ANNUAL REVIEWS

Annual Review of Microbiology

Virulence and Pathogenicity of Chytrid Fungi Causing Amphibian Extinctions

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Annu. Rev. Microbiol. 2021. 75:31.1–31.21

The *Annual Review of Microbiology* is online at micro.annualreviews.org

https://doi.org/10.1146/annurev-micro-052621-124212

Keywords

amphibian, infectious disease, fungus, chytridiomycosis, chytrid, evolution, *Batrachochytrium*

Abstract

Ancient enzootic associations between wildlife and their infections allow evolution to innovate mechanisms of pathogenicity that are counterbalanced by host responses. However, erosion of barriers to pathogen dispersal by globalization leads to the infection of hosts that have not evolved effective resistance and the emergence of highly virulent infections. Global amphibian declines driven by the rise of chytrid fungi and chytridiomycosis are emblematic of emerging infections. Here, we review how modern biological methods have been used to understand the adaptations and counteradaptations that these fungi and their amphibian hosts have evolved. We explore the interplay of biotic and abiotic factors that modify the virulence of these infections and dissect the complexity of this disease system. We highlight progress that has led to insights into how we might in the future lessen the impact of these emerging infections.

Contents

1. INTRODUCTION

Recognition that a phylum of fungi previously believed to be innocuous, the Chytridiomycota, includes pathogens with the inherent ability to destroy entire species and to radically alter ecosystem functioning was a key discovery in the field of microbial epidemiology. The finding that Earth's amphibian species were declining at unprecedented rates, and often in pristine habitats, spurred a global search for the underlying cause (45). Spanning three decades of research and collaboration, **Figure 1** charts the main sequence of events that led to the discovery and description of two species of chytrids that infect amphibians, *Batrachochytrium dendrobatidis* and *Batrachochytrium salamandrivorans*. These zoosporic aquatic fungi infect the cutaneous tissues of susceptible species of amphibians to cause the disease chytridiomycosis. The balance of evidence suggests that the evolutionary origins of both *B. dendrobatidis* and *B. salamandrivorans* lie across a region East Asia where they are endemic and appear to have been infecting amphibians for over 50 million years (75, 90). In this region they are widespread yet rare and patchily distributed, and a lack of observable disease or associated declines suggests that the Asian amphibian hosts have achieved a stable enzootic association with these infectious fungi. However, genomic analysis has shown that lineages of *B. dendrobatidis* have emerged out of Asia at least five times across the last century, with *B. salamandrivorans* more recently invading Europe in the 2000s (recently reviewed in 45). It appears that the expanding global trade in amphibians unintentionally caused both *Batrachochytrium* species to be widely vectored (90), and these epidemiological sparks have ignited waves of infection leading to the decline of at least 501 species (6.5% of all amphibian species) alongside presumed extinction of 90 worldwide (119). These losses place *Batrachochytrium* high in the ranks of the most impactful pathogens ever encountered.

2. THE SPECTRUM OF *BATRACHOCHYTRIUM* PATHOGENICITY AND VIRULENCE

Despite the infamously aggressive nature of amphibian chytridiomycosis, enormous heterogeneity underpins the *Batrachochytrium*-host interactions that cause this disease, a complexity that is often underappreciated. In this review we examine the key factors that determine whether an

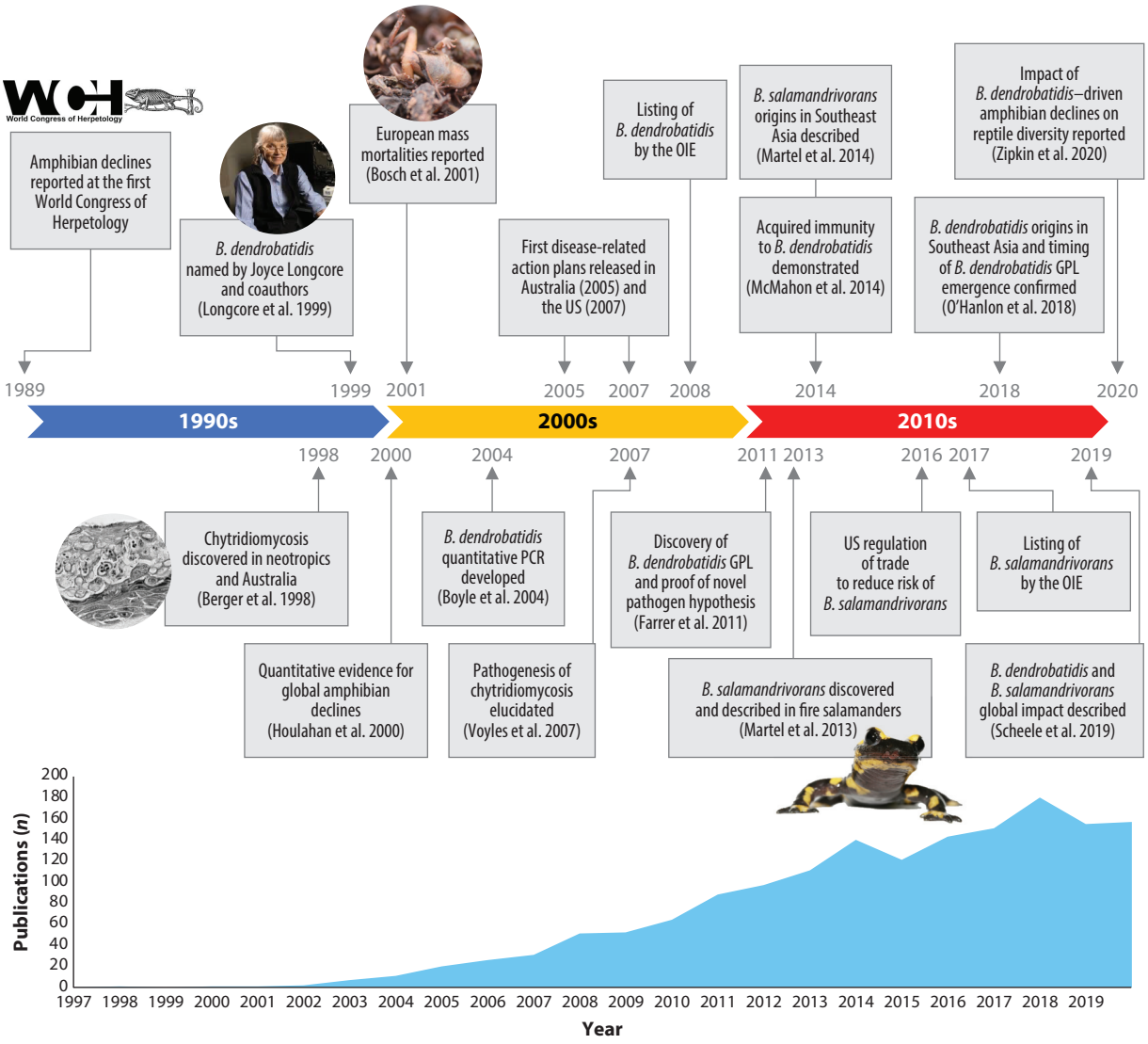

Figure 1

Time line of milestones in the discovery of *Batrachochytrium* following recognition of global amphibian declines (58). The disease amphibian chytridiomycosis was described in 1998 (8) and its etiological agent, *B. dendrobatidis*, named in 1999 (68) along with a key molecular diagnostic (18). The inset micrograph shows heavy infection with sporangia (*arrowhead*) in a wild frog, *Litoria caerulea*, from Australia (8). *B. salamandrivorans* was discovered and named in 2013 (76). Graph shows the number of publications on *Batrachochytrium* per year. Abbreviations: GPL, global panzootic lineage; OIE, World Organization for Animal Health. Micrograph adapted with permission from Reference 8; copyright 1998 National Academy of Sciences, U.S.A.

amphibian survives or dies when exposed to a pathogenic chytrid. Here, we differentiate pathogenicity (the intrinsic ability to cause disease) from virulence (the expression of pathogenicity across a group of host and pathogen genotypes). These terms can be related as follows: Pathogenicity (number of individuals with chytridiomycosis divided by the number exposed) equals infection (number of infected individuals divided by the number exposed) times virulence

(number of individuals with chytridiomycosis divided by the number infected). This relationship captures the key facets of the *Batrachochytrium*-amphibian system: (*a*) the multiple-host nature of the system reflecting high interspecific variation in pathogenicity among *Batrachochytrium* species and (*b*) variation in the virulence within *Batrachochytrium* caused by intrinsic (genetic) and extrinsic biotic and abiotic factors. Following a consideration of the key factors that govern pathogenicity, a key question that we address is, under what conditions do highly virulent interactions occur, leading to more severe epidemiological scenarios and eventually driving a proportion of species to local extirpation or total extinction?

3. SIMILARITIES AND DIFFERENCES IN THE PATHOPHYSIOLOGY OF *BATRACHOCHYTRIUM* INFECTIONS

The parasitic stage of amphibian chytrids has adapted to a very specific niche, parasitizing epithelial cells in the amphibian epidermis. Although the pathogenesis of infection by *B. dendrobatidis* and that of *B. salamandrivorans* show some similarities, marked differences between the two result in strongly divergent lesions and pathophysiology.

Initial infection of the amphibian skin requires recognition of and adhesion to the outer skin layers by infectious zoospores in the aquatic environment. Flagellate motile zoospores of both species lack a cell wall and are released through a discharge tube that protrudes from the amphibian skin surface (68, 95, 135). *B. dendrobatidis* zoospores are chemotactic. This leads them to swim either toward skin compounds including sugars, proteins (including keratin), and amino acids (84, 86, 136) or away from molecules that may signify hostile environments, such as the bacterial metabolites 2,4-diacetylphloroglucinol and indole-3-carboxaldehyde (63). Notably, *B. salamandrivorans* produces a second type of infectious spore that is nonmotile, has a cell wall, and floats at the water-air interface (128). These encysted *B. salamandrivorans* spores are thought to infect salamanders through passive adherence followed by germination and initiation of invasion.

Attachment of zoospores to the host skin is poorly understood, but at least for *B. dendrobatidis*, it is accompanied by pseudopod-mediated motility that may mediate physical attachment (98, 135). Comparative genomics has predicted the presence of adhesion proteins such as vinculin, fibronectin, fasciclin, and chitin-binding molecules that are expected to contribute to this process (1, 67, 79, 111, 112, 136). Strikingly, genome comparisons between *Batrachochytrium* and closely related, free-living chytrids show that both *B. dendrobatidis* and *B. salamandrivorans* exhibit large expansions of genes encoding carbohydrate-binding module family 18 (CBM-18) proteins (1, 42). Although unproven, that these genes contain secretion signals suggests that their activity is extracellular and may be involved in either host recognition through chitin-binding and adhesion (66) or dampening chitin-recognition mechanisms in the host.

Unlike *B. salamandrivorans*, *B. dendrobatidis* zoospores are preloaded with an aggressive cocktail of proteases (22, 42, 110). Their presence indicates that *B. dendrobatidis* zoospores are able to initiate proteolytic activity likely involved in cell invasion immediately, while *B. salamandrivorans* needs to synthesize these hydrolytic proteins de novo following adherence to the amphibian host. This difference is likely linked to the subsequent divergence between *B. dendrobatidis* and *B. salamandrivorans* in their mechanisms of pathogenesis. No evidence for specialized invasion structures has been documented for *B. salamandrivorans*, but *B. dendrobatidis* forms a germ tube that physically invades keratinocyte cells in the epithelia and allows the pathogen to become intracellular (7, 135) (**Figure 2**). This process is superficially similar to hyphal invasion by other fungal pathogens, where the generation of substantial hydrostatic pressure accompanied by the secretion of hydrolytic enzymes drives cell entry (87, 131). After developing an intracellular sporangium, *B. dendrobatidis* may penetrate toward deeper epithelial layers or migrate toward the epidermal

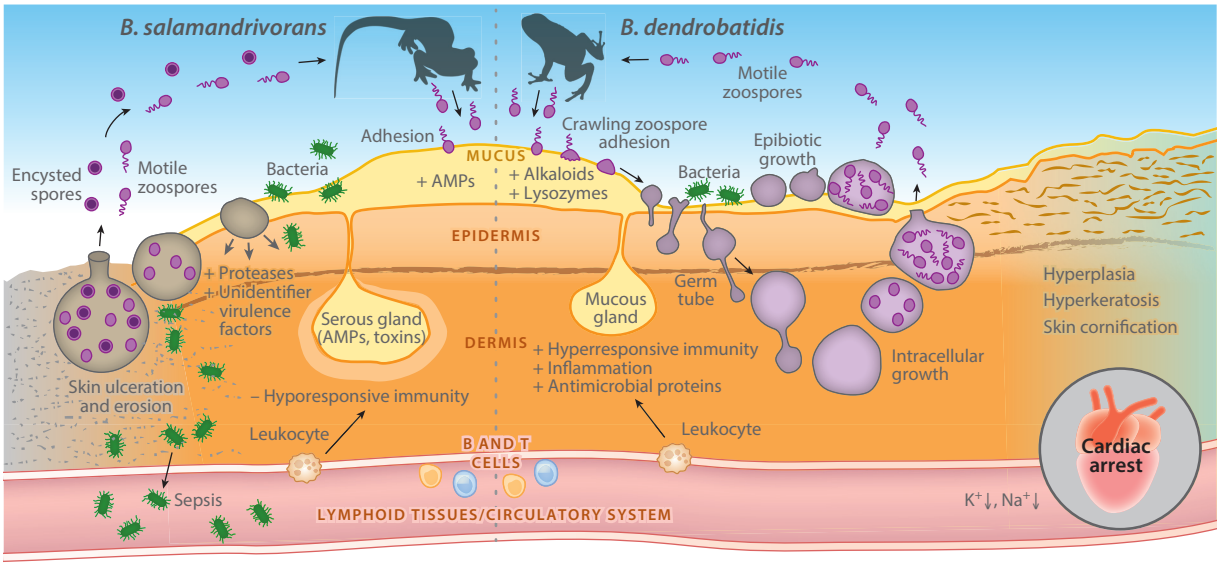

Figure 2

Similarities and differences in the pathophysiology of *Batrachochytrium dendrobatidis* and *Batrachochytrium salamandrivorans* skin infection. (*Right*) *B. dendrobatidis* is associated with germ tube invasion leading to a thickened, cornified dermis caused by hyperkeratosis. (*Left*) In contrast, *B. salamandrivorans* is associated with skin erosion and ulceration, with the likely involvement of bacterial sepsis. Frontline defense against both species is provided by the skin, where serous glands secrete mucus containing AMPs and toxins. Both innate immunity and adaptive immunity play a role in resistance to infection by *Batrachochytrium* and are strikingly absent in fire salamanders with lethal infections of *B. salamandrivorans* (128). Abbreviation: AMP, antimicrobial peptide.

surface, where it releases infectious zoospores that continue the cycle of infection. Invasion of the keratinocytes results in apoptosis of the host cell (66, 140), and epidermal cell death is positively associated with infection loads and morbidity (19).

As remarked above, pathogenesis and pathophysiology markedly diverge between these fungi. While epidermal invasion by *B. dendrobatidis* into the stratum corneum and its subsequent proliferation result in epidermal hyperplasia and hyperkeratosis (7), *B. salamandrivorans* infection erodes the epidermis across its full thickness (76) (**Figure 2**). The net result with *B. dendrobatidis* is loss of the physiological functions of the skin and with *B. salamandrivorans* loss of its barrier function. Since the amphibian skin is crucial for electrolyte and fluid homeostasis and for respiration, severe *B. dendrobatidis* infection results in lethal metabolic disorders and eventually heart failure (24, 74, 141). In comparison, loss of the skin barrier due to *B. salamandrivorans* is associated with lethal septicemic conditions, with opportunistic bacteria causing secondary infections in moribund animals (5, 13) and eventually death. Although not yet proven in natural environments, it is possible that the microbial dysbiosis that accompanies infection by *B. salamandrivorans* is a major determinant of its pathogenicity. If true, then treating this dysbiosis may be a viable route to mitigating the infection.

Severe lesions resulting in host death are typically observed in postmetamorphic stages, despite susceptibility of aquatic amphibian larval stages to infection. However, measurable costs to exposure accrue in amphibian larvae (53) and are evident even when fulminate infections do not occur. *B. dendrobatidis* readily infects frog (anuran) tadpoles, with a trophism for the protein keratin in the highly keratinized mouthparts. Subsequently, shifting patterns of keratinization during larval development result in expanding sites of infection, with increased colonization of other body

parts such as the legs in froglets near metamorphosis (53, 80). Infection of mouthparts may or may not result in depigmentation and oral deformities (35, 92). If so, this might reduce feeding efficiency (93). While costs to larval exposure and infection have been clearly demonstrated in the laboratory (53), their impact in nature remains unclear. Since *B. dendrobatidis* and *B. salamandrivorans* infections can be maintained through metamorphosis, they pose a threat to the survival of their metamorphosing hosts (82) and to the terrestrial amphibian community. Therefore, the current consensus is that while infections of larval amphibian stages are largely subclinical, these life-history stages play an important role in disease ecology as disease reservoirs.

Comparing more broadly across the fungal kingdom, *Batrachochytrium* infections in amphibians show similarities in pathogenicity with other epithelium-related emerging infections (46). Snake fungal disease caused by *Ophidiomyces ophiodiicola* erodes snake skin tissue, while *Pseudogymnoascus destructans* in bats causes white nose syndrome, a skin infection that has resulted in severe population declines of several North American bat species. In common with disease caused by *B. dendrobatidis* and *B. salamandrivorans*, the outbreak of white nose syndrome in North American bats is due to recent introduction of an exotic fungus, in this case from a Eurasian origin that is the center of the region where it is endemic (36), while the pathogeography of snake fungal disease remains more cryptic. Infection of the bat's skin results in infection-intensity-dependent physiological disorders that include disturbance of fluid and electrolyte homeostasis; acidosis; and frequent, costly arousal from hibernation (139). As is seen in chytridiomycosis, the severity of infection by *P. destructans* [and probably *O. ophiodiicola* (72)] is determined by the outcome of the host-pathogen-environment interaction. A remarkable similarity among these infections is the importance of the host thermal ecology to fungal pathogenicity. Both for the chytrid fungi and for *P. destructans*, disease and death of the host are observed to occur within the thermal envelopes that govern the optimal growth of these fungi. Chytrid fungi do not tolerate temperatures above 20°C (*B. salamandrivorans*) (76) and 26°C (*B. dendrobatidis*) (96) very well, a partial explanation for the current geographical range of chytridiomycosis-driven declines. Similarly, *P. destructans*'s critical maximum temperature is 20°C (139), which explains why the disease is known to affect only hibernating bats, whose body temperatures approach those of their hibernacula. Coevolution of *Batrachochytrium* and *P. destructans* in their regions of origin likely armed enzootic hosts against the more harmful effects of these fungi, including their growth under conducive environmental conditions, while simultaneously leading to the acquisition of fungal pathogenicity that allows coexistence with the host. However, efforts to understand fungal pathogenesis mainly focus on disease, while the host-pathogen-environment interactions that allows host-pathogen coexistence remain poorly understood. Understanding the ecoevolutionary adaptations underpinning this arms race may well open new avenues for disease mitigation. Such understanding requires model systems for study.

4. MODEL SYSTEMS FOR STUDYING *BATRACHOCHYTRIUM* PATHOGENICITY

Evidence accumulated to date shows that amphibian population declines follow incursion of *Batrachochytrium* into naive and susceptible host communities; however, this occurs only when these host communities are embedded in a conducive environment (45). Identifying the host, pathogen, and environmental factors that govern the epidemiology of these diseases requires study systems that can provide proofs of concept that range from molecular interactions of subcellular fractions to community-level effects of infection.

In vitro systems have the advantages of standardization, reproducibility, high-throughput techniques, simultaneous comparison of a multitude of experimental conditions, fewer ethical concerns, and the potential to dissect the host-pathogen interaction in a tightly controlled manner.

However, extrapolation to the level of the individual, let alone the host community, requires proper validation in vivo and preferably in situ within the context of the host's ecological niche.

In the laboratory, multi-locus sequence typing and whole-genome sequencing enable linkages between genetic and phenotypic diversity in vitro (44). These associations can then inform an understanding of how both are proxies for differentiation between isolates with different measurable biological traits that may impact virulence in vivo and in situ (42). Further, enzymatic and gene expression profiles of chytrid cultures grown under different conditions yield information on potential virulence factors, the relevance of which can be confirmed using recombinantly produced or purified chytrid compounds (85, 96, 112, 130). More-complex models such as host cell or tissue cultures allow simultaneous mapping of host and pathogen responses (22, 134, 140, 145). Adding a live host tissue to the system increases biological relevance and has provided insights into gene regulation in the presence of host skin compounds (154), the potential of discrete host defenses to control chytrid infection [for example the skin microbiome and various compounds of skin secretions, such as antimicrobial peptides (AMPs)], and key mechanisms of pathogen adhesion and invasion (135). The recent development of protocols to genetically transform chytrids (including *Batrachochytrium*) lays important foundations for the development of gene modification to create knockout mutants (83, 129). It is likely only a matter of time before CRISPR-Cas9 is developed to provide unprecedented opportunities to investigate, and manipulate, virulence and pathogenicity in these chytrids.

Final proof of pathogenicity, however, requires a model of the complex environment at the level of the intact host. This includes the presence of intact barriers to infection, such as epidermal mucus, keratinized skin layers, and their associated array of innate and acquired defenses. These environments are difficult or impossible to replicate in vitro, favoring the development of either alternative animal models or amphibian skin tissue explants. Nematode *Caenorhabditis elegans* models have yielded promising results, although the observed pathogenicity may be due to toxic chytrid products rather than active fungal infection (10, 124). The most relevant models use vertebrates in which active infections are established. Zebrafish larvae have been successfully used to demonstrate key features of *B. dendrobatidis* pathogenicity in a nonamphibian host, and the advantage of using fish is that it is not restricted by licenses governing animal use in research as long as they are still in their nonfeeding larval stage. Moreover the toolbox available for zebrafish larvae is highly developed, making this an exciting model for fundamental research (66). The proof of the pudding, however, is in the eating: The most pertinent models for pathogenicity are intact postmetamorphosis amphibians.

Careful selection of species and life stage maximizes the model's relevance. Although amphibian models are most relevant to natural environments, a plethora of known (e.g., life stage, species, temperature, dose) and unknown (e.g., perhaps nutrition, prior infections, environmental determinants, captivity) factors confound interpretation of results and reduce reproducibility (5, 116, 128). For example, skin secretions comprising toxins sequestered from prey items (29, 30) and microbiomes (5) of animals collected from natural environments are different from those of animals in captivity, leading to ambiguity when linking in vitro results to in situ disease dynamics. Stringent standardization of experimental conditions facilitates comparison within and between studies. However, this may limit the breadth of conclusions and should take into account the introduction of biases, for example, thermal mismatch (116) when selecting a single experimental temperature to compare infection dynamics among species with different thermal ecologies.

The final test of pathogenicity includes the addition of community-level effects and requires complex experimental setups including biotic and abiotic compounds of the amphibian ecosystem. Even the most complex mesocosm setup, however, falls short of the natural ecosystem, and validation of experimental results by observations or interventions in nature remains the gold standard.

5. FACTORS GOVERNING THE VIRULENCE OF *BATRACHOCHYTRIUM*

Virulent infections by *Batrachochytrium* appear to be mainly a function of pathogen load, with lethal infections typically associated with high pathogen burdens and major changes in skin morphology and function (147). In turn, the pathogen load is determined by complex interaction between host factors, *Batrachochytrium* virulence factors, and the environmental context over time.

Textbook cases of chytridiomycosis are characterized by a rapid buildup of infection to high levels (often approaching 10,000 genomic equivalents in swab samples), with marked skin pathology that results in death (8, 145, 147). Combined with a high prevalence, such cases account for the outbreaks and mass die-offs that have generated broad attention for this disease. Measures of mortality, however, do not always translate to extinction risk (116), and for most species that are threatened by chytridiomycosis, individual mortality (let alone mass die-offs) has never been witnessed. This, however, may in part be due to the secretive lifestyle of most amphibians leading to underdetection of mortality. A second explanation is that a very low prevalence may still result in population level effects if the probability of disease-induced mortality is sufficiently high when infection occurs (133). A third possibility is that unseen costs incurred by largely subclinical infections that are highly prevalent affect fitness and hence decrease individual survival (126). These mechanisms may result in unobserved silent declines in a large number of amphibian taxa and warrant future fine-grain ecological studies on amphibian demographic trends.

Laboratory trials and field data with both chytrids show tolerance to high infection loads in several anuran (frog) and urodele (salamander) species (6, 51, 75, 102, 103, 105, 121, 149). Infection-tolerant animals can behave as supershedders of infectious inocula that maintain a high infection pressure in the amphibian community. Currently, it is not clear how such high chytrid burdens can be maintained in tolerant animals in the absence of obvious lesions, disease, or death. Part of the explanation could be the adoption of a different infection strategy by the fungus. In ex vivo assays, Van Rooij et al. (135) demonstrated that *B. dendrobatidis* is capable of epibiotic growth suggesting that locally abundant however noninvasive populations of *B. dendrobatidis* may occur. In tolerant urodeles, *B. salamandrivorans* is found in the superficial epidermal cell layers, which does not result in the breaching of epidermal integrity. This limited evidence suggests that, by limiting fungal proliferation to the most superficial skin layers, tolerant amphibians succeed in reducing the damage to skin integrity and physiology.

Relatively high infection loads with no records of mass mortality events and no negative effects on population dynamics have been observed in species that are prone to chytridiomycosis-driven declines elsewhere (115). This observation may be attributable, at least partially, to the complex population genetic structure of *B. dendrobatidis*, whereby populations of amphibians are infected by lineages and genotypes of *B. dendrobatidis* with different virulence. The European midwife toad (*Alytes obstetricans*) is emblematic for this scenario in Europe, with mass mortality events leading to widespread declines in Iberian and Pyrenean populations while northern populations widely coexist with *B. dendrobatidis* (16, 26, 127). Abiotic variables such as altitude strongly predict risk of death by chytridiomycosis (148); however, recent research has further shown that genotypes of the *B. dendrobatidis* global panzootic lineage (*Bd*GPL) infecting more northerly populations of these amphibians are of lower virulence to those found elsewhere (54). These observations suggest that the virulence of *B. dendrobatidis* in *A. obstetricans* is, alongside the modifying effect of the environment, a product of high-intensity infections due to subpopulations of highly virulent and infectious lineages of *B. dendrobatidis*. However, as demonstrated by Doddington et al. (34) in natural populations of *Alytes muletensis*, the environmental context of infection in this highly susceptible species is key. In Mallorca, Spain, the temperature of the animal's environment predicts whether it will thrive or die from chytridiomycosis (34). Infections in Mallorca are caused

by *Bd*CAPE, a lineage with low virulence in ex situ challenge experiments (34, 43, 90). However, these in vivo challenge experiments do not predict population trends in nature, which are determined by the thermal profile of the geographical locations of infections. Clearly, both the context within which infection occurs as well as the genotype of the infecting isolate are critical to the realization of *B. dendrobatidis* virulence.

6. HOST FACTORS MODULATING PATHOGENICITY

Susceptibility to chytridiomycosis shows marked phylogenetic clustering across amphibia (91, 116, 119), and both fungi threaten the survival of entire genera of anurans and urodeles. Further, *B. dendrobatidis* and *B. salamandrivorans* have markedly different host spectra. While *B. dendrobatidis* infects caecilians, urodeles, and anurans but mainly impacts anuran populations (119), *B. salamandrivorans* is a urodele pathogen with occasional and asymptomatic infections in anurans (88, 128). However, infection and disease susceptibility to *Batrachochytrium* infection can be highly variable within a given species (see 12). While for some species such as *A. obstetricans*, juvenile stages shortly after metamorphosis are considered most vulnerable, in other species it seems that it is primarily adult stages that are affected by disease (116, 119, 128). Acquired immunity due to prior infections (54, 55, 108), coinfections (3, 54), the host MHC genotype (62, 69, 117, 118), body condition and size (52), behavioral traits (for example basking or the use of retreat sites) (31, 99, 106, 113), life stage, and age (80) and variation in antimicrobial defenses (e.g., the skin microbiome, antimicrobial skin secretions such as AMPs) (9, 109, 150) are all host characteristics that have been linked to disease resistance.

The collection of compounds that can be rinsed off the outer surface of an amphibian is named the mucosome and contains a variety of known and unknown compounds: bacteria and their metabolites (57, 64, 152), AMPs and toxins (mainly in anurans) (94), mucus components, and antibodies (101). These compounds are partly secreted by the skin's abundant serous and mucous glands and may provide a first barrier or a portal of entry for pathogens. Moreover, the continuous production and shedding of mucus may also remove pathogens from the host surface, as is well known to occur in the gastrointestinal tract. Although the mucosome may play a central role in the amphibian defense against *Batrachochytrium* and its antifungal activity has been associated with decreased susceptibility to *B. dendrobatidis* and *B. salamandrivorans* (2, 125, 151, 153), pinpointing and isolating antifungal factors in the host mucosome is challenging. Research into the chytrid-mucosome interaction (including, notably, the largely overlooked compounds in mucus that may play a crucial role in pathogen adhesion) is likely to offer novel insights into host-pathogen coevolution and may result in the development of novel tools for disease control.

AMPs represent a highly diversified innate defense system in the amphibian skin that protects against microbial attack. The composition and activity of the AMP cocktail on anuran skin correlate with susceptibility to *B. dendrobatidis* in vitro, and several amphibian AMPs efficiently kill *B. dendrobatidis* (153). As is found for the microbiome, exposure to *B. dendrobatidis* in turn affects AMP activity; for example, it induces genes that are associated with the production of AMPs such as caerulein (104). A recent study hints at adaptation of chronically infected amphibian populations toward more efficient antifungal AMP production (144) and points toward a mechanistic basis for how species impacted by chytridiomycosis in nature may recover.

The composition of amphibian-associated microbial communities is associated with population-level disease dynamics, with *B. dendrobatidis* driving changes in bacterial communities during natural disease dynamics (4, 59). In turn, these microbes may reduce the pathogenicity of *B. dendrobatidis* and alleviate severity of infection, opening avenues for disease mitigation through bioaugmentation (14). However, the microbiome also includes a variety of opportunistic

bacterial pathogens that may take advantage of disruption of the epidermal barrier and contribute to chytridiomycosis-related death due to both *B. dendrobatidis* (5) and *B. salamandrivorans* (5, 13). The inhibitory effects of cutaneous bacteria are largely attributed to antifungal bacterial metabolites. A remarkable paucity of bacteria, however, was demonstrated on salamander skin in a recent study (13), and may render direct antifungal action of such compounds (which typically are concentration dependent) less likely for such species. While this may at least in part explain why in vitro results sometimes translate poorly to in vivo protection, the demonstrated impact of captivity on amphibian skin-microbiome structure needs to be considered (5). We encourage inclusion of microbial density and antifungal compound concentration as key parameters for analysis in future studies (137) and exploration of additional, more indirect mechanisms (such as immune priming) through which bacterial communities may affect amphibian susceptibility to chytridiomycosis. The development of novel models, including axenic animals, would greatly facilitate such research opportunities.

Differential immune responses are also known to drive differential susceptibility to infection by *Batrachochytrium*, with both innate and adaptive immunity playing a role (55, 107). Infection with *B. dendrobatidis* results in marked host immune responses that vary with host susceptibility (39, 40, 97). As noted above, host susceptibility is largely dictated by effective innate immunity comprising skin AMP repertoire and responses. While adaptive immunity is highly functional in amphibians, it appears to be suboptimal in protecting against both *B. dendrobatidis* (reviewed in 101, but see 81) and *B. salamandrivorans* (128). Farrer et al. (42) found a marked difference in how the transcriptome of a susceptible host (the crocodile newt *Tylototriton wenxianensis*) responded to the two pathogens. While *B. dendrobatidis* evoked a large host response, with marked changes in genes involved in epidermal cornification, electrolyte and fluid homeostasis, and innate and adaptive immunity, the newts showed little response to the highly destructive *B. salamandrivorans* infection. Here, the hyperimmune response seen during *B. dendrobatidis* infection contrasts with the hyporesponsive state seen during *B. salamandrivorans* infection and appears to be a fundamental difference in immunity mounted against the two species. This difference may be due to direct manipulation of host immunity by *B. dendrobatidis*, as the infection appears to actively modulate host adaptive immunity; *B. dendrobatidis* culture supernatant inhibits lymphocyte proliferation and induces apoptosis (47) via an unknown intermediary. However, adaptive immunity against *B. dendrobatidis* is not entirely nonfunctional, and that conferred by prior, nonlethal infections with *B. dendrobatidis* can protect against virulent infection by *B. dendrobatidis* and even *B. salamandrivorans*, at least in some amphibian species (25, 54, 81). In northern Europe, the widespread occurrence of low-virulence *Bd*GPL isolates confers protection against incursion by the epidemic, highly virulent isolates, perhaps even naturally vaccinating these exposed populations (54). Similarly, prior infection by these low-virulence *Bd*GPL isolates protected some, but not all, salamanders against virulent infection by *B. salamandrivorans*. These findings stress an underrecognized aspect of *Batrachochytrium* epidemiology, suggesting that instead of posing an acute threat, low-virulence chytrids may protect amphibian communities from losses due to chytridiomycosis.

Amphibians are ectotherms, and consequently, at suboptimal temperatures their immune responses may be dysregulated, resulting in increased *B. dendrobatidis* infection loads. Within a species's thermal niche, temperature-dependent patterns of gene expression dictate immune defenses (104). These temperature-dependent trade-offs between pathogen growth and host immune response lead to complex relationships in nature. The thermal-mismatch hypothesis posits that virulence is dictated by the performance gap between host and pathogen vital rates. Evidence for thermal mismatch leading to compromised immunity is seen in vivo (27) and in situ (26) where seasonal variation is linked to more aggressive outbreaks of *B. dendrobatidis*–driven chytridiomycosis. In the salamander *Plethodon cinereus*, lower temperatures resulted in more

pronounced activation of innate immune pathways, with increased fungal pathogenicity, a feature that was also seen in the clawed frog *Xenopus tropicalis* (104). At higher temperatures, adaptive immune genes were more highly expressed, which tended to increase host survival (37). Although susceptible hosts show the largest changes in gene expression, this response does not protect them from disease or death. Zamudio et al. (154) hypothesized that this response may be too late in susceptible hosts to successfully curb infection. Genes involved in skin integrity seem to be differentially regulated, with upregulation in less susceptible and downregulation in highly susceptible species (reviewed in 154). An alternative explanation is that the immune system overreacts or is dysregulated, negatively affecting the host (42, 55).

7. UNTEMPERED VIRULENCE RESULTS IN EXCESSIVE PROLIFERATION AND PATHOGENICITY

Highly virulent lineages of *B. dendrobatidis* and *B. salamandrivorans* account for chytridiomycosis-driven amphibian losses (43, 75, 119). Extinction hot spots in the Americas and Australia and *B. dendrobatidis*–associated declines in Europe have been driven by the incursion of the hypervirulent *Bd*GPL (90). Asia, considered the cradle of evolution for the *Batrachochytrium*, has been spared the declines driven by chytridiomycosis. This observation can be explained by host-pathogen coevolution increasingly armoring amphibian chytrids against increasingly efficient amphibian defenses (48). Within the native enzootic system, the dynamic and continuously evolving balance between pathogen virulence and host defense allows coexistence. However, deploying the full armamentarium of acquired virulence factors in a naive environment can result in uncontrolled chytrid growth, with dose-dependent pathological effects and with the potential to culminate in mass die-offs, population declines, and extinctions.

Most of what is known regarding *Batrachochytrium* virulence has been deduced from comparative genomic studies against related free-living saprotrophic chytrids. *B. dendrobatidis* shows lineage-specific gene regulation, and increased expression of chitin-binding proteins, fungalysin metalloproteases, and crinkler-like (CRN) genes may be good predictors of virulence (38, 42, 77, 110). The CBM-18 family is markedly expanded in *B. dendrobatidis* (1), where it may serve a role in fungal adhesion to host skin or dampening chitin-recognition mechanisms in the host (42). Proteolysis by the expanded M36 metalloprotease gene family in both *Batrachochytrium* species is likely key to the process of invasion (22). Metalloproteases are greatly expanded in *B. salamandrivorans* (42), concordant with its aggressive necrotic pathology, and both *B. salamandrivorans* ($n = 110$) and *B. dendrobatidis* ($n = 35$) have expanded M36 families compared to the free-living saprotrophic chytrids *Spizellomyces punctatus* ($n = 2$) and *Homolaphlyctis polyrhiza* ($n = 3$). Patterns of expression should preferably be assessed in the presence of host compounds (110) or in infected hosts (38, 42), which typically yields very different results compared to assessment in culture media. Gene expression in *B. dendrobatidis* itself varies alongside the susceptibility of the hosts it infects (38). Genes involved in ciliary structure and function are overexpressed when *B. dendrobatidis* infects a host (*Atelopus zeteki*) that is highly susceptible to lethal infection. Both species of *Batrachochytrium* show the presence of CRN genes, which show close homology to known virulence effectors in pathogens belonging to the *Phytophthora* and *Lagenidium* genera of oomycetes (41). CRN-like genes are highly expanded in the *B. dendrobatidis* genome (162 compared to only 11 in *B. salamandrivorans*), and *B. dendrobatidis* zoospores showed increased expression of CRN genes upon exposure to host tissue (41). In comparison, *B. salamandrivorans* zoospores were associated with decreased expression, indicating that CRN genes are possibly of greater interest in the early infection stage of *B. dendrobatidis*, but not *B. salamandrivorans*. However, the functions of these genes are unknown, and their roles in pathogenesis are unproven. Observations such as these can be extended to

other amplified gene-tribes in *Batrachochytrium* showing that substantial genomic dark matter exists, and that likely plays yet-unexplained roles in governing their virulence and pathogenicity (41). Although this is paradoxical at first sight, significant advances in understanding virulence will most likely come from research across regions where ancient *Batrachochytrium* species were endemic, as it is here that coevolution will have matched pathogen adaptations with effective host responses.

8. ENVIRONMENT AND *BATRACHOCHYTRIUM* VIRULENCE

While chytridiomycosis has had a widespread impact in terms of biodiversity loss, extinction hot spots themselves are highly localized. The widespread occurrence of potential hosts and virulent *B. dendrobatidis* genotypes suggests that environmental factors are key modifiers of disease dynamics in regions without prior host-pathogen coevolution. As noted above, temperature is key for ectothermic amphibians to survive challenge by *Batrachochytrium*. Specifically, the thermal-mismatch hypothesis (27) predicts increased virulence at the extremes of the host species's thermal niche owing to attenuated host defenses (reviewed in 154). Observations suggest that this effect is especially pronounced for temperatures at the low end of the range of thermal tolerances, likely owing to host immune suppression (73, 100), and may drive the pronounced seasonal differences in disease dynamics observed in natural populations (26, 70, 71). However, in tropical climates, where amphibians are continuously exposed to a thermal environment that could be expected to promote optimal physiological and immunological functioning, the fungal thermal optima may be equally important. The optimal temperatures for growth of *B. dendrobatidis* range from 17°C to 25°C (96; but see 142, which reports growth between 2°C and 27°C), and the optimal temperature for *B. salamandrivorans* is approximately 15°C (76). Host preferences for higher temperatures (116) or increased body temperature during infection due to behaviors such as basking (114) may shift the balance in favor of the host. Any prediction of *Batrachochytrium* virulence should thus take into account the microclimate conditions that influence the thermal ecology of the host (11, 65).

Chytridiomycosis-driven extinction events require the presence of reservoirs of inocula that buffer the pathogen against density-dependent limitation (20). Postmetamorphic stages of disease-tolerant species clearly are reservoirs of infection, and amphibian community composition is an important driver of infection dynamics (91, 128), as are long-lived tadpoles with subclinical infections (20). However, our knowledge of the full extent of *Batrachochytrium* reservoirs is imperfect. Despite the collapse of amphibian communities and the decrease in amphibian hosts in chytrid-infested ecosystems (28), chytridiomycosis-induced mortality continues, suggesting a role for nonamphibian pathogen reservoirs. Crustaceans have been proposed as alternative *B. dendrobatidis* hosts, having demonstrated their capacity to infect tadpoles (79). In Spain, the occurrence of *B. dendrobatidis* DNA in crayfish guts is related to the presence of *B. dendrobatidis* in amphibians (89). However, Betancourt-Roman et al. (10) could not reproduce *B. dendrobatidis* persistence in crayfish, bringing into question the role of invertebrates as alternative hosts. Elucidating the role of nonamphibian hosts in *B. dendrobatidis* infection dynamics will contribute to the design of mitigation protocols.

Similarly, an intriguing gap in our knowledge is the extent to which *Batrachochytrium* can behave as saprotrophs. If they are able to grow and reproduce in nature, the environmental infectious pathogen load may be an important driver of disease dynamics, maintaining high prevalence of infection in host communities even when hosts become rare (15, 122). Certainly, survival of *Batrachochytrium* in the environment appears possible for epidemiologically relevant periods, and *B. salamandrivorans*–contaminated soil remains infective for at least 48 h for salamanders (128). These stages can survive in sterile water for four (*B. salamandrivorans*) to seven (*B. dendrobatidis*) weeks and in sterile river sand for up to three months (60, 128); however, they lose motility and

settle within hours (96). Viability in the field is difficult to assess but has been shown to depend on environmental factors like canopy cover, ultraviolet radiation, water flow, water depth, water temperature, pH, and the presence of zooplankton or other organisms that actively prey on fungal spores (23, 56, 60, 122, 123, 132). While rapid encystment by *Batrachochytrium* may produce environmentally resilient life stages, this suggests rapid loss of infectivity and raises questions about the contribution of mere zoospore persistence to establishing a significant environmental chytrid reservoir (33, 128). The encysted spores of *B. salamandrivorans* are infectious, float at the water-air interface, survive for long periods in water, and are more resistant to predation, maintaining an infected environment even in the absence of reservoir hosts for up to 31 days. While encysted spores such as these have never been seen for *B. dendrobatidis*, a saprotrophic aspect to its lifestyle with environmental proliferation and de novo production of infectious spores would explain several key features of chytrid epidemiology, including extinction events and poor recolonization of systems from which amphibians have been extirpated (21, 68, 119). Both *B. dendrobatidis* and *B. salamandrivorans* have indeed been shown to grow saprotrophically on a number of sterile organic substrates such as feathers and reptilian scales in lab trials (49, 60, 128), but investigation of saprotrophic life stages in nature is hindered by the inability to isolate them from environments that are heavily contaminated by fungi and bacteria, suggesting that (as is seen with amphibian skin microbiomes) environmental microbes present a significant challenge to *Batrachochytrium*. Environmentally resilient life stages and reservoirs would be consistent with findings that neither of the *Batrachochytrium* species shows signs of attenuation even several years after invasion (128, 144), suggesting virulence is not penalized. The discovery of environmental reservoirs could radically change our view of chytrid epidemiology; amphibians would be incidental hosts for an otherwise saprophytic fungus. This would render elimination of environmental reservoirs crucial to chytrid mitigation efforts, and it is notable that the only successful mitigation to date required environmental chemical disinfection (17).

Identification of factors that determine the longevity and importance of environmental reservoirs is key to understanding the epidemiology of chytridiomycosis. Infectious stages of *Batrachochytrium* are part of the aquatic food web, and the abundance and diversity of zooplankton preying on chytrid spores dictate infection rates in tadpoles (122). This may partially explain the persistence of amphibians in highly specific niches in the neotropics, such as bromeliad leaf ponds, that offer little possibility of survival for the infectious life stage of *B. dendrobatidis* (15). Conversely, favorable environments with high infection pressure may result in the high prevalence and infection intensity that are associated with mass die-offs (147). Accordingly, the manipulation of trophic webs may be a valuable tool to reduce environmental infection pressure and temper *Batrachochytrium* pathogenicity (32).

9. STRUCTURE OF POSTEPIZOOTIC AMPHIBIAN COMMUNITIES AND THE PATH AHEAD

Although the future of amphibians is uncertain, it is highly unlikely that *Batrachochytrium* will ever disappear from the regions they have newly invaded. Recovery of amphibian communities will thus require acquisition of traits that allow host-pathogen coexistence in the long term. Several species that were once considered lost are being rediscovered, and *B. dendrobatidis* appears to have invaded most of the world. Few truly naive communities still exist (Papua New Guinea is probably the biggest area where *Batrachochytrium* does not occur, and the infection status of Madagascar remains unclear). *B. dendrobatidis* can now be found in almost any region of the planet, and the worst may well be behind us (119, 120). However, in the affected communities, even 20 years after *B. dendrobatidis*'s incursion, few signs herald the recovery of populations to

preoutbreak levels. The overall abundance of amphibians in the worst-hit areas remains very low and, globally, signs of postepizootic recovery have been observed in only 60 of 501 (12%) species of amphibians that have suffered a decline (119). Since the virulence of *Bd*GPL does not seem to have attenuated over time, evolution of the host to tolerate these fungi appears crucial in reestablishing the once abundant communities in the cloud forests of Latin America and Australia (144). Strikingly, a large-scale rebound of frog populations has been observed in the US Sierra Nevada mountain range (Yosemite National Park, California) after decades of declines (61). Removal of environmental stressors (introduced fish) was significantly associated with recovery, yet the largest effect may be due to the frogs having adapted to survive *B. dendrobatidis* infections. Encouragingly, similar signs of recovery have been observed elsewhere (144) and selection for genetic markers that are associated with disease resistance is increasingly being found (78, 117). Although such adaptations may at present seem insufficient to restore abundance to pre-decline levels, they may rescue amphibian populations over the longer term and undo some of the wider ecosystem-level impacts of chytridiomycosis (155).

However, extinction is forever. We are unlikely to see full recovery of the spectacular amphibian diversity in the impacted tropical and montane regions of the planet. But will we? Certainly, human interventions may reduce extinction risk and promote long-term survival, provided such interventions result in transferable (across generations) and durable (for example, environmentally safe) modifications that permanently increase the resilience of host communities against chytridiomycosis. A variety of management options exist (reviewed in 50), and some have been trialed with a degree of success in nature (17, 146). Augmented evolution of animals selected for tolerance to infection is an enticing possibility (138) and will be increasingly feasible as the CRISPR revolution proceeds. Further, de-extinction through technological means, while it has never been achieved, remains a possibility with the help of cryopreserved cadavers of members of species that have been lost to chytridiomycosis. Ultimately, for any method to be successful, amphibians need to be presented with an intact ecosystem where breeding dynamics can compensate for the impact of disease. Given current trends in rates of biodiversity loss due to habitat destruction, and the future trends expected because of global heating, disease mitigation must be secured through effective habitat conservation and by strengthening of transnational biosecurity (46, 143).

DISCLOSURE STATEMENT

The authors are not aware of any affiliations, memberships, funding, or financial holdings that might be perceived as affecting the objectivity of this review.

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