Chytridiomycosis and symbiosis:

Context-dependency in amphibian disease and conservation

Thesis submitted by

Joshua H. Daskin, B.S. *cum laude*, Brandeis University

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STATEMENT ON THE CONTRIBUTION OF OTHERS TO THIS THESIS

My supervisors, Dr. Ross Alford, Dr. Lin Schwarzkopf, and Dr. Jan Mattias Hagman, assisted with experimental design, statistical, editorial, and financial support for all the research described in this thesis. Sara Bell conducted PCR to prepare bacterial DNA for sequencing. Dr. Robert Puschendorf discussed the experimental design and hypothesis formation for Chapter Five with me. Betsy Roznik, Sarah Sapsford, and Kiyomi Yasumiba assisted in capture and processing of frogs during field work at Windin Creek. Sara Bell, Falk Ortlieb, Martha Silva Velasco, and Kiyomi Yasumiba helped with bacterial challenge assay set-ups. Liz Tynan and Sarah Fann provided editorial assistance for Chapters One and Three and Five, respectively.

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The table below provides more specific information on the contribution of others to individual chapters of this thesis being prepared for publication.

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DECLARATION ON ETHICS

The research presented and reported in this thesis was conducted within the guidelines for research ethics outlined in the National Statement on Ethics Conduct in Research Involving Humans (1999), the Joint NHMRC/AVCC Statement and Guidelines on Research Practice (1997), the James Cook University Policy on Experimentation Ethics. Standard Practices and Guidelines (2001), and the James Cook University Statement and Guidelines on Research Practice (2001). The proposed research methodology received clearance from the James Cook University Animal Ethics Review Committee (approval number A1316) and the Queensland Department of Environment and Resource Management (permit WITK05922209).

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Abstract

Symbioses, long-term direct relationships between individuals of multiple species, are well known from many taxa and biomes. Although they are often thought of as static, symbioses are frequently dependent on environmental or ecological context. While there is a substantial amount of information on context-dependence in many symbioses, there little is known of how symbioses important to wildlife disease and conservation vary.

One disease of conservation importance that may be strongly affected by context-dependent symbioses is chytridiomycosis. This amphibian disease is caused by the fungal pathogen *Batrachochytrium dendrobatidis* (*Bd*) and has caused population declines and extinctions on several continents. *Bd*'s growth rate depends on environmental context, as evidenced by its distinct *in vitro* thermal optimum (17-25°C). The majority of *Bd*-driven declines have occurred in relatively cool climates (at high-elevation and during winter in tropical regions), demonstrating that environmental context affects disease outcome. However, many cool weather declines have occurred at temperatures well below *Bd*'s thermal optimum, where chytridiomycosis might be expected to be less severe. Two possible explanations for this pattern are that *Bd* in natural environments may adapt to low temperatures by increasing fecundity, or that production of anti-*Bd* skin peptides, a component of many amphibians' innate immunity, may be down-regulated in cool environments. Still a third possible explanation for the high incidence of *Bd*-driven declines at temperatures below the fungus's *in vitro* thermal optimum is that symbiotic bacteria that also inhabit amphibian skin and that can reduce the severity of chytridiomycosis may have severely reduced activity or density at cooler temperatures. However, no information exists on how the composition or antifungal activity of amphibians' anti-*Bd* bacterial assemblages respond to environmental contexts.

Eventually, antifungal bacterial symbiont populations may be augmented on amphibians' skin for management of chytridiomycosis. Currently, there is no broadly effective treatment or preventative available for wild amphibians at risk of chytridiomycosis, but supplementing amphibians' natural protective microflora, to enhance the protective effect they lend (termed "bioaugmentation,") is being explored. If these bacteria's anti-*Bd* activity varies with environmental and ecological context, bioaugmentation will require careful selection of robustly antifungal bacteria.

I identified 16 bacterial species previously not known to act as anti-*Bd* symbionts on the rainforest tree frogs *Litoria serrata* and *L. nannotis*. On average, *in vitro* anti-*Bd* activity of symbionts (both newly identified and previously known) from all bacterial classes was reduced at cooler temperatures characteristic of areas where declines driven by chytridiomycosis have been most severe. Such context-dependency in anti-*Bd* activity of amphibian symbionts may partially explain the association of past *Bd*-driven declines with cool temperatures, especially below the fungus's thermal optimum. It could also affect selection of bacteria for bioaugmentation and disease management.

Bd is a member of the little-studied and phylogenetically-basal Chytridiomycota, which are only distantly related to the better-studied higher fungi. Little is known of chytrids' interactions with bacteria. However, many higher fungi induce or inhibit the antifungal activity of antagonistic bacteria, altering the outcome of microbial symbioses and affecting agricultural biocontrol schemes. If *Bd* has mechanisms to defend against antifungal bacteria, they might adversely affect the success of bioaugmentation in natural amphibian populations. I found that *Bd* metabolites can affect the anti-*Bd* activity of antifungal bacterial symbionts isolated from *Litoria serrata*, *L. rheocola*, and *L. nannotis*. The activity of most symbionts was not affected, suggesting that although bacteria that are candidates for bioaugmentation will need to be screened for responses to *Bd* metabolites, most will not be affected. Activity of *Bd* metabolites should therefore not pose a severe problem for the management of chytridiomycosis by bioaugmentation, although *Bd* may still harbor non-chemical defense mechanisms not yet detected or evaluated.

While many amphibian species have declined due to *Bd*, some populations persist in an apparent commensal relationship with *Bd*. For example, in the Australian Wet Tropics, *Litoria nannotis* declined sharply at high elevation rain forest sites due to *Bd* in the early 1990s, and has not recovered. However *L. nannotis*, and one population of *L. lorica*, the latter previously believed extinct due to *Bd*, were recently found in high elevation dry forests, apparently healthy, but infected with *Bd* at high prevalence. One explanation previously suggested for persistence with *Bd* in dry forests is that exposure to sun-warmed rocks frogs perch on in these habitats may be keeping *Bd* infections below the lethal threshold attained at cooler rain forest sites. I tested whether short-term

exposure to elevated temperatures can hamper *in vitro Bd* growth. One hour daily at 33°C, but not at 28°C (representing exposure to heavily and more moderately warmed rocks in dry forests, respectively) reduced *in vitro Bd* growth below that in a constant 15°C regime (representing cool rain forest habitats). The reduction in *Bd* growth over the fungus's first generation after inoculation was small but may translate into maintenance of *Bd* infection intensities below a lethal threshold in natural settings over multiple generations of *Bd* growth.

My work provides early evidence of the role context-dependency may play in determining the outcome of the amphibian-*Bd*-bacteria symbiosis. If observed reductions in the anti-*Bd* activity of amphibians' bacterial symbionts at cool temperatures *in vitro* translate to wild frogs' symbionts, then corresponding losses in amphibian protection from *Bd* could account for the relatively frequent occurrence of amphibian declines at cool temperatures below *Bd*'s thermal optimum. In what was, to my knowledge, the first study of a chytrid fungus's effects on antagonistic bacteria, I found that metabolites of *Bd* were not active against the majority of *Litoria* spp. bacterial symbionts tested, suggesting that most antagonistic bacteria are not affected by any defenses involving metabolites. In future work based on the present study, chemical methods could be used to measure *in situ* concentrations of antifungal compounds on amphibian skin, and to identify the composition of *Bd*'s metabolites. Future studies should also clarify the roles of warm microhabitats, amphibian immunity, and animal behavior in driving the apparent commensal nature of the amphibian-*Bd* symbiosis in tropical Australia's upland dry forests and elsewhere.

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Chapter One—Context-dependent symbioses and their potential role in wildlife disease research

Abstract

Symbioses are well known across taxa and habitats. These relationships are often context-dependent, changing nature (mutualistic, commensal, or parasitic) with environmental and ecological context. Such variation in the formation and outcome of symbioses can alter the ecology and fitness of hosts, reduce the effectiveness of agricultural biocontrols, and affect human health. I review context-dependent symbioses and their importance from across disciplines. Despite the accumulated knowledge on context-dependent symbioses, they have been little-studied in the fields of wildlife disease and conservation biology. Using coral disease as a case study, I illustrate the potential that studies of context-dependent symbioses hold for improved understanding of wildlife disease outbreaks and subsequent population declines. Other wildlife diseases, particularly chytridiomycosis (with which the remainder of this thesis deals) also have host-symbiosis-context connections that are only poorly understood to date, but that may be important to outbreak investigation and conservation management.

1.1 Introduction

The 'First Law of Ecology' is "It depends" (Perlman 2007), and symbioses, long-term direct relationships between individuals of two or more species, are no exception to this rule. The character (i.e., whether it is mutualistic, commensal, or parasitic) and outcome (i.e., the survival or persistence of participant individuals or populations) of symbiotic relationships depends strongly on environmental and ecological context (Bronstein 1994). Recognition of the importance and frequency of context-dependent symbioses has contributed to the development of evolutionary theory, deepened our understanding of many species' ecologies, improved application of agricultural management practices, and begun to contribute to human medicine. In this chapter, I will review the accumulated evidence from across disciplines demonstrating the extent and significance of variation in symbioses, and conclude by highlighting the opportunity to incorporate the effects of context-dependent symbioses into the study

of wildlife diseases, one area that has not fully recognized the pervasive effects of symbiotic variation.

1.2 Ecology of context-dependent symbionts

1.2.1 Temperature and other environmental drivers of context-dependent symbioses

The effects of temperature on symbioses have been investigated in a wide range of symbioses, probably at least in part because climate change is looming, and may alter the ecology and conservation status of many species (Bernstein 2007). Also, temperature is a ubiquitous and easily manipulated variable relevant to all organisms. In general, its effects on a given symbiosis depend on the intersection of the individual symbionts' thermal profiles, which are determined in turn by their genetics, physiology, and behavior (Thomas & Blanford 2003). For example, in a factorial experiment crossing symbiont type and temperature, different species of facultatively symbiotic bacteria from the aphid *Acyrthosiphon pisum* conferred different levels of resistance to heat stress as measured by fecundity (Guay et al. 2009). Similarly, changing the mean temperature of the wood inhabited by bark beetles *Dendroctonus frontalis* altered the ratio of transmission of two mutualistic fungi from adult beetles to larva (Hofstetter et al. 2007). The beetle and one of the fungi, *Ophiostoma minus*, are serious pests of forests and plantation trees, and can be lethal to pine trees (*Pinus* spp.) (Gorton & Webber 2000). Thus, the context-dependent frequency of transmission of the fungi from adult to offspring in this system has applied consequences beyond the symbionts directly involved. Tree pest mite assemblages on *D. frontalis* hosts also vary with beetle larval incubation temperature (Hofstetter et al. 2007).

Temperature can also affect the outcome of a host's interaction with natural enemies. Aphid resistance to parasitism by the wasp *Aphidius ervi* varied with temperature, depending on which facultative bacterial symbionts were present (Guay et al. 2009). In particular, when aphids hosted a combination of two different symbionts, their resistance to parasitism at elevated temperatures increased greatly. In plants, the virulence of most bacterial pathogens increased at cooler temperatures (Smirnova et al. 2001). Ultimate causes for this pattern are unclear, but the authors

suggested that cooler air promoted formation of liquid films on the surface of host leaves, facilitating bacterial invasion through stomata (Smirnova et al. 2001). The proximate causes for environmentally- or ecologically-induced pathogenesis in symbiotic bacteria, though, can often be inferred from known mechanisms of bacterial responses to temperature. They include up-regulation of heat- or cold-shock protein synthesis (Smirnova et al. 2001), reorganization of membrane lipid structure, and protein conformation changes (Shivaji & Prakash 2010).

Seasonal effects on symbioses are closely allied to the effects of temperature. Koren and Rosenberg (2006) identified large, community-level shifts in bacterial assemblages of the coral *Oculina patagonica* in winter and summer, although the most abundant bacteria were always *Vibrio* spp. This relationship held when culture-independent molecular methods were used to analyze the *O. patagonica* bacterial flora.

Although not as well studied, environmental variables other than temperature are also likely influence the ecology of symbioses. For example, coral microbiota are sensitive to pH, dissolved oxygen levels and nutrient flux (Thurber et al. 2009 and see Wildlife Disease section below).

1.2.2 Ecological drivers of variable symbioses

The presence of species in addition to primary symbionts, interlopers perhaps, can drive context-dependency in symbioses in sometimes unexpected ways. An example is the familiar leaf-cutter ants and their relatives (tribe Attini) from the Neotropics, long-known for cultivating obligate fungal mutualists for consumption within their nests (Mueller et al. 2001). Contrary to the once-accepted belief that non-mutualist microbes are excluded from colonies by worker ants, a parasitic fungus (*Escovopsis* sp.) has been isolated from nests of multiple species of Attine ants and is especially common among the higher leaf-cutters, *Atta* spp. and *Acromyrmex* spp. (Currie et al. 1999a). The fungus can cause rapid and complete destruction of the ants' garden, and consequently collapse of the colony (Currie et al. 1999a).

In fact, an entire suite of microorganisms aside from the garden fungus is present on ants and in their nests, and contributes to the success or failure of the colony

(Currie 2001). All 22 species of Attine ants surveyed were host to Actinomycete bacteria in the genus *Pseudonocardia*, which produce compounds that selectively inhibit *Escovopsis* but not garden fungi (Currie et al. 1999b; Poulsen & Currie 2010). In addition, a group of black yeasts may inhibit the anti-*Escovopsis* function of these *Pseudonocardia* (Little & Currie 2008). The yeast decreased in vitro growth of *Pseudonocardia*, grew better in their presence, and was associated with greater losses of garden fungus biomass on infection with *Escovopsis*. Interestingly, the yeast did not affect antibiotic production by *Pseudonocardia*, suggesting that its *Escovopsis*-promoting effects may be due to competition with the bacteria (Little & Currie 2008).

In another example of symbiont community mediated outcomes, laboratory experiments with bacteria isolated from pollock (*Pollachius* sp.) showed that *Lactobacillus plantarum* only exhibited activity against *Vibrio* spp. pathogenic to fish hosts when grown in the presence of *Bacillus thuringiensis* culture filtrate (Ringo & Gatesoupe 1998). *B. thuringiensis* is a commonly employed biocontrol bacterium in agriculture and forestry and presumably produced a metabolite that induced expression of anti-*Vibrio* activity in *L. plantarum*. Although this precise pairing of symbionts may not occur under natural conditions, similar interactions may occur among microbes in the wild and these experiments were targeted at development of aquaculture probiotics.

Short-term host needs can also drive symbiotic variation. In European hoopoes *Upupa epops* and green woodhoopoes *Phoeniculus purpureus*, females exude a dark, pungent secretion from their uropygial gland, but only in the short time when incubating eggs during the nesting season (Martin-Vivaldi et al. 2009). These secretions are produced by symbiotic bacteria of the genus *Enterococcus*, and inhibit bacterial pathogens, probably protecting eggs and/or nestlings (Martin-Vivaldi et al. 2010; Soler et al. 2008). It is not known how the birds regulate the change in secretion type on beginning their short nesting period, but non-nesting females and all males produce clear or white secretions, without bacteria, and inactive against pathogens (Martin-Vivaldi et al. 2009). Hormonal changes in females may induce bacteria, present at low levels year round, to flourish in the breeding season.

1.3 Evolution and symbiotic variation

1.3.1 Symbiont plasticity and host adaptation

Perhaps unsurprisingly, given the role of variation in biological evolution, extensive work has been done on the evolution of context-dependent symbioses and on their evolutionary consequences. Recently, it has even been suggested that the combination of a host and its symbiont community, the "hologenome," should be considered the unit of natural selection because of the flexible, novel functions and fitness benefits provided by symbionts (Rosenberg et al. 2007b; Rosenberg et al. 2010). Although the specifics of this hologenome theory have been criticized (Leggat et al. 2007; Rosenberg et al. 2007a; Thurber et al. 2009), the role of microbial symbionts in the evolution of animals and many of their cellular features is firmly cemented (Fraune & Bosch 2010). Several field studies, and some laboratory experiments, support the hologenome theory of evolution's requirement that microbial symbionts' vast array of genotypes and relatively quick responses allow adaptation and improved fitness of the host and symbionts. Four examples follow. The wasp *Trichogramma cordubensis* forms obligate symbioses with bacteria of several species of *Wolbachia*, and experimental exposure of consecutive wasp generations to elevated temperature selected for bacterial genotypes that conferred heat stress resistance, benefiting both the bacteria and *T. cordubensis* (Pintureau et al. 1999). The same study presented evidence that reproductive modes of natural populations of *Trichogramma spp*. and the distribution of their *Wolbachia* spp. symbionts may be adapted to warmer summers (Pintureau et al. 1999). Similarly, the prevalence of symbiotic bacteria of the genus *Spiroplasma* has recently increased in *Drosophila neotestacea* from eastern North America, presumably because of the protective effect the bacteria lend to *D. neotestacea* against the sterilizing effects of the common parasitic nematode *Howardula aoronymphium* (Jaenike et al. 2010). Likewise, at least preliminary evidence from reef-building corals shows that host resistance to disease may be improved, or even driven by symbiont assemblage shifts (Reshef et al. 2006), although the virulence of bacteria associated with corals can also increase with exposure to abiotic stresses (Thurber et al. 2009). Finally, in the American prairie grass Big Bluestem (*Andropogon gerardii*), population-level

adaptation to local soil conditions measured by total grass biomass can be attributed, in part, to the benefits of mycorrhizal fungi in low-nutrient, but not in higher-nutrient, soils (Schultz et al. 2001).

1.3.2 Effects of context-dependency on symbiogenesis

When the costs and benefits of involvement in a symbiosis differ according to environmental or ecological conditions, the stability of the symbiosis is likely to vary. Mutualisms, for example, may be viewed not as altruistic interactions involving endlessly-giving partners, but as two or more organisms trying to invest as little as possible in benefiting each other without losing their own benefits. Ecological (e.g., life stage) or environmental (e.g., temperature) context can change the needs or abilities of the species involved (Bronstein 1994) and certain thresholds may need to be met for the "goods" exchanged in symbioses to be available. For example, bacteria often must reach a critical density for quorum sensing to initiate antibiotic production (Miller & Bassler 2001), and potential hosts may not form relationships with potentially protective antibiotic-producers unless they reach this critical mass often enough in a given environment. In aphids, *Acyrthosiphon pisum*, individuals may host any of at least three facultative bacterial endosymbionts. In vitro, the insects receive differing degrees of protection from heat shock depending on the symbiont present and on the stage of the aphid's development (Russell & Moran 2006). Fitness costs and benefits are likewise associated with heat shock resistance, making symbiosis with certain bacteria more or less beneficial, and hence more or less likely to form, under different environmental conditions. Field evidence for shifting dominance among the three symbionts tested is equivocal, probably because the costs and benefits of hosting a given symbiont are influenced by many more factors than just temperature (Russell & Moran 2006).

1.4 Context-dependency in agricultural symbionts and biocontrol

Because of the importance of agriculture to society and the crucial role of microbial root symbionts in nutrient uptake by plants (Petrini 1986), symbioses in agricultural systems have been among the most heavily-studied. A single species of

symbiont can exhibit mutualistic, commensal, and parasitic relationships, making context-dependency in the benefits obtained by plant hosts, and in the development of disease, common. Biocontrol schemes used in agriculture can be thought of as induced symbioses or parasitism, and so are included below.

Multiple stages in plant-microbe symbioses are subject to variation. Morphological changes and development of host plant root systems to "welcome" nitrogen-fixing bacteria are triggered by chemical nodulation factors released by the bacteria and recognized by the plant (Long 1996). Low soil temperatures, though, can inhibit the synthesis of these compounds in the bacterium *Bradyrhizobium japonicum*. This is one of several mechanisms by which low temperatures can inhibit formation of rhizobia-legume symbioses (Duzan et al. 2006). After formation of the symbiosis, symbionts can shift their behavior. Several species of the fungal plant symbiont genus *Colletotrichum* can range from mutualistic to pathogenic depending on which host species is colonized (Redman et al. 2001). Redman et al. (2001) speculate that plant hosts may regulate fungal gene expression and indicate that simple classification of an organism as a mutualist, pathogen, or commensal may depend heavily on the choice of metrics used to measure cost and benefit.

Production of antimicrobial compounds by biocontrol agents can also be strongly affected by environmental and ecological context. The fungal sponge symbiont *Letendraea helminthicola* produced maximal quantities of compounds active against marine fouling organisms only within certain pH, salinity, and temperature windows (Yang et al. 2007). For salinity and pH, bioactive chemical production was maximized outside the windows for maximum growth of the fungus, requiring careful calibration of culture conditions for efficient use of this fungus as a biological control. Temperature, pH, biocontrol strain and density, and host plant all act independently to influence production of antibiotics by biocontrol bacteria [reviewed by Raajimakers et al. (2002)]. Expression of antibiotic-producing genes in *Pseudomonas fluorescens*, a commonly used biocontrol bacterium and one that regularly forms natural symbioses with animals and plants in which the hosts benefit from bacterial antibiosis and other mechanisms of pathogen control (Haas & Defago 2005; Woodhams et al. 2007) is down-regulated at 35°C compared to 30°C (Humair et al. 2009). Another

Pseudomonas sp., active against soil-borne pathogens and isolated from the roots of sugar beets, produced significantly different amounts of 2,4-diacetylphloroglucinol depending on temperature, carbon source, and culture surface area to volume ratio (Shanahan et al. 1992). However, pH as well as iron and oxygen concentrations did not alter 2,4-DAPG production.

Animal pest biocontrol is also subject to environment-induced variation. For example, two fungi used to control Orthopteran pests can range from highly effective to entirely ineffective depending on environmental temperature (Blanford & Thomas 2000).

The target organism's response also causes variable outcomes in agricultural biocontrol schemes. *Fusarium* spp. fungi are major pathogens of multiple crop species and can cause opportunistic infections in humans (Gupta et al. 2000; Summerbell & Schroers 2002; Zhang et al. 2006). In agriculture, *Fusarium* infestations are treated or prevented by applying *Pseudomonas* spp. and other bacteria and fungi, (Larkin & Fravel 1998, 1999) and *Fusarium*-resistant genetically-modified crops are being developed (Bakan et al. 2002). When tested against 76 plant-pathogenic strains of *F. oxysporum*, though, 2,4-diacetylphloroglucinol (2,4-DAPG), a primary antibiotic metabolite of *Pseudomonas* spp., was ineffective at halting growth of 14% of strains (Schouten et al. 2004). The same study found that strains of *F. oxysporum* varied greatly in their production of fusaric acid—an inhibitor of 2,4-DAPG production (Schouten et al. 2004). Thus, two kinds of pathogen defense have the potential to influence the success of biological control of *Fusarium*: susceptibility of the pathogen to 2,4-DAPG, and ability of the pathogen to inhibit 2,4-DAPG synthesis (Tarkka et al. 2009). Duffy, Schouten, and Raajimakers (2003) reviewed the role of plant pathogen defenses against biocontrols, highlighting the many mechanisms (antibiotic metabolism and resistance, pathogen-produced toxins, alteration of biocontrol agent gene expression, environmental alteration for competitive benefit) and the fact that most studies of biocontrol have focused only on the effect of the control agent on the target organism, and not vice versa. They identified cases of both pathogen inhibition and stimulation of biocontrol activity in the literature.

1.5 Human medicine and disease

Biomedical scientists and physicians have begun to examine the importance for disease development and progression of variation in the behavior and community ecology of human microbial symbionts. Recently, it has been proposed that the role of the immune system is not to recognize and respond to non-self material as has been traditionally taught, but rather to recognize disturbances to the mutualistic and commensal human microbiota (Eberl 2010). Such a frame shift would be an acknowledgement of the integrated function of humans and their symbionts, both defensive and nutritional, and of the substantial role of symbiotic homeostasis in preventing and signaling disease.

In the absence of dietary-, pharmaceutical, or disease-driven disturbance, the human microbiota can be stabile through time (Costello et al. 2009). Manipulation of the human microbiota for disease prevention or treatment is regularly practiced through the use of probiotics. Evidence suggests that patients suffering from some chronic gastrointestinal disorders may benefit from probiotics (Kruis et al. 1997; Nobaek et al. 2000). Researchers have also successfully targeted a range of other disorders, including eczema and dairy allergies (Majamaa & Isolauri 1997), with probiotics. It is clear that human microbial symbiont communities can be incredibly responsive and variable, shifting metagenomic activity in the course of a day when exposed to dietary changes (Turnbaugh et al. 2009) and that their variability or lack thereof plays a crucial role in human health.

Shifts in given symbionts from commensal or mutualistic to pathogenic behavior are perhaps the most studied variations in human disease symbioses. *Staphylococcus epidermidis* is an exceedingly common member of the human skin microbiota (Cogen et al. 2008), and is thought to prevent pathogen colonization both directly by antibiotic production (Ekkelenkamp et al. 2005) and indirectly by "priming" the immune system to deal with other microbes (Cogen et al. 2008). The mutualistic nature of *S. epidermidis* can be lost and virulent disease initiated, though, when the organism gains entry to internal tissues (Cogen et al. 2008; Oduwole et al. 2010). In recent years, *S. epidermidis* has been recognized as a common and critically-

important cause of nosocomial infections (Vuong & Otto 2002), despite its potential to act as a mutualist. In similar fashion, the pathogenic bacterium *Parachlymydia acanthamoeba* lyses its ameobal host at human body temperature but remains lysogenic at cooler temperatures (Grueb et al. 2003). When the infected amoeba moves from the cooler nasal cavity, where it and its intracellular *P. acanthamoeba* symbionts are common commensals, to the warmer respiratory tract, it is believed that large numbers of the bacteria are released causing pneumonia, bronchitis and other respiratory diseases (Corsaro & Greub 2006). The contextually-dependent changes that occur in *P. acanthamoeba*- and *S. epidermidis*-human interactions when the microbes move between tissues are analogous to species' responses to moving among habitats or hosts in other systems.

1.6 Emerging diseases of wildlife

Acknowledgement of the role of context-dependent symbioses has been slower to emerge in the literature on conservation and wildlife disease than in the fields reviewed above. In general, wildlife diseases only cause extinctions of a given host when a living or environmental reservoir for the pathogen is available, or when the population in question is already small and threatened by other factors (de Castro & Bolker 2005). This is well-established and my argument does not challenge this notion. Rather, I suggest that changes to or differences among symbionts at the individual, population, or species levels in hosts may be crucial in determining persistence and survival following infection. This is a largely unexplored possibility and admittedly somewhat speculative, but nonetheless is one worth exploring with respect to progression, distribution, and outcome of diseases of conservation importance. The role of symbionts in defense against pathogens in general (White & Torres 2009), their potential to respond rapidly to environmental change often associated with disease and/or population declines (Rosenberg et al. 2010), and the often difficult to predict distribution of wildlife diseases justify investigation of context-dependency in symbioses affecting wildlife disease.

1.6.1 Coral disease investigation and context-dependent symbioses

An exception to the lack of investigation of the role of symbionts and their variation in development, progression, and potential prevention of wildlife diseases is research on several diseases of corals considered to be drivers of recent reef declines (Harvell et al. 2007). The long-known obligate coral-algae symbiosis seems to have predisposed researchers to continue probing the impact of symbionts when disease concerns emerged. Below, I present a general summary of what is known of variation in coral microbe communities, their genetic expression, and their effects on coral health as a case study of the sort of information that could be overlooked if wildlife hosts are considered only in isolation, without their symbionts.

In the case of at least one syndrome, coral bleaching, symbiotic algae, bacteria, and fungi appear to have a crucial role (Rosenberg et al. 2007b). Bleaching is the potentially deadly loss of corals' symbiotic algae (Hoegh-Guldberg 1999), and is caused by some combination of environmental stress and pathogenic microbes (Rosenberg et al. 2007b; Rowan 2004). The massive degradation of reefs due to bleaching in the Caribbean, Australia, and elsewhere (Hoegh-Guldberg 1999) illustrates the effects of this particular alteration of the close link between corals, their symbionts, and their survival. In addition to bleaching, the invasion of coral tissues by pathogens that cause other diseases may be assisted by the effects of raised seawater temperature on symbiont behavior and presence (Rosenberg et al. 2007a). *In vitro* antibiotic activity of bacteria and mucous from corals in the genus *Acropora* was reduced at higher temperatures, potentially causing a reduced protective effect in wild corals (Shnit-Orland & Kushmaro 2009). In the marine sponge *Rhopaloeides odorabile*, symbiotic bacterial assemblages remained stable between 27°C and 31°C, but changed dramatically when water temperature was raised to 33°C (Webster et al. 2008). The altered, high-temperature community was closely related to those of bleached corals, while lower temperature sponge symbionts were characteristic of healthy *R. odorabile*. Similarly, relative abundances of both eukaryotic and bacterial symbiont taxa changed and the expression of bacterial and fungal genes associated with disease was increased on exposure of the coral *Porites compressa* and its symbionts to thermal, oxygen, pH, and nutrient stressors (Thurber et al. 2009). It is worth noting, too, that simply moving corals from the wild to control aquaria caused

significant symbiont assemblage shifts from the time zero field collection state (Thurber et al. 2009). Although community-level treatment-associated changes were distinguished from control aquaria changes by the induction of disease-associated genes and taxa, the highly responsive nature of symbionts is highlighted by the effect of the control treatment.

In addition to evidence of stressor-induced shifts towards a disease state, a positive role for such rapid responses by coral symbionts has been proposed. As a precursor to the more general Hologenome theory of evolution (see above section on Evolution and Context-Dependent Symbioses) Reshef et al. (2006) suggested that the diverse communities of coral symbionts enable rapid response to disease or environmental changes. According to this coral probiotic hypothesis, the ability of some corals to develop disease resistance despite not having an antibody-mediated immune response is conferred by shifting symbiont communities which match the prevailing conditions and provide temporally-variable immunity-like services. Recently, the potential to manipulate the microbial communities of corals for disease management in ways similar to the biocontrol schemes used in agriculture has been tested. A range of options including inoculation and transplantation of hatchery-reared corals with beneficial bacteria or pathogen-targeting bacteriophages was reviewed by Teplitski and Ritchie (2009). In one case, phages cultured from natural reef material and applied to coral prevented infection by the bacterial bleaching agent *Vibrio coralliilyticus* for at least two months (Efrony et al. 2007) establishing the potential for a medium- to long-term protective effect.

1.6.2 Context-dependent symbioses and other wildlife diseases

Although more is known about how variation in symbiont activity leads to disease in corals than for any other group of wildlife, some authors continue to call for better basic understanding of context-dependency in coral-microbe relationships (Teplitski & Ritchie 2009). The same, if not greater, potential exists for context-dependent symbiosis research on non-coral wildlife diseases. For example, bats in the northeastern United States have recently suffered catastrophic declines, due to a condition termed white-nose syndrome (WNS) (Boyles & Willis 2010). The fungus

Geomyces destructans is associated with the deaths of >500,000 bats in the affected area, but is present, apparently as a commensal symbiont, in some European bats, as well (Puechmaille et al. 2010). Modeling of bat die-offs and physiology, and *G. destructans*' cool *in vitro* thermal optimum, suggest that providing artificially-warmed roosting sites for hibernating bats could ameliorate the effects of WNS (Boyles & Willis 2010; Gargas et al. 2009). Preliminary studies of bats' bacterial symbionts have also begun, seeking anti-*Geomyces* bacteria and any changes to the microflora in diseased animals (Amelon & Knudsen 2010). Despite the recent increase in study of emerging diseases of wildlife, many with conservation or human health relevance (Woolhouse et al. 2005), there has been relatively little effort (but see Belden & Harris 2007) to realize the potential that studies of wildlife symbionts can have for understanding disease.

* * * * *

To this point I have established the importance of context-dependent symbioses in evolution, ecology, agriculture and human disease (Table 1.1). I have also demonstrated through a case study of coral diseases the potential benefits of studying symbiosis variation for wildlife disease and conservation medicine, although little such work has been done to date. Below, I investigate the potential role of context-dependency in amphibian-bacterial symbioses with respect to the past and future of chytridiomycosis, a pandemic emerging infectious disease of amphibians caused by the fungal pathogen *Batrachochytrium dendrobatidis* (*Bd*) (Kilpatrick et al. 2010). The following studies will both inform on management of chytridiomycosis and act as a catalyst for future studies of context-dependent symbioses in other wildlife diseases with applied conservation or public health importance.

Table 1.1—Disciplines and phenomena influenced by context-dependent symbioses. The examples here are not exhaustive; see text for more detail and more examples.

Discipline	Illustrative Phenomena	Reference
Ecology	Introduction of natural pests & pathogens	(Hofstetter et al. 2007)
Evolution	Symbiont-assisted adaptation to novel environments	(Rosenberg et al. 2010)
Agriculture	Crop pest biocontrol effectiveness	(Duffy et al. 2003; Raaijmakers et al. 2002)
Human medicine	Induction of commensal microbe pathogenicity	(Cogen et al. 2008; Oduwole et al. 2010)
Wildlife disease	Coral symbiont activity and health	(Shnit-Orland & Kushmaro 2009)

The next chapter reviews the history of chytridiomycosis, the discovery of symbiotic bacteria that inhibit *Bd*, and the clear potential for context-dependency in these amphibian-bacterial symbioses. Chapter Three investigates the role of environmental temperature in regulating the antifungal activity of symbiotic bacteria from three species of tropical Australian Hylid frogs which varied in their response to *Bd* invasion. The fourth chapter examines whether or not metabolites of *Bd* can induce or inhibit antifungal activity of amphibian bacterial symbionts. Chapter Five assesses the possible effects on the amphibian-*Bd* symbiosis of various thermal regimes experienced by the same tropical Australian treefrogs and their symbionts in multiple microhabitats. Finally, Chapter Six is a general discussion and summation of my findings.

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Chapter Two—Chytridiomycosis and context-dependent symbioses

Abstract

Globally, amphibians are threatened by the fungal pathogen *Batrachochytrium dendrobatidis* (*Bd*) and the disease it causes, chytridiomycosis. A striking feature of *Bd*-associated declines is the relatively narrow range of environmental conditions under which they have occurred. Some amphibians are known to host bacteria on their skin that inhibit *Bd* and that may provide some protection from chytridiomycosis. Currently, there is no broadly effective treatment or preventative available for wild populations at risk of infection, but augmentation of amphibians' natural protective microflora is being considered as a possible management technique. In other host-pathogen systems, microbial protection against disease is known to vary because the nature of protective symbioses can depend on environmental context. No information exists on the responses of bacterial anti-*Bd* activity to environmental context. However, information on such responses may explain some of the environmental dependence of past declines, and is essential if biocontrol strategies for mitigating the effects of chytridiomycosis are to be developed.

2.1 Introduction

According to the Global Amphibian Assessment and more recent analyses (Hoffmann et al. 2010; Stuart et al. 2004), over one third world's amphibian species are under threat of extinction, the highest proportion of any vertebrate group. The standard threats to biodiversity at large—habitat loss, chemical pollutants, overharvesting and climate change—certainly apply to amphibians (Collins 2010; Gascon et al. 2007), but declines, extirpations, and extinctions of over 200 species have been attributed to the recently-emerged fungal pathogen *Batrachochytrium dendrobatidis* (*Bd*) (Skerratt et al. 2007), which causes chytridiomycosis, a

contagious cutaneous disease (Berger et al. 1998). In this chapter, I briefly review the history of chytridiomycosis, and then consider amphibian declines and disease management with particular attention to amphibians' protective bacterial symbionts. Context-dependent symbioses are known to be important in ecology, evolution, medicine, and biocontrol, as detailed in Chapter One. I argue that variation in these symbiont communities and their activity could account, in part, for patterns of amphibian decline and persistence, and may be key to future management efforts.

2.2 History of chytridiomycosis and anti-*Bd* bacterial symbionts

2.2.1 Chytridiomycosis history and patterns

Batrachochytrium dendrobatidis was originally described from a captive blue poison dart frog *Dendrobates azureus* in the United States (Longcore et al. 1999). Simultaneously, chytridiomycosis was discovered in captive and wild Australian hylids and myobatrachids, Central American hylids, centrolenids, leptodactylids and bufonids, and captive dendrobatids and hylids from North America (Berger et al. 1998; Pessier et al. 1999). It has since become apparent that amphibian declines at least as early as the late 1970s were probably caused by *Bd* epidemics (Laurance et al. 1996, 1997). The fungus has now spread to six continents, infecting 350-plus amphibian species (Fisher et al. 2009). Still, a few large areas with high amphibian diversity remain free of *Bd* (Andreone et al. 2008; Skerratt et al. 2010; Weldon et al. 2008), or have not yet experienced population crashes following arrival of the fungus and infection of wild amphibians (Bai et al. 2010; Hossack et al. 2010). It is unclear why only some species and populations crash, but the phenomenon is likely best explained by a range of organismal, ecological, and environmental factors (Alford 2010).

One clear pattern, though, is a strong connection between declines and environmental temperature. Prevalence and intensity of *Bd* infection, and the severity of *Bd*-caused amphibian declines, have been worst at high elevations and in cooler seasons, particularly in the tropics where the majority of extirpations have occurred (Berger et al. 2004; Kriger & Hero 2008; Lips 1998; Woodhams & Alford 2005). To date, the fungus's relatively cool *in vitro* optimal temperature window, centered on

17-25^{o}C (Piotrowski et al. 2004), has been regarded as the cause for this cool-weather pattern of declines (Kriger & Hero 2007), but amphibian immunity and symbiont activity may contribute to explaining the pattern, too. (See *Potential role of context-dependency in chytridiomycosis and amphibian conservation* below).

2.2.2 Discovery and protective effect of anti-Bd bacterial symbionts

In 2006, bacterial symbionts that inhibit the growth of *Bd in vitro* were discovered on two species of Plethodontid salamanders (Harris et al. 2006). Such bacteria have since been found on multiple amphibian species over a wide range of geographic distributions, phylogenies, habitats, and host chytridiomycosis susceptibilities (Table 2.1.1). Multiple research groups are investigating bacteria on amphibians in Europe, Latin America, the United States and Australia.

Table 2.1.1—Species with documented cutaneous bacterial symbionts active *in vitro* against *Batrachochytrium dendrobatidis*

Species	**Family**	**Habitat Type**	**Regional Distribution**	***Bd*-associated declines?**	**Reference**
Litroia nannotis	Hylidae	Stream-breeding tree frog	Tropical North Queensland	Y	(Chapter Three; Bell in prep.)
Litoria serrata	Hylidae	Stream-breeding tree frog	Tropical North Queensland	Y, but recovered	(Chapter Three; Bell in prep.)
Litoria rheocoloa	Hylidae	Stream-breeding tree frog	Tropical North Queensland	Y	(Chapter Three; Bell in prep.)
Rana catesbeiana	Ranidae	Pond-breeder	Eastern North America	N	(Walke & Belden 2010)
Rana muscosa	Ranidae	Pond-breeder	Western USA	Y	(Woodhams et al. 2007)
Rana palustris	Ranidae	Pond-breeder	Eastern North America	N	(Walke & Belden 2010)
Rana sierrae	Ranidae	Pond-breeder	Western USA	Y	(Lam et al. 2010; Woodhams et al. 2007)
Hemidactylium scutatum	Plethodontidae	Terrestrial adults and nests, pond larvae	Eastern North America	N	(Harris et al. 2006)
Plethodon cinereus	Plethodontidae	Fully terrestrial	Eastern North America	N	(Harris et al. 2006)
Notophthalmus viridescens	Salamandridae	Aquatic larvae, adults; terrestrial juveniles	Eastern North America	N	(Walke & Belden 2010)

Although these discoveries are not the first known microbial symbionts of amphibians, studies of anti-*Bd* bacteria have already surpassed the depth of previous research on the relationships between amphibians and cutaneous microbial symbionts (Box 2.1).

Box 2.1—Previous study of amphibians' microbial symbionts

Prior to the discovery of anti-*Bd* bacteria on *Hemidactylium scutatum* and *Plethodon cinereus* (Harris et al. 2006), there had been only limited investigation of amphibians' cutaneous symbionts. Bettin and Grevin (1986) published the first report of bacteria isolated from the skin of an amphibian, *Salamandra salamandra*. They identified only three species, *Pseduomonas fluorescens*, *Acinetobacter lwolfi*, and *Providencia stuartii*, but did suggest that cutaneous microbes could improve salamanders' disease immunity. They also suggested that antimicrobial peptides on the skin could select for a distinctive native microflora. A decade-and-a-half later, Austin (2000) found that the skin of southern zigzag salamanders *Plethodon ventralis* can harbor a microbial flora quite different from that of their environment's leaf-litter. Culp et al. (2007) found that several amphibians from the Eastern U.S. harbored bacterial communities that were a subset of environmental assemblages, although they sampled only a small number of animals. Austin (2000) also found that several bacteria from *P. ventralis* skin inhibited the growth of *Cunninghamella echinulata*, an intestinal and fecal fungal symbiont of the salamander, which is taxonomically similar to pathogenic fungi found on the amphibian's eggs. Austin suggested that parental nest attendance activity may spread beneficial bacteria from adults to eggs and that skin bacteria communities in *P. ventralis* may have evolved to protect eggs from the pathogens they are exposed to on laying.

Eukaryotic microbes also form symbioses with amphibians. Some amphibian embryos benefit from the growth of algae on and within eggs, which both increases oxygen availability (Bachmann et al. 1986) and provides protection from UV radiation (Marco & Blaustein 2000). Finally, it was recently reported that algae form an intracellular symbiosis with embryos of the salamander *Ambystoma maculatum* (Petherick 2010), presumably exchanging nutrients as in extracellular algae-salamander relationships (Goff & Stein 1978; Hutchison & Hammen 1958).

Cutaneous bacteria are likely to protect at least some amphibians from *Bd*, as shown by studies of the sister species *Rana muscosa* and *R. sierrae*. Woodhams et al.

(2007) surveyed the bacterial flora of one population each of *R. muscosa* and *R. sierrae* [both referred to as *R. muscosa* prior to taxonomic revision by Vredenberg et al. (2007)] in the Sierra Nevada mountains of California. Just months after sampling, the *R. muscosa* population was rapidly and completely extirpated due to newly-arrived *Bd* (Woodhams et al. 2007). In the *R. sierrae* population, though, *Bd* had become endemic and had coexisted with a healthy frog population for six years. Compared to the *R. sierrae* population, the *R. muscosa* population had a lower proportion of individuals hosting one or more species of bacteria that inhibited *Bd* when tested *in vitro* (62% vs. 85% of individuals), as well as lower infection prevalence and lighter infection burdens, on average (Woodhams et al. 2007). Woodhams et al. (2007) also found that cutaneous antimicrobial peptides from the extirpated population were stronger (inhibited *Bd* at lower concentration) than were those of the persisting population (Woodhams et al. 2007), suggesting that the difference in persistence was not related to differences in antimicrobial peptides. A later field study sampled microbiota in 2006 from a previously unstudied *Bd*-free *R. muscosa* population and re-sampled bacteria from the persisting population of *R. sierrae.* In 2006, both the persisting *R. sierrae* and the naïve *R. muscosa* population had high proportions of individuals with at least one species of anti-*Bd* bacteria present (80% and 79%, respectively; Lam et al. 2010). When *Bd* invaded the *R. muscosa* population in 2007, the frogs persisted, possibly due to the high proportion of frogs with anti-*Bd* bacteria. The authors note that based on models of disease spread and immunity, populations with 80% of frogs harboring anti-*Bd* bacteria could achieve herd immunity.

Although several types of antagonism and competition occur between bacteria and fungi (Tarkka et al. 2009), so far the only well-supported mechanism of bacterial inhibition of *Bd* is by antibiosis. *Janthinobacteria lividum* produces the anti-*Bd* metabolites violacein and indole-3-carboxaldehyde, both of which are found on wild *Plethodon cinereus* at biologically relevant concentrations (Brucker et al. 2008b). Additionally, the salamander symbiont *Lysobacter gummosus* produces 2,4-diacetylphloroglucinol (Brucker et al. 2008a), a compound long known to inhibit fungal plant pathogens when produced by *Pseudomonas* spp. (Schouten et al. 2004;

Shanahan et al. 1992). In general, *Pseudomonas* spp. are well-documented producers of a range of antifungal chemicals, and occur on amphibians in North America and Australia (Chapter 3; Bell in prep.; Woodhams et al. 2007).

Removal experiments have added to the evidence that naturally-occurring microbial symbionts contribute to amphibian defenses against *Bd*. When wild-caught *Plethodon cinereus* were experimentally infected with *Bd*, those that previously had their natural microbial flora reduced in antibiotic and hydrogen peroxide baths suffered greater weight loss, a sign of greater disease progression (Retallick & Miera 2007), than did individuals with intact cutaneous microflora (Becker & Harris 2010). *P. cinereus* is relatively insensitive to *Bd* and before one month's time all salamanders in this experiment had cleared the infection, suggesting, unsurprisingly, that bacteria are not the sole source of immunity in *P. cinereus*. Antimicrobial peptides (Fredericks & Dankert 2000) or innate and adaptive immune responses (Richmond et al. 2009) may work in tandem with protective bacteria in this and other species.

2.3 Past management of chytridiomycosis and the potential for bioaugmentation

2.3.1 Past management schemes—unsuccessful or unsustainable

A range of management and treatment options for chytridiomycosis have been trialed and studied (Woodhams et al. 2011). In captivity, chytridiomycosis can often be treated and cleared effectively. Treatment with a variety of antifungal drugs, generally administered by bathing affected individuals, is the current preferred method (Pessier & Mendelson 2010), although such measures are not effective across all amphibian species (Berger et al. 2009; Pessier & Mendelson 2010) or life stages (Garner et al. 2009). Exposure of infected individuals to elevated temperature kills *Bd* in two hylid species, *Litoria chloris* (Woodhams et al. 2003) and *Pseudacris triseriata* (Retallick & Miera 2007), but more trials with more species are needed to confirm that this effect occurs across all amphibians, and it may be impossible in species with low heat tolerance. It is still recommended that heat be combined with drug therapy (Pessier & Mendelson 2010).

In Europe, at least one attempt has been made to eliminate *Bd* from a natural population. On the Spanish island of Mallorca, a pond home to an infected population of the midwife toad *Alytes obstetricans* was drained to dry out and kill any fungi following removal and treatment of tadpoles with an antifungal drug (Lubick 2010). When the pond refilled with rain, though, the fungus returned, too, albeit at lower levels. A second round of draining and tadpole treatment may be tried in 2011, in hopes of lowering infection burdens further (Lubick 2010).

There have been two vaccines trialed for chytridiomycosis, although two very different measures were used to evaluate their effectiveness. The first showed no reduction in infection intensity or prevalence, and no improved survival, in *Rana muscosa* (Stice & Briggs 2010). The second trial, which included an additional round of vaccination, higher numbers of heat-killed *Bd* cells in the vaccine, and animals housed at temperatures more encouraging of immune reactions, found much-increased production of *Bd*-specific antibodies in African clawed frogs *Xenopus laevis* for up to four weeks following immunization (Ramsey et al. 2010). Production of antibodies, though, is not a guarantee of improved post-infection survival if, for example, they can not access fungal cells within amphibian skin. To show Ramsey et al.'s vaccine provides effective protection from *Bd*, it is necessary to compare post-infection survival with and without immunization, as in Stice and Briggs's (2010) trial. Such a study should employ an amphibian species more susceptible to chytridiomycosis than *X. laevis* (Parker et al. 2002; Weldon et al. 2004), to maximize the likelihood of detecting improved survival.

2.3.2 Bioaugmentation for active disease management

The variable success and time- and resource-intensive nature of all the methods discussed above means there is currently no broadly effective curative or preventative treatment available for chytridiomycosis in wild populations (Becker et al. 2009; Harris et al. 2009b). Substantial excitement has been generated, though, by the potential for disease management by augmentation of the natural cutaneous microbiota of amphibians; i.e., increasing the abundance of antifungal bacteria to enhance their protective effects and improve success of epidemic prevention and

amphibian reintroductions (Harris et al. 2009a; Harris et al. 2009b; Lam et al. 2010; Woodhams et al. 2007).

Three laboratory studies have demonstrated that addition of anti-*Bd* bacteria can reduce the effects of *Bd* infection. *Plethodon cinereus* individuals inoculated with either of the bacteria *Pseudomonas reactans* or *Janthinobacteria lividum* prior to experimental infection with *Bd* lost less weight than did individuals that were not inoculated (Becker et al. 2009; Harris et al. 2009b). *J. lividum* addition was also tested in *Rana muscosa*, one of several amphibians from which this bacteria has been isolated. *R. muscosa* is more susceptible to *Bd* than is *P. cinereus*, and high mortality of control frogs experimentally infected with *Bd* occurred, but individuals inoculated with *J. lividum* prior to *Bd* exposure experienced 100% survival, suffered significantly less weight loss, and did not develop detectable *Bd* infections (Harris et al. 2009a). The frogs in this experiment had their native microflora reduced by bathing in hydrogen peroxide before experimental treatments (Harris et al. 2009a), a procedure that may have assisted in greater *J. lividum* colonization of the frogs' skin. Thus, laboratory evidence from bacterial addition trials, along with the bacterial removal and field studies discussed above (Becker & Harris 2010; Lam et al. 2010; Woodhams et al. 2007) suggests that augmentation of amphibians' microflora with anti-*Bd* bacteria could be used to stop or slow the spread of chytridiomycosis. Ideally, application of anti-*Bd* bacteria to a substantial fraction of the amphibians in a population would alter the balance of the skin microbe assemblage, so that a higher proportion of individuals carry anti-*Bd* bacteria over a medium or long-term period. A self-perpetuating symbiont pool would ideally spread among individual amphibian hosts, providing herd immunity. Bacterial additions might also be applied at regular intervals to high-priority amphibian populations or habitats, if long-term or self-perpetuating bacterial protection is not achieved from a single application. So far, the only field trial of bioaugmentation was an addition of *Janthinobacteria lividium* to some populations of yellow-legged frogs in the region in which the field studies of Woodhams et al. (2007) and Lam et al. (2010) were carried out (Rex 2010). Results of this experiment are not yet available. As discussed in Chapter Six, continued field

trials and mesocosm-scale experiments are critical if bioaugmentation is to be employed on a meaningful scale for conservation.

2.4 Potential role of context-dependency in chytridiomycosis and bioaugmentation

2.4.1 Limitations of amphibian-bacterial symbiosis research to date

Studies of amphibians' microbial symbionts and their interactions with *Bd* have primarily taken place *in vitro*, mostly employing either of two challenge assay methods to identify anti-*Bd* bacterial symbionts under tightly controlled laboratory conditions. The first method, developed by Timothy James and used by Harris et al. (2006) in the original documentation of anti-*Bd* bacteria, involves co-culture growth assays in which *Bd* is grown in a lawn across an agar plate and the bacteria to be tested is streaked alongside *Bd*. As both microbes grow, if a zone of inhibition is apparent between *Bd* and the bacteria, it is deemed to be inhibitory. Non-inhibitory bacteria are overgrown by the fungus. While this method is intuitive, inexpensive, and relatively speedy once bacteria are available in pure culture, it does have drawbacks. Perhaps most importantly, the technique itself is quite tricky to employ, requiring enough moisture on the agar plate for *Bd* growth, but not so much that the bacteria is unable to establish (Harris, R. and Bell, S., pers. comm., July 2010). Additionally, although evaluation of the results of this method are intuitive, they are not quantitative, meaning there is little way to describe the strength of inhibition, and the amount of bacteria in an assay is not standardized.

The second challenge assay method is as yet unpublished, having been developed by Sara Bell in the two years prior to the experiments described Chapters Three and Four. In brief, a known quantity of live *Bd* zoospores is inoculated into liquid medium with a crude extract of a pure bacterial isolate (Bell in prep.). *Bd* growth is then monitored by spectrophotometry as in established methods for assaying the anti-*Bd* activity of amphibian skin peptides (Rollins-Smith et al. 2003; Rollins-Smith et al. 2002; Woodhams et al. 2006). This method is more time consuming, as extracts must first be produced from live bacterial cultures. It also requires more specialized equipment and training. The advantages of this method are that a quantitative

measure of growth inhibition is obtained for each isolate tested, and known quantities of bacterial extract and *Bd* are used; both provide more detailed information on antifungal activity than James' method. While these laboratory methods brought the phenomenon of amphibian-bacterial symbiosis and its potential to affect the outcome of *Bd* infection to light, they have limitations as they have been employed to date, especially for bioaugmentation in wild populations. The sterile, constant conditions of the lab remove the potential influence of any environmental or ecological drivers of amphibian-microbe symbioses. In the James method, live bacteria only interact with *Bd* or its products at the zone of contact between the pure cultures (Harris et al. 2006), limiting the chances for interspecific interaction. In the Bell method, live bacteria never interact with the fungus (Bell in prep.). Bacteria are known to alter the expression of antifungal genes depending on their interactions with fungi (Duffy et al. 2003) and inhibitory bacteria could be missed in the Bell assay if exposure to *Bd* induces antifungal activity. Alternatively, if *Bd* inhibits the action of these bacteria, poor bioaugmentation candidates could be misidentified as effective. Both laboratory assays also necessarily miss any effects of interspecific interactions among bacteria, and experiments to date have not incorporated the effects of other environmental variables (temperature, pH, etc…). The possibility that anti-*Bd* activity may depend on environmental and microbial community context could, in the worst case, negate the apparent protective activity of bacteria not yet studied *in situ*, which includes almost all of those reported. In the more likely case that observed *in vitro* production of antifungal compounds by amphibian bacterial symbionts varies but is not completely lost in the field, such changes in symbiont behavior could still be crucially important for use of symbionts in bioaugmentation. For instance, it will be necessary to understand the robustness of the antifungal action of candidate bacteria when selecting isolates for use in bioaugmentation trials or for larger-scale attempts at disease management.

2.4.2 Variation in bacterial symbiont defenses—past declines and future recovery

Context-dependency in amphibian-bacterial symbioses could help explain patterns of past chytridiomycosis-driven declines, as well as contribute to

bioaugmentation development. Other microbes, including antibiotic-producing symbionts, respond to temperature and other environmental variation, often leading to fitness consequences for hosts (Hofstetter et al. 2007; Prado et al. 2010; Shanahan et al. 1992). If bacterial production of antibiotics or abundance of protective bacteria on frogs is reduced at cooler temperatures, the association of worse declines with these conditions (Berger et al. 2004; La Marca et al. 2005; Woodhams & Alford 2005) could be partly explained. Also, in species that declined at cooler, higher elevation sites, frogs with the most robustly-antifungal bacteria may be most successful in recolonizing upland sites from persisting lowland populations. Anecdotal reports of such lowland-to-upland movement exist, but these migrations have not been studied carefully.

The lack of information on potential variation in activity of anti-*Bd* bacteria is largely due to the young state of amphibian-microbe research, but is also symptomatic of how microbes have been studied in the past. Others have noted that traditional microbiological and microbial ecology studies have involved *in vitro* study of bacteria and fungi far-removed from their native environments, thus eliminating all relevant context (Plowright et al. 2008; Straight & Kolter 2009). More recently, symbioses examined in the field or in more complex laboratory set-ups have revealed important consequences of more realistic environmental and ecological conditions for symbiosis outcomes (Little & Currie 2008; Rudgers et al. 2010; Thurber et al. 2009). Many of these examples were reviewed in Chapter One.

2.4 Conclusions

The study of variation in the defensive properties of amphibians' antifungal bacterial symbionts holds great opportunities for understanding patterns of past *Bd*-driven declines and for improving targeted prevention of future extinctions. Currently, there are no data on how abundance or activity of these bacteria change with environment or ecology, *in vitro* or in the wild, despite the importance of this potential variation. In fact, the truly short history of amphibian microbial symbiont research, both before and since the discovery of *Bd*-inhibiting bacteria, means that our understanding of the dynamics and drivers of symbiont-provided amphibian

immunity is extremely limited (Box 2.2). Nonetheless, bioaugmentation remains a viable option for chytridiomycosis management and amphibian conservation. The experiments that follow begin to explore context-dependency in anti-*Bd* activity with variable environmental and ecological circumstance.

Box 2.2: Key questions in amphibian microbial symbiont research

Diversity of amphibians and their symbionts

Among the thousands of amphibian species worldwide, fewer than a dozen species' microbiotas have been studied (see Table 2.1 above). These species belong to only four families and live on only two continents. To better understand the role of cutaneous microbes in natural populations, it will be necessary to sample the microbiota of a far greater number of species. The approach of Woodhams et al. (2007) and Lam et al. (2010) of sampling the microbiota before and after arrival of *Bd* to understand bacterial impacts on infection outcome is also a good one, and will maximize the ability to detect individual bacterial species, or microbial community attributes that successfully defend against the fungus in natural environments.

Uninfected populations can be found both on the local scale within otherwise infected landscapes and on the larger regional/national scale, including some spots with high amphibian diversity (Andreone et al. 2008; Skerratt et al. 2010; Weldon et al. 2008). However, given that prevention of the spread of *Bd* has been largely unsuccessful (Fisher et al. 2009; Kusrini et al. 2008; Wollenberg et al. 2010), it is likely to continue spreading to many currently uninfected locales. Focus should be on identifying potential bioaugmentation bacteria in these high-priority uninfected amphibian communities, to prepare managers for quicker action using locally-native amphibian symbionts, if and when it becomes necessary.

Formation of amphibian-bacteria symbioses

It is not clear how the microflora of amphibian skin is established. In amphibians with parental care and nest attendance, adults likely pass microbes from their skin to offspring (Banning et al. 2008; Harris et al. 2006; Lauer et al. 2007). However, non-brooding species such as *Rana muscosa* and several *Litoria* species can host anti-*Bd*

bacteria (see Table 2.1), and there have been few studies of how their microbiotas are obtained. They could acquire microbes from substrates and water bodies they inhabit, and may select for certain bacteria by way of anti-microbial peptides secreted from skin glands (Bettin & Greven 1986; Brucker et al. 2008b). Microbes may also be passed among individuals by direct contact, a phenomenon that could aid in producing herd immunity through bioaugmentation. No studies have assessed mechanisms of bacterial attachment to amphibian skin, but their mucous covering likely plays a role in facilitating microbe attachment.

Term of protection after application

Laboratory bacterial addition studies have investigated only short-term protection from *Bd* infection, when inoculation with *Bd* occurs within two or three days following inoculation with protective bacteria (Becker et al. 2009; Harris et al. 2009a; Harris et al. 2009b). In the wild, bioaugmentation will need to provide protection for months, and preferably across generations to avoid necessitating repeated bacterial application to frogs or their habitats. The yellow-legged frog field trial currently underway is the first to test for such long term protection (Lubick 2010), and more such studies in the wild or in captive environments are needed to understand the true potential and limitations of bioaugmentation.

Density of anti-Bd symbionts and the nature and concentration of their metabolites

Because the majority of amphibian microbial symbiont studies have been *in vitro*, little is known of the density of anti-*Bd* bacteria on amphibian skin. Additionally, only Brucker et al. (2008a; 2008b) have measured the concentration or described the nature of anti-*Bd* metabolites on wild hosts. Symbiotic microbes on other hosts are known to produce a wide range of bioactive substances (Piel 2004). Use of molecular and chemical techniques to understand the concentrations of metabolites and their producers could inform on thresholds required for effective protection from *Bd* in the field—a concept already suggested by other authors (Becker et al. 2009).

Non-target effects of bioaugmentation

Finally, resistance to bioaugmentation is certain to appear from those concerned about the non-target effects of adding even native bacteria to amphibians and their habitats. Researchers, therefore, cannot confine their work to amphibian-bacteria-fungus interactions. Mesocosm studies involving aquatic plants or insects may be valuable, although some inference may be possible from studies of the large-scale use of biocontrol bacteria already practiced in agricultural settings. Limited non-target effects are seen there (Winding et al. 2004), although crop fields are admittedly less complex than any native ecosystem.

A version of this chapter will be submitted to Biological Conservation with authors Daskin, J.H., Bell, S., Schwarzkopf, L., and Alford, R.A.

Chapter Three—Defensive bacterial symbionts of three *Litoria* species and the effects of temperature on their activity against *Batrachochytrium dendrobatidis*

Abstract

It may be possible to prevent or reverse amphibian declines caused by the fungal pathogen *Batrachochytrium dendrobatidis* (*Bd*) by supplementing populations of amphibians' cutaneous antifungal bacteria. However, little is known about the activity of such symbionts outside the laboratory, in natural environments. Typically, the activity of antibiotic-producing bacteria depends upon environmental context. This affects host health and fitness and the usefulness of symbionts in biocontrol schemes. We need to understand how the responses of anti-*Bd* bacteria to environmental conditions will affect their use in *Bd* management. I identified anti-*Bd* bacterial symbionts from *Litoria serrata* and *L. nannotis*, rainforest tree frogs endemic to Australia. I demonstrated that many symbionts show reduced anti-*Bd* activity *in vitro* at the cool temperatures that characterize these frogs' habitats in winter. Reduced effectiveness of anti-*Bd* bacteria at low temperatures may partially explain the association between past *Bd*-driven declines and cool temperatures, and help predict future amphibian declines. A more complete understanding of such context-dependency will be crucial in developing techniques for biocontrol of chytridiomycosis.

3.1 Introduction

Since the late 1970s, over 200 amphibian species have experienced rapid and extensive population declines and extinctions due to chytridiomycosis and the fungal pathogen that causes it, *Batrachochytrium dendrobatidis* (*Bd*) (Fisher et al. 2009; Laurance et al. 1996, 1997). Currently, no effective treatment or preventative for this disease is available for broad use in wild populations. However, bioaugmentation, supplementing populations of anti-*Bd* bacteria on skin of threatened amphibians,

could be a viable option for disease management, if wild animals gain increased protection as they can in laboratory trials (Harris et al. 2009; Lam et al. 2010; Woodhams et al. 2007).

Previous work on anti-*Bd* bacteria has been done under static, simplified laboratory conditions, but other symbiotic bacteria, including other producers of antibiotics, vary in abundance and activity with environmental context (Bronstein 1994; Little & Currie 2008; Prado et al. 2010). Variations related to environmental conditions can have dramatic evolutionary, ecological, and medical impacts (Grueb et al. 2003; Jaenike et al. 2010; Rosenberg et al. 2010; Smirnova et al. 2001), and can affect the success of biocontrol programs (Duffy et al. 2003; Humair et al. 2009; Schouten et al. 2004; Chapter One). Bacteria selected for anti-*Bd* activity observed in the laboratory may lose it on exposure to the more variable conditions present in nature. Therefore, optimal selection of bacteria for use in the management of chytridiomycosis requires knowledge of amphibian bacterial symbionts' responses to environmental conditions.

In addition to its relevance to bioaugmentation, environmentally-induced variation in the protection afforded to amphibian hosts by bacterial symbionts could partially explain patterns of past chytridiomycosis-driven declines. In the tropics, where the impacts of chytridiomycosis have been most severe, higher elevations and cooler seasons have been associated with higher prevalence of infection, more intense infections, and more frequent declines (Berger et al. 2004; Kriger & Hero 2008; Lips 1998; Woodhams & Alford 2005). *Bd*'s relatively cool thermal optimum (17-25°C) (Piotrowski et al. 2004) has been suggested to explain this pattern (Kriger & Hero 2008), but many declines have occurred well below this temperature window, where chytridiomycosis might be expected to be less severe. *Bd* in natural environments may adapt to low temperatures by increasing fecundity (Woodhams et al. 2008), or production of anti-*Bd* skin peptides, a component of many amphibians' innate immunity, may be down-regulated in cool environments (Rollins-Smith 2001; Rollins-Smith et al. 2002). A third possible explanation for the high incidence of *Bd*-driven declines at temperatures below the fungus's *in vitro* thermal optimum, though, is that symbiotic bacteria that also inhabit amphibian skin and can reduce the severity

of chytridiomycosis may have severely reduced activity or density at cooler temperatures. However, no information exists on how the composition or antifungal activity of amphibians' anti-*Bd* bacterial assemblages respond to environmental contexts.

To examine how the interaction between *Bd* and bacteria is affected by the temperature at which the bacteria are cultured, I initially set out to identify a suite of bacterial symbionts from the Australian hylid frogs *Litoria serrata* and *L. nannotis.* I then evaluated and characterized the antifungal activity of these and other *Litoria*-associated bacteria across a range of temperatures experienced by their amphibian hosts. This is the first study of context-dependency of anti-*Bd* activity in the microbial symbionts of amphibians.

3.2 Methods

3.2.1 Collection and isolation of bacteria

Bacteria were collected from six *Litoria serrata* and six *L. nannotis* caught at Windin Creek in Wooroonooran National Park (~750 m a.s.l., S 17°21′57″ E 145°42′54″) on the night of February 27, 2010. Each frog's legs and dorsum were swabbed twice with a sterile cotton swab after rinsing to remove non-resident bacteria (Lauer et al. 2007; Lauer et al. 2008). Each frog's swab was streaked onto a new Difco R2A agar plate. Using a second swab, the dorsal surfaces of the frog's body and its forefeet, legs, and hindfeet were sampled for *Bd*, according to Hyatt et al. (2007). Each frog was weighed and its snout-vent length (SVL) was measured. All field procedures included prevention of cross-contamination between individuals, using new plastic bags and gloves for capture and handling of each animal.

At the laboratory, each morphologically-distinct bacterium was isolated into pure colonies, described, and stored on R2A agar slopes, and at -80°C both in TSYE broth and on ceramic Microbank microbeads (Microbank, Inc.) (adapted from Lauer et al. 2007). Slides of each isolate were Gram-stained with crystal violet and viewed by light microscopy to ensure purity.

3.2.2 Baseline challenge assay

To determine which bacterial isolates inhibited growth of *Bd in vitro* and which did not, challenge assays were performed using a method developed by S. Bell (in prep.). Bacteria were inoculated into one mL ½-strength TGhL broth for 48-hours growth at 23°C. Each liquid culture was then centrifuged for five minutes at 9000 rpm, and the supernatant was filtered through a .22 μm syringe filter. This left a crude extract of bacterial metabolites, and no living cells.

In 96-well assay plates, 1.0×10^5 live *Bd* zoospores (isolate Gibbo River, L. Les donna, 06-LB-1) in 50 μL ½-strength TGhL were inoculated into each of five replicates of 50 μL of each bacterial crude extract. Positive and negative controls consisted of 1.0×10^5 live and heat-killed *Bd* zoospores, respectively, in 100 μL ½-strength TGhL. Plates were kept at 23°C and progress of *Bd* growth in each extract was monitored daily using spectrophotometry at 492 nm (Rollins-Smith et al. 2002). Monitoring continued until the positive control's growth plateaued.

Mean optical densities (OD_{492}) for each isolate on each day were transformed to correct for initial extract coloration (by subtracting the extracts' mean initial OD_{492}) and absorbance of inoculated zoospores (by subtracting the mean change in negative control OD_{492}), and standardized against the similarly corrected value for the positive control. To calculate the endpoint, or EP, I subtracted one from the corrected and standardized values. This returned a value of zero for the positive control, negative values for isolates in which *Bd* grew less well than in the positive control, and positive values for extracts in which *Bd* grew better than in the positive control (Equation 3.1).

Equation 3.1

$$EP = [(\text{Sample } OD_{492} - \text{Initial Sample } OD_{492} - \text{Final Negative Control } OD_{492}) / (\text{Positive Control } OD_{492} - \text{Initial Positive Control } OD_{492} - \text{Final Negative Control } OD_{492})] - 1$$

Any contaminated replicates were removed from the calculation after observation by light microscopy.

3.2.3 Identification of bacteria

DNA from each isolate that inhibited *Bd* in the baseline challenge assays was isolated, first by three freeze-thaw cycles of 10 minutes each at -80°C and 70°C, then,

if freeze-thaw cycles did not yield sufficient DNA for PCR, using Qiagen's (Hilden, Germany) DNeasy kit. DNA was amplified using universal bacterial 8F and 1492R primers, then sequenced by Macrogen, Inc. (Seoul, South Korea). Sequences were matched to the NCBI GenBank database (http://ncbi.nlm.nih.gov) to identify bacteria.

3.2.4 Experimental challenge assay

Following baseline assays, another challenge assay was performed to test for temperature-induced changes in anti-*Bd* activity of bacterial symbionts identified as inhibitory in the baseline assay. In addition to the bacteria from frogs sampled in February 2010, inhibitory bacteria isolated by Bell according to the same methods were included in experimental assays. These symbionts came from one *Litoria serrata*, one *L. nannotis*, and three *L. rheocola* sampled at Windin Creek in the Austral winter of 2009.

Each bacterium was inoculated from Microbank beads into 10 mL of ½-strength TGhL at 23°C for 48 hours growth. 500 µL of each bacterial solution was then inoculated into one mL of ½-strength TGhL at each of 8, 13, 18, 23, 28, and 33°C (Figure 3.1). Temperatures were chosen to approximate the range of temperatures experienced by *Litoria* in the Australian wet tropics (Rowley 2006).

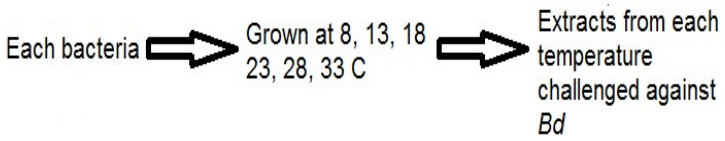

Figure 3.1—Experimental setup. Bacteria from three *Litoria* species were grown at each of six temperatures.

Cultures were grown for two to five days, based on the time to maximal bacterial concentration for each isolate-temperature combination in previous pilot studies, not presented here. Growing to maximum concentration should minimize any differences in metabolite production that otherwise could have been produced by quorum sensing mechanisms (Miller & Bassler 2001). Following growth at each temperature, the production of bacterial crude extracts and challenge assay were carried out as described for the baseline assay, with 2.9 X 10^4 zoospores initially inoculated in each

100 μL assay well. Approximately one third of cultures were viewed by light microscope during the assay to observe morphological changes in *Bd*.

3.2.5 Statistical analysis

A two-way factorial ANOVA was used to analyze the effects of temperature, bacterial class, and their interaction on the anti-*Bd* activity of tested bacteria. Type III sums of squares were calculated, as there were unequal numbers of bacteria across classes. All analyses were performed in SPlus (version 8.0, Insightful, Corporation). Again, any contaminated replicates were removed from the analysis after observation by light microscopy. Thus, the final number of observations was not equal to five replicates times the number of inhibitory bacteria tested.

3.3 Results

3.3.1 Summer frog and Bd sampling

The twelve frogs (six *Litoria serrata*, six *L. nannotis*) sampled in summer weighed between 3.5-18.0 g (median 8.2 g), and measured 40-61 mm (median 45 mm) SVL. Six frogs (two *L. serrata*, four *L. nannotis*) were infected with *Bd*, one *L. nannotis* had a deformed right foot, and one *L. nannotis* was gravid. Morphometrics and infection details of frogs sampled in winter are reported elsewhere (Bell in prep.).

3.3.2 Baseline challenge assay

The positive control reached maximum growth on days three and five of the two baseline assays, respectively. Mean EPs of the 97 isolates from the 12 frogs sampled in summer exhibited a non-normal distribution (Figure 3.2), with a clear cutoff (< -0.8) for inhibitory isolates. This value is similar to the cutoff for anti-*Bd* extract EPs observed by Bell (in prep.) using the same method.

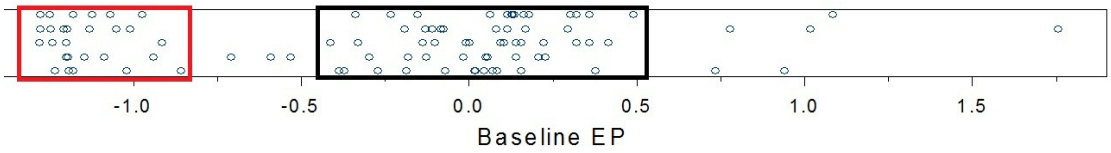

Figure 3.2—Endpoints (EP) for 97 extracts of amphibian bacterial symbionts in baseline challenge assays against *Bd*. Open circles represent extract EPs and are raised and lowered vertically to reduce symbol overlap. Red and black boxes denote inhibitory and inactive extracts, respectively.

Twenty-eight isolates were considered inhibitory by this standard; *Bd* in these isolates' extracts grew poorly, or not at all (Figure 3.3). The 28 inhibitory isolates came from four infected and four uninfected frogs.

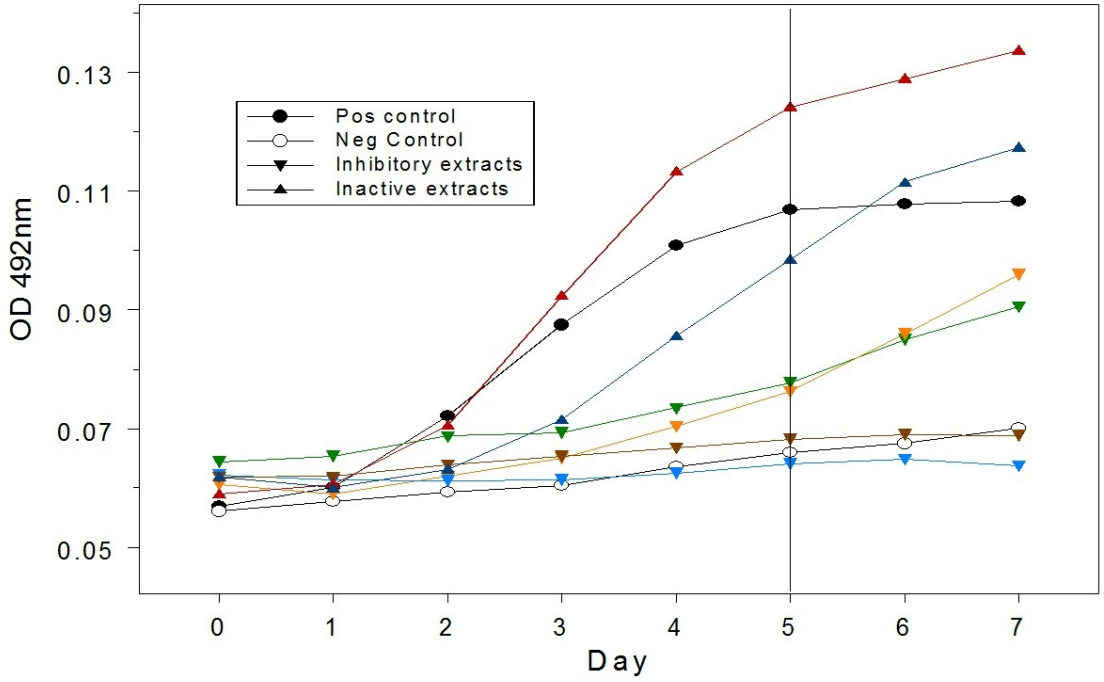

Figure 3.3—Example growth curves of positive control, negative control, inactive, and inhibitory extracts from a baseline challenge assay. Higher optical density at 492 nm (OD_{492}) indicates greater *Bd* growth. Day 0 OD_{492} varies with original extract color, which is corrected for in EP calculations (see Equation 3.1). The vertical line at Day = 5 denotes the end of the challenge assay, at the plateau of the positive control.

3.3.3 Identification of inhibitory bacteria

After removing duplicate isolates that came from the same individual frog and matched identical GenBank entries, 19 distinct isolations of inhibitory bacteria remained from summer samples. These 19 matched the 16 distinct operational taxonomic units (OTUs) listed in Table 3.1. Pseudomonads constituted seven of the 16 OTUs and nine of 19 distinct isolations from frogs, making them the most

common group among summer inhibitory bacteria. Winter inhibitory isolates included in experimental assays are also listed in Table 3.1.

Table 3.1—Inhibitory bacteria in experimental challenge assay

GenBank ID^ [Accession Number, % match]	Frog species and infection status
From frogs sampled in summer	
Actinobacteria	
Microbacterium sp. HY14(2010) [HM579805, 99.5%]	LN,Y
Bacilli	
Bacillus thuringiensis isolate CCM15B [FN433029, 99.8%]	LN,Y
Lysinibacillus sp. 210_8 [FJ938119, 99.3%]	LS,N
β-Proteobacteria	
Tetrathiobacter mimigardefordensis strain DPN7 [AY880023, 96.6%]	LS,N
Uncultured *Silvimonas* sp. clone ntu63 [EU159476, 98.5%]	LS,N
Flavobacteria	
Uncultured bacterium clone nbw1150f04c1 [GQ082309, 99.1%]; *Chryseobacterium hispanicum* type strain VP48 [AM159183, 98.7]	LS,N
γ-Proteobacteria	
Pseudomonas fluorescens strain d3 16S [HQ166099, 99.7%]	LS,N
Pseudomonas koreensis strain SSG10 [HM367598, 99.8%]	LN*,Y
Pseudomonas koreensis strain SSG5 [HM367599, 99.9%]	LN,N
Pseudomonas mosselii strain WAB1873 [AM184215, 99.7%]	LN,Y
Pseudomonas mosselii strain R10 [DQ073452, 99.6%]	LN,Y
Pseudomonas putida strain 31920-1 [FJ932760, 99.6%]	LN,Y
Pseudomonas sp. SBR3-slima [EU043328, 99.3%]	LN,Y
Serratia marcescens strain C1 [GU220796, 99.9%]	LS,N**
Uncultured bacterium clone nbw969a06c1 [GQ043359, 98.4%]; *Xanthomonas* sp. CC-AFH5 [DQ490979, 98.1%]	LN,Y
Uncultured bacterium clone P7D82-747 [EF509545, 99.6%]; *Stenotrophomonas* sp. TSG4 [HM135101, 99.6%]	LN,Y
From frogs sampled in winter	
β-Proteobacteria	
Bacterium H2 [AY345552, 99.1%]; *Iodobacter* sp. CdM7 [FJ872386, 98.8%]	LR,Y
Flavobacteria	
Chryseobacterium sp. CH33 [GU353129, 99.1%]	LN,Y
γ-Proteobacteria	
Hafnia alvei [AB519795, 99.9%]	LN,Y
Pseudomonas fluorescens strain 1408 [GU726880, 99.9%]	LS,N
Pseudomonas fluorescens strain: KU-7 [AB266613, 98.9%]	LR,Y
Pseudomonas koreensis strain Ps 9-14 [NR_025228, 99.9%]	LR,Y

Pseudomonas putida strain PASS3-tpnb [EU043325, 99.7%]	LR,Y
Pseudomonas sp. CBCN33 [EF427863, 99.6%]	LN,Y
Pseudomonas tolaasii strain NCPPB 2325 [AF320990, 100%]	LS,Y
Stenotrophomonas maltophilia strain 6B2-1 [AB306288, 99.9%]	LR,Y
Stenotrophomonas maltophilia strain YLZZ-2 [EU022689, 99.6%]	LS,N
Stenotrophomonas sp. 7-3 [EU054384, 99.6%]	LR,Y
Stenotrophomonas sp. B4M-T [GQ478276, 99.8%]	LR,Y

LN = *Litoria nannotis*, *LR = L. rheocola*, *LS = L. serrata*

^ Where the best GenBank match was an unnamed bacterium, the closest named match is included to give the best possible sense of likely phylogenetic breadth.

Two isolates from two additional LN, one infected, one uninfected, matched the same GenBank ID.

** Another isolate that matched to the same GenbankID was found on an infected LN.

3.3.4 Experimental challenge assay

Bd in positive controls reached maximum growth on day four of the experimental challenge assay. Different bacterial isolates appeared to show a range of responses to temperature, with antifungal activity increasing or decreasing systematically, changing erratically, or remaining steady across the range of temperatures tested (Figure 3.4).

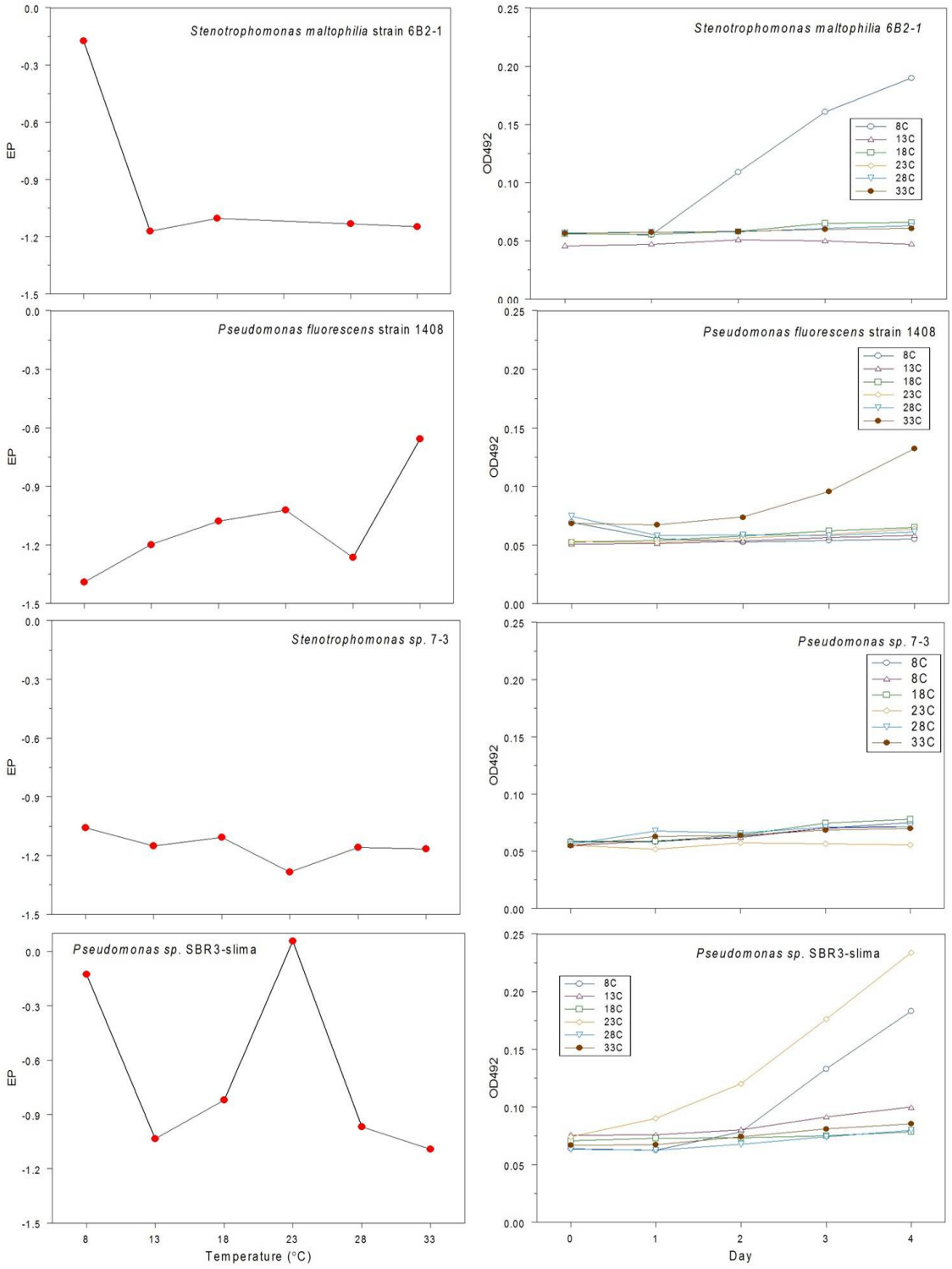

Figure 3.4—Paired challenge assay endpoints (EP) vs. temperature and *Bd* growth curves (optical density at 492 nm, OD) from the experimental challenge assay for each of four bacteria. EPs and ODs are mean values for all replicates of illustrated bacteria. Higher EP at a given temperature corresponds to greater OD492, a surrogate for *Bd* growth.

Across all tested bacteria, antifungal activity tended to be lowest at 8°C (Figure 3.5). If the EP < -0.8 cutoff from the baseline assay is used to define an extract as inhibitory, approximately 40% of extracts exhibited lost antifungal activity at 8°C, more than in other temperature treatments.

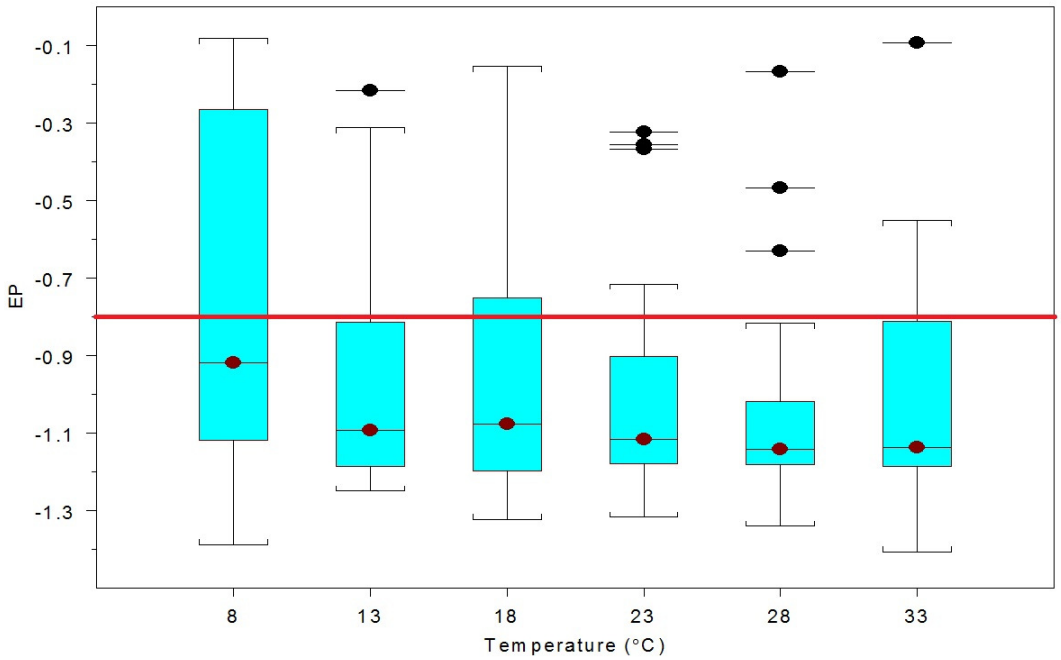

Figure 3.5—Mean challenge assay endpoints (EP) of bacterial extracts for 29 bacteria vs. the temperature at which they were produced. Extracts with EPs > -0.8 (denoted by the horizontal line) lost anti-*Bd* activity.

The ANOVA demonstrated that temperature, bacterial class, and to a marginal extent, their interaction, had significant effects on the antifungal activity (EP) of bacteria from four of the classes tested (Table 3.2). β-Proteobacteria were excluded from the factorial ANOVA because none of these isolates' extracts grown in the 13°C treatment remained uncontaminated in the challenge assay, leaving an empty cell in the full factorial design. There were too few bacteria in this class to perform a separate one-way ANOVA to formally test for effects of temperature on the β-Proteobacteria's EP, but the median EP for the group was greatest at 8°C (Figure 3.6).

Table 3.2—ANOVA table for the effects of temperature and bacterial class on antifungal activity of *Litoria* species bacteria symbionts.

	df	SS	MS	F	p-value
Temperature	5	1.49	0.30	3.76	< 0.005
Class	3	2.42	0.81	10.23	< 0.001
Temperature:Class	15	1.97	0.13	1.66	0.068
Residuals	119	9.40	0.08		

In general, antifungal activity was reduced at 8°C, and, with the exception of the β-Proteobacteria tested, was greater at 28°C and 33°C (Figure 3.6). The anti-*Bd* activity of Flavobacteria and Actinobacteria was diminished at 18°C and 23°C, respectively. Finally, the β-Proteobacteria had greatest anti-*Bd* activity between 18°C and 28°C.

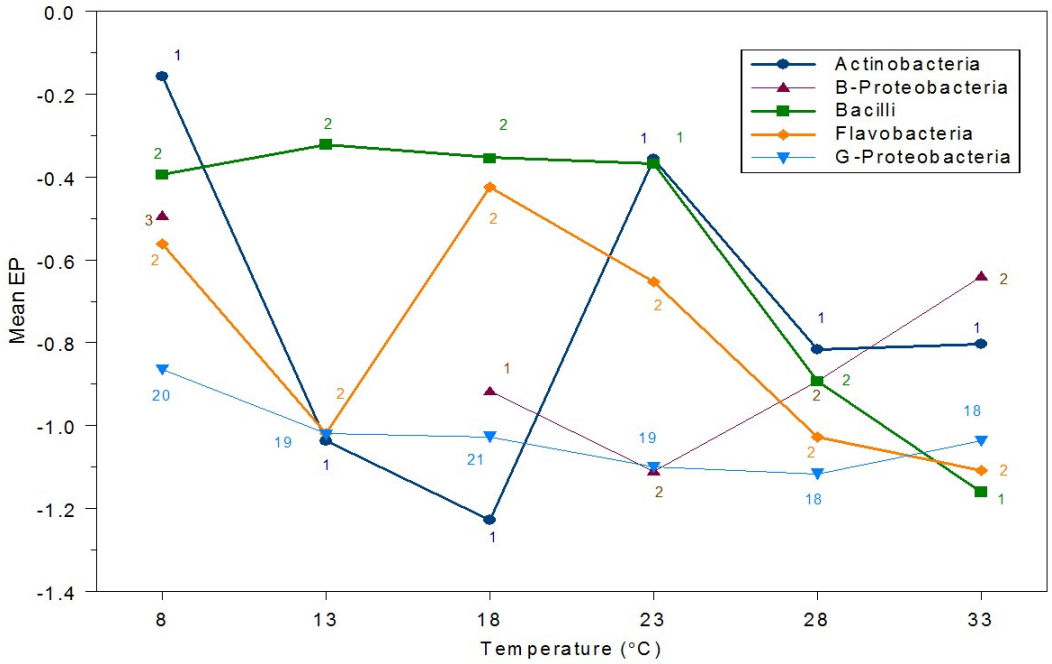

Figure 3.6—Interaction between temperature and bacterial class in mean endpoint (EP) of tested bacteria. Greater EP = reduced anti-*Batrachochytrium dendrobatidis* activity. Numbers adjacent to each point indicate the number of bacterial OTUs tested that contributed to the mean EP presented. Each OTU-temperature combination was replicated one to five times.

Across temperatures, the EPs of bacteria sampled in summer were more variable than those of frogs sampled in winter (Figure 3.7).

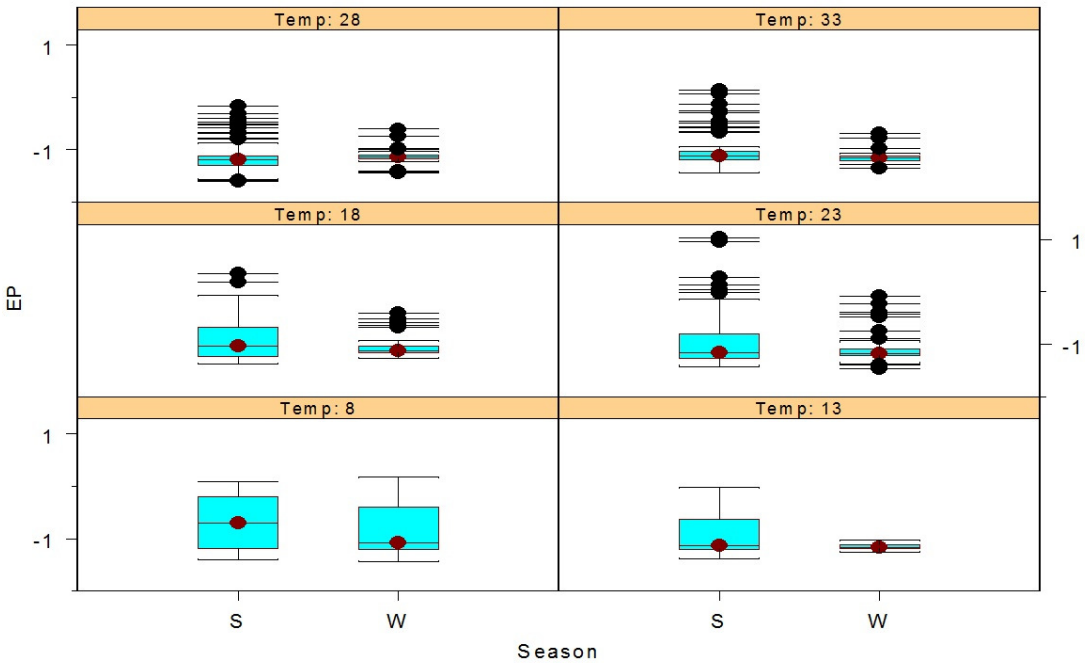

Figure 3.7—Challenge assay endpoints (EP) by season of bacterial collection and temperature treatment. Lower endpoints indicate greater activity against *Batrachochytrium dendrobatidis*.

Cultures observed by light microscopy during the experimental challenge assay showed that *Bd* responded in various ways to the extracts of different inhibitory bacteria. For example, in the extract of *Pseudomonas fluorescens* strain 1408 grown at 8°C, the zoospores of *Bd* appeared dead and deformed, whereas the extract of *Tetrathiobacter mimigardefordensis* strain DPN7 grown at 33°C stunted *Bd* sporangia growth. In the extract from *Chryseobacterium* sp. *CH33* grown at 8°C, zoospores developed into sporangia that died without producing viable zoospores. Similar variation existed across observed cultures.

3.4 Discussion

3.4.1 The challenge assays and variable antifungal activity

The clearest pattern that emerged from the present study was reduced antifungal activity of amphibians' defensive bacterial symbionts at low environmental temperatures. This was evident across all bacterial classes (Figure 3.6) and could be a contributing factor in high elevation chytridiomycosis-driven declines of the three *Litoria* species sampled here (McDonald & Alford 1999; Richards et al. 1993; Woodhams & Alford 2005). Winter temperatures at Windin Creek and other high

elevation habitats of threatened Australian hylids can dip below 8°C in winter (Puschendorf 2009, and Sapsford unpublished). Therefore, frogs may experience decreased bacterial protection from *Bd* in winter, when die-offs occur most often. If the composition of *Litoria* microbiotas does shift with season, it is likely that warm-adapted summer bacteria would respond more strongly to cool temperatures, as seen here (Figure 3.7). Winter bacteria, though, did not lose antifungal activity at warm temperatures. This pattern fits with the hypothesis that reduced antifungal activity in winter, but not summer, could, in part, explain cool-weather chytridiomycosis-driven declines. It is less clear why bacteria sampled in summer were more variable across temperatures than were bacteria from winter sampling.

Although I can only speculate from the limited number of frogs and species sampled in this study, other amphibians may also be more vulnerable to decline due to reduced bacterial protection from *Bd* at cool temperatures. Anti-*Bd* bacteria have been found on a wide geographic and phylogenetic range of amphibians (Harris et al. 2006; Lam et al. 2010), and the pattern that chytridiomycosis is more virulent at cool temperatures is also widespread (Berger et al. 2004; Kriger & Hero 2007; Lips 1998; Woodhams & Alford 2005). Additionally, many bacteria alter rates of production of antibiotics in response to environmental temperatures (Humair et al. 2009; Kavitha & Vijayalakshmi 2009; Raaijmakers et al. 2002). Larger scale, longitudinal studies across environmental gradients and between seasons, characterizing the diversity and abundance of amphibian bacterial symbionts, and their metabolites, are required to test this hypothesis in the amphibian-*Bd*-bacteria symbiosis.

The Flavobacteria and Actinobacteria tested did not on average show unidirectional responses to temperature; rather they lost anti-*Bd* activity at the lowest temperature, and again at moderate temperatures. The production of different antibiotics at different temperatures could explain the non-linear response in the groups. Many bacteria are known to produce more than one antibiotic (Raaijmakers et al. 2002), and some, including Actinobacteria, regulate their production through multiple genes (Cundliffe 2006).

Complete understanding of the behavior of *Bd* and bacterial extracts in the challenge assay procedure employed here is still being refined. Based on mean EP,

three summer and one winter (14% of all tested) bacteria that inhibited *Bd* in the baseline assay lost inhibitory activity at the baseline 23°C in the experimental assay. It is unclear what the cause of this transition was, although the three-and-a-half-fold difference in *Bd* concentration between the experimental and baseline assays is a possibility, if different concentrations of *Bd* respond differently to a given bacterial extract.

3.4.2—Pseudomonas bacteria as anti-Bd symbionts and bioaugmentation candidates

In the search for potential bioaugmentation candidates, a focus on the activity of Gamma Proteobacteria, and specifically *Pseudomonas* species, seems warranted. Seven of 16 distinct operational taxonomic units (OTUs) from my summer sampling were Pseudomonads, and it is becoming clear that Pseudomonads are widespread antifungal symbionts of amphibians. *Litoria* symbionts sampled in winter (Bell in prep.) and tested here, include many anti-*Bd* Pseudomonads. Woodhams et al. (2007) found 50% of anti-*Bd* bacterial OTUs from *Rana muscosa* and *R. sierrae* in California were *Pseudomonas* species, and the salamanders *Hemidactylium scutatum* and *Plethodon cinereus* also harbor antifungal Pseudomonad symbionts (Lauer et al. 2007; Lauer et al. 2008). Many *Pseudomonas* species produce antibiotics as plant symbionts and soil-dwelling bacteria, are used as antifungal biocontrol agents in agriculture (Haas & Defago 2005; Raaijmakers et al. 1997), and the present data suggest they may be promising candidates for management of chytridiomycosis by bioaugmentation.

3.4.3 Management implications and future research

The fact that some symbionts change activity across temperatures means that only the most robustly-antifungal isolates should be used in bioaugmentation. Antifungal activity observed in static, simplified laboratory conditions could be lost upon exposure to variable field environments. Using antifungal bacteria with inconsistent activity across temperatures in bioaugmentation attempts could cost managers time and resources, and could create the illusion that bioaugmentation is less effective than it could be, if more appropriate isolates were used. Even closely related bacteria may

respond differently to environmental variations, as illustrated by two *Stenotrophomonas* spp. in this study (Figure 3.4). One retained strongly antifungal activity across the entire 8-33°C range, whereas the other lost antifungal activity at 8°C.

Additionally, the bacteria tested in the experimental challenge assay were selected based on their inhibitory activity at 23°C in the baseline assay. It is likely that a somewhat different subset of the entire sampled bacterial community would have been recognized as inhibitory if the baseline assay had been run at either a higher or lower temperature. One of my aims, however, was to document context-dependency missed by standard challenge assays, which are run at 23°C. It remains possible that some bacteria from the skin of the *Litoria spp.* sampled here are effectively antifungal at low temperature and were not selected because they are not inhibitory at 23°C.

Only very limited work has assessed the metabolic products of amphibian symbionts (but see Becker et al. 2009; Brucker et al. 2008a; Brucker et al. 2008b). Based on the varied responses of *Bd* to extracts from different bacteria, that I observed microscopically, and the wide phylogenic range represented in addition to the Pseudomonads, it is likely that a variety of anti-fungal compounds were produced by the bacteria I tested. Future work should seek to identify these compounds, to make it possible to measure of their concentrations on amphibian skin. A few bacteria (Figure 3.2) may produce metabolites that actually stimulate *Bd* growth. This is a potentially interesting result, and one that should be explored further.

There is an urgent need to continue extending research on amphibian anti-*Bd*-bacteria to more realistic *in vivo* and *in situ* conditions. The present study focused on changes to the bacterial production of anti-*Bd* metabolites, but not on potential context-dependent changes to the fungus, itself. It is a step towards understanding the environmentally-induced variation in the amphibian-*Bd*-bacteria symbiosis, but no study has yet assessed both *Bd* and bacterial responses to varying temperature. Additionally, no mesocosm or field study of bioaugmentation has been completed to date, and a host of questions remain surrounding the best methods of application of beneficial bacteria, non-target effects, and the term of protection afforded (see Box 2.1). Transitioning *Bd*-bacteria research into *in vivo* systems will also make results

more reliable for bioaugmentation application in similar natural environments, even if interpretation of specific experimental treatments is complicated. Given the opportunity to apply bioaugmentation for protection of many amphibian species globally, it is most important to develop effective management protocols, while identifying robust bacteria commonly found on target species' skin. This is, perhaps, more important than a heavy focus on questions of basic microbial ecology of amphibian skin.

A version of this chapter will be submitted to PLoS One with authors Daskin, J.H., Bell, S., and Alford, R.A.

Chapter Four—Limited activity of *Batrachochytrium dendrobatidis* metabolites against antifungal amphibian symbionts

Abstract

Laboratory studies have identified many amphibian bacterial symbionts with activity against the virulent amphibian pathogen *Batrachochytrium dendrobatidis* (*Bd*). If their natural populations on amphibian skin can be supplemented, a process termed "bioaugmentation," such bacteria could be used for biocontrol of *Bd* and management of chytridiomycosis. Few of these bacteria have been studied *in situ*, though, and little is known of interspecific interactions in the amphibian skin microflora.

One possible challenge to bioaugmentation in natural amphibian populations is that *Bd* may have mechanisms to defend against the compounds produced by antifungal bacteria. Other fungal pathogens employ a range of defenses against antagonistic bacteria, and similar activity in *Bd* could decrease the effectiveness of protective amphibian symbionts. However, no study has assessed effects of *Bd* or other Chytridiomycete fungi on activity of antagonistic bacteria. I tested the affects of co-culturing bacterial symbionts of three *Litoria* spp. frogs with a solution of *Bd* metabolites on the bacteria's anti-*Bd* activity. *Bd* metabolites only had a significant affect on the anti-*Bd* activity of five out of 35 bacterial symbionts isolated from the Australian hylids *Litoria serrata*, *L. rheocola*, and *L. nannotis*. Two of 27 bacteria that inhibited *Bd* in baseline assays lost anti-*Bd* activity, and three of eight that were inactive in baseline assays gained anti-*Bd* activity on exposure to *Bd* metabolites. Because few anti-*Bd* bacteria lost their antifungal activity, *Bd* metabolites may not be a major hurdle preventing the management of chytridiomycosis by bioaugmentation.

4.1 Introduction

Chytridiomycosis, the fungal disease caused by *Batrachochytrium dendrobatidis* (*Bd*), has caused declines and extinctions in several hundred amphibian species

(Skerratt et al. 2007). To date, no effective preventative or treatment is available for large-scale use in wild animals, but supplementing the native populations of amphibians' antifungal bacterial symbionts (a process termed "bioaugmentation") could be a viable way to manage chytridiomycosis (Harris et al. 2009a; Harris et al. 2006; Woodhams et al. 2007). Several studies have identified amphibian bacterial symbionts with antifungal activity by challenging pure cultures of the bacteria or their metabolites against pathogenic fungi, including *Bd* (Culp et al. 2007; Harris et al. 2006; Woodhams et al. 2007; Bell in prep; Chapter 3). Some of the metabolites of skin bacteria that show anti-*Bd* activity have been identified (Becker et al. 2009; Brucker et al. 2008b; Harris et al. 2009a). One recent study identified a protease in *Bd*'s metabolites thought to be active in *Bd*'s invasion of amphibian skin (Moss et al. 2010). However, no work has explicitly considered two-way bacteria-*Bd* interactions, or more specifically, *Bd*'s effects on amphibians' antifungal bacteria.

Despite the lack of information on *Bd*'s defenses, the ability of other fungal pathogens, mainly higher Ascomycetes and Basidiomycetes, to defend against antagonistic microbes is well documented (Duffy et al. 2003; Schouten et al. 2004). *Fusarium oxysporum*, a common crop pathogenic fungus, can down-regulate both fungal and bacterial biocontrol organisms' antibiotic production by secretion of its own metabolites (e.g., fusaric acid) (Bacon et al. 1996; Lutz et al. 2003; Schouten et al. 2004). Fungi from several genera can metabolize mycotoxic plant oils (Kinderlerer 1993) and or modify the target sites for bacterial action (Morrissey & Osbourn 1999). Other fungi sequester or remove antagonistic compounds (Duffy et al. 2003), and biocontrol effectiveness in both agriculture and aquaculture can be hindered by pathogen defense (Crowe & Olsson 2001; Ringo & Gatesoupe 1998). There is no known study of a Chytridiomycete's defenses against bacterial antagonism, but bioaugmentation for chytridiomycosis management may be affected by potential *Bd* defenses.

In previous studies, anti-*Bd* bacteria grown in culture have typically had only very limited exposure to *Bd* or its products. One challenge assay method employs pure cultures of *Bd* and bacteria that only meet at the point of challenge (Harris et al. 2006), while another never allows live bacteria to interact with the fungus (Bell in

prep.; Chapter 3). If longer exposures to *Bd* inhibit antifungal activity, poor bioaugmentation candidates could be misidentified as effective. Alternatively, if antifungal activity is induced in some bacteria upon exposure to *Bd*, inhibitory bacteria could be missed using current assay methods.

To examine possible reciprocal interactions between *Bd* and bacteria, I set out to characterize the *in vitro* effects of *Bd*'s metabolites on anti-*Bd* activity of bacterial symbionts from the Australian hylid frogs *Litoria nannotis*, *L. rheocola*, and *L. serrata*. This study is the first to explicitly consider two-way interactions between *Bd* and the potentially protective symbionts of amphibians.

4.2 Methods

4.2.1 Collection and identification of bacteria

The bacteria tested here (Table 4.1) were isolated and identified from *Litoria serrata*, *L. rheocola* and *L. nannotis* (Chapter 3). Briefly, frogs from Windin Creek, Wooroonooran National Park, QLD (~750 m a.s.l., S 17°21′57″ E 145°42′54″) were swabbed in the field for bacterial collection. All field procedures included prevention of cross-contamination between individuals, using new plastic bags and gloves for capture and handling of each animal. Bacteria were then isolated in pure culture in the laboratory (adapted from Lauer et al. 2007), and their baseline anti-*Bd* activity was determined using a standard challenge assay (Chapter 3). DNA sequencing by Macrogen Inc. (Korea) allowed matching of bacteria to the NCBI GenBank database for identification.

Eight additional bacterial isolates, inactive against *Bd* in the baseline assay, were also included with the antifungal symbionts. These were included to test whether the metabolites of *Bd* could initiate production of anti-*Bd* activity, if present during the pre-extract-production culture. These eight baseline inactive isolates were isolated from the same frogs by the same methods as the inhibitory bacteria (Chapter 3). They were chosen from the larger pool of inactive symbionts, based on morphology, to try to maximize the phylogenetic spread of bacteria exposed to *Bd* metabolites in these experiments. A total of 35 bacteria were included in the present experiment.

Table 4.1—Bacteria tested in the present experiment, and their activity in the baseline challenge assays of Chapter 3.

GenBank ID^ [Accession Number]	Baseline activity
From frogs sampled in summer	
Actinobacteria	
Micrococcus luteus strain 6J-4A strain 6J-4a [EU379295]	Inactive
A-Proteobacteria	
Uncultured soil bacterium clone SBANT43 [HM596217]; *Sphingomonas* sp. isolate K74 [AJ009709]	Inactive
Bacilli	
Bacillus thuringiensis isolate CCM15B [FN433029]	Inhibitory
Lysinibacillus sp. 210_8 [FJ938119]	Inhibitory
Bacillus cereus [GU171381]	Inactive
B-Proteobacteria	
Tetrathiobacter mimigardefordensis strain DPN7 [AY880023]	Inhibitory
Uncultured *Silvimonas* sp. clone ntu63 [EU159476]	Inhibitory
Flavobacteria	
Uncultured bacterium clone nbw1150f04c1 [GQ082309]; *Chryseobacterium hispanicum* type strain VP48 [AM159183]	Inhibitory
Flavobacterium sp. WB2.3-9 [AM934644]	Inactive
γ-Proteobacteria	
Enterobacter aerogenes strain 1-WCH [FJ811872]	Inactive
Pseudomonas fluorescens strain d3 16S [HQ166099]	Inhibitory
Pseudomonas koreensis strain SSG10 [HM367598]	Inhibitory
Pseudomonas koreensis strain SSG5 [HM367599]	Inhibitory
Pseudomonas mosselii strain WAB1873 [AM184215]	Inhibitory
Pseudomonas mosselii strain R10 [DQ073452]	Inhibitory
Pseudomonas sp. SBR3-slima [EU043328]	Inhibitory
Serratia marcescens strain C1 [GU220796]	Inhibitory
Uncultured bacterium clone 6s2 [DQ068850]; *Enterobacter aerogenes* strain 1-WCH [FJ811872]	Inactive
Uncultured bacterium clone nbw969a06c1 [GQ043359]; *Xanthomonas* sp. CC-AFH5 [DQ490979]	Inhibitory
Uncultured bacterium clone P7D82-747 [EF509545]; *Stenotrophomonas* sp. TSG4 [HM135101]	Inhibitory
Pseudomonas sp. lm10 [EU240462]	Inactive
Uncultured Gamma proteobacterium clone RSC_RRA09 [GU205302]; *Pectobacterium cypripedii* strain EH59 [GU339286]	Inactive
From frogs sampled in winter	
B-Proteobacteria	
Bacterium H2 [AY345552]; *Iodobacter* sp. CdM7 [FJ872386]	Inhibitory
Flavobacteria	
Chryseobacterium sp. CH33 [GU353129]	Inhibitory
γ-Proteobacteria	
Hafnia alvei [AB519795]	Inhibitory
Pseudomonas fluorescens strain 1408 [*GU726880*]	Inhibitory
Pseudomonas fluorescens strain KU-7 [*AB266613*]	Inhibitory
Pseudomonas koreensis strain Ps 9-14 [*NR_025228*]	Inhibitory
Pseudomonas putida strain PASS3-tpnb [EU043325]	Inhibitory

Pseudomonas sp. CBCN33 [EF427863]	Inhibitory
Pseudomonas tolaasii strain NCPPB 2325 [AF320990]	Inhibitory
Stenotrophomonas maltophilia strain 6B2-1 [AB306288]	Inhibitory
Stenotrophomonas maltophilia strain YLZZ-2 [EU022689]	Inhibitory
Stenotrophomonas sp. 7-3 [EU054384]	Inhibitory
Stenotrophomonas sp. B4M-T [GQ478276]	Inhibitory

^ Where the best GenBank match was an unnamed bacterium, the closest named match is included to give the best possible sense of likely phylogenic breadth.

4.2.2 Production of Bd metabolites

Bd (isolate Lles donna 2) in ½-strength TGhL broth was passed weekly and kept at 23°C, the optimal temperature for *Bd* growth (Piotrowski et al. 2004). On the maximum growth day (the seventh after passing), eight mL of this solution was removed and placed in a centrifuge tube. The solution contained 1.38 X 10^5 live zoospores and 3.5 X 10^4 live sporangia per mL, estimated by counting on a haemocytometer. Given the growth of *Bd* in culture and the presence of live fungi in the solution, it is assumed that *Bd* metabolites were present in the broth. This solution was centrifuged for five minutes at 9000 rpm to pellet the fungi, and the supernatant, containing *Bd* metabolites, was filtered through 0.22 µm syringe filters to ensure removal of live fungi from the remaining metabolite solution.

4.2.3 Experimental growth treatments

Each of the 35 bacteria was inoculated from ceramic microbeads (Microbank, Inc.) at -80°C into 10 mL of ½-strength TGhL broth at 23°C. After 48 hours growth, 500 µL from each liquid culture were inoculated into (1) one mL ½-strength TGhL, and (2) 200 µL of the *Bd* metabolite solution, plus 800 µL ½-strength TGhL. The first condition was identical to previous challenge assays that disregarded potential fungal effects on bacteria, and the second tested for induction or inhibition of bacterial anti-*Bd* activity upon exposure to *Bd* metabolites. A control condition, identical to the *Bd* metabolite treatments, but with no bacteria, was also produced.

4.2.4 Experimental challenge assays

Following 48 hours growth of bacterial culture treatments at 23°C, bacterial crude extracts were produced for challenge assays (Bell in prep; Chapter 3). Liquid cultures and controls were centrifuged and filtered to remove all live bacteria. 7.80 X 10^4

zoospores of *Bd* (isolate Lles donna 2) in 50 µL ½-strength TGhL were inoculated into five replicates of 50 µL of each bacterial extract. Positive and negative controls consisted of 7.80 X 10^4 live and heat-killed *Bd* zoospores, respectively, in 100 µL ½-strength TGhL. The *Bd* metabolite control was also inoculated with live zoospores. Progress of *Bd* growth in each extract was monitored daily using spectrophotometry (Rollins-Smith et al. 2002), until the growth of the positive control plateaued. The endpoint, EP, for each extract-*Bd* combination was calculated (Equation 4.1). See Chapter 3 for a detailed explanation of the calculation of endpoints.

Equation 4.1
EP = [(Final Sample OD_{492} - Initial Sample OD_{492} - Final Negative Control OD_{492}) / (Final Positive Control OD_{492} - Initial Positive Control OD_{492} - Final Negative Control OD_{492})] - 1

To distinguish EPs from a given bacterium grown in the two experimental treatments, I refer to EPs from extracts produced in the *Bd*-metabolite treatment as EP_c, for "EP co-culture."

4.2.5 Statistical analyses

If no systematic overall (across isolates) change in endpoint occurred between treatments, i.e., if there were no significant effects of *Bd*'s metabolites on anti-*Bd* effects of bacteria, then the slope of the least-squares regression line for EP_c on EP should equal one. If there was a significant influence of *Bd* metabolites on the anti-*Bd* effects of bacteria, then the slope of this regression line should be greater or less than one. I therefore used a regression of EP_c against EP to examine the influence of *Bd* metabolites on anti-*Bd* effects of bacteria. I used the 95% confidence intervals for the slope to determine whether it was significantly different from one.

A previous experiment demonstrated that bacterial extracts with endpoints < -0.8 are inhibitory, and those with endpoints > -0.5 do not inhibit *Bd* growth (Chapter 3). Thus, for individual isolates, I considered EP-EP_c differences meaningful when there was a shift from a value considered inhibitory to one considered inactive. For each isolate that displayed a biologically meaningful EP-EP_c shift, I used a t-test to check for statistical significance, too.

All analyses were performed in SPlus 8.0 for Windows (Insightful Corporation, 2007).

4.3 Results

Because the difference between *Pseudomonas* sp. SBR3-slima's EP_c and EP was greater than three SD from the mean of this quantity for all bacteria tested, least squares linear regression lines were fit to the EP_c-EP data both with and without this bacterium's data. With all bacteria included, EP explained 62% of the variation in EP_c (Table 4.2) and the 95% confidence interval for the slope of the least squares regression line (0.51-0.91) did not include one, the value expected if *Bd* metabolites had no significant overall influence on the anti-*Bd* effects of bacteria. When *P.* sp. SBR3-slima was removed, EP explained 75% of the variation in EP_c (Table 4.3), and the 95% confidence interval for the slope of the least squares regression line (0.73 - 1.13) included one.

Table 4.2—Least squares regression of EP_c on EP for all tested bacteria.

	Value	*Standard Error*	t_{32}	*P*
Intercept	-0.28	0.11	-2.64	< 0.05
EP	0.71	0.097	7.28	< 0.001
R^2	0.62			

Table 4.3—Least squares regression of EP_c on EP with the outlier *Pseudomonas* sp. SBR3-slima removed from the dataset.

	Value	*Standard Error*	t_{32}	*P*
Intercept	-0.10	0.10	-1.03	0.31
EP	0.93	0.096	9.72	< 0.001
R^2	0.75			

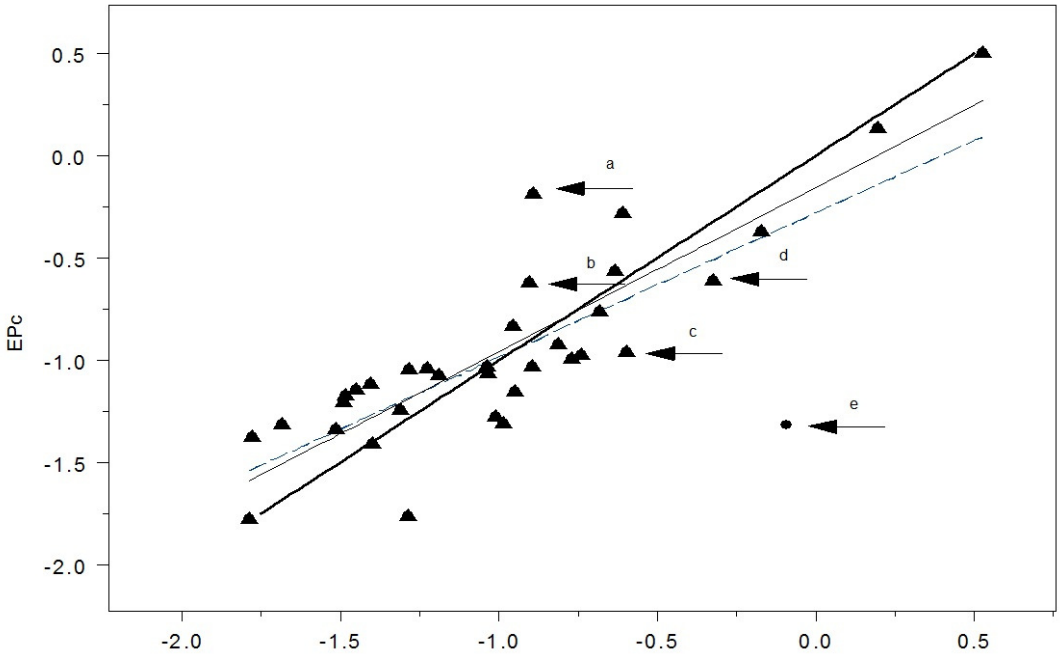

Figure 4.1—Regression of EP_c on EP. The dark solid line is the 1:1 line, with the slope expected if there is no affect of co-culture with *Bd* metabolites on the anti-*Bd* activity of tested bacteria. The dashed line is the least squares regression line for all tested bacteria, and the thin solid line is that for all bacteria excluding *Pseudomonas* sp. SBR3-slima, point e denoted with a circle. Arrows indicate five isolates whose extracts transitioned from inhibitory to inactive or vice versa. a = *Pseudomonas mosselii* strain R10, b = *Bacillus cereus*, c = *Micrococcus luteus* strain 6J-4A, d = *Tetrathiobacter mimigardefordensis*, and e = *Pseudomonas* sp. SBR3-slima. Indicated points above the line denote isolates whose extracts lost anti-*Bd* activity when produced from cultures grown with *Bd* metabolites, while those below the line gained anti-*Bd* activity.

Exposure to *Bd* metabolites did not alter the anti-*Bd* activity of most bacterial isolates, i.e., *Bd* growth curves (OD_{492} vs. time) were generally similar whether *Bd* metabolites were present or absent in the broth in which the bacteria that produced the tested extracts were grown. However, exposure to *Bd* metabolites did shift the activity

of five bacterial isolates from inhibitory to inactive, or vice versa (four indicated in Figure 4.1). *Pseudomonas mosselii* strain R10 (EP = -0.89, EP_c = -0.18; t_4 = -6.34, p < 0.005) and *Bacillus cereus* (EP = -0.90, EP_c = -0.62; t_4 = -9.25, p < 0.001) both lost inhibitory activity when grown in the presence of *Bd*'s metabolites, and partial inhibitory activity was induced in *Micrococcus luteus* strain 6J-4A following the *Bd* metabolite treatment (EP = -0.60, EP_c = -0.96; t_4 = -0.74, p = 0.50) (Figure 4.2).

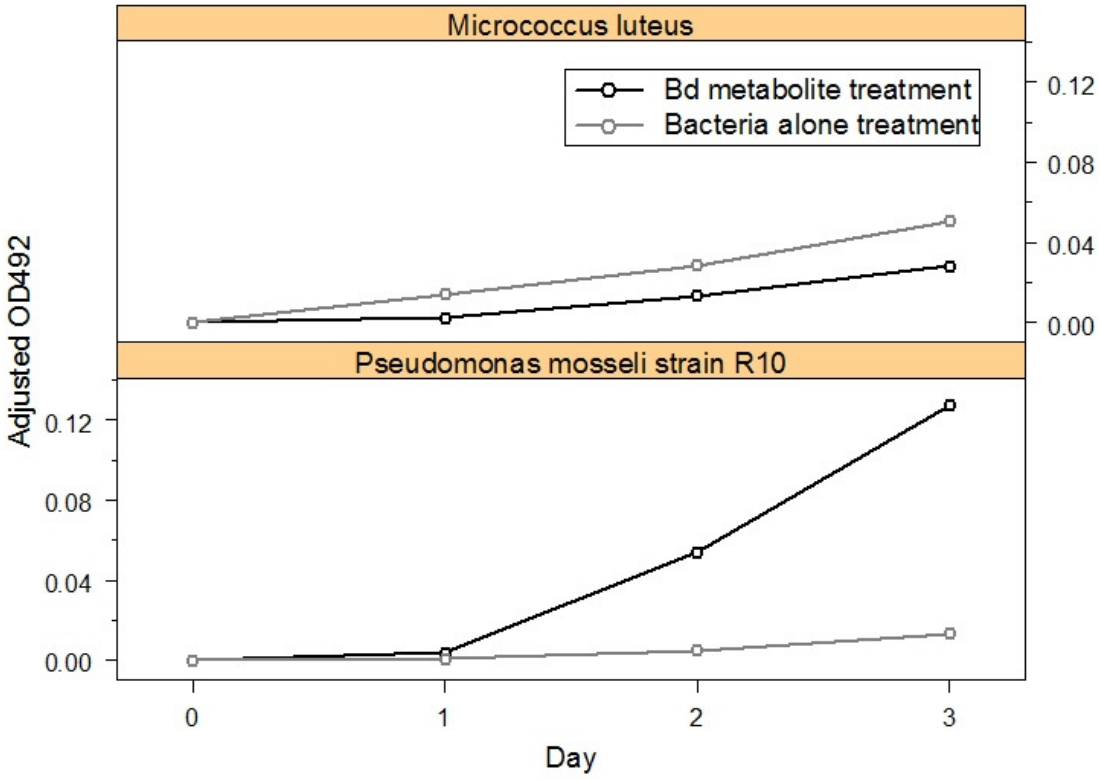

Figure 4.2—Growth curves for *Bd* in extracts of two bacteria that (top) lost and (bottom) gained anti-*Bd* activity after growth in *Bd* metabolites. Adjusted OD_{492} is mean OD_{492} – mean initial OD_{492} to correct for extract color. All standard errors are smaller than symbols.

Pseudomonas sp. SBR3-slima and *Tetrathiobacter mimigardefordensis* strain DPN7 also exhibited inhibitory activity only after growth with *Bd* metabolites. EP_c for *T. mimigardefordensis* (-0.61) did not reach the -0.8 threshold, but examination of *Bd* growth curves showed partial inhibition of fungal growth. Both *T. mimigardefordensis* and *P.* sp. SBR3-slima were inhibitory in the prior baseline assay (Chapter 3), so their loss of anti-*Bd* activity without exposure to *Bd* metabolites (i.e., in the control) was unexpected. Other EP_c - EP combinations had greater magnitudes, but did not include transition from antifungal activity to inactive, or vice versa.

Bd growth was strongly inhibited when cultured in the *Bd*-metabolites control broth (EP of -1.02).

4.4 Discussion

4.4.1 Activity of Bd metabolites

Bd metabolites did not defend strongly against antifungal bacterial symbionts of the three *Litoria* spp. sampled here. On average, the antifungal activity of these bacterial symbionts was not significantly affected by exposure of bacterial cultures to *Bd* metabolites. Two lines of evidence suggest that *Bd*'s metabolites are inactive compounds, perhaps mainly waste products. First, in almost all the bacteria tested here, antifungal activity was not affected when bacteria were grown in the presence of *Bd* metabolites. Of 35 bacteria tested, only *Micrococcus luteus* strain 6J-4A, *Pseudomonas mosselii*, and *Bacillus cereus* clearly responded to the presence of *Bd* metabolites. These bacteria had their baseline activity reversed, *M. luteus* strain 6J-4A and *B. cereus* from inactive to inhibitory, and *P. mosselii* from inhibitory to inactive. The responses of *Pseudomonas* sp. SBR3-slima and *Tetrathiobacter mimigardefordensis* strain DPN7 were less easy to interpret. Both were inhibitory in the original baseline challenge assay (Chapter 3), but inactive when grown alone (i.e., without *Bd* metabolites) in the present experiments. It is unclear whether the transition of interest was this unexpected loss of inhibitory activity, or the fact that they regained (at least partial) anti-*Bd* activity following exposure to *Bd* metabolites. Either way, only between three and five of 35 (8.5-14.3%) isolates appeared to respond to *Bd* metabolites.

Second, growth of *Bd* was itself strongly inhibited by *Bd* metabolites. If the metabolites were largely waste products normally not present in the fungus's immediate environment, it is unsurprising that live *Bd* in broth with these metabolites added were killed. The small differences between EP and EP_c suggest that extensive bacterial growth in 48-hour TGhL cultures dwarfed any similar metabolite-produced inhibitory effect in the bacterial extracts. Future work should identify the chemical makeup of *Bd*'s metabolites, to test this waste product hypothesis.

Chytrid fungi are mainly known from their role as parasites of freshwater algae (Kagami et al. 2007), and no prior studies have assessed the response of chytrids to microbial antagonism. Although many fungi that are pathogenic to agricultural crops produce defensive compounds (Duffy et al. 2003; Schouten et al. 2004), *Bd* is only distantly related to the Ascomycota and Basidiomycota (Rosenblum et al. 2010), to which most of these species belong. Thus, any defensive mechanisms of *Bd* may be quite different from those of the better-studied higher fungi.

4.4.2 Implications for chytridiomycosis management

The finding that metabolites of *Bd* do not affect anti-*Bd* activity in the majority of baseline inhibitory bacteria tested is encouraging for management of chytridiomycosis by bioaugmentation. It suggests that the majority of candidate bacteria are not likely to be subject to reduction of anti-*Bd* activity when they are exposed to *Bd in vivo*. However, because some bacteria did reduce levels of anti-*Bd* activity, these results strongly suggest that the possibility of inhibition of bacteria by *Bd* should be evaluated early in the process of screening candidate bacteria. The results of the present study are also a step towards confirming that challenge assay techniques are not misidentifying candidates for bioaugmentation, despite the assays' highly simplified conditions relative to natural environments.

Intriguingly, up to three of the eight baseline inactive bacteria tested gained some measure of anti-*Bd* activity. This may point to a response by some bacteria to the presence of *Bd* metabolites. If some bacteria previously identified *in vitro* as inactive against *Bd* are able to upregulate antifungal activity on greater exposure to the fungus, bioaugmentation efforts may be facilitated.

Although my results indicate that only a few bacteria are likely to lose anti-*Bd* activity when metabolites produced by the fungus are present, *Bd* may also employ a range of non-chemical defenses, such as evolution of resistance to anti-*Bd* bacterial antagonism. These could complicate efforts to achieve lasting protection of amphibians by bioaugmentation. Many questions remain about how the basic ecological aspects of the amphibian-*Bd*-bacteria symbiosis might affect bioaugmentation. Future research should focus on refining the actual implementation

of bioaugmentation while remaining mindful of the potential pitfalls of *Bd* defense strategies and other microbial interactions not reproduced during *in vitro* work (see Box 2.2). Given the continuing risk posed by *Bd* to many amphibian species, and the proof of concept already demonstrated for bioaugmentation by both *in vitro* and *in vivo* studies (Harris et al. 2009a; Harris et al. 2006; Bell in prep; Chapter 3), well-designed mesocosm and *in situ* experiments could allow protection of threatened amphibians, while addressing outstanding questions about amphibian-bacteria symbioses.

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Chapter 5—Short-term exposure to warm microhabitats may explain amphibian persistence with *Batrachochytrium dendrobatidis*

Abstract

The nature and outcomes of symbiotic interactions often depend on the responses of symbionts to environmental conditions. Many amphibian species have declined due to chytridiomycosis, caused by the pathogenic fungus *Batrachochytrium dendrobatidis (Bd*), but many others persist despite high prevalence of infection by *Bd,* indicating that the virulence of the pathogen is lower or that the fungus may even act as a commensal rather than a parasite in some hosts. In the Australian Wet Tropics, chytridiomycosis caused *Litoria nannotis* to decline to local extinction at high elevation rain forest sites in the early 1990s. Although the species is recolonizing many sites, no population has yet fully recovered. *Litoria lorica* disappeared from all known sites in the early 1990s and was thought to be globally extinct, but a new population was discovered in 2008 in an upland dry forest habitat, which it shares with *L. nannotis*. All frogs of both species observed during three population censuses were apparently healthy, although most were infected with *Bd*. Sun-warmed rocks that frogs perch on in dry forest streams may keep *Bd* infections below the lethal level threshold that occurs at cooler rain forest sites. I tested the hypothesis that short-term exposure to elevated temperatures can hamper *Bd* growth. One hour daily at 33°C, but not at 28°C (representing exposure to heavily and moderately warmed rocks in dry forests) reduced *in vitro Bd* growth below that in the constant 15°C regime (representing rain forest habitats). This reduction in growth was observed over just four days and one generation of *Bd*'s lifecycle. During exponential growth towards a lethal threshold, such changes to the growth rate could allow survival of frogs in dry forests. Still, other possible causes of the apparent low virulence of *Bd* in tropical Australia's upland dry forests may interact with frog perch temperatures and should be investigated.

5.1 Introduction

The nature and outcome of symbioses depend on individual symbionts' responses to environmental and ecological conditions (Thomas & Blanford 2003). For instance, the distribution, abundance, life history strategy, and virulence of microbial symbionts can all be influenced by the environment, and can in turn affect the development and outcome of disease (Grueb et al. 2003; Patz et al. 1996; Thurber et al. 2009; Woodhams et al. 2008). In the case of chytridiomycosis, a fungal disease of amphibians responsible for hundreds of species' declines and extinctions (Kilpatrick et al. 2010; Skerratt et al. 2007), cooler temperatures are associated with worse impacts, especially in the tropics where the majority of affected species reside (Berger et al. 2004, Woodhams & Alford 2005, Kriger & Hero 2008). It has been suggested that this pattern is due to the relatively cool thermal optimum of the fungal pathogen, *Batrachochytrium dendrobatidis* (*Bd*) (Kriger & Hero 2007; Piotrowski et al. 2004) , but the response of *Bd* to realistic thermal regimes experienced by persisting and declining populations in the wild has not been tested.

In the Australian Wet Tropics, the waterfall frog *Litoria nannotis* experienced severe population declines due to *Bd* at upland rain forest sites in the early 1990s (Hero & Rettalick 2004; McDonald & Alford 1999). Recently, apparently healthy populations of *L. nannotis* with high prevalence of *Bd* infections were found at upland dry forest sites in close proximity to rain forest sites at which the species had declined (Puschendorf 2009). The closely related *L. lorica*, believed for 17 years to be extinct due to chytridiomycosis, was rediscovered persisting with *Bd* at one of these dry forest sites (Puschendorf 2009). Both species inhabit waterfalls and torrents, spending their days in and under the water and emerging onto rocks late in the day for their nocturnal activity periods (Puschendorf 2009; Rowley & Alford 2007a, b). Because dry forest sites have less canopy cover than rain forest sites, rocks are exposed to sunlight and can reach up to 40°C during the day. They can remain above 30°C when frogs emerge (Puschendorf 2009). It is likely that infections must reach a threshold intensity before mortality occurs (Carey et al. 2006), and dry forest frogs may reduce infection intensity by effectively "basking" on warm rocks at night, killing fungus.

To test this hypothesis, I carried out an *in vitro* experiment in which I grew *Bd* under thermal regimes simulating the thermal environments *L. nannotis* and *L. lorica* experience at upland rain forest and dry forest sites. This is the first study to test *Bd*'s response to temperature regimes experienced by diseased and persisting amphibian populations.

5.2 Methods

Following four days growth, *Bd* (isolate Lles donna 2) was flushed from ½-strength TGhL agar plates with three mL ½-strength TGhL and filtered to remove sporangia. 3.5 X 10^4 zoospores were inoculated into 100µL ½-strength TGhL in each of 30 wells of each of three 96-well assay plates (modified from Rollins-Smith et al. 2002). All plates were kept at 15°C for the first 24 hours. Thereafter, one plate each was kept at (1) 15°C 24 hours a day to simulate steady, cool conditions at rain forest sites, (2) 15°C 23 hours a day with one hour at 28°C to simulate exposure to moderately-warmed rocks in dry forests, and (3) 15°C 23 hours a day with one hour at 33°C to simulate more heavily-warmed rocks in dry forests. These temperatures were selected based on data from temperature loggers placed on rocks and in frog retreat sites at wet and dry forest streams where *L. nannotis* declined or persists (Puschendorf 2009).

The growth of *Bd* cultures was measured by spectrophotometry at 492 nm (Rollins-Smith et al. 2002) at the outset, and every 24 hours thereafter, immediately after the higher-temperature plates were exposed to their treatments. Cultures were also monitored visually using an inverted light microscope to observe growth and check for any contamination.

The initial OD_{492} for each well was subtracted from each subsequent reading to give the adjusted OD_{492}, as change since time zero. To test for differences in *Bd* growth among treatments, a one-way ANOVA with Tukey's post-hoc tests was performed on the final adjusted OD_{492} for each treatment. Analysis was performed in SPlus 8.0 for Windows (Insightful Corporation, 2007).

5.3 Results

After four days, cultures in all treatments had developed into dense sporangial aggregations. Tukey tests (Figure 5.1) indicated that the 33°C spike treatments had significantly lower *Bd* growth (mean adjusted OD_{492}) than those in either the steady 15°C control or the 28°C spike treatment (Table 5.1 and Figure 5.1). The control and 28°C treatment cultures did not have significantly different *Bd* growth.

Table 5.1—ANOVA table for change in OD_{492} at day 4 of *Bd* cultures exposed to three temperature regimes (see text).

	df	Sum of squares	Mean squares	F	p-value
Treatment	2	0.0074	0.0037	10.11	< 0.001
Residuals	87	0.032	0.00037		

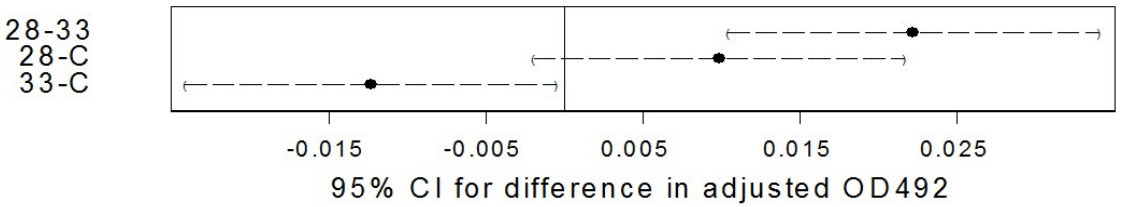

Figure 5.1—95% confidence intervals by Tukey's post-hoc tests for difference in mean adjusted OD_{492} between temperature regimes. 15 = constant 15°C; 28 = 28°C for 1 hour/day, 15°C for 23 hours/day; 33 = 33°C for 1 hour/day, 15°C for 23 hours/day.

Bd growth curves for the three treatments were broadly similar (Figure 5.2), and light microscopy of cultures during the trial did not reveal any observable difference in culture growth or density, or development time of sporangia.

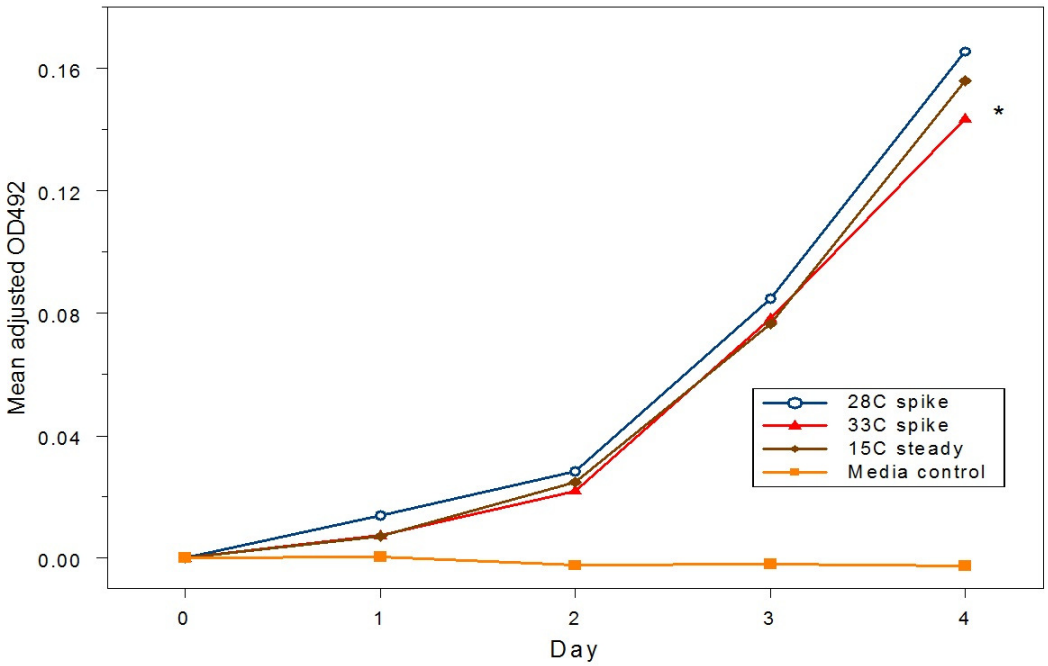

Figure 5.2—Adjusted OD_{492} (OD_{492} – initial OD_{492}) vs. day of the trial. Higher OD_{492} corresponds to greater *Bd* growth. * = significantly lower than 15°C steady and 28°C spike treatments. Standard errors are all smaller than symbols.

5.4 Discussion

Although the reduction of *Bd* growth with short-term elevation of temperature in the 33°C treatment was statistically significant, it was relatively small and visual observation of cultures under light microscopy showed that zoospores developed into mature sporangia at similar rates across the three treatments. Still, there is a plausible mechanism by which the observed changes could translate to the large decreases in *Bd* virulence observed at dry forest field sites. In previous experiments with *Bd* in 96-well plates, fungal growth peaked after zoospores developed into sporangia, presumably due to nutrient and space limitation (Chapter 3, Daskin et al. in prep.), and the present experiment encompassed only a single *Bd* lifecycle. Carey et al. (2006) demonstrated that in highly susceptible species, the development of chytridiomycosis can be explained as a consequence of unregulated exponential growth of the population of *Bd* on the host. After multiple generations of exponential growth, even small differences in the growth rate parameter can make very large differences in population size. So, it is possible that over longer periods more

substantial differences in *Bd* growth between "dry forest" and "rain forest" temperature treatments might have developed. If the temperature responses of the *Bd* that occurs at the dry forest field site are similar to those of the *Bd* isolate used in this experiment, night "basking" on warmed rocks could explain the persistence of *Litoria nannotis* and *L. lorica* with *Bd* in dry forest habitats.

There are at least two alternative explanations for *Litoria nannotis* and *L. lorica* persistence with *Bd* at dry forest sites. First, frogs at two dry forest sites spent more time under running water than did those at rain forest sites (Puschendorf 2009). This may have flushed away zoospores that would otherwise re-infect frogs and boost infection intensity towards a lethal threshold. Second, warmer temperatures may stimulate amphibian immune defenses (Andre et al. 2008; Ramsey et al. 2010; Ribas et al. 2009), but there are few studies on the effects of short-term warming on immune function.

It remains unclear why the amphibian-*Bd* symbiosis is shifted at least partway along the axis from host-pathogen to host-commensal in dry forest *Litoria* populations. Short-term temperature spikes over longer time scales (several weeks) than possible in the present experiment may reduce *Bd* growth rates enough to prevent lethal infection intensities. Using equipment that can read larger culture quantities spectrophotometrically, or *in vivo* experiments, multiple generations of *Bd* should be exposed to temperature shocks. Additional future work should test the effect of short-term temperature spikes on amphibian immunity. Also, experimental infections of frogs in stagnant and running water could be used to simulate dry and rain forest behavioral differences mentioned above. Understanding the specific factors driving context-dependency in *Litoria-Bd* symbioses would improve prediction of future chytridiomycosis-driven declines, and location of refugia for threatened species, like that already found for *Litoria lorica*.

Chapter Six—General Conclusions and Future Directions

6.1 Context-dependent symbioses, amphibian declines and wildlife disease

In Chapter Three, I showed that, on average, bacterial symbionts of three *Litoria* species from the Australian Wet Tropics have reduced activity against the pathogen *Batrachochytrium dendrobatidis* (*Bd*) at temperatures characteristic of cool-weather *Bd*-driven declines. This is the first evidence that context-dependency in the protection afforded to amphibians by their antifungal bacterial symbionts may be a factor in the cool-temperature chytridiomycosis-driven amphibian declines common in Australia and elsewhere (Berger et al. 1998; Berger et al. 2004; Kriger & Hero 2007; Woodhams & Alford 2005). It has been suggested in the literature (Kriger & Hero 2007) that the relatively cool optimal temperature window for *in vitro Bd* growth [17-25^{o}C; (Piotrowski et al. 2004)] may be responsible for this pattern. However, this does not account for the many declines that have occurred at times and places where temperatures were below the lower limit of the optimal range. It is possible (Woodhams et al. 2008) that *Bd* may compensate for lower metabolic activity and longer generation times at temperatures below its thermal optimum by increasing fecundity, which could maintain virulence. Rates of production of antimicrobial peptides, a major element of the innate immune defenses of frogs, could decrease at lower temperatures (Rollins-Smith 2001; Rollins-Smith et al. 2002). Another possibility, not previously raised in the literature, is that another component of the innate immune defenses of amphibians, antifungal activity of amphibians' bacterial symbionts, could be suppressed in cool environments, as it was in my laboratory trials. This would lead to less effective protection of frogs from chytridiomycosis and could, in part, account for the occurrence of amphibian declines in these conditions.

I also investigated other aspects of context-dependency in the amphibian-*Bd*-bacteria symbiosis. In Chapter Four, I showed that metabolites produced *in vitro* by *Bd* can affect anti-*Bd* activity of some bacterial symbionts. No previous study explicitly assess the effects of a Chytridiomycete fungus on antagonistic bacteria. Its results indicate that the effects of *Bd* metabolites on bacterial symbionts are unlikely

to limit their potential for use in bioaugmentation for chytridiomycosis management. However, it will be important to evaluate the effects of *Bd* on any candidate bacteria, because some will be affected.

Finally, based on the *in vitro* growth assay presented in Chapter Five, short-term exposure to warm perch sites available in dry forests, but not wet forests, could account for the persistence of *Litoria nannotis* and *L. lorica* with high prevalence of *Bd* infection at dry forest sites, although other possible explanations remain.

6.2 Future directions

6.2.1 Environmental context, antifungal bacteria, and chytridiomycosis

When antifungal compounds produced by amphibians' symbionts are identified using chemical methods, techniques exist to quantify their concentrations on the skin of living amphibians (Becker et al. 2009; Brucker et al. 2008b). Using these techniques to study the effects of temperature on the anti-*Bd* activity amphibians' bacterial symbionts by sampling their activity *in situ* would complement the laboratory studies described here, and would allow preliminary evaluation of the hypothesis that context-dependent antifungal activity may have played a role in past decline patterns. Bacterial protection from *Bd* could be correlated with environmental conditions and population persistence. I would expect populations at higher elevations and in other cooler habitats to have reduced cutaneous antifungal activity, all else being equal. Shifting abundance and composition of amphibian microflora could also affect chytridiomycosis susceptibility, and could be assayed using high-throughput genetic techniques.

Longer-term and *in vivo* studies should be carried out to confirm whether or not short-term exposure to high temperatures can create differential survival of *Litoria nannotis* and *L. lorica* with *Bd* in dry and wet forest habitats. Variation in protective bacteria, antifungal skin peptides, frog behavior, and environmental conditions are all likely to affect the outcome of chytridiomycosis to different degrees in different populations and species (Kriger & Hero 2007; Rollins-Smith 2009; Rowley & Alford 2007a), and may also affect the *Litoria* species discussed in Chapter Five.

6.2.2 Bd biology

Interspecific interactions of the Chytridiomycota and other phylogentically-basal fungi are poorly studied, but my results suggest that *Bd* may not employ chemical mechanisms similar to those of higher fungi, which can defend againt antagonistic activity of bacteria, including biocontrol agents (Tarkka et al. 2009; Chapter One). Three of eight tested bacteria had antifungal activity initiated by exposure to *Bd* metabolites, but this is too small a sample size to extrapolate to the wider amphibian-associated bacterial community. It is possible that *Bd* grown in more naturalistic conditions, for example co-cultured with bacteria, might produce more effective chemical defenses. It is also possible that *Bd* has non-chemical defense mechanisms (Duffy et al. 2003) that could have important effects on amphibian-microbe symbioses and potential bioaugmentation programs.

6.2.3 Implications for chytridiomycosis management by bioaugmentation

My finding that low temperature inhibits the anti-*Bd* activity of many bacterial symbionts of *Litoria species* indicates that in the development of bioaugmentation as a method for mitigating the effects of chytridiomycosis, selecting robustly antifungal bacteria will be crucial. Otherwise, antifungal activity observed *in vitro* could be lost on exposure to variable environmental and ecological conditions.

Bioaugmentation should now be trialed in more natural settings, perhaps in mesocosms, and certainly in wild amphibians, if further chytridiomycosis-driven declines are to be prevented. Proof-of-concept for bioaugmentation has been demonstrated in simplified laboratory environments in *Rana muscosa*, *Plethodon cinereus*, and *Hemidactylium scutatum* (Harris et al. 2009a; Harris et al. 2009b), and is supported by correlations of the degree of resistance to chytridiomycosis with the presence of anti-*Bd* bacteria in wild *R. muscosa* and *R. sierrae* (Lam et al. 2010). Development of bioaugmentation methods is especially urgent for amphibian diversity hotspots, such as Madagascar and Southeast Asia, where dozens or hundreds of extinctions could be prevented when *Bd* eventually spreads to naïve populations (Kusrini et al. 2008; Weldon et al. 2008). It is not yet clear how susceptible African and Asian amphibians are to chytridiomycosis, but having bioaugmentation and other

management protocols prepared for *Bd*'s nearly inevitable arrival is critical to conservation success. Trials in natural settings will also allow naturally-varying environmental conditions to affect the host-pathogen-microbe system, potentially complicating interpretation of results, but improving their reliability.

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