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Review

Interactions between *Batrachochytrium dendrobatidis* and its amphibian hosts: a review of pathogenesis and immunity

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Abstract

The fungus *Batrachochytrium dendrobatidis* (*Bd*) causes a lethal skin disease of amphibians, chytridiomycosis, which has caused catastrophic amphibian die-offs around the world. This review provides a summary of host characteristics, pathogen characteristics and host-pathogen responses to infection that are important for understanding disease development.

Keywords: Amphibian declines; *Batrachochytrium dendrobatidis*; Chytridiomycosis; Pathogenesis; Amphibian immunity

1. Introduction- global amphibian declines

At the World Congress of Herpetology in 1989, herpetologists started discussing unusual disappearances of amphibian populations in protected areas that had been noted since the late 1970's [1]. Due to a lack of high-quality, long-term census data, it was difficult to determine if anecdotal observations reflected normal population fluctuations or true declines [2]. However, evidence of genuine declines began to mount. In Australia, where amphibian disappearances were reported for multiple species [3], researchers proposed that an introduced pathogen was responsible for the declines [4]. Initially this suggestion was met with skepticism because the available data were considered insufficient to support the hypothesis. Further investigation led to the discovery of *Batrachochytrium dendrobatidis* (hereafter *Bd*), a novel fungus identified in histological sections from the skin of dead frogs found in Australia, North America, and Central America [5,6]. Since its discovery *Bd* has been linked with amphibian mass mortality events, severe population declines and even species extinctions occurring around the world [7,8]. In some regions were *Bd* is now considered endemic, mortality rates remain high in infected amphibian populations [9] and although many species have persisted beyond initial outbreaks, some have dramatically reduced abundances and distributions [10,11].

The description of *Bd* required the creation of a monotypic genus in Chytridiomycota, a phylum of fungi not previously known as pathogens of vertebrates [6]. Preliminary genetic work found little variation among globally collected isolates, suggesting that the *Bd* outbreak is due to an asexual pandemic clone [12]. More recent molecular analyses indicate that phylogenetic structure exists among isolates collected from globally wide spread sources [13–15]. The origin of *Bd* is still unknown and hypotheses regarding possible sources, including Africa, Asia, or North America, are currently being debated [14–16]. Meanwhile, amphibian declines attributed to *Bd* continue to be reported (e.g., North America [11], Central America [17] and Asia [18]). As a result of its global impact, the World Organization for Animal Health (OIE) recently listed *Bd* as a notifiable pathogen [19], the first to be included for its threat to biodiversity.

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Many important studies on the ecology and epidemiology of chytridiomycosis have been recently reviewed [20,21]. However, gaining a thorough understanding of disease development in individual hosts has been hampered by a lack of essential information. Even after a decade of research, fundamental questions about the biology of chytridiomycosis remain unanswered. Here we present a synthesis of basic biological knowledge concerning the host, the pathogen and their responses during infection.

2. Amphibian characteristics

2.1. Anatomy of amphibian epidermis

Many characteristics of amphibian skin differ from those of other terrestrial vertebrates. Most notably, the amphibian epidermis is permeable and physiologically active - a key organ involved in maintaining homeostasis. Therefore, an in-depth understanding of the anatomy, physiology, and unique cutaneous properties of amphibian skin is useful for the study of chytridiomycosis.

The epidermis consists of multiple (5–7) layers of epithelial cells and specialized cell types [22]. Epidermal layers include (from deep to superficial): *stratum germinativum, stratum spinosum, stratum granulosum, and stratum corneum*. The *stratum germinativum* is the basal layer where cellular division generates new cells that migrate superficially and differentiate into various cell types [22]. The cells of the *stratum corneum* are differentiated as keratinocytes, meaning they contain the cytoskeletal protein keratin [22]. These cells have become a focal point of chytridiomycosis research because *Bd* is found in keratinized cells and in less mature cells in the deeper epidermal layers [23].

Because frog skin is a moist, nutritive substrate on which microorganisms can flourish, secretory glands in the superficial layers of epidermis allow amphibians to maintain proper skin functioning while providing a defense against potential pathogens [24]. Two types of glands are abundant on the surface of frog skin: mucous and serous (also known as granular or poison) glands. Mucous secretions prevent desiccation, facilitate temperature control and protect from abrasive damage [24–26]. Serous glands are the primary source of chemicals active against predators and against invasion by microorganisms [22,24]. Just as the amphibian integument differs over the body surface of individuals and among species [22], serous glands and their secretions are heterogeneous in abundance, anatomical distribution, morphology and functional characteristics [27–29].

2.2. Physiology of amphibian epidermis

Amphibian skin is permeable to water and is a site of regulated transport for ions (electrolytes) and respiratory gases [22,30,31]. In order to maintain osmotic balance, amphibians must sustain a hyperosmotic internal environment relative to the hypoosmotic external environment. This is accomplished by active regulation of transport of multiple electrolytes (including sodium, magnesium, potassium, chloride) across the surface of the skin [23,30,31]. A steady inward flux of sodium must be consistent despite its movement against an electrochemical gradient. This is accomplished via a cyclic AMP-regulated pathway, the sodium potassium pump, which exchanges potassium ions for sodium ions, thereby regulating intracellular and extracellular ion concentrations. Water flow across the skin results when an osmotic gradient is established by electrical currents, induced by an exchange of these ions [31].

The regulatory properties of frog skin are primarily determined by electrolyte transport in the flask-shaped, mitochondrial-rich cells (MR cells) of the *stratum granulosum* [32]. However, as a multi-layered composition of many cell types, amphibian epidermal cells "work" together to maintain required concentrations of electrolytes and water balance [33]. The permeability of frog skin varies over the body surface of an individual and also among species [22]. In some amphibian taxa, for example in bufonids, osmotic permeability is greatest in an area of the ventral integument commonly referred to as the pelvic patch, where there is dense cutaneous vasculature [27]. Despite some variation in permeability, amphibian skin (across all species) is a central organ in maintaining water and electrolyte equilibrium [22,30].

Healthy amphibians regularly molt by shedding their outer keratinized layer, as a replacement *stratum corneum* forms [34]. The molting process is dependent on species-specific physiology and behavior [34–36]. The frequency of molting also varies by species and can vary from a few days to several weeks depending on multiple factors including temperature, age, size and sex [34].

3. Characteristics of *Batrachochytrium dendrobatidis*

3.1. Morphology, growth and development

Orders and genera in the phylum Chytridiomycota are classified by ultrastructural morphology of the zoospore, especially the flagellar apparatus [37] and molecular characters [38]. *Batrachochytrium dendrobatidis* was originally isolated from, and named for, a blue poison dart frog (*Dendrobates azureus*) [6]. Multiple *Bd* isolates from various amphibian species have been brought into pure culture [39]. *Bd* has two main life stages. The dispersal stage is the infectious zoospore, which moves with a posterior flagellum [6,40]. The zoospore encysts, absorbs the flagellum and develops rhizoids [40]. The maturing thallus develops into a zoosporangium (i.e. container for zoospores) in which the cytoplasm cleaves and forms flagellated zoospores [40]. A discharge tube forms and at maturity the plug dissolves and the zoospores are released into the external environment to continue the life cycle [6,40] (Fig. 1).

3.2. Nutrient utilization

To date our understanding of *Bd* nutrient utilization is incomplete [41–43]. It is unclear whether *Bd* can persist in the

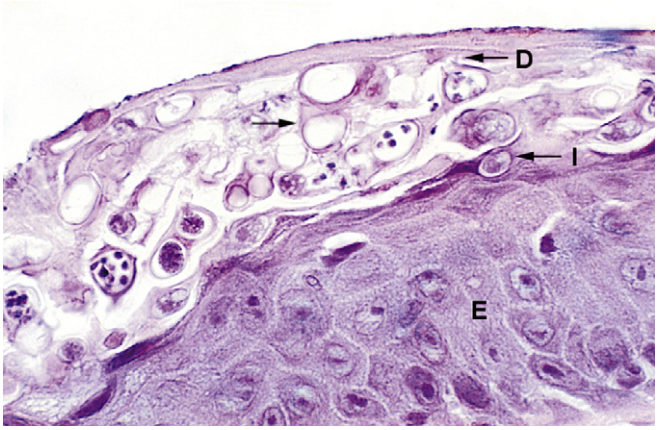

Fig. 1. Histological section of infected epidermis in an adult *Litoria caerulea* frog. Note homogenous immature stage (I), larger multinucleate stages, zoosporangium with discharge tube (D) containing zoospores, and empty zoosporangium after zoospores have discharged (arrow). E = epidermis. Stained with hematoxylin and eosin. From Berger et al. [55].

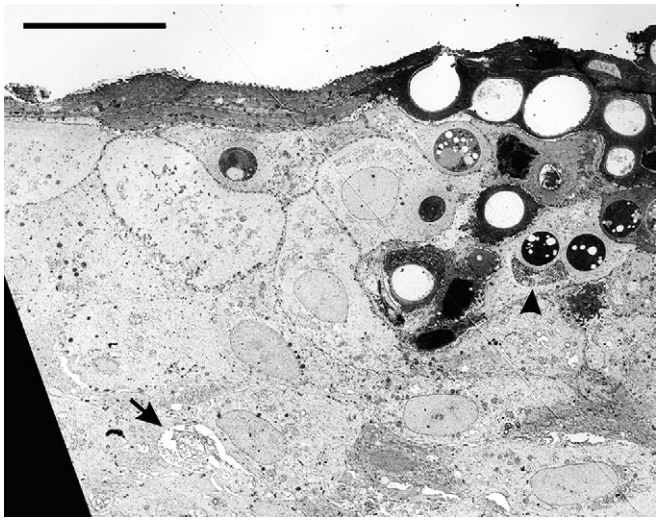

Fig. 2. TEM (HPF/FS) of infected epidermis in an adult *Litoria gracilenta* frog. Some nuclei of infected cells are degenerate and chromatolytic (arrowhead), and necrosis and dissolution of cells is shown in the deeper epidermis (arrow). Scale bar = 18 μm. Frog Berger et al. [40].

environment as a saprobe, utilizing non-amphibian organic materials, a question that has important implications for the evolution of *Bd* virulence and the epidemiology of chytridiomycosis [44]. Additionally, determining what amphibian nutrient sources are utilized by *Bd* may explain the mechanism of epidermal cell colonization, a key step in pathogenesis [42,43].

It is a common misconception that *Bd* uses only keratin as a nutrient source. Although *Bd* is found in keratinizing epidermis of frog skin [6,23] and tadpole mouthparts [45], it invades and grows initially in deeper epidermal cells containing prekeratin, and has completed much of its development by the time cells are completely keratinized [40]. It does not grow well on pure keratin of non-amphibian origin [41,43]. *In vitro Bd* grows on various simple and complex media. Mixtures of different concentrations have included peptonized milk, tryptone, gelatin hydrolysate, lactose, glucose, asparagine, yeast extract, malt extract, peptone, sucrose, maltose, sorbitol, glycerol [6,41,42]. Additional substrates have included sterilized snakeskin, ground feather meal and sloughed amphibian skin [6,41,42].

Growth and development rates of *Bd* in culture can vary greatly among nutrient conditions. Even in standardized nutrient media, growth rates can be inconsistent [41]. Currently *Bd* isolates are most commonly cultured on 1% tryptone agar or liquid medium [6]. Serial culturing influences growth and development (and infectivity and virulence) of many pathogens and some researchers have expressed concern regarding selective pressures on *Bd* in culture [12,39,42].

4. Pathogenesis

4.1. Colonization

Pathogen colonization, regarded as a first step in pathogenesis, occurs in the cells of the host epidermis [6,40]. The reproductive life cycle of *Bd* is presumed to be the same in frog skin as it is in culture; the *Bd* thalli live and mature within cells of the *stratum granulosum* and *stratum corneum* [6,40] (Figs. 1 and 2). It is unclear exactly how cell entry is achieved. Longcore et al. [6] hypothesized that a zoospore might encyst on the exterior surface of an epidermal cell and insert *Bd* nuclear material via a germ tube. Following cell entry, *Bd* develops sporangia within the epidermal cells, filling the cells. Up to three sporangia have been seen within the one host cell [40]. Infected skin layers move from deep to more superficial, coinciding with the normal directional movement and sloughing of epidermal cells. Berger et al. [40] observed that the rate of development of *Bd* was timed such that the final stages of development, formation of discharge tube, occurred when infected epidermal cells were most superficial. Also, discharge tubes are oriented toward the external environment, suggesting that *Bd* may be well adapted to amphibian skin [40].

Some evidence indicates that *Bd* enzymatic activity may be involved with the colonization process. The initial penetration of *Bd* into amphibian epidermal cells probably requires digestive enzymes, which may also be responsible for the dissolution of cellular cytoplasm [40] (Fig. 2). In culture *Bd* secretes extracellular proteases that degrade casein and gelatin [41] and proteolytic enzyme activity has been detected for multiple *Bd* isolates [12,41,42]. At the molecular level, genomic research into *Bd* has revealed differential expression patterns in gene families such as serine protease and fungalysin metallopeptidase [43], two enzymes that are involved in infection processes (enzymatic penetration of host cells) in other pathogens [46].

4.2. Intensity of infection

The reproductive biology of *Bd* appears to be a critical factor in disease development and mortality. When maintained in *in*

vitro high-nutrient conditions, exponential growth and a peak in zoospore production is followed by a decrease in zoospore production and activity, presumably due to exhaustion of nutrient resources [47] or possibly a buildup of inhibitory metabolites. In frog skin, however, growth limitations due to nutrient exhaustion are unlikely [47]. Local re-infection on an individual host can therefore lead to an exponential increase in *Bd* load and development of severe infections and mortality [11,48]. In infected frogs, clinical signs of disease and mortality occur in individuals with the highest zoospore loads [11,49,50], suggesting that zoospore production and intensity of infection are critical for pathogenesis.

Rates of growth and development of *Bd* are influenced by temperature. The optimal temperature range appears to be approximately 17–23 °C, but *Bd* can grow at lower temperatures [41,47]. Cultures of *Bd* incubated at 30 °C and higher die [41]. Woodhams et al. [47] observed and modeled how *Bd* reproductive life history traits were influenced by temperature. At lower temperatures (7–10 °C) motile *Bd* zoospores took longer to encyst, mature and produce propagules than *Bd* zoospores maintained in warmer temperatures (17–23 °C). This shift in life history may enable *Bd* to maintain a relatively high long-term growth rate across a range of temperatures [47].

Population-level observations in nature confirm that temperature plays an important role in chytridiomycosis outbreaks [51,52]. In tropical regions amphibian declines predominantly occur in upland sites where temperatures are cool [51], and low temperature is significantly related to high prevalence of *Bd* [52]. The importance of temperature also prompted the hypothesis that global climate change might create optimal thermal conditions for disease spread (i.e. the chytrid thermal optimum hypothesis) [53]. However, this hypothesis is controversial [54] and most of the central postulates (e.g. homogenous thermal conditions contribute to *Bd* proliferation) have not yet been experimentally tested.

4.3. Pathophysiology

Primary pathological abnormalities include epidermal hyperplasia and hyperkeratosis (thickening of the *stratum corneum*) [5,23,50] (Figs. 1 and 2). Other pathological changes in the epidermis include cytoplasmic degeneration and vacuolation in scattered cells in deeper layers [40]. Significant changes in internal organs have not been observed [5,50].

Epidermal damage caused by *Bd* appears to disrupt critical cutaneous functioning [50]. Although the cellular and biochemical mechanisms of pathogenesis have not yet been thoroughly investigated, Voyles et al. [50] demonstrated that clinically diseased frogs had marked inhibition of electrolyte transport across isolated ventral skin samples. These epidermal disruptions coincided with two other critical pathophysiological changes: reductions in plasma electrolyte concentrations and deterioration of cardiac electrical functioning leading to aystolic cardiac arrest [50]. Blood samples that were collected from clinically diseased individuals, which were tested for a wide range of biochemical and hematological parameters, indicated significantly reduced plasma sodium and potassium concentrations. In contrast, plasma albumin, hematocrit and urea levels were stable, suggesting that the cardiac electrical deterioration was due to a loss of electrolytes from circulation, rather than a change in water volume that could occur with increased water uptake [50]. Changes in levels of carbon dioxide were not detected in clinically diseased frogs [49], but hypoxia (low blood oxygen saturation) has not been conclusively ruled out as a possible cause of cardiac electrical functioning in amphibians with chytridiomycosis [50].

Typical clinical signs of severe chytridiomycosis include: lethargy, inappetence, cutaneous erythema, irregular skin sloughing, abnormal posture (hind legs abducted), and loss of righting reflex [50,55] (Fig. 3). The significance of irregular skin sloughing is unclear. In one study, salamanders (*Ambystoma tigrinum*) sustained *Bd*-infections more than 60 days after experimental exposure, seemingly without adverse affects, but with a notable increase in the sloughing of skin [56]. Because *Bd* was found in sloughed skin, it was hypothesized that the increase in sloughing might assist amphibians in shedding the infection [56]. In support of this hypothesis Berger et al. [51] suggested that increased molt frequency might be a reason frogs can clear infection at higher temperatures. However, it is also possible that *Bd* causes changes in molting patterns that are detrimental. During normal molting events alterations in water permeability and electrolyte transport cause temporary changes sodium permeability, sodium excretion and net sodium loss [57], but the effects of *Bd* on this process have not been investigated.

5. Host defenses

5.1. Innate and acquired immune responses

Although amphibians have highly competent immune systems, several studies suggest that many species lack a robust immune response to *Bd* infection [23,58,59]. Initial research focused on the innate immune responses to *Bd* infection and, more specifically, on the effectiveness of anti-microbial peptides (AMPs) produced in epidermal granular glands (recently reviewed in [60]). Because they are secreted at the skin surface, AMPs represent a first-line defense against *Bd* infection [60].

Many of the AMPs that have been collected from frog skin impede *Bd* growth in *in vitro* studies; laboratory assays have demonstrated that both heterogonous mixtures and their purified peptide constituents inhibit *Bd* growth to different degrees [60]. To date at least 40 AMPs have been purified, characterized and tested to establish a minimum inhibitory concentration (MIC) for effectiveness against *Bd* [60]. The mechanism of AMPs inhibition is unknown but Rollins-Smith et al. [61] hypothesized that secreted peptides may disrupt the integrity of cellular membranes of *Bd*, perhaps at all life stages, but especially at the zoospore phase in which *Bd* lacks cell walls.

Although some AMPs appear to be highly fungicidal against *Bd in vitro*, it is less clear how effective AMPs are *in vivo*. Because the diversity of AMP repertoire differs among amphibian species [58,60], some researchers have suggested

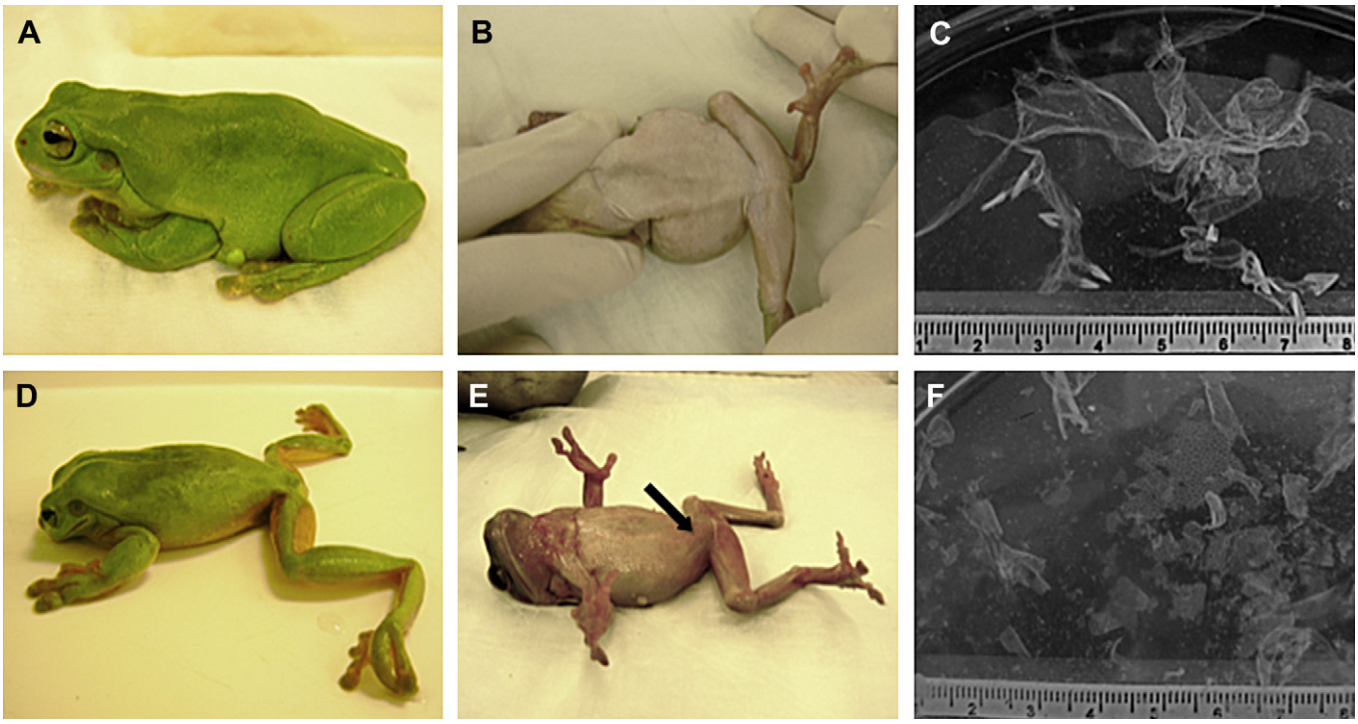

Fig. 3. Appearance and behavior of *Litoria caerulea* and the pattern of skin shedding were altered in animals experimentally infected with *Batrachochytrium dendrobatidis*. A, B) Lateral and ventral views of a healthy frog. C) A single large skin slough from a healthy frog. D) A frog with severe chytridiomycosis showing abnormal posture with head lowered and hind legs abducted. E) A frog in dorsal recumbency that is unable to reorient and has cutaneous erythema (arrow) in the ventral epidermis. F) Multiple small skin sloughs collected from the water in the container of a frog with severe chytridiomycosis. From Voyles et al. [50].

that the species-specific potency of AMPs may equate to differential resistance to chytridiomycosis in wild amphibians [58]. In one exposure experiment, the survival rate in groups of different species of frogs appeared to be correlated with the effectiveness of skin peptide mixtures (when assessing the MIC of natural mixtures *in vitro*) [58]. However, in order to establish the true efficacy of AMPs in amphibians in the wild, more research is needed to investigate the variability of AMP repertoires within and among amphibian populations and species, what stimulates AMP production and release from the granular glands and what additional factors influence the microbial interactions taking place at the skin surface. Important interactions may include abiotic factors such as temperature [62] and biotic factors such as the presence of other cutaneous skin bacteria [63].

The most substantial evidence of an acquired immune response to *Bd* infection comes from studies of the frog species *Silurana* (*Xenopus*) *laevis* [64]. This species has historically been used as a model to study amphibian immunity and appears to be less susceptible to chytridiomycosis in the wild (i.e. populations of *Xenopus laevis* have persisted with *Bd*-infections with scant evidence of disease [65]). Therefore, the immune defenses of *X. laevis* are of interest for chytridiomycosis research (reviewed in [66]). One study used multiple techniques to determine if compromising innate immunity (via depletion of AMPs) and acquired immune functions (via suppression of lymphocyte function using sub-lethal X-irradiation) altered the resistance of *X. laevis* to chytridiomycosis [64]. The results suggested that *X. laevis* frogs that were experimentally immuno-suppressed became more susceptible to *Bd* infection [64]. This study also detected *Bd* antigen-binding immunoglobulins in the skin mucus of *X. leavis*, suggesting the involvement of an additional component of the immune system [64].

A related but susceptible species *Silurana (Xenopus) tropicalis* was used to investigate the transcriptional response to *Bd* infection in amphibian skin, liver and spleen [59]. Rosenblum et al. [59] found that immunogenetic responses to *Bd* in the early stages of infection were remarkably weak. In the late stages of infection (once frogs developed clinical signs of disease), there was a greater overall transcriptional response to infection but only a small number of genes related to immune function showed increased expression in clinically diseased frogs and some immune function genes showed suppressed activity [59]. An independent study using the same *Xenopus tropicalis* system also found a lack of an adaptive immune-genetic response [67]. One possible explanation for the finding of minimal amphibian immunity to chytridiomycosis is that *Bd* may evade or suppress the immune responses that would typically occur with pathogen invasion in amphibian hosts [43], however further research is needed to determine whether *Bd* employs such tactics. Collectively these studies of amphibian immune responses support the assumption that resistance to *Bd* infection (and/or elevated intensity of infection) and disease is probably highly species-specific.

Experiments that have attempted to stimulate acquired immune responses have produced mixed results depending on the methods. Two studies report no difference in survival

between control frogs and those injected with dead *Bd* (heat or formalin-killed) in immunization trials [66,68]. However, other experiments showed that susceptible amphibian species that were experimentally infected, treated for *Bd* and then subsequently re-exposed, had increased survival rates compared to amphibians that were completely naïve to *Bd* infection (reviewed in [69]). One study detected elevated *Bd*-specific antibodies following treatment with heat-killed *Bd* in *X. leavis* [64], though it is not yet clear if a similar treatment regime would produce the same result in a more susceptible species. These results suggest that the amphibian immune system has the capacity to develop some protection against disease and mortality induced by *Bd*, but the usefulness of immunization techniques will require further investigation.

6. Conclusions

The decline of amphibians caused by chytridiomycosis has been described as "the most spectacular loss of vertebrate biodiversity due to disease in recorded history" [8], but the underpinning mechanisms of these extirpations have puzzled researchers ever since *Bd* was first discovered. Understandably, most studies to date have focused on the ecology and epidemiology of chytridiomycosis [20,21], and this research has yielded many important insights into the disease. However, a more complete understanding of the basic biology of the host, the pathogen and their responses in a shared environment will be central to future investigations.

Several recent reviews have provided proposals for future research directions on disease ecology [21], epidemiology [20], host immunity [60] and immunogenetic responses [68,69] to infection. We suggest that further research on pathogenesis, at the molecular and cellular levels, should also be a top priority to understand chytridiomycosis and fungal pathogenicity in general. In this review we have identified several areas for future work. For example, it is unresolved how *Bd* colonizes and disrupts amphibian epidermal cellular machinery. What are the properties of frog skin that make it conducive to fungal colonization? And what are the factors that allow *Bd* to cause severe epidermal pathology without eliciting a robust immune response? Because few fungi are known to be highly pathogenic in vertebrates, there are no similar disease systems for comparison and it has therefore been difficult to answer these questions. There have been, however, some recent advancements in molecular techniques that provide the potential to address many uncertainties about chytridiomycosis. As reviewed by Rosenblum et al. [70], the development of molecular toolkits will accelerate important studies on *Bd* genetics and functional genomics for comparative studies among *Bd* strains as well as among other pathogenic and non-pathogenic fungi. In addition to questions concerning the functional responses of pathogen and host to infection, we may also be able to address persistent questions regarding *Bd* origin and patterns of spread [69].

With limited examples of disease-induced extinctions, chytridiomycosis is an important model system for understanding newly emerging infectious diseases that have broad host specificity. But also, the importance of chytridiomycosis research for amphibian conservation cannot be overlooked. Resolving the determinants of interspecific disease susceptibility or resistance will identify those species in the greatest need of conservation intervention. Additionally, an in-depth understanding of the pathophysiology of chytridiomycosis should facilitate the development of treatment options for species that suffer high rates of mortality. Thus, the practical implications of this research is especially important as conservation organizations look for safe and effective mitigation strategies to confront this unprecedented threat to amphibian biodiversity.

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