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Global Amphibian Declines, Disease, and the Ongoing Battle between *Batrachochytrium* Fungi and the Immune System

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Abstract: In the approximately 30 years since the recognition of the crisis of global amphibian declines, much has been learned about the likely causes. Among the leading causes are several amphibian diseases including the disease termed chytridiomycosis caused by the chytrid fungi *Batrachochytrium dendrobatidis* (*Bd*) and *Batrachochytrium salamandrivorans* (*Bsal*). Here, I briefly review the fundamentals of amphibian immunity, amphibian immune defenses against the chytrid fungi, and the host–pathogen interactions that often favor the pathogen to the detriment of the host. Because amphibians are ectotherms, climate and temperature have a major impact on the amphibian immunity. Thus, I discuss current information about the role that temperature and unpredictable weather events may play in disease and immune responses to the chytrids. Because much research on amphibian declines is directed toward finding management solutions to protect threatened amphibians, I conclude by drawing attention to some of the most promising and novel mitigation strategies that are being proposed.

Key words: Adaptive immunity; Amphibian; Chytrid; Climate; Immune evasion; Innate immunity; Temperature

In evolutionary terms, the adaptive immune system (lymphocyte-mediated immunity) arose within the first primitive jawed fishes approximately 500 million years ago. The major features are B and T lymphocytes with a great variety of antigen receptors (B cell receptors [BCRs] in B cells and T cell receptors [TCRs] in T cells), a major histocompatibility complex (MHC), networks of interacting molecules called chemokines and cytokines, specialized primary lymphoid organs for development of lymphocytes, and secondary lymphoid organs to generate protective responses (reviewed in Flajnik and Kasahara 2010; Flajnik 2018). These features are shared by all jawed vertebrates, including amphibians. In addition, all animals including invertebrate species have innate defenses that include phagocytic cells, antimicrobial peptides (AMPs), and other defensive proteins such as lysozymes (reviewed in Buchmann 2014). Amphibians use both innate defenses and adaptive defenses to effectively protect against viral, bacterial, and fungal pathogens. Amphibian immunity, amphibian disease ecology, immune changes at metamorphosis, and other aspects of this subject have been previously reviewed. Whenever possible, I cite these reviews so readers can find additional information, and I distinguish the primary literature from the reviews. This current article is intended to draw attention to very recent findings that advance our understanding of immune defenses against the chytrid fungi *Batrachochytrium dendrobatidis* (*Bd*) and *Batrachochytrium salamandrivorans* (*Bsal*). Although amphibians have complex and robust immune defenses, many factors can favor or impair an effective immune response against a specific pathogen. Cold temperatures and stress impair amphibian immune defenses, whereas warm temperatures and nonstressful conditions favor effective defenses (reviewed in Rollins-Smith 2017). In addition, there is a continuing coevolution of host and pathogen interactions, and some pathogens, including the chytrid fungi, have developed strategies to evade amphibian immune responses to survive and often kill their hosts (Fites et al. 2013, 2014; Rollins-Smith et al. 2015, 2019). Here, I review the fundamentals of amphibian immunity, the basic biology of chytrid fungi, and the host–pathogen interactions, with a focus on the effects of temperature on those interactions.

Fundamentals of Amphibian Immunity

Innate Immune Defenses

Amphibians have immune defenses that are nearly as complex as more highly studied mammalian species. Some features of the immune defenses are shared with other warm- and cold-blooded vertebrates, and some are more unique to amphibians. Much of what is known results from studies in South African Clawed Frogs, *Xenopus laevis*, and these studies have been described in previous reviews (Du Pasquier et al. 1989; Robert and Ohta 2009; Rollins-Smith and Woodhams 2012; Flajnik 2018). With some exceptions, the hematopoietic system of all vertebrates is highly conserved; thus, the blood cells that protect from viruses, bacteria, fungi, and other pathogens are shared (reviewed in Jagannathan-Bogdan and Zon 2013). These cell types include phagocytic cells such as macrophages (Mϕs), neutrophils, Langerhans cells in the skin, and dendritic cells that form the first line of innate defenses. Phagocytic cells capable of directly killing pathogens include Mϕs, neutrophils, basophils, and eosinophils (Hadji-Azimi et al. 1987). Although phagocytosis is mostly limited to these professional antigen-presenting cells, B lymphocytes in mammalian species and *X. laevis* are also capable of phagocytosis (Li et al. 2006; Parra et al. 2012). In addition to these sentinel phagocytic cells, amphibians have natural killer (NK) cells that can kill tumor targets without specific priming (Horton et al. 2000) and are important for a rapid response to virus infection (Morales et al. 2010). NK cells belong to a class of cells termed innate lymphoid cells (ILCs). They are mainly tissue-resident lymphocytes without the diverse set of antigen receptors that are found on conventional T and B cells. ILCs can react quickly to a danger threat. These cells also include invariant NK T cells and mucosa-associated

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invariant T (MAIT) cells (reviewed in Vivier et al. 2016, 2018). ILCs have a central role in innate immune responses and also have important roles in adaptive or acquired immune responses. Recently, it was shown that *X. laevis* has a set of innate-like T lymphocytes (iT cells) that seem to recognize conserved pathogen-associated antigens presented by distinct MHC Class I–like molecules (Edholm et al. 2013; Rhoo et al. 2019; reviewed in Edholm et al. 2016). Interactions of iT cells may be especially important in the immune responses of tadpoles, which have a limited conventional T cell recognition capability. It has been suggested that developing amphibians with a more limited set of lymphocytes might use iT cells that can act rapidly as a predominant response (Flajnik 2018). Further study of iT cells and MAIT cells in amphibians is an area of important future research (Rhoo and Robert 2019).

The complement system is composed of >30 serum proteins that were originally described for their ability to complement (help) antibody-dependent killing. They function in a series of activation events (usually cleavage of the next protein), leading to activation of a membrane attack complex and direct killing of pathogens. All three recognized pathways of the complement system, the classical (requiring antibody-binding), the alternative (direct binding to pathogens), and the lectin-dependent pathway (requiring carbohydrate-binding proteins from the liver to bind pathogens) are present in amphibians (reviewed in Fujita et al. 2004; Nonaka and Kimura 2006).

The mucus of amphibian skin is an important barrier for infection by *Bd* and *Bsal*. Contained in the mucus are proteolytic enzymes produced by the host as well as the community of bacteria that inhabit the host. Also present are lysozymes (Ostrovsky et al. 1976; Zhao et al. 2006; Zhang et al. 2009; Yu et al. 2013; Savage et al. 2014; Xiao et al. 2014) that target the peptidoglycan layer of bacteria and the chitin in the cell wall of fungi (Callewaert and Michiels 2010). The microbial community (microbiome) of the skin is now considered to be an important component of the innate immune defenses of amphibians (reviewed in Rebollar et al. 2016; Walke and Belden 2016). Also see the article by Rebollar et al. (2020) describing the amphibian skin microbiome and the potential for the use of antifungal microbes as probiotics to protect amphibians against chytrid pathogens. Some of the most important defensive molecules in the mucus of amphibians are AMPs. These short and generally hydrophobic peptides are encoded as prepropeptides (reviewed in Nicolas et al. 2003) and synthesized and stored as mature or near-mature peptides (Brunetti et al. 2018) in the granular glands of many amphibian species. They are released in high concentrations into the mucus when amphibians are alarmed or injured (reviewed in Rollins-Smith and Conlon 2005; Rollins-Smith 2009; Landram and Nicolas 2016; Varga et al. 2019). To summarize, amphibians have many of the same innate immune components as mammalian species, and they may have an expanded set of iT cells that recognize antigens presented by nonclassical MHC molecules.

Adaptive Immune Defenses

The adaptive immune system consists of multiple subsets of lymphocytes, phagocytic cells, and antigen-presenting cells that originate in the primary lymphoid organs (thymus, bone marrow, and liver) and dwell at various times within the secondary lymphoid organs (primarily the spleen in amphibians, but lymphoid accumulations can occur at other sites) and the blood (Du Pasquier et al. 1989). Amphibians lack lymph nodes (Robert and Ohta 2009), and the sites used for hematopoiesis may differ from mammals and other vertebrate groups, but the functions are highly conserved. Other notable differences are the lack of follicular dendritic cells and germinal centers within B cell zones. Instead, amphibians seem to have a dendritic cell that has dual properties of conventional dendritic cells involved in antigen presentation to T cells as well as properties of follicular dendritic cells involved in germinal center formation and class switch recombination in mammals (Neeley et al. 2018). The immune cells communicate with a network of immune-specific mediators (mostly small- to intermediate-sized proteins) called interleukins, chemokines, and cytokines (Qi and Nie 2008; Robert and Ohta 2009; Grayfer and Robert 2013, 2015; Grayfer et al. 2014; Koubourli et al. 2018). The functions of the adaptive immune system are intimately connected and regulated by a genetic region called the MHC (Du Pasquier et al. 1989). The adaptive immune system is highly complex with elaborate redundancies to ensure that vertebrate species are elegantly protected from all types of pathogens. The essential components are B lymphocytes with a diverse array of antigen receptors producing antibodies, T lymphocytes with conventional rearranging αβ receptors, and an MHC. These central features are shared by amphibians, and they have been previously described in greater detail by others (Du Pasquier et al. 1989; Robert and Ohta 2009; Rollins-Smith and Woodhams 2012; Colombo et al. 2015; Flajnik 2018; Grogan et al. 2018b). It is also important to note that although they may not yet have been characterized, many of the possible subsets of cells and critical molecules essential to the function of the immune system of mammals are likely to be found in amphibians as well. For example, the ortholog of the interferon-γ gene in the frog *Xenopus tropicalis* was discovered by exploiting conserved genome synteny (Savan et al. 2009). That is, by searching the genomes of the amphibians for which the entire genome has been sequenced [e.g., *Ambystoma mexicanum*, *X. laevis*, *X. tropicalis*, *Rana* (*Lithobates*) *catesbeiana*, *Rhinella marina*], immune genes can be found by their location in relation to the genomes of other well-characterized species. There are some notable exceptions. Amphibians and fish seem to lack granulocyte–macrophage colony-stimulating factor and the cytokine interleukin (IL)-3 (Pazhakh and Lieschke 2018), but other related factors may be able to replace them in hematopoiesis and lymphocyte differentiation.

An adaptive immune response begins with recognition of a pathogen by the resident and patrolling professional antigen-presenting cells (e.g., Mϕs, dendritic cells, Langerhans cells in the skin, Kupffer cells in the liver; Robert and Ohta 2009). Pathogens have conserved pattern motifs called pathogen-associated molecular patterns that can be recognized by specific pattern recognition receptors on the immune cells (reviewed in Schenten and Medzhitov 2011). The phagocytic cells digest the antigens and display them in association with Class I and Class II MHC molecules. The diverse TCRs are focused around these MHC molecules during development in the thymus, and they only recognize

specific peptides that are displayed associated with the MHC. Innate-like T cells, in contrast, have a more limited set of antigen-recognition receptors, and they seem to find their antigens presented by nonclassical MHC Class I–like molecules (Edholm et al. 2013; reviewed in Edholm et al. 2016). *Xenopus laevis* has at least 20 such nonclassical MHC molecules (Flajnik et al. 1993). Following successful MHC–TCR–antigen engagement, the activated T cells differentiate into helper T cell subsets that provide cytokines for development of effective antibody responses (Blomberg et al. 1980) and cytotoxic T cells that directly kill virus-infected cells (Morales and Robert 2007; reviewed in Grayfer et al. 2012). Unlike most T cells that require MHC presentation, B cells and a subset of so-called γδ T cells can directly recognize antigens (reviewed in Edholm et al. 2016), and B cells respond by activation and production of antibodies.

Amphibians have three classes of secreted antibodies: IgM, IgY, and IgX. IgM has a pentameric or hexameric structure and is the first type of antibody secreted in response to a pathogen to neutralize it. IgY is similar to the IgG of mammals and is secreted as a monomer in response to a secondary challenge requiring T cell help (Bernard et al. 1981). IgX is also secreted as a pentamer or hexamer and is thought to be most abundant at mucosal surfaces (reviewed in Flajnik 2018).

Chytridiomycosis—The Global Challenge

Batrachochytrium dendrobatidis

Bd and the disease chytridiomycosis was first identified and characterized approximately 20 years ago (Berger et al. 1998; Longcore et al. 1999; Pessier et al. 1999). At the time of its discovery, it was a surprise that this unique fungus could be the cause of significant amphibian population declines. Until its discovery, chytrid fungi had never been linked to pathogenesis in any vertebrate species. Transmission occurs when swimming zoospores attach to and enter keratinized mouthparts of tadpoles or skin cells of adult amphibians. The process of infection by zoospores has been carefully documented by histology and electron microscopy. Briefly, zoospores attach, settle, and rapidly form a germ tube that conducts the contents of the zoospore into healthy epithelial cells within 24 h of skin exposure. The developing zoospore gives rise to an intracellular chytrid thallus within the host cell that develops into a mature zoosporangium over a period of 4–5 d at 22°C and eventually kills the host cell (Greenspan et al. 2012; Van Rooij et al. 2012; reviewed in Van Rooij et al. 2015). As infected skin cells move toward the skin surface, the zoosporangium matures, a discharge papilla opens, and mature zoospores swim out (Berger et al. 2005). Unlike other fungal pathogens that move from the site of infection to other organs, *Bd* remains confined to the skin. From the first description of *Bd*, it was recognized that chytrids detected in the skin are generally not associated with classic lymphocytic cell infiltration (Berger et al. 1998; Pessier et al. 1999). Since *Bd* was discovered, it has been associated with declines of hundreds of species and the likely extinction of many species (Stuart et al. 2004; Skerratt et al. 2007; Scheele et al. 2019), and it has been mapped to nearly every amphibian habitat worldwide (see www.bd-maps.net). The full genomes of *Bd* and the sister species *Bsal* (described below) have been sequenced, and they are very closely related (Farrer et al. 2017). Among the genes expressed in these two pathogenic species that are not found in nonpathogenic chytrids are *Batrachochytrium*-specific proteases (especially metalloproteases; Joneson et al. 2011; Farrer et al. 2017) and other elaborated chitin-binding cell wall proteins (Abramyan and Stajich 2012; Farrer et al. 2017). Both sets of expanded proteins are likely involved in virulence of this pathogen. Although the optimal temperature for growth of *Bd* in culture is approximately 17–25°C, the fungus also thrives at cool temperatures (7–10°C) and releases more zoospores per zoosporangium at such cold temperatures (Piotrowski et al. 2004; Woodhams et al. 2008).

Batrachochytrium salamandrivorans

Although *Bd* has been known to amphibian biologists since 1998, a second chytrid fungus was not recognized until 2013 when it was associated with the decline of Fire Salamanders, *Salamandra salamandra*, in Belgium and The Netherlands in 2013 (Martel et al. 2013, 2014). Although present in Europe and Asia (Martel et al. 2013, 2014; Spitzen-van der Sluijs et al. 2016; González et al. 2019), it has not been detected in North America (Bales et al. 2015; Klocke et al. 2017; Parrott et al. 2017). At present, this species seems to be more specialized for infection and pathogenesis leading to death in tailed amphibians (salamanders and newts), but it can also infect anuran species (Nguyen et al. 2017; Stegen et al. 2017). Because North America is home to a wide diversity of unique endemic species of salamanders that are at risk for infection, there is great concern that this pathogen will be accidentally introduced into North American and lead to the declines of native species (Yap et al. 2015; reviewed in Yap et al. 2017). One worrisome finding related to possible *Bsal* spread in Europe and elsewhere is the observation that some salamanders in captivity have asymptomatic *Bsal* infections (Sabino-Pinto et al. 2018). The optimal temperature for growth of *Bsal* is approximately 10–15°C (Martel et al. 2013), a temperature somewhat lower than that for growth of *Bd*. It survives at 5°C and dies at or above 25°C (Blooi et al. 2015). Unlike *Bd*, *Bsal* has a nonmotile, environmentally resistant zoospore that may permit greater survival in moist environments such as soil (Stegen et al. 2017). Much less is known about the pathogenesis of *Bsal* than *Bd*. However, because the two chytrid species are more closely related to each other than to any other chytrid fungi (Martel et al. 2013; Farrar et al. 2017), it is thought that mechanisms of virulence and immune evasion may be similar. Although amphibians in North America seem to be free of *Bsal* infections, many species are chronically infected with *Bd*, including Eastern Newts (*Notophthalmus viridescens*; Rothermel et al. 2008, 2016; Longo et al. 2019). Because *Bd* is widespread globally, it is possible that both *Bd* and *Bsal* coexist in some Asian amphibian habitats. Only one study has documented such coexistence (Laking et al. 2017). If *Bsal* is accidentally introduced into wild populations in North America, it will likely infect many amphibians persisting with active *Bd* infections. A recent laboratory study of coinfections of Eastern newts with both *Bd* and *Bsal* showed that the dual infection was much more harmful than either independent infection. While the newts were able to clear the *Bd* infection, they were still susceptible and more likely to die from a subsequent *Bsal* infection (Longo et al. 2019). This

suggests that immune defenses that protect against *Bd* may not limit *Bsal* infections, and coinfections may overwhelm the immune defenses. The problem of possible coinfections with both *Bd* and *Bsal* in this and other amphibian species is a worrisome gap in our knowledge about pathogenesis and immune defenses in this situation, and study of the effects of coinfections in additional species is an important area for future research.

Challenge of Temperature Variation for Immune Defenses against *Bd* and *Bsal*

Because amphibians are ectotherms, their physiological functions change with temperature. Most functions would be expected to speed up at warmer temperatures and slow down at cooler temperatures. Changes in immune responses are also altered by temperature, and the literature describing changes in amphibian immune responses with temperature are briefly reviewed here. Although many of these studies are >10 years old, they are fundamental for understanding basic changes in the immune system resulting from temperature changes experienced by amphibians. It is useful to think about the effects of temperature on immune defenses as (1) effects of cold, (2) effects of extreme heat, and (3) effects of temperature fluctuations.

Studies of anuran species that undergo a defined period of hibernation show that the immune system involutes seasonally and is restored when animals emerge from hibernation (Plytycz and Bigaj 1983; Plytycz et al. 1991; Cooper et al. 1992; Miodoński et al. 1996). The shrinkage in lymphoid populations in all organs of the immune system is thought to be due to a decrease in new immune cell production rather than migration or programmed death (Cooper et al. 1992). Eastern Newts also have fewer lymphocytes and eosinophils during winter (Raffel et al. 2006). Complement factors in blood are reduced, and lymphocyte proliferation is also reduced under conditions of hibernation (Green and Cohen 1977; Maniero and Carey 1997). The natural involution of the immune system in hibernation may be physiologically valuable to conserve energy when animals are fasting and metabolism is reduced, but it also results in a lag of weeks before the immune system is restored in the spring (Cooper et al. 1992), allowing for a period of increased vulnerability to pathogens. Changes in immune function also occur with temporary reductions in temperature. Antibody responses and skin graft responses are reduced or delayed when anuran species and newts were maintained at temperatures below the optimal temperature of the host (Cohen 1966; Cone and Marchalonis 1972; Lin and Rowlands 1973; Jozkowicz and Plytycz 1998).

There is almost no literature describing the effects of extreme heat on immune function in amphibians. However, there are a few studies that suggest extreme heat induces a stress response characterized by elevated corticosteroid hormones (Juráni et al. 1973; Jessop et al. 2013; Narayan and Hero 2014). Even temporarily elevated corticosteroid levels would be expected to inhibit immune functions (reviewed in Rollins-Smith 2017). Many amphibian species, especially those in tropical regions, may be at risk for extinction if climate change increases the temperature of their local water bodies beyond their critical thermal maximum (CT_{max}; von May et al. 2019). A few studies have examined effects of an ongoing infection with *Bd* on thermal tolerance. Infection with the fungal pathogen reduced the maximum heat tolerance (CT_{max}) of adult Australian *Litoria spenceri* frogs by approximately 4°C, but the heat tolerance was improved by acclimation (Greenspan et al. 2017). Tadpoles of European Midwife Toads, *Alytes obstetricans*, also had a reduced CT_{max} when infected with *Bd*, but the more mature toadlets did not show the same reduced CT_{max} response (Fernándes-Loras et al. 2019). Studies of highly susceptible Panamanian Golden Frogs, *Atelopus zeteki*, suggest that this species has declined because of a coincidence of disease caused by *Bd* and high temperatures (Cohen et al. 2017, 2019a). One common thread in these studies is the idea that as climate change shifts amphibian hosts away from their optimal temperatures, the probability of increased susceptibility to infectious diseases might increase. This idea is expressed as the tolerance mismatch hypothesis (Nowakowski et al. 2016) or the thermal mismatch hypothesis (Cohen et al. 2017, 2019a, b).

In the tolerance mismatch hypothesis, tolerance refers to environmental tolerance and predicts that hosts that can tolerate higher temperatures at which *Bd* growth is slowed will remain and possibly control infections at subclinical levels because they can reach environmental niches that are incompatible with the thermal limits of *Bd*. Thus, as temperatures warm, the prevalence and pathogen burdens would be reduced in those species with a high CT_{max}. Other species with low thermal tolerance would have overwhelming infections, die, and disappear from the community.

In the thermal mismatch hypothesis, the authors suggest that pathogens can adapt more quickly than their hosts; thus, the prediction is made that hosts adapted to warmer climates would be most susceptible to *Bd* when temperatures shift to cold temperatures, whereas hosts adapted to colder temperatures would be more susceptible when conditions shift to unusually warm conditions. From an immunologist point of view, it makes sense that many amphibian species in temperate climates would be at a disadvantage for infection and increased *Bd* burdens when temperatures are cold because *Bd* can continue to survive in the skin and produces more zoospores per zoosporangium at cold temperatures (Woodhams et al. 2008; Voyles et al. 2017) and the immune defenses, in general, are diminished in the cold.

Data analyzed by proponents of both the tolerance mismatch hypothesis and the thermal mismatch hypothesis support the observation that amphibians under warm conditions have higher prevalence and *Bd* pathogen burdens when shifted to cold conditions (Nowakowski et al. 2016; Cohen et al. 2017, 2019a, b). This is supported by many previous studies that are described below. If unusually warm conditions induce stress in cold-adapted species, immune defenses might be reduced, allowing pathogen persistence. Further research is needed to understand the effects of warm temperature spikes on chytridiomycosis.

In addition to climate warming that may frequently exceed the thermal tolerance of specific amphibian species, there is concern about the unpredictability of thermal events. Eastern Newts exposed to *Bd* and then shifted to a colder temperature had higher pathogen burdens than those acclimated to the colder temperature prior to exposure (Raffel et al. 2015). Similarly, a species of tropical frog in Australia also had slightly higher *Bd* burdens when shifted from a baseline 26°C to 21°C compared with colder frogs at

16°C shifted to 21°C (Greenspan et al. 2017). A warm-to-cold shift seems to be more advantageous for this pathogen. The declines of species in the genus *Atelopus* linked to *Bd* have been largely attributed to temperature variability associated with climate change (Rohr and Raffel 2010; Cohen et al. 2019a). All of these studies suggest that there is a complex interaction between a pathogen and the physiological response to temperature shifts that may disadvantage the host. In general, temperature shifts and their effects on immune responses to a specific pathogen will depend on the characteristics of each pathogen or parasite and the life stage of the amphibian. There is a significant gap in our understanding of the immune responses to *Bd* following temperature shifts up or down, and more research is needed.

Chytrids and Cold

Both pathogenic chytrid species, *Bd and Bsal*, thrive at temperatures as cold as 4–7°C (Piotrowski et al. 2004; Woodhams et al. 2008; Voyles et al. 2012, 2017; Martel et al. 2013). Disease outbreaks of chytridiomycosis are associated with high elevations and cooler temperatures for many species in several environments (Bradley et al. 2002; Berger et al. 2004; McDonald et al. 2005; Drew et al. 2006; Kriger et al. 2007; Savage et al. 2011; Olson et al. 2013; reviewed in Fisher et al. 2009; Rollins-Smith and Woodhams 2012; Rollins-Smith 2017). Many species of amphibians affected by *Bd* show a seasonal change in prevalence and infection loads, with increased prevalence and pathogen burdens and increased mortality in colder conditions (Berger et al. 2004; McDonald et al. 2005; Russell et al. 2010; Savage et al. 2011; Olson et al. 2013; Phillott et al. 2013; Sapsford et al. 2015; Grogan et al. 2016). Similar decreases in survival at low temperature were observed in laboratory experiments (Andre et al. 2008). Thus, for many species for which environmental temperatures have a wide range, there may be an annual cycle of increased infection burdens in colder months that are reduced during warmer months. These cycles correlate with reduced adaptive immune defenses in the cold that significantly improve during warmer months. AMP defenses are also affected by the cold (Matutte et al. 2000; Robak et al. 2019). Ongoing studies suggest that AMP concentrations also vary seasonally in Southern Leopard Frogs (*Lithobates sphenocephalus*, or *Rana sphenocephala*; E. Hall and L. Rollins-Smith, personal observations). Furthermore, juvenile Southern Leopard Frogs experimentally depleted of their peptides are poorly able to restore the depleted peptides at a cold temperature (14°C) compared with frogs experimentally depleted of their peptides and held at a warm temperature (26°C). When exposed to *Bd*, the cold frogs had higher mortality and greater pathogen burdens (Robak et al. 2019).

Immune Defenses Against Chytrid Fungi

This subject has been recently reviewed in greater detail than I cover here (Grogan et al. 2018b). Many of the gaps in our current understanding of immune defenses against *Bd* and *Bsal* were mentioned in that review, and I highlight some of them here as well. This current review has a greater focus on the mechanisms of immune evasion by the chytrids.

It All Starts in the Skin Mucus and Epithelium

The first barriers to infection by *Batrachochytrium* zoospores are the sets of chemicals present in the mucus, including conventional hydrophobic AMPs in many, but not all, anuran species (reviewed in Rollins-Smith and Conlon 2005; Rollins-Smith 2009; Rollins-Smith et al. 2011; Landram and Nicolas 2016; Varga et al. 2019). Additional antifungal factors produced by symbiotic bacteria and other fungi include small metabolites, proteolytic enzymes (Brucker et al. 2008a, b; Kearns et al. 2017; Woodhams et al. 2018), alkaloids (reviewed in Daly et al. 2005; Rodríguez et al. 2017; Luddecke et al. 2018), lysozymes (Ostrovsky et al. 1976; Zhao et al. 2006; Zhang et al. 2009; Yu et al. 2013; Savage et al. 2014; Xiao et al. 2014), and mucosal antibodies (Ramsey et al. 2010).

For anuran amphibians that have a complex set of effective AMPs in the mucus (e.g., *X. laevis* and *R. catesbeiana*), these peptides are likely to be a major deterrent to *Bd* infections because zoospores must continually emerge onto the surface of the skin and reinfect other adjacent sites on the skin. Depletion of AMPs by norepinephrine injections resulted in increased *Bd* burdens in *X. laevis* (Ramsey et al. 2010) and death in juvenile Northern Leopard Frogs (Pask et al. 2013). Other studies have repeatedly shown a strong correlation between effective AMP defenses and resistance to chytridiomycosis (Woodhams et al. 2006a, b, 2007; Voyles et al. 2018).

The community of bacteria and fungi that inhabit the mucosal surface undoubtedly interact with the amphibian chytrids, and there is likely to be competition and inhibition by the established microbial community. As the fungus grows epibiotically on the skin or when the new zoospores must emerge and reinfect, they would continue to encounter the inhibitory metabolites produced by the microbial community (Brucker et al. 2008a, b). Thus, the nature of that microbial community is important for deterrence (Rebollar et al. 2020). A recent study of the microbiome of Fire Salamanders with or without *Bsal* infections showed that the presence of *Bsal* altered the microbiome. Healthy salamanders had a community of anti-*Bsal* bacteria, and the experimental increase of these anti-*Bsal* bacteria by daily additions slowed disease progression. However, the abundance of these helpful community members was not sufficient alone to inhibit *Bsal* infections (Bletz et al. 2018). Thus, the skin microbiome is critically important for defense against both pathogenic chytrid fungi.

B cells in the skin can produce antibodies that reach the mucus (Ramsey et al. 2010), but the nature of the B cell compartment and the mechanisms by which the antibodies reach the skin are unknown. *Xenopus laevis* exposed to *Bd* and allowed to recover had *Bd*-specific antibodies in the mucus (Ramsey et al. 2010), and it is likely that other species that are exposed to nonlethal numbers of zoospores and clear the infections also produce anti-*Bd* antibodies. Systemic immunization by injection of killed *Bd* into the peritoneum or subcutaneously was not effective in inducing protective antibody responses in several species (Rollins-Smith et al. 2009; Stice and Briggs 2010; Poorten et al. 2016). Thus, having circulating antibodies is not necessarily protective for

the skin. However, several rounds of exposures to live or dead *Bd* across the skin and clearance by heat resulted in reduced pathogen burdens and increased numbers of splenocytes, suggesting activation of an immune defense across the skin (McMahon et al. 2014). Whether protective mucosal antibody responses can be induced remains an important question for future research.

If zoospores survive the chemical barriers of the skin mucus, they then infect the skin epithelium. As *Bd* modifies the infected cells, antigen presenting cells (Mϕ, dendritic cells, and Langerhans cells) would be expected to recognize a foreign invader and directly target the invader or induce lymphocyte activation. Activated lymphocytes would then mount an effector response (antibodies, effector T cells, and additional phagocyte recruitment). Immune clearance of fungi is generally thought to be mediated by T cells. T cells must recognize fungal antigens presented in the context of MHC. Current evidence from RNA-seq studies suggests that MHC antigens are upregulated in the skin following *Bd* exposures (Ellison et al. 2014a, b; Grogan et al. 2018a), and survival of some species and populations was correlated with specific MHC Class II loci suggesting positive selection (Savage et al. 2011, Savage and Zamudio 2016; Bataille et al. 2015). However, a puzzling feature of *Bd* infection is the apparent lack of inflammation and lymphocyte recruitment (Berger et al. 1998; Pessier et al. 1999). This puzzle has led to the hypothesis that the fungus is able to thwart the immune defenses in the skin and evade clearance.

When Immunity Fails, Where Is the Brake (Break)?

The fungal counter-defenses used by *Bd* to evade immunity will be described below. One important question is why has this unique host–pathogen relationship in which the host accommodates sometimes substantial chytrid infection burdens, persisted over time for somewhere between 50–120 years or longer (O'Hanlon et al. 2018). One hypothesis is that the fungus may have evolved as a commensal organism able to induce an immunosuppressive local environment. This may benefit the host by avoiding a deleterious inflammatory response because the functions of the skin are vital for survival. The fungal cells within the skin environment may experience stress, including possible oxidative stress, due to mediators released by Mϕs or neutrophils. Under stress, the fungal cells may synthesize increased amounts of inhibitory metabolites (spermidine and MTA) as protection (Rollins-Smith et al. 2019). From recent RNA-sequencing (RNA-seq) studies in highly susceptible species, we have learned that the expression of many immune-related genes is upregulated in infected skin compared with the skin of naïve and unexposed frogs (Ellison et al. 2014a, b; Grogan et al. 2018a). The prominently upregulated genes include some that are proinflammatory, such as IL-1β, IL-6, and members of the tumor necrosis factor family, as well as some that are immunosuppressive molecules such as IL-10. Although many of these immune-related genes are expressed, susceptible species of frogs still die from chytridiomycosis, suggesting that the immune response is highly dysregulated (Ellison et al. 2014a, b; Grogan et al. 2018a). Other frog species that are more resistant to chytridiomycosis seem to have reduced inflammatory responses in the skin (Ellison et al. 2014b). Although *X. laevis* and *X. tropicalis* can be infected by *Bd*, these species do not generally develop lethal chytridiomycosis unless severely stressed (Ribas et al. 2009; Ramsey et al. 2010; Tinsley et al. 2015). Thus, it seems that either an inflammatory response or an immunosuppressive response can develop, and which is the predominant response in the skin may determine whether the host will survive or die. It is also the case that the chytrid infection itself may be stressful (reviewed in Rollins-Smith 2017), and efforts to rebalance the physiological state may induce upregulation of corticosteroids that exacerbate the disease symptoms and inhibit immune defenses (Kindermann et al. 2012; Peterson et al. 2013; Gabor et al. 2015). Immune responses within the skin compartment and the effects of fungal products on these responses is another area in need of further research.

Chytrid Fungi Counter-Defenses

Fungi that infect other vertebrates have complex interactions with their hosts, and host immune defenses usually keep them from overwhelming the host unless the host is immunocompromised (reviewed in Wüthrich et al. 2012). However, many pathogenic fungi have developed strategies to evade immune destruction (reviewed in Collette and Lorenz 2011; Cheng et al. 2012; Wüthrich et al. 2012). There is now substantial evidence that *Bd* can interfere with effective immune clearance (Ribas et al. 2009; Rosenblum et al. 2009, 2012; Woodhams et al. 2012; Fites et al. 2013, 2014; Ellison et al. 2014a, b). One mechanism of evasion is the use of the germ tube to essentially sneak from the outside into skin cells, evading the initial immune recognition (Greenspan et al. 2012; Van Rooij et al. 2012). In addition, others have shown that *Bd* expresses a rich array of metalloproteases (Joneson et al. 2011; Farrer et al. 2017) and chitin-binding cell wall proteins (Abramyan and Stajich 2012; Farrer et al. 2017), each of which may play a role in pathogenesis. Our recent work has shown that *Bd* also releases a mixture of small metabolites that individually, or in concert, inhibit lymphocyte functions and induce apoptosis (Fites et al. 2013; Rollins-Smith et al. 2015, 2019). In addition to the induction of lymphocyte apoptosis, *Bd* also seems to induce apoptosis of skin cells (Brannelly et al. 2017). Inhibitory products released by *Bd* include kynurenine (KYN), methylthioadenosine (MTA), and the polyamine spermidine (Rollins-Smith et al. 2015, 2019). MTA is a by-product of spermidine synthesis, and the two can synergize to inhibit lymphocyte proliferation at reduced concentrations for each metabolite (Rollins-Smith et al. 2019). In mammalian systems, KYN promotes the development of regulatory T cells (Tregs; Mezrich et al. 2010). If Tregs develop in the skin compartment, this would also have the effect of suppressing an effector T cell response against *Bd*. Whether Tregs play a role in immune evasion by *Bd* is another significant gap in our knowledge of this host pathogen interaction.

In addition to the inhibitory metabolites produced by *Bd*, the use of an agent that interferes with chytrid cell wall development, nikkomycin Z (an inhibitor of chitin synthase), suggested that components of the cell wall may also be involved in immune suppression. Cells pretreated with nikkomycin Z were less able to inhibit lymphocyte proliferation, and supernatants produced by these cells were less inhibitory (Fites et al. 2013). These observations are

supported by some additional experiments showing that enriched cell wall preparations alone (lacking the internal components of the chytrid cells) also inhibit lymphocyte proliferation (L. Rollins-Smith and J. Lee, personal observations). Collectively, these studies demonstrate that *Bd* has abundant mechanisms to interfere with immune clearance. Although the interactions of *Bsal* with immune cells have not been documented, preliminary studies in our lab suggest that *Bsal* can also release inhibitory metabolites including MTA and spermidine (L. Rollins-Smith, T. Umile, and K. Minbiole, personal observations).

Concluding Remarks and Outstanding Questions

Although much has been learned about the immune defenses of amphibian species against *Bd* and *Bsal*, many important questions remain. From my point of view, the mucosal defenses are critical because infection and reinfection is a continuous process across the skin surface (Berger et al. 2005; Van Rooij et al. 2015). These defenses include AMPs, microbial proteases, lysozymes, and other factors. For some species, the mixture of AMPs seems to be especially important for defense (Woodhams et al. 2006a, b, 2007; Voyles et al. 2018). It is still unclear whether mucosal antibodies play an important role in protection from infection and reinfection by chytrid zoospores. If the immune system can be primed to produce protective mucosal antibodies, perhaps a vaccine strategy can be developed to protect vulnerable amphibians in captivity (McMahon et al. 2014).

Another important area of research that needs further development is the interactions of *Bd* and *Bsal*, the targeted skin cells, and antigen-presenting cells such as Mϕs and dendritic cells. Interactions between skin cells infected by zoospores and antigen-presenting cells are not yet well characterized. It is unclear what may be going on between skin cells and chytrid pathogens to promote or suppress development of an immune response. Do the skin cells send out alarm signals? If not, why not? Perhaps the default response is immunosuppression or failure of recruitment of antigen-presenting cells to the skin to protect the integrity of the skin because the skin is critical for survival.

Another outstanding question concerns the role of MHC Class I and Class II in the response to chytrid infections. We do not yet have a clear understanding of the roles of these molecules in recognition and activation of an anti-*Bd* response and whether wild populations with favorable MHC types are being selected.

In spite of the modeling efforts to understand possible effects of unpredictable climate change presented in the tolerance mismatch hypothesis and the thermal mismatch hypothesis, it is still unclear what the effects of cold or heat stress and unpredictable temperature fluctuations will have on disease outcomes. Both amphibians and the chytrid pathogens have distinct temperature comfort zones. Further research on thermal mismatch and disease is needed.

Finally, if *Bsal* is accidentally introduced into new amphibian habitats, it is unclear what would be the consequences of coinfections with *Bd* and *Bsal*. Immunity against one species might result in some cross-protection against the other species or dual infections may overwhelm natural defenses as suggested by initial coinfection studies in the laboratory (Longo et al. 2019). All of these questions are of great importance for a better understanding of immune defenses against chytrid pathogens and should be explored in future research.

We have come a very long way in the past 20 years in understanding the pathogenesis and immune defenses against chytrid pathogens of amphibians. As our understanding increases, new strategies for protecting and preserving amphibian populations will continue to be introduced. There are many promising strategies proposed to mitigate amphibian declines due to chytridiomycosis (many introduced in Woodhams et al. 2011). They include manipulation of microbial communities in the skin (Bletz et al. 2013; Rebollar et al. 2016, 2020); vaccination (McMahon et al. 2014); antifungal treatment of hosts (Drawert et al. 2017); modulating the environmental temperature, for example, by altering canopy cover (Woodhams et al. 2003; Becker et al. 2012; Hettyey et al. 2019); reintroductions with assisted selection of more resistant hosts (Muths et al. 2001; Harding et al. 2015; Duarte et al. 2017); and management of amphibian communities to reduce pathogen carriers or reduce the density of infected hosts as suggested by some host pathogen models (Briggs et al. 2010; Canessa et al. 2019). All of these proposed strategies deserve further study to give wildlife managers tools to improve amphibian survival.

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