial Chemotherapy,* 48**,** 775-9.

LASS-FLÖRL, C., FUCHS, D., LEDOCHOWSKI, M., SPETH, C., DIERICH, M. P. & WURZNER, R. 2003. Antifungal properties of 5-hydroxytryptamine (serotonin) against *Candida* species *in vitro*. *Journal of Medical Microbiology,* 52**,** 169-71.

LE QUANG TRONG, Y. 1975. Etude de la peau et des glandes cutanees de quelques amphibiens du genre *Ptychadena*. *Annales de l'Universite d'Abidjan, Série E: Écology,* 8**,** 31-52.

LEE, J., KANG, Y., CHANG, J., SONG, J. & KIM, B.-K. 2020. Determination of Serotonin Concentration in Single Human Platelets through Single-Entity Electrochemistry. *ACS Sensors,* 5**,** 1943-1948.

LEE, J. D. & KOLATTUKUDY, P. E. 1995. Molecular cloning of the cDNA and gene for an elastinolytic aspartic proteinase from *Aspergillus fumigatus* and evidence of its secretion by the fungus during invasion of the host lung. *Infection & Immunity,* 63**,** 3796-803.

LEON-PONTE, M., AHERN, G. P. & O'CONNELL, P. J. 2007. Serotonin provides an accessory signal to enhance T-cell activation by signaling through the 5-HT7 receptor. *Blood,* 109**,** 3139-46.

LESURTEL, M., GRAF, R., ALEIL, B., WALTHER, D. J., TIAN, Y., JOCHUM, W., GACHET, C., BADER, M. & CLAVIEN, P.-A. 2006. Platelet-Derived Serotonin Mediates Liver Regeneration. *Science,* 312**,** 104.

LIEW, N., MAZON MOYA, M. J., WIERZBICKI, C. J., HOLLINSHEAD, M., DILLON, M. J., THORNTON, C. R., ELLISON, A., CABLE, J., FISHER, M. C. & MOSTOWY, S. 2017. Chytrid fungus infection in zebrafish demonstrates that the pathogen can parasitize non-amphibian vertebrate hosts. *Nature Communications,* 8**,** 15048.

LIPS, K. R., BREM, F., BRENES, R., REEVE, J. D., ALFORD, R. A., VOYLES, J., CAREY, C., LIVO, L., PESSIER, A. P. & COLLINS, J. P. 2006. Emerging infectious disease and the loss of biodiversity in a Neotropical amphibian community. *Proceedings of the National Academy of Sciences of the United States of America,* 103**,** 3165-3170.

LIPS, K. R., DIFFENDORFER, J., MENDELSON, J. R., III & SEARS, M. W. 2008. Riding the wave: reconciling the roles of disease and climate change in amphibian declines. *PLOS Biology,* 6**,** e72.

LIU, P. & STAJICH, J. E. 2015. Characterization of the carbohydrate binding module 18 gene family in the amphibian pathogen *Batrachochytrium dendrobatidis*. *Fungal Genetics and Biology,* 77**,** 31-39.

LLEWELYN, V., BERGER, L. & GLASS, B. D. 2020. Can models of percutaneous absorption based on in vitro data in frogs predict in vivo absorption? *PLoS One*.

LLEWELYN, V. K., BERGER, L. & GLASS, B. D. 2018. Regional variation in percutaneous absorption in the tree frog Litoria caerulea. *Environmental Toxicology and Pharmacology,* 60**,** 5-11.

LOCKSHIN, R. A. & WILLIAMS, C. M. 1965. Programmed cell death—I. cytology of degeneration in the intersegmental muscles of the Pernyi silkmoth. *Journal of Insect Physiology,* 11**,** 123-133.

LONGCORE, J. E., PESSIER, A. P. & NICHOLS, D. K. 1999. *Batrochochytrium dendrobatidis* gen. et sp. nov., a chytrid pathogenic to amphibians. *Mycologia.*

LUSHCHAK, V. I. 2012. Glutathione Homeostasis and Functions: Potential Targets for Medical Interventions. *Journal of Amino Acids,* 2012**,** 736837.

LUSHCHAK, V. I. 2014. Free radicals, reactive oxygen species, oxidative stress and its classification. *Chemico-Biological Interactions,* 224**,** 164-175.

MADEIRA, F., PARK, Y. M., LEE, J., BUSO, N., GUR, T., MADHUSOODANAN, N., BASUTKAR, P., TIVEY, A. R. N., POTTER, S. C., FINN, R. D. & LOPEZ, R. 2019. The EMBL-EBI search and sequence analysis tools APIs in 2019. *Nucleic acids research,* 47**,** W636-W641.

MALLET, J. 2007. Hybrid speciation. *Nature,* 446**,** 279-283.

MARICHAL, P., GORRENS, J., LAURIJSSENS, L., VERMUYTEN, K., VAN HOVE, C., LE JEUNE, L., VERHASSELT, P., SANGLARD, D., BORGERS, M., RAMAEKERS, F. C. S., ODDS, F. & VANDEN BOSSCHE, H. 1999. Accumulation of 3-ketosteroids induced by itraconazole in azole-resistant clinical *Candida albican*s isolates. *Antimicrobial Agents and Chemotherapy,* 43**,** 2663-2670.

MARTEL, A., BLOOI, M., ADRIAENSEN, C., VAN ROOIJ, P., BEUKEMA, W., FISHER, M. C., FARRER, R. A., SCHMIDT, B. R., TOBLER, U., GOKA, K., LIPS, K. R., MULETZ, C., ZAMUDIO, K. R., BOSCH, J., LÖTTERS, S., WOMBWELL, E., GARNER, T. W. J., CUNNINGHAM, A. A., SPITZEN-VAN DER SLUIJS, A., SALVIDIO, S., DUCATELLE, R., NISHIKAWA, K., NGUYEN, T. T., KOLBY, J. E., VAN BOCXLAER, I., BOSSUYT, F. & PASMANS, F. 2014. Recent introduction of a chytrid fungus endangers western palearctic salamanders. *Science,* 346**,** 630-631.

MARTEL, A., VAN ROOIJ, P., VERCAUTEREN, G., BAERT, K., VAN WAEYENBERGHE, L., DEBACKER, P., GARNER, T. W. J., WOELTJES, T., DUCATELLE, R., HAESEBROUCK, F. & PASMANS, F. 2011. Developing a safe antifungal treatment protocol to eliminate *Batrachochytrium dendrobatidis* from amphibians. *Medical Mycology,* 49**,** 143-149.

MATTHES, S., MOSIENKO, V., POPOVA, E., RIVALAN, M., BADER, M. & ALENINA, N. 2019. Targeted Manipulation of Brain Serotonin: RNAi-Mediated Knockdown of Tryptophan Hydroxylase 2 in Rats. *ACS Chemical Neuroscience,* 10**,** 3207-3217.

MAURER, M., OPITZ, M., HENZ, B. M. & PAUS, R. 1997. The mast cell products histamine and serotonin stimulate and TNF-alpha inhibits the proliferation of murine epidermal keratinocytes in situ. *The Journal of Dermatological Science,* 16**,** 79-84.

MAXWELL, S. L., FULLER, R. A., BROOKS, T. M. & WATSON, J. E. 2016. Biodiversity: the ravages of guns, nets and bulldozers. *Nature,* 536**,** 143-5.

MAYR, A., HINTERBERGER, G., DIERICH, M. P. & LASS-FLÖRL, C. 2005. Interaction of serotonin with *Candida albicans* selectively attenuates fungal virulence in vitro. *International Journal of Antimicrobial Agents,* 26**,** 335-337.

MCDONALD, K. R. 1992. Distribution patterns and conservation status of north Queensland rainforest frogs. Brisbane: Queensland Dept. of Environment and Heritage.

MCMAHON, T. A., BRANNELLY, L. A., CHATFIELD, M. W. H., JOHNSON, P. T. J., JOSEPH, M. B., MCKENZIE, V. J., RICHARDS-ZAWACKI, C. L., VENESKY, M. D. & ROHR, J. R. 2013. Chytrid fungus *Batrachochytrium dendrobatidis* has nonamphibian hosts and releases chemicals that cause pathology in the absence of infection. *Proceedings of the National Academy of Sciences,* 110**,** 210-215.

MCMAHON, T. A., SEARS, B. F., VENESKY, M. D., BESSLER, S. M., BROWN, J. M., DEUTSCH, K., HALSTEAD, N. T., LENTZ, G., TENOURI, N., YOUNG, S., CIVITELLO, D. J., ORTEGA, N., FITES, J. S., REINERT, L. K., ROLLINS-SMITH, L. A., RAFFEL, T. R. & ROHR, J. R. 2014. Amphibians acquire resistance to live and dead fungus overcoming fungal immunosuppression. *Nature,* 511**,** 224-227.

MEHDI, K. & PENNINCKX, M. J. 1997. An important role for glutathione and γ-glutamyltranspeptidase in the supply of growth requirements during nitrogen starvation of the yeast *Saccharomyces cerevisiae*. *Microbiology,* 143**,** 1885-1889.

MEZRICH, J. D., FECHNER, J. H., ZHANG, X., JOHNSON, B. P., BURLINGHAM, W. J. & BRADFIELD, C. A. 2010. An interaction between kynurenine and the aryl hydrocarbon receptor can generate regulatory T Cells. *The Journal of Immunology,* 185**,** 9.

MIAUD, C., DEJEAN, T., SAVARD, K., MILLERY-VIGUES, A., VALENTINI, A., CURT GRAND GAUDIN, N. & GARNER, T. W. J. 2016. Invasive north American

bullfrogs transmit lethal fungus *Batrachochytrium dendrobatidis* infections to native amphibian host species. *Biological Invasions,* 18**,** 2299-2308.

MILLER, M. A., MCGOWAN, S. E., GANTT, K. R., CHAMPION, M., NOVICK, S. L., ANDERSEN, K. A., BACCHI, C. J., YARLETT, N., BRITIGAN, B. E. & WILSON, M. E. 2000. Inducible resistance to oxidant stress in the protozoan *Leishmania chagasi*. *Journal of Biological Chemistry,* 275**,** 33883-9.

MISSALL, T. A., LODGE, J. K. & MCEWEN, J. E. 2004. Mechanisms of resistance to oxidative and nitrosative stress: implications for fungal survival in mammalian hosts. *Eukaryotic Cell,* 3**,** 835.

MÖLKÄNEN, T., TYYNELÄ, J., HELIN, J., KALKKINEN, N. & KUUSELA, P. 2002. Enhanced activation of bound plasminogen on *Staphylococcus aureus* by staphylokinase. *FEBS Letters,* 517**,** 72-78.

MOREHOUSE, E. A., JAMES, T. Y., GANLEY, A. R., VILGALYS, R., BERGER, L., MURPHY, P. J. & LONGCORE, J. E. 2003. Multilocus sequence typing suggests the chytrid pathogen of amphibians is a recently emerged clone. *Molecular Ecology,* 12**,** 395-403.

MORGAN, J. A. T., VREDENBURG, V. T., RACHOWICZ, L. J., KNAPP, R. A., STICE, M. J., TUNSTALL, T., BINGHAM, R. E., PARKER, J. M., LONGCORE, J. E., MORITZ, C., BRIGGS, C. J. & TAYLOR, J. W. 2007. Population genetics of the frog-killing fungus *Batrachochytrium dendrobatidis*. *Proceedings of the National Academy of Sciences,* 104**,** 13845-13850.

MOSS, A. S., CARTY, N. & SAN FRANCISCO, M. J. 2010. Identification and partial characterization of an elastolytic protease in the amphibian pathogen *Batrachochytrium dendrobatidis*. *Diseases of Aquatic Organisms,* 92**,** 149-58.

MURRAY, K. A., RETALLICK, R. W. R., PUSCHENDORF, R., SKERRATT, L. F., ROSAUER, D., MCCALLUM, H. I., BERGER, L., SPEARE, R. & VANDERWAL, J. 2011. Assessing spatial patterns of disease risk to biodiversity: implications for the

management of the amphibian pathogen, *Batrachochytrium dendrobatidis*. *Journal of Applied Ecology,* 48**,** 163-173.

MYERS, J. M., RAMSEY, J. P., BLACKMAN, A. L., NICHOLS, A. E., MINBIOLE, K. P. & HARRIS, R. N. 2012. Synergistic inhibition of the lethal fungal pathogen *Batrachochytrium dendrobatidis*: the combined effect of symbiotic bacterial metabolites and antimicrobial peptides of the frog *Rana muscosa*. *Journal of Chemical Ecology,* 38**,** 958-65.

NELDON-ORTIZ, D. L. & QURESHI, M. A. 1991. Direct and microsomal activated aflatoxin B1 exposure and its effects on turkey peritoneal macrophage functions in vitro. *Toxicology & Applied Pharmacology,* 109**,** 432-42.

NEWMAN, S. F., SULTANA, R., PERLUIGI, M., COCCIA, R., CAI, J., PIERCE, W. M., KLEIN, J. B., TURNER, D. M. & BUTTERFIELD, D. A. 2007. An increase in S-glutathionylated proteins in the Alzheimer's disease inferior parietal lobule, a proteomics approach. *Journal of Neuroscience Research,* 85**,** 1506-1514.

NORDLIND, K., SUNDSTRÖM, E. & BONDESSON, L. 1992. Inhibiting effects of serotonin antagonists on the proliferation of mercuric chloride stimulated human peripheral blood T lymphocytes. *International Archives of Allergy Immunology,* 97**,** 105-8.

NPWS 2001. Recovery plan for the spotted tree frog (*Litoria spenceri*). Hurstville, New South Wales: NSW National Park and Wildlife Service.

O'HANLON, S. J., RIEUX, A., FARRER, R. A., ROSA, G. M., WALDMAN, B., BATAILLE, A., KOSCH, T. A., MURRAY, K. A., BRANKOVICS, B., FUMAGALLI, M., MARTIN, M. D., WALES, N., ALVARADO-RYBAK, M., BATES, K. A., BERGER, L., BÖLL, S., BROOKES, L., CLARE, F., COURTOIS, E. A., CUNNINGHAM, A. A., DOHERTY-BONE, T. M., GHOSH, P., GOWER, D. J., HINTZ, W. E., HÖGLUND, J., JENKINSON, T. S., LIN, C.-F., LAURILA, A., LOYAU, A., MARTEL, A., MEURLING, S., MIAUD, C., MINTING, P., PASMANS, F., SCHMELLER, D. S., SCHMIDT, B. R., SHELTON, J. M. G.,

SKERRATT, L. F., SMITH, F., SOTO-AZAT, C., SPAGNOLETTI, M., TESSA, G., TOLEDO, L. F., VALENZUELA-SÁNCHEZ, A., VERSTER, R., VÖRÖS, J., WEBB, R. J., WIERZBICKI, C., WOMBWELL, E., ZAMUDIO, K. R., AANENSEN, D. M., JAMES, T. Y., GILBERT, M. T. P., WELDON, C., BOSCH, J., BALLOUX, F., GARNER, T. W. J. & FISHER, M. C. 2018. Recent Asian origin of chytrid fungi causing global amphibian declines. *Science,* 360**,** 621-627.

OIE. 2019. *OIE-listed diseases 2019* [Online]. World Organisation for Animal Health. Available: http://www.oie.int/animal-health-in-the-world/oie-listed-diseases-2019/ [Accessed 2019].

OLSON, D. H. & RONNENBERG, K. L. 2014. Global mapping project. *FrogLog.*

OSBORNE, W. 1989. Distribution, relative abundance and conservation status of corroboree frogs, *Pseudophrne corroboree moore* (Anura, Myobatrachidae). *Wildlife Research,* 16**,** 537-547.

PALEGO, L., BETTI, L., ROSSI, A. & GIANNACCINI, G. 2016. Tryptophan Biochemistry: Structural, Nutritional, Metabolic, and Medical Aspects in Humans. *Journal of amino acids,* 2016**,** 8952520-8952520.

PARSONS, R. H. & MOBIN, F. 1991. Water flow across the pectoral and ventral pelvic patch in *Rana catesbeiana*. *Physiological Zoology,* 64**,** 812-822.

PASMANS, F., ZWART, P. & HYATT, A. D. 2004. Chytridiomycosis in the central American bolitoglossine salamander *Bolitoglossa dofleini*. *Veterinary Record,* 154**,** 153-153.

PENNINCKX, M. J. 2002. An overview on glutathione in *Saccharomyces* versus non-conventional yeasts. *FEMS Yeast Research,* 2**,** 295-305.

PERKHOFER, S., NIEDEREGGER, H., BLUM, G., BURGSTALLER, W., LEDOCHOWSKI, M., DIERICH, M. P. & LASS-FLÖRL, C. 2007. Interaction of 5-

hydroxytryptamine (serotonin) against *Aspergillus* spp. in vitro. *International Journal of Antimicrobial Agents,* 29**,** 424-429.

PESSIER, A. P. 2008. Management of disease as a threat to amphibian conservation. *International Zoo Yearbook,* 42**,** 30-39.

PETERSON, A. C. & MCKENZIE, V. J. 2014. Investigating differences across host species and scales to explain the distribution of the amphibian pathogen *Batrachochytrium dendrobatidis*. *PLoS ONE,* 9**,** e107441.

PHILIPPE, B., IBRAHIM-GRANET, O., PRÉVOST, M. C., GOUGEROT-POCIDALO, M. A., SANCHEZ PEREZ, M., VAN DER MEEREN, A. & LATGÉ, J. P. 2003. Killing of *Aspergillus fumigatus* by alveolar macrophages is mediated by reactive oxidant intermediates. *Infection and Immunity,* 71**,** 3034-3042.

PICCO, A. M. & COLLINS, J. P. 2007. Fungal and viral pathogen occurrence in Costa Rican amphibians. *Journal of Herpetology,* 41**,** 746-749.

PIOTROWSKI, J. S., ANNIS, S. L. & LONGCORE, J. E. 2004. Physiology of *Batrachochytrium dendrobatidis*, a chytrid pathogen of amphibians. *Mycologia,* 96**,** 9-15.

PIOVIA-SCOTT, J., POPE, K., JOY WORTH, S., ROSENBLUM, E. B., POORTEN, T., REFSNIDER, J., ROLLINS-SMITH, L. A., REINERT, L. K., WELLS, H. L., REJMANEK, D., LAWLER, S. & FOLEY, J. 2014. Correlates of virulence in a frog-killing fungal pathogen: evidence from a California amphibian decline. *The ISME Journal,* 9**,** 1570.

PRIYA, V. K., SARKAR, S. & SINHA, S. 2014. Evolution of tryptophan biosynthetic pathway in microbial genomes: a comparative genetic study. *Systems and synthetic biology,* 8**,** 59-72.

PUSCHENDORF, R. & BOLANOS, F. 2006. Detection of *Batrachochytrium dendrobatidis* in *Eleutherodactylus fitzingeri*: effects of skin sample location and histologic stain. *Journal of Wildlife Diseases,* 42**,** 301-6.

RACHOWICZ, L. J., HERO, J.-M., ALFORD, R. A., TAYLOR, J. W., MORGAN, J. A. T., VREDENBURG, V. T., COLLINS, J. P. & BRIGGS, C. J. 2005. The novel and endemic pathogen hypotheses: competing explanations for the origin of emerging infectious diseases of wildlife. *Conservation Biology,* 19**,** 1441-1448.

RACHOWICZ, L. J., KNAPP, R. A., MORGAN, J. A. T., STICE, M. J., VREDENBURG, V. T., PARKER, J. M. & BRIGGS, C. J. 2006. Emerging infectious disease as a proximate cause of amphibian mass mortality. *Ecology,* 87**,** 1671-1683.

RACHOWICZ, L. J. & VREDENBURG, V. T. 2004. Transmission of *Batrachochytrium dendrobatidis* within and between amphibian life stages. *Diseases of Aquatic Organisms,* 61**,** 75-83.

RAMSDALE, M. 2006. Programmed cell death and apoptosis in fungi. *In:* BROWN, A. J. P. (ed.) *Fungal Genomics.* Berlin, Heidelberg: Springer Berlin Heidelberg.

RAMSEY, J. P., REINERT, L. K., HARPER, L. K., WOODHAMS, D. C. & ROLLINS-SMITH, L. A. 2010. Immune defenses against *Batrachochytrium dendrobatidis*, a fungus linked to global amphibian declines, in the South African clawed frog, *Xenopus laevis*. *Integrative and Comparative Biology,* 51**,** 10.

REED, K. D., RUTH, G. R., MEYER, J. A. & SHUKLA, S. K. 2000. *Chlamydia pneumoniae* infection in a breeding colony of African clawed frogs (*Xenopus tropicalis*). *Emerging Infectious Diseases,* 6**,** 196-199.

RIBAS, L., LI, M.-S., DODDINGTON, B. J., ROBERT, J., SEIDEL, J. A., KROLL, J. S., ZIMMERMAN, L. B., GRASSLY, N. C., GARNER, T. W. J. & FISHER, M. C. 2009. Expression profiling the temperature-dependent amphibian response to infection by *Batrachochytrium dendrobatidis*. *PLoS ONE,* 4**,** e8408.

RICHARDS, S. J. 1993. A guide to the identification of declining frogs and their tadpoles in the Wet Tropics Biogeographical Region, Queensland. Queensland Department of Environment and Heritage.

RICHARDS, T. A., SOANES, D. M., JONES, M. D. M., VASIEVA, O., LEONARD, G., PASZKIEWICZ, K., FOSTER, P. G., HALL, N. & TALBOT, N. J. 2011. Horizontal gene transfer facilitated the evolution of plant parasitic mechanisms in the oomycetes. *Proceedings of the National Academy of Sciences,* 108**,** 15258-15263.

ROBERTS, A. A., BERGER, L., ROBERTSON, S. G., WEBB, R. J., KOSCH, T. A., MCFADDEN, M., SKERRATT, L. F., GLASS, B. D., MOTTI, C. A. & BRANNELLY, L. A. 2018. The efficacy and pharmacokinetics of terbinafine against the frog-killing fungus (*Batrachochytrium dendrobatidis*). *Medical Mycology***,** myy010-myy010.

RODRIGUEZ, D., BECKER, C. G., PUPIN, N. C., HADDAD, C. F. B. & ZAMUDIO, K. R. 2014. Long-term endemism of two highly divergent lineages of the amphibian-killing fungus in the Atlantic Forest of Brazil. *Molecular Ecology,* 23**,** 774-787.

ROLLINS-SMITH, L. A. 2009. The role of amphibian antimicrobial peptides in protection of amphibians from pathogens linked to global amphibian declines. *Biochimica et Biophysica Acta (BBA) - Biomembranes,* 1788**,** 1593-1599.

ROLLINS-SMITH, L. A., CAREY, C., CONLON, J. M., REINERT, L. K., DOERSAM, J. K., BERGMAN, T., SILBERRING, J., LANKINEN, H. & WADE, D. 2003. Activities of temporin family peptides against the chytrid fungus (*Batrachochytrium dendrobatidis*) associated with global amphibian declines. *Antimicrobial Agents & Chemotherapy,* 47**,** 1157-60.

ROLLINS-SMITH, L. A. & CONLON, J. M. 2005. Antimicrobial peptide defenses against chytridiomycosis, an emerging infectious disease of amphibian populations. *Developmental & Comparative Immunology,* 29**,** 589-98.

ROLLINS-SMITH, L. A., FITES, J. S., REINERT, L. K., SHIAKOLAS, A. R., UMILE, T. P. & MINBIOLE, K. P. C. 2015. Immunomodulatory metabolites released by the frog-killing fungus *Batrachochytrium dendrobatidis*. *Infection and Immunity,* 83**,** 4565-4570.

ROLLINS-SMITH, L. A., RAMSEY, J. P., PASK, J. D., REINERT, L. K. & WOODHAMS, D. C. 2011. *Amphibian immune defenses against chytridiomycosis: impacts of changing environments* [Online]. Oxford University Press. [Accessed].

ROLLINS-SMITH, L. A., RAMSEY, J. P., REINERT, L. K., WOODHAMS, D. C., LIVO, L. J. & CAREY, C. 2009. Immune defenses of *Xenopus laevis* against *Batrachochytrium dendrobatidis*. *Frontiers in Bioscience (Scholar Edition),* 1**,** 68-91.

ROLLINS-SMITH, L. A., REINERT, L. K., MIERA, V. & CONLON, J. M. 2002. Antimicrobial peptide defenses of the Tarahumara frog, *Rana tarahumarae*. *Biochemical & Biophysical Research Communications,* 297**,** 361-7.

ROLLINS-SMITH, L. A., RUZZINI, A. C., FITES, J. S., REINERT, L. K., HALL, E. M., JOOSSE, B. A., RAVIKUMAR, V. I., HUEBNER, M. I., AKA, A., KEHS, M. H., GILLARD, B. M., DOE, E., TASCA, J. A., UMILE, T. P., CLARDY, J. & MINBIOLE, K. P. C. 2019. Metabolites involved in immune evasion by *Batrachochytrium dendrobatidis* include the polyamine spermidine. *Infection and Immunity,* 87**,** e00035-19.

ROLLINS-SMITH, L. A., WOODHAMS, D. C., REINERT, L. K., VREDENBURG, V. T., BRIGGS, C. J., NIELSEN, P. F. & MICHAEL CONLON, J. 2006. Antimicrobial peptide defenses of the mountain yellow-legged frog (Rana muscosa). *Developmental & Comparative Immunology,* 30**,** 831-842.

ROSEGHINI, M., ERSPAMER, V., ERSPAMER, G. F. & CEI, J. M. 1986. Indole-, imidazole- and phenyl-alkylamines in the skin of one hundred and forty american amphibian species other than bufonids. *Comparative Biochemistry and Physiology Part C: Comparative Pharmacology,* 85**,** 139-147.

ROSEGHINI, M., FALCONIERI ERSPAMER, G. & SEVERINI, C. 1988. Biogenic amines and active peptides in the skin of fifty-two African amphibian species other than bufonids. *Comparative Biochemistry & Physiology C,* 91**,** 281-6.

ROSENBLUM, E. B., FISHER, M. C., JAMES, T. Y., STAJICH, J. E., LONGCORE, J. E., GENTRY, L. R. & POORTEN, T. J. 2011. A molecular perspective: biology of the emerging pathogen *Batrachochytrium dendrobatidis*. *Diseases of Aquatic Organisms,* 92**,** 131-47.

ROSENBLUM, E. B., POORTEN, T. J., JONESON, S. & SETTLES, M. 2012. Substrate-specific gene expression in *Batrachochytrium dendrobatidis*, the chytrid pathogen of amphibians. *PLoS ONE,* 7**,** e49924.

ROSENBLUM, E. B., POORTEN, T. J., SETTLES, M., MURDOCH, G. K., ROBERT, J., MADDOX, N. & EISEN, M. B. 2009. Genome-wide transcriptional response of *Silurana* (*Xenopus*) *tropicalis* to infection with the deadly chytrid fungus. *PLoS ONE,* 4**,** e6494.

ROSENBLUM, E. B., STAJICH, J. E., MADDOX, N. & EISEN, M. B. 2008. Global gene expression profiles for life stages of the deadly amphibian pathogen *Batrachochytrium dendrobatidis*. *Proceedings of the National Academy of Sciences USA,* 105**,** 17034-9.

ROWLEY, J. J. & ALFORD, R. A. 2007. Behaviour of Australian rainforest stream frogs may affect the transmission of chytridiomycosis. *Diseases of Aquatic Organisms,* 77**,** 1-9.

ROWLEY, J. J. L. & ALFORD, R. A. 2013. Hot bodies protect amphibians against chytrid infection in nature. *Scientific Reports,* 3**,** 1515.

RUGGERI, J., RIBEIRO, L. P., PONTES, M. R., TOFFOLO, C., CANDIDO, M., CARRIERO, M. M., ZANELLA, N., SOUSA, R. L. M. & TOLEDO, L. F. 2019. First case of wild amphibians infected with ranavirus in Brazil. *Journal of Wildlife Diseases,* 55.

RULAND, A. & KREUTER, J. 1991. Transdermal permeability and skin accumulation of amino acids. *International Journal of Pharmaceutics,* 72**,** 149-155.

SADRI, A.-R., JESCHKE, M. G. & AMINI-NIK, S. 2016. Advances in Liver Regeneration: Revisiting Hepatic Stem/Progenitor Cells and Their Origin. *Stem cells international,* 2016**,** 7920897-7920897.

SARMIENTO-RAMIREZ, J. M., ABELLA, E., MARTIN, M. P., TELLERIA, M. T., LOPEZ-JURADO, L. F., MARCO, A. & DIEGUEZ-URIBEONDO, J. 2010. *Fusarium solani* is responsible for mass mortalities in nests of loggerhead sea turtle, *Caretta caretta*, in Boavista, Cape Verde. *FEMS Microbiology Letters,* 312**,** 192-200.

SAVAGE, A. E. & ZAMUDIO, K. R. 2011. MHC genotypes associate with resistance to a frog-killing fungus. *Proceedings of the National Academy of Sciences,* 108**,** 16705-16710.

SCHEELE, B. C., HUNTER, D. A., BRANNELLY, L. A., SKERRATT, L. F. & DRISCOLL, D. A. 2016. Reservoir-host amplification of disease impact in an endangered amphibian. *Conservation Biology,* 31**,** 592-600.

SCHEELE, B. C., PASMANS, F., SKERRATT, L. F., BERGER, L., MARTEL, A., BEUKEMA, W., ACEVEDO, A. A., BURROWES, P. A., CARVALHO, T., CATENAZZI, A., DE LA RIVA, I., FISHER, M. C., FLECHAS, S. V., FOSTER, C. N., FRIAS-ALVAREZ, P., GARNER, T. W. J., GRATWICKE, B., GUAYASAMIN, J. M., HIRSCHFELD, M., KOLBY, J. E., KOSCH, T. A., LA MARCA, E., LINDENMAYER, D. B., LIPS, K. R., LONGO, A. V., MANEYRO, R., MCDONALD, C. A., MENDELSON, J. I., PALACIOS-RODRIGUEZ, P., PARRA-OLEA, G., RICHARDS-ZAWACKI, C. L., RODEL, M.-O., ROVITO, S. M., SOTO-AZAT, C., TOLEDO, L. F., VOYLES, J., WELDON, C., WHITFIELD, S. M., WILKINSON, M., ZAMUDIO, K. R. & CANESSA, S. 2019. Amphibian fungal panzootic causes catastrophic and ongoing loss of biodiversity. *Science,* 363**,** 1459-1463.

SCHEELE, B. C., SKERRATT, L. F., GROGAN, L. F., HUNTER, D. A., CLEMANN, N., MCFADDEN, M., NEWELL, D., HOSKIN, C. J., GILLESPIE, G. R., HEARD, G. W., BRANNELLY, L., ROBERTS, A. A. & BERGER, L. 2017. After the epidemic: ongoing declines, stabilizations and recoveries in amphibians afflicted by chytridiomycosis. *Biological Conservation,* 206**,** 37-46.

SCHLOEGEL, L. M., TOLEDO, L. F., LONGCORE, J. E., GREENSPAN, S. E., VIEIRA, C. A., LEE, M., ZHAO, S., WANGEN, C., FERREIRA, C. M., HIPOLITO, M., DAVIES, A. J., CUOMO, C. A., DASZAK, P. & JAMES, T. Y. 2012. Novel, panzootic and hybrid genotypes of amphibian chytridiomycosis associated with the bullfrog trade. *Molecular Ecology,* 21**,** 5162-77.

SCHNEIDER, C. A., RASBAND, W. S. & ELICEIRI, K. W. 2012. NIH image to ImageJ: 25 years of image analysis. *Nature Methods,* 9**,** 671-675.

SCHNEIDER, U., SCHWENK, H.-U. & BORNKAMM, G. 1977. Characterization of EBV-genome negative "null" and "T" cell lines derived from children with acute lymphoblastic leukemia and leukemic transformed non-Hodgkin lymphoma. *International Journal of Cancer,* 19**,** 621-626.

SEARLE, C. L., MENDELSON, J. R., GREEN, L. E. & DUFFY, M. A. 2013. Daphnia predation on the amphibian chytrid fungus and its impacts on disease risk in tadpoles. *Ecology and Evolution,* 3**,** 4129-4138.

SEKI, T., KIKUYAMA, S. & YANAIHARA, N. 1989. Development of *Xenopus laevis* skin glands producing 5-hydroxytryptamine and caerulein. *Cell and Tissue Research,* 258**,** 483-489.

SEKI, T., KIKUYAMA, S. & YANAIHARA, N. 1995. *Morphology of the skin glands of the crab-eating frog Rana cancrivora*, BIOONE.

ŞENGEZER-İNCELI, M., SÜREN, S., MURATHANOĞLU, O. & KAPTAN, E. 2004. Immunohistochemical detection of serotonin in the skin of frogs (*Rana ridibunda*) kept at different temperatures. *Biologia,* 59**,** 273-281.

SERAFEIM, A., GRAFTON, G., CHAMBA, A., GREGORY, C. D., BLAKELY, R. D., BOWERY, N. G., BARNES, N. M. & GORDON, J. 2002. 5-Hydroxytryptamine drives apoptosis in biopsylike Burkitt lymphoma cells: reversal by selective serotonin reuptake inhibitors. *Blood,* 99**,** 2545-2553.

SHAPARD, E. J., MOSS, A. S. & SAN FRANCISCO, M. J. 2012. *Batrachochytrium dendrobatidis* can infect and cause mortality in the nematode *Caenorhabditis elegans*. *Mycopathologia,* 173**,** 121-6.

SHLEZINGER, N., GOLDFINGER, N. & SHARON, A. 2012. Apoptotic-like programed cell death in fungi: the benefits in filamentous species. *Frontiers in Oncology,* 2**,** 97-97.

SIES, H. 1999. Glutathione and its role in cellular functions. *Free Radical Biology and Medicine,* 27**,** 916-921.

SIMBERLOFF, D. 2014. Biological invasions: what's worth fighting and what can be won? *Ecological Engineering,* 65**,** 112-121.

SINGH, S. 2004. Trials of new antiarrhythmic drugs for maintenance of sinus rhythm in patients with atrial fibrillation. *Journal of Interventional Cardiac Electrophysiology,* 10 Suppl 1**,** 71-6.

SKERRATT, L. F., BERGER, L., CLEMANN, N., HUNTER, D. A., MARANTELLI, G., NEWELL, D. A., PHILIPS, A., MCFADDEN, M., HINES, H. B., SCHEELE, B. C., BRANNELLY, L. A., SPEARE, R., VERSTEEGEN, S., CASHINS, S. D. & WEST, M. 2016. Priorities for management of chytridiomycosis in Australia: saving frogs from extinction. *Wildlife Research,* 43**,** 105-120, 16.

SKERRATT, L. F., BERGER, L., HINES, H. B., MCDONALD, K. R., MENDEZ, D. & SPEARE, R. 2008. Survey protocol for detecting chytridiomycosis in all Australian frog populations. *Diseases of Aquatic Organisms,* 80**,** 85-94.

SKERRATT, L. F., BERGER, L., SPEARE, R., CASHINS, S., MCDONALD, K. R., PHILLOTT, A. D., HINES, H. B. & KENYON, N. 2007. Spread of chytridiomycosis has caused the rapid global decline and extinction of frogs. *Ecohealth,* 4**,** 125-134.

SLOMINSKI, A. T., KIM, T.-K., KLESZCZYŃSKI, K., SEMAK, I., JANJETOVIC, Z., SWEATMAN, T., SKOBOWIAT, C., STEKETEE, J. D., LIN, Z., POSTLETHWAITE, A., LI, W., REITER, R. J. & TOBIN, D. J. 2020. Characterization of serotonin and N-acetylserotonin systems in the human epidermis and skin cells. *Journal of pineal research,* 68**,** e12626-e12626.

SOTO-AZAT, C., CLARKE, B. T., POYNTON, J. C. & CUNNINGHAM, A. A. 2010. Widespread historical presence of *Batrachochytrium dendrobatidis* in African pipid frogs. *Diversity and Distributions,* 16**,** 126-131.

STERNBACH, H. 1991. The serotonin syndrome. *American Journal of Psychiatry,* 148**,** 705-13.

STERNBERG, E. M., TRIAL, J. & PARKER, C. W. 1986. Effect of serotonin on murine macrophages: suppression of Ia expression by serotonin and its reversal by 5-HT2 serotonergic receptor antagonists. *The Journal of Immunology,* 137**,** 276-282.

STEVENSON, L. A., ALFORD, R. A., BELL, S. C., ROZNIK, E. A., BERGER, L. & PIKE, D. A. 2013. Variation in thermal performance of a widespread pathogen, the amphibian chytrid fungus *Batrachochytrium dendrobatidis*. *PLoS ONE,* 8**,** e73830.

STICE, M. J. & BRIGGS, C. J. 2010. Immunization is ineffective at preventing infection and mortality due to the amphibian chytrid fungus *Batrachochytrium dendrobatidis*. *Journal of Wildlife Diseases,* 46**,** 70-7.

STOCKWELL, M. P., CLULOW, J. & MAHONY, M. J. 2012. Sodium chloride inhibits the growth and infective capacity of the amphibian chytrid fungus and increases host survival rates. *PLoS ONE,* 7**,** e36942.

STRAUSS, A. & SMITH, K. G. 2013. Why does amphibian chytrid (*Batrachochytrium dendrobatidis*) not occur everywhere? an exploratory study in Missouri ponds. *PLoS ONE,* 8**,** e76035.

STUKENBROCK, E. H. & MCDONALD, B. A. 2008. The origins of plant pathogens in agro-ecosystems. *Annual Review of Phytopathology,* 46**,** 75-100.

SUN, B., LI, T., XIAO, J., LIU, L., ZHANG, P., MURPHY, R. W., HE, S. & HUANG, D. 2016. Contribution of multiple inter-kingdom horizontal gene transfers to evolution and adaptation of amphibian-killing chytrid, *Batrachochytrium dendrobatidis*. *Frontiers in Microbiology,* 7.

SUN, G., YANG, Z., KOSCH, T., SUMMERS, K. & HUANG, J. 2011. Evidence for acquisition of virulence effectors in pathogenic chytrids. *BMC Evolutionary Biology,* 11**,** 195.

SYMONDS, E. P., TROTT, D. J., BIRD, P. S. & MILLS, P. 2008. Growth characteristics and enzyme activity in *Batrachochytrium dendrobatidis* isolates. *Mycopathologia,* 166**,** 143-147.

TECOTT, L. H. 2007. Serotonin and the orchestration of energy balance. *Cell Metabolism,* 6**,** 352-61.

THE UNIVERSITY OF QUEENSLAND, A. 2017. *Using ImageJ to quantify blots* [Online]. Available: https://di.uq.edu.au/community-and-alumni/sparq-ed/sparq-ed-services/using-imagej-quantify-blots [Accessed 2019].

THEKKINIATH, J., ZABET-MOGHADDAM, M., KOTTAPALLI, K. R., PASHAM, M. R., SAN FRANCISCO, S. & SAN FRANCISCO, M. 2015. Quantitative proteomics of an amphibian pathogen, *Batrachochytrium dendrobatidis*, following exposure to thyroid hormone. *PLOS ONE,* 10**,** e0123637.

THEKKINIATH, J. C., ZABET-MOGHADDAM, M., SAN FRANCISCO, S. K. & SAN FRANCISCO, M. J. 2013. A novel subtilisin-like serine protease of

Batrachochytrium dendrobatidis is induced by thyroid hormone and degrades antimicrobial peptides. *Fungal Biology,* 117**,** 451-61.

THOMAS, C. D., CAMERON, A., GREEN, R. E., BAKKENES, M., BEAUMONT, L. J., COLLINGHAM, Y. C., ERASMUS, B. F. N., DE SIQUEIRA, M. F., GRAINGER, A., HANNAH, L., HUGHES, L., HUNTLEY, B., VAN JAARSVELD, A. S., MIDGLEY, G. F., MILES, L., ORTEGA-HUERTA, M. A., TOWNSEND PETERSON, A., PHILLIPS, O. L. & WILLIAMS, S. E. 2004. Extinction risk from climate change. *Nature,* 427**,** 145-148.

TOLEDO, R. C. & JARED, C. 1995. Cutaneous granular glands and amphibian venoms. *Comparative Biochemistry and Physiology Part A: Physiology,* 111**,** 1-29.

TROFA, D., AGOVINO, M., STEHR, F., SCHÄFER, W., RYKUNOV, D., FISER, A., HAMARI, Z., NOSANCHUK, J. D. & GÁCSER, A. 2009. Acetylsalicylic acid (aspirin) reduces damage to reconstituted human tissues infected with Candida species by inhibiting extracellular fungal lipases. *Microbes and Infection,* 11**,** 1131-1139.

USSING, H. H. 1949. The active ion transport through the isolated frog skin in the light of tracer studies. *Acta Physiologica Scandinavica,* 17**,** 1-37.

VAN NUETEN, J. M., JANSSENS, W. J. & VANHOUTTE, P. M. 1985. Serotonin and vascular reactivity. *Pharmacological Research Communications,* 17**,** 585-608.

VAN ROOIJ, P., MARTEL, A., D'HERDE, K., BRUTYN, M., CROUBELS, S., DUCATELLE, R., HAESEBROUCK, F. & PASMANS, F. 2012. Germ tube mediated invasion of *Batrachochytrium dendrobatidis* in amphibian skin Is host dependent. *PLoS ONE,* 7**,** e41481.

VAN ROOIJ, P., MARTEL, A., NERZ, J., VOITEL, S., VAN IMMERSEEL, F., HAESEBROUCK, F. & PASMANS, F. 2011. Detection of *Batrachochytrium dendrobatidis* in Mexican bolitoglossine salamanders using an optimal sampling protocol. *Ecohealth,* 8**,** 237-43.

VANABLE, J. W. 1964. Granular gland development during *Xenopus laevis* metamorphosis. *Developmental Biology,* 10**,** 331-357.

VOYLES, J., JOHNSON, L. R., BRIGGS, C. J., CASHINS, S. D., ALFORD, R. A., BERGER, L., SKERRATT, L. F., SPEARE, R. & ROSENBLUM, E. B. 2012. Temperature alters reproductive life history patterns in *Batrachochytrium dendrobatidis*, a lethal pathogen associated with the global loss of amphibians. *Ecology & Evolution,* 2**,** 2241-9.

VOYLES, J., JOHNSON, L. R., BRIGGS, C. J., CASHINS, S. D., ALFORD, R. A., BERGER, L., SKERRATT, L. F., SPEARE, R. & ROSENBLUM, E. B. 2014. Experimental evolution alters the rate and temporal pattern of population growth in *Batrachochytrium dendrobatidis*, a lethal fungal pathogen of amphibians. *Ecology and Evolution,* 4**,** 3633-3641.

VOYLES, J., YOUNG, S., BERGER, L., CAMPBELL, C., VOYLES, W. F., DINUDOM, A., COOK, D., WEBB, R., ALFORD, R. A., SKERRATT, L. F. & SPEARE, R. 2009. Pathogenesis of chytridiomycosis, a cause of catastrophic amphibian declines. *Science,* 326**,** 582-585.

WALKER, M. J., MCARTHUR, J. D., MCKAY, F. & RANSON, M. 2005. Is plasminogen deployed as a Streptococcus pyogenes virulence factor? *Trends in Microbiology,* 13**,** 308-313.

WANG, H., ZHOU, N., ZHANG, R., WU, Y., ZHANG, R. & ZHANG, S. 2014. Identification and localization of gastrointestinal hormones in the skin of the bullfrog *Rana catesbeiana* during periods of activity and hibernation. *Acta Histochemica,* 116**,** 1418-1426.

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***,** 105688.

WELDON, C., DU PREEZ, L. H., HYATT, A. D., MULLER, R. & SPEARE, R. 2004. Origin of the amphibian chytrid fungus. *Emerging Infectious Diseases,* 10**,** 2100-2105.

WELSH, D. J., HARNETT, M., MACLEAN, M. & PEACOCK, A. J. 2004. Proliferation and Signaling in Fibroblasts. *American Journal of Respiratory and Critical Care Medicine,* 170**,** 252-259.

WHITFIELD, S. M., GEERDES, E., CHACON, I., BALLESTERO RODRIGUEZ, E., JIMENEZ, R. R., DONNELLY, M. A. & KERBY, J. L. 2013. Infection and co-infection by the amphibian chytrid fungus and ranavirus in wild Costa Rican frogs. *Diseases of Aquatic Organisms,* 104**,** 173-8.

WHITFORD, W. G. & HUTCHISON, V. H. 1965. Gas exchange in salamanders. *Physiological Zoology,* 38**,** 228-242.

WILLIAMS, J. H., PHILLIPS, T. D., JOLLY, P. E., STILES, J. K., JOLLY, C. M. & AGGARWAL, D. 2004. Human aflatoxicosis in developing countries: a review of toxicology, exposure, potential health consequences, and interventions. *American Journal of Clinical Nutrition,* 80**,** 1106-1122.

WOLFE, J. M. & RICE, E. L. 1979. Allelopathic interactions among algae. *Journal of Chemical Ecology,* 5**,** 533-542.

WOODHAMS, D. C., ARDIPRADJA, K., ALFORD, R. A., MARANTELLI, G., REINERT, L. K. & ROLLINS-SMITH, L. A. 2007. Resistance to chytridiomycosis varies among amphibian species and is correlated with skin peptide defenses. *Animal Conservation,* 10**,** 409-417.

WOODHAMS, D. C., ROLLINS-SMITH, L. A., REINERT, L. K., LAM, B. A., HARRIS, R. N., BRIGGS, C. J., VREDENBURG, V. T., PATEL, B. T., CAPRIOLI, R. M., CHAURAND, P., HUNZIKER, P. & BIGLER, L. 2020. Probiotics modulate a novel amphibian skin defense peptide that is antifungal and facilitates growth of antifungal bacteria. *Microbial Ecology,* 79**,** 192-202.

YADAV, A. K., DESAI, P. R., RAI, M. N., KAUR, R., GANESAN, K. & BACHHAWAT, A. K. 2011. Glutathione biosynthesis in the yeast pathogens *Candida glabrata* and *Candida albicans*: essential in *C. glabrata*, and essential for virulence in *C. albicans*. *Microbiology,* 157**,** 484-95.

YE, J., COULOURIS, G., ZARETSKAYA, I., CUTCUTACHE, I., ROZEN, S. & MADDEN, T. L. 2012. Primer-BLAST: A tool to design target-specific primers for polymerase chain reaction. *BMC Bioinformatics,* 13**,** 134.

YORIO, T. & BENTLEY, P. J. 1977. Asymmetrical permeability of the integument of tree frogs (Hylidae). *The Journal of Experimental Biology,* 67**,** 197.

YOSHIE, S., IWANAGA, T. & FUJITA, T. 1985. Coexistence of bombesin and 5-hydroxytryptamine in the cutaneous gland of frog, *Bombina orientalis*. *Cell and Tissue Research,* 239**,** 5.

YOUNG, S., WHITEHORN, P., BERGER, L., SKERRATT, L. F., SPEARE, R., GARLAND, S. & WEBB, R. 2014. Defects in host immune function in tree frogs with chronic chytridiomycosis. *PLOS ONE,* 9**,** e107284.

YOUNG, T. J., OLIVER, G. P., PRYDE, D., PERROS, M. & PARKINSON, T. 2003. Antifungal activity of selective serotonin reuptake inhibitors attributed to non-specific cytotoxicity. *Journal of Antimicrobial Chemotherapy,* 51**,** 1045-1047.

ZHAI, B., WU, C., WANG, L., SACHS, M. S. & LIN, X. 2012. The antidepressant sertraline provides a promising therapeutic option for neurotropic cryptococcal infections. *Antimicrobial Agents and Chemotherapy,* 56**,** 3758.