The amphibian complement system and chytridiomycosis

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Abstract
Understanding host immune function and ecoimmunology is increasingly important at a time when emerging infectious diseases (EIDs) threaten wildlife. One EID that has emerged and spread widely in recent years is chytridiomycosis, caused by the fungal pathogen *Batrachochytrium dendrobatidis* (*Bd*), which is implicated in unprecedented amphibian declines around the world. The impacts of *Bd* have been severe for many amphibian species, but some populations have exhibited signs of persistence, and even recovery, in some regions. Many mechanisms may underpin this pattern and amphibian immune responses are likely one key component. Although we have made great strides in understanding amphibian immunity, the complement system remains poorly understood. The complement system is a nonspecific, innate immune defense that is known to enhance other immune responses. Complement activation can occur by three different biochemical pathways and result in protective mechanisms, such as inflammation, opsonization, and pathogen lysis, thereby providing protection to the host. We currently lack an understanding of complement pathway activation for chytridiomycosis, but several studies have suggested that it may be a key part of an early and robust immune response that confers host resistance. Here, we review the available research on the complement system in general as well as amphibian complement responses to *Bd* infection. Additionally, we propose future research directions that will increase our understanding of the amphibian complement system and other immune responses to *Bd*. Finally, we suggest how a deeper understanding of amphibian immunity could enhance the conservation and management of amphibian species that are threatened by chytridiomycosis.

KEYWORDS
amphibians, *Batrachochytrium dendrobatidis*, complement, chytridiomycosis, ecoimmunology, ectotherms, emerging infectious disease, innate immunity
INTRODUCTION

Emerging infectious diseases (EIDs) in wildlife are increasing in incidence and threatening the world’s biodiversity (Jones et al., 2008). At the same time, the relatively new field of ecoimmunology is advancing our understanding of how host immunity functions in natural environmental conditions and in the context of an organism’s ecology and evolutionary biology (Martin et al., 2011; Ferguson et al., 2018). The immune system is composed of diverse cells, tissues, and physiological mechanisms, that constitute the adaptive (pathogen-specific) as well as innate (nonspecific) components (Janeway et al., 2001). In addition, the innate system includes mechanisms that function to enhance or “complement” other immune responses (e.g., the complement system), enabling robust host defenses against infectious agents (reviewed in Carroll, 2004). Although disentangling these diverse mechanisms is challenging, it is also integral to understanding and forecasting pathogen emergence, disease transmission, and the impacts of disease outbreaks on host populations and communities (Grogan, Robert, et al., 2018). This is particularly important for ectotherms whose physiology and immune efficacy are temperature-sensitive and inextricably linked to environmental conditions (Huey & Kingsolver, 1989; Raffel et al., 2006).

One EID in ectothermic hosts that has drawn considerable attention is amphibian chytridiomycosis (Berger et al., 1998). Chytridiomycosis is caused by the fungal pathogen *Batrachochytrium dendrobatidis* (*Bd*; Longcore et al., 1999). *Bd* has two life stages, an infectious zoospore stage, and a mature sporangium stage that produces additional zoospores to be released into the external environment (Longcore et al., 1999). *Bd* infects amphibian skin (Figure 1), causing hyperkeratosis, disruption of osmoregulatory functioning, and ultimately death (Carver et al., 2010; Marcum et al., 2010; Voyles et al., 2007, 2009; Wu et al., 2018, 2019). The emergence of chytridiomycosis has precipitated severe amphibian die-offs around the world, causing declines in an estimated 500 species, as well as putative extinctions in up to 90 species (Olson et al., 2013; Scheele et al., 2019). The impact of chytridiomycosis on amphibian biodiversity has been so severe that researchers have dubbed its impact, “...the most spectacular loss of vertebrate biodiversity due to disease in recorded history” (Skerratt et al., 2007).

While the impacts of *Bd* emergence have been indisputably destructive, some populations and species have persisted, and in some cases even recovered, following initial outbreaks (Knapp et al., 2016; Scheele et al., 2017, 2019; Voyles et al., 2018). Because there is some evidence that *Bd* can maintain high pathogenicity (i.e., the ability to cause severe disease and death) for many years following emergence, some researchers have suggested that persistence and increased survival rates may be largely due to host—rather than pathogen—factors (Knapp et al., 2016; Voyles et al., 2018). These host traits could include host life history characteristics (Lips et al., 2003), behavior (Richards-Zawacki, 2010), genetics (Luquet et al., 2012), reproductive potential (Muths, 2003), and various immune defenses (Conlon, 2004; reviewed in Rollins-Smith et al., 2011; Rollins-Smith & Woodhams, 2012). Although these factors are not mutually exclusive and interact in complex and additive ways (Robak & Richards-Zawacki, 2018; Robak et al., 2019), host immune defenses are likely to be one of the most important aspects influencing susceptibility or resistance to *Bd* infection, disease development, and subsequent population recoveries (reviewed in Rollins-Smith, 2017). Thus, research investigating host immunity at the individual level (e.g., adaptive and innate immune responses) can help inform what

![Figure 1](https://www.wileyonlinelibrary.com)
processes could lead to amphibian survival and recovery at the population and species levels.

The amphibian adaptive immune system resembles that of other immunologically competent, jawed vertebrates (Rollins-Smith & Woodhams, 2012). It is composed of lymphoid tissues (e.g., thymus and spleen) and humoral- and cell-mediated immune factors (Rollins-Smith & Woodhams, 2012; Reviewed in Grogan, Robert, et al., 2018). Although adaptive immune components are thought to be highly functional in amphibians, several early studies suggest that adaptive immune responses may not protect against Bd infection in many amphibian species (Berger et al., 2005; Rollins-Smith et al., 2009; Rosenblum et al., 2009; Ramsey et al., 2010; Stice & Briggs, 2010, Rosenblum et al., 2012; Cashins et al., 2013; but see McMahon et al., 2014). Several possible mechanisms could explain the lack of protective adaptive responses to Bd in infected amphibians (Fites et al., 2013; Grogan, Cashins, et al., 2018; Grogan, Robert, et al., 2018). First, Bd appears to produce compounds that inhibit or “paralyze” amphibian lymphocyte responses (Fites et al., 2013), suggesting that Bd suppresses the adaptive immune system. Second, some hosts have been shown to express high levels of gene expression for immune factors in late stages of infection (Ellison et al., 2014, 2015), suggesting that a possible immune dysregulation or immunopathology may contribute to disease development in susceptible species (Grogan, Cashins, et al., 2018; Grogan, Robert, et al., 2018).

Because Bd is a pathogen that colonizes epidermal cells, investigations on nonspecific, innate defenses typically prioritize immune factors that are available at the skin surface (Woodhams et al., 2007; Figure 1). For example, amphibian skin itself provides a physical barrier that can be sloughed off, potentially preventing pathogen colonization (Ohmer et al., 2015; Wu et al., 2019). In addition, skin secretions provide a protective layer of mucus that is chemically complex, containing multiple components that are biologically active against Bd (Woodhams et al., 2007; Figure 1). For example, mucus constituents include antimicrobial peptides (AMPs), lysozyme, antibodies, and a diverse cutaneous microbiome (König et al., 2015; Meng et al., 2013; Rollins-Smith et al., 2009; Woodhams et al., 2007; Reviewed in Bletz, Perl, et al., 2017; Bletz, Myers, et al., 2017; Grogan, Robert, et al., 2018; Figure 1). While innate defenses at the skin surface have been relatively less attention. Focusing on other nonspecific defenses is important because early and robust innate immune responses, including complement activation, may be key to resisting Bd infection and protecting against lethal disease (Grogan, Cashins, et al., 2018; Grogan, Robert, et al., 2018).

Previous publications have extensively reviewed general amphibian immunity (Grogan, Robert, et al., 2018; Kohli et al., 2019), innate skin secretions (Woodhams et al., 2007), as well as the amphibian cutaneous microbiome (Bletz, Perl, et al., 2017; Bletz, Myers, 2017). Here, we focus on the possible role of the amphibian complement system in facilitating immune responses to Bd infection. We review the biological processes that result in complement activation, the available research on complement activation in response to Bd and chytridiomycosis, and, finally, we discuss how future research could inform our understanding of amphibian complement responses to chytridiomycosis.

2 | THE COMPLEMENT SYSTEM

Following pathogen colonization, a host’s immune system will initiate several different responses, one of which is the complement cascade (Janeway et al., 2001). The complement cascade is a biochemical immune process mediated by an estimated 50 proteins found in blood plasma (Dodds & Matsushita, 2006; Janeway et al., 2001). These proteins are excreted by the liver (Figure 1), white blood cells (Lubbers et al., 2017), fibroblasts (Garred et al., 1990), and keratinocytes (Timár et al., 2007). The majority circulate as inactive proteins (i.e.,zymogens) until the detection of a pathogen causes them to be cleaved, activating biochemical pathways that are collectively known as the complement cascade (Lubbers et al., 2017).

The complement cascade has three pathways that differ in how they are triggered and include the classical, lectin, and alternative pathways (Janeway et al., 2001; Figure 2). All three pathways eventually lead to the production of C3-convertase, an enzyme that cleaves the C3 protein into C3a and C3b components (Janeway et al., 2001; Ricklin, 2016). The cleavage of the C3 protein results in three downstream outcomes: (1) inflammation, (2) opsonization, and (3) pathogen cell lysis (Janeway et al., 2001; Figure 2). The first outcome, inflammation, results when C3a acts as a chemoattractant of phagocytes to the location of the pathogen (Janeway et al., 2001; Lubbers et al., 2017). The second outcome, opsonization, occurs when C3b covalently binds to the pathogen’s cell membrane and “tags” the pathogen for destruction by phagocytes (Janeway et al., 2001; Ricklin, 2016). The third outcome, pathogen cell lysis, occurs when C3b binds to C3-convertase, creating a C3b/C3-convertase complex (also called C5-convertase), which cleaves protein C5 into C5a and C5b (Janeway et al., 2001; Ricklin, 2016). The production of the protein C5b will lead to polymerization with additional proteins to produce the membrane attack complex (MAC), which forms pores in the pathogen’s cell membrane, causing it to lyse and neutralize the pathogen (Janeway et al., 2001; Lubbers et al., 2017).

The complement system is primarily known to enhance other immune factors (Janeway et al., 2001). As a result, several mechanistic studies have focused on resolving how a lack of complement proteins alters downstream immune functioning, leading hosts to succumb to the disease (Diamond et al., 1974). For example, in mammalian sera lacking complement proteins, the phagocytic capacity of neutrophils diminished by 40%–60% (Diamond et al., 1974). In addition, mice that are deficient in complement component C3 have curtailed activation and regulation of B cells (reviewed in Carroll, 1998). Taken together, these studies provide key insights into the function of the complement system. First, the complement system provides an important link between the innate and the adaptive branches of the immune system (Carroll, 2004). Second, a
compromise or limitation in the complement system may severely hamper the development of a robust and effective immune protection against pathogens and disease.

3 | AMPHIBIAN COMPLEMENT

Studies on the function of amphibian complement relative to endotherms began with simple hemolytic assays in the 1960s (Legler & Evans, 1966). Since then, it has been established that amphibians have a robust complement system that is similar to other vertebrates (Alexander & Steiner, 1980; DiLabio et al., 1983). The amphibian complement system is heat-labile and operates more effectively at lower temperatures (Koppenheffer, 1987). Complement proteins of salamanders, frogs, and toads are comparable in structure and function to those of mammals (Avila & Lambris, 1990; Fujii et al., 1985; Sekizawa et al., 1984; Weinheimer et al., 1971). For example, amphibian complement proteins have shown activation of and synergistic interactions with complement proteins from guinea pigs, rabbits, pigs, and humans, suggesting the complement system is highly conserved across taxa and may have evolved in ectotherms (Alexander & Steiner, 1980; Gewurz et al., 1966). To date, few studies have focused on understanding how amphibian complement responds to infectious pathogens (Price et al., 2015). Some evidence suggests that complement does not respond to infections with Ranavirus, a virus that is also known to cause amphibian declines (Eaton et al., 2010; Price et al., 2015).

4 | COMPLEMENT RESPONSES TO FUNGAL PATHOGENS

Although the majority of studies focus on complement responses to bacteria and viruses, the complement system also plays an important role in host defense against fungal pathogens (reviewed in Kozel, 1996; Speth et al., 2008). For example, experiments in mammals have shown that the complement system is activated in response to pathogenic ascomycetes such as *Saccharomyces cerevisiae*, *Candida albicans*, and *Aspergillus spp.* (reviewed in Kozel, 1996). Additionally, the host ability to survive infection by the pathogenic basidiomycete,
Cryptococcus neoformans, is highly dependent on the complement system (Speth et al., 2008). Specifically, exposure experiments using C3 and C5 knockout mice demonstrated an increased susceptibility to cryptococcosis, leading to greater disease-induced mortality relative to wild-type mice (Speth et al., 2008). As such, complement appears to be an important aspect of host defense and survival against a variety of pathogenic fungi (Kozel, 1996; Speth et al., 2008).

We currently do not understand much about the role of the complement system in infections with fungi belonging to the phylum Chytridomycota (Barr, 2001). This lack of information may be due to a historic association between Chytridomycota fungi and plants, algae, and invertebrates, but not vertebrates (Barr, 2001). However, due to the recent emergence of multiple vertebrate pathogens (e.g., Bd and Batrachochytrium salamandrivorans; Martel et al., 2013) in this phylum, understanding host complement responses to these fungal pathogens is increasingly important to provide a holistic understanding of host defenses against fungal diseases, such as chytridiomycosis.

5 | COMPLEMENT ACTIVITY AND BD

Research on complement activation in response to chytridiomycosis is predominantly comprised of studies that use two main approaches: (1) in vitro challenge assays known as bacterial killing assays (BKAs) or (2) molecular techniques to quantify the production of RNA transcripts (i.e., transcriptomics) using microarrays or RNAseq, in response to Bd infection (reviewed in Grogan, Robert, et al., 2018). While each of these approaches have their respective benefits and drawbacks, they have collectively offered some key insights into amphibian host responses to Bd.

6 | IN VITRO CHALLENGE ASSAYS TO INVESTIGATE AMPHIBIAN COMPLEMENT AND BD

BKAs have classically been used to measure innate immune activity, including the complement response in vitro (de Assis et al., 2013; Liebl & Martin, 2009; Matson et al., 2006; Millet et al., 2007). In BKAs, host blood or blood serum is inoculated into a growth media with a known concentration of bacteria (e.g., Escherichia coli; Matson et al., 2006). The bacterial growth in the presence of the blood products is calculated and compared with a control consisting of bacteria and no blood products (Liebl & Martin, 2009; Matson et al., 2006). A result with comparatively lower bacterial growth indicates destruction of the bacteria by the blood plasma constituents, which can include complement proteins, lysozyme, natural antibodies, and other microbialidal factors (Jacobs & Fair, 2016; Liebl & Martin, 2009; Matson et al., 2006; Ochsbein et al., 1999). Therefore, a higher bacteria-killing ability is frequently used as a proxy for inferring complement activation in infected hosts (Dlabač et al., 1983; Legler & Evans, 1966; Virta et al., 1998).

To investigate amphibian innate immune responses to Bd infection, investigators have integrated BKA measurements with traditional Bd exposure experiments using a variety of host species (Gervasi et al., 2014; Savage et al., 2016; Venesky et al., 2011; Table 1). By exposing naïve hosts to a known dose of Bd and collecting blood samples at multiple time points following inoculation, researchers are able to measure immune responses over time throughout the course of infection (Gervasi et al., 2014; Savage et al., 2016). For example, in lowland leopard frogs (Lithobates yavapaiensis), Savage et al. (2016) reported a higher bacteria-killing ability of blood plasma in individuals that did not show clinical signs of disease compared with individuals that developed the clinical disease and died. In a comparison between two frog species, Gervasi et al. (2014) observed increased bacteria-killing ability in Bd-infected cascades frogs (Rana cascadae) compared with infected pacific tree frogs (Pseudacris regilla). No mortality occurred in Bd-inoculated R. cascadae as compared with a 16% mortality in Bd exposed P. regilla (Gervasi et al., 2014). The investigators concluded that increased innate immune activity, as measured with a BKA, was a possible mechanism of resistance in R. cascadae (Gervasi et al., 2014). Taken together, these studies suggest that blood products from frogs that are infected with Bd, but do not show clinical signs of disease, or subsequently recover from Bd infection, have higher innate immune activity than blood products from infected frogs that succumb to disease (Gervasi et al., 2014; Savage et al., 2016).

BKAs are valuable because they evaluate an individual host’s ability to limit microbial growth, but there are important caveats and limitations to this approach (Beck et al., 2017; Savage et al., 2016). Depending on the methods that are used, BKAs may not differentiate between broad-spectrum functions of the immune system or the presence and function of specific blood constituents (e.g., lysozyme or complement proteins; de Assis et al., 2013; Figure 3). For example, BKA studies that use whole blood instead of blood plasma do not eliminate the inhibitory contributions of neutrophils, natural killer cells, and other lymphocytes, and therefore cannot distinguish between cellular or humoral immune inhibitory contributions (de Assis et al., 2013; Figure 3). In addition, lysozyme activity, which varies in its efficacy depending on the bacterial species that are used (Masschalck & Michiels, 2003), is also an important factor to consider for BKA studies. Therefore, the methods used for BKAs may lack specificity in evaluating complement activity per se (de Assis et al., 2013; Matson et al., 2006).

Investigators using BKAs in other organisms have optimized procedures to study innate immune activity (Beck et al., 2017; Jacobs & Fair, 2016; Liebl & Martin, 2009; Matson et al., 2004, 2006; Millet et al., 2007). Yet, studies of innate immune activity during Bd infection using BKAs have implemented inconsistent methods (e.g., using whole blood [Gervasi et al., 2014; Matson et al., 2006; Venesky et al., 2011] vs. blood serum [Matson et al., 2006; Savage et al., 2016; Figure 3]). Therefore, we suggest adopting standardized procedures that ensure repeatability to better draw conclusions about amphibian complement across studies (Figure 3). For example, stress from handling and restraint of host animals can skew BKA results (Gomes
et al., 2012). Therefore, it is important to minimize the time spent between animal capture and blood collection (Matson et al., 2006; Neuman-Lee & French, 2014; De Assis et al., 2015; Becker et al., 2019). In addition, because plasma and blood lose bacteria-killing ability over time, it is beneficial to perform BKAs quickly after blood collection, appropriately store blood samples immediately after collection, and limit the number of freeze/thaw cycles (Mollnes et al., 1988; Beck et al., 2017; Becker et al., 2019; Jacobs & Fair, 2016). Furthermore, due to differing growth rates among bacterial species depending on culturing conditions, bacterial growth protocols should be optimized before performing the BKA (Beck et al., 2017; Liebl & Martin, 2009). Finally, it is important to note that the efficacy, timing,
and intensity of the complement response may differ by host species. Therefore, researchers should avoid extrapolating findings to other species (Beck et al., 2017; Becker et al., 2019; Jacobs & Fair, 2016).

Recent technological advances in genetics, genomics, and transcriptomics have provided a molecular toolkit for understanding host responses to pathogen infection (Byrne et al., 2016; Rosenblum et al., 2010). Molecular techniques such as microarrays and RNAseq are generally used to uncover which genes are being transcribed from DNA into messenger RNA (Kogenaru et al., 2012). The majority of studies using molecular approaches to understand immune responses to Bd infection have combined traditional exposure experiments with postexposure tissue sample collection for nontargeted profiling of immune-related loci (reviewed in Grogan, Robert, et al., 2018). A common goal is identifying the genetic pathways that are up or downregulated in response to Bd infection by harvesting skin (the site of Bd infection) and/or lymphoid tissues (e.g., liver and spleen). However, because tissue sample collection is terminal for the host, a variety of experimental designs have been implemented to capture post-inoculation immune changes in amphibians that are thought to be susceptible or resistant to chytridiomycosis (Grogan, Robert, et al., 2018).

Molecular approaches have allowed investigators to gain some insights into the timing of complement activation in response to Bd. Early transcriptomics used standard exposure experiment protocols in susceptible and Bd-naïve frogs (Xenopus (Silurana) tropicalis; Ribas et al., 2009; Rosenblum et al., 2009, 2012). Following Bd inoculation, investigators sacrificed frogs for tissue collection at standard time points following Bd inoculation and compared the transcriptional responses between infected and control individuals within the same species (Ribas et al., 2009; Rosenblum et al., 2009, 2012). These studies suggested significant downregulation of complement genes at multiple time points of disease development (~3–7 days post Bd exposure; Ribas et al., 2009; Rosenblum et al., 2009, 2012). In Xenopus (Silurana) tropicalis, the decreased expression of complement was pronounced and consistent across postexposure time points, prompting speculation that complement suppression may be a “characteristic feature of chytridiomycosis” (Rosenblum et al., 2009). However, the investigators stressed the importance of broadening the taxonomic focus to include species that appeared to be resistant to Bd infection (Rosenblum et al., 2012).

In subsequent studies, with the ability to develop de novo transcriptomes for nonmodel species, investigators have implemented similar experimental approaches, but comparing within and among populations or species thought to be differentially resistant to chytridiomycosis (Grogan, Cashins, et al., 2018; Poorten & Rosenblum, 2012).
2016; Price et al., 2015). For example, Price et al. (2015) found an upregulation of complement pathway genes after 4 days in infected frogs compared with control frogs in a resistant species Lithobates (Rana) temporaria. Poorten and Rosenblum (2016) also found relative differences in upregulation of complement genes in resistant and susceptible species Chaunus (Bufo) marinus and Anaxyrus (Bufo) boreas, although the responses differed by tissue (i.e., skin vs. liver) and were collected in the late stages of infection (18 days postinoculation). Ellison et al. (2014) compared transcriptional responses in four species and reported complement pathway activation in Atelopus zeteki, A. glypbus, and Craugastor fitzingeri in later stages of infection (~22–55 days postinoculation). Lastly, Grogan, Cashins, et al. (2018) compared gene expression responses among populations of Litoria verreauxii alpina that had either persisted beyond initial Bd emergence (thought to have occurred locally in the 1980s; Osborne et al., 1999) and were considered resistant, or were apparently naïve and susceptible (Grogan, Cashins, et al., 2018). The investigators found an early upregulation of the alternative complement pathway (i.e., the pathway activated when complement proteins encounter foreign molecules on the surface of pathogens) in the skin of frogs from a phenotypically resistant population after four days. In contrast, those individuals with a less resistant phenotype did not show an upregulation of complement gene transcription until 14 days post-infection (Grogan, Cashins, et al., 2018). Although their findings contrasted somewhat with results from later time points, the investigators suggested that an early and pronounced innate response may explain the patterns in resistance among frog populations (Grogan, Cashins, et al., 2018). However, more research is needed to determine if this proposed mechanism is consistent across taxa.

More recent studies provide evidence of upregulation of complement pathways (Ellison et al., 2014, 2015; Grogan, Cashins, et al., 2018), which contrasts with findings from initial studies where complement pathways were downregulated (e.g., Ribas et al., 2009; Rosenblum et al., 2009, 2012). One favored explanation for this discrepancy is that contrasting results may be due to interspecific differences in susceptibility or resistance (Ellison et al., 2014; Rosenblum et al., 2012). However, other differences among these studies that could explain mixed results include variation in sampling regime (e.g., sample collection time points), tissue types, pathogen isolate, inoculation dose and duration, experimental conditions (e.g., temperatures), and other factors that play an important role in host-pathogen interactions. As such, we recommend standardizing experimental designs of future studies by the use of uniform Bd exposure methods, tissue sample collection techniques, and testing frequencies to provide comparable protocols among species. Standardizing methods and techniques will considerably improve our understanding of amphibian complement responses to Bd and many other amphibian pathogens (e.g., Bsal).

### 8 FUTURE DIRECTIONS

We have much to learn about the amphibian complement system and immunity to Bd. Part of the challenge involved with understanding how complement responds to Bd can be attributed to the variation in amphibian host responses to Bd across multiple biological scales (i.e., at the individual, population, and species levels; Venesky et al., 2014). Optimizing methods to be comparable and repeatable will help determine if a complement response limits Bd infection and protects against disease and mortality and is an important next step for chytridiomycosis research (Grogan, Robert, et al., 2018). We suggest several highly focused research approaches that can provide insights on the complement system and other amphibian innate immune defenses against Bd.

#### 8.1 Evaluation of complement activation

Experiments using BKAs should incorporate methods that reduce or eliminate targeted components of blood and quantify the infection outcomes. For example, methods that use only blood serum, rather than whole blood, will be more specific for complement activity because the serum is separated from immune cells found in whole blood (e.g., leukocytes; Gervasi et al., 2014; Janeway et al., 2001). Additionally, BKAs that incorporate heat-inactivated serum or cobra venom factor (CVF) will deactivate complement activity, leaving other potential bacterial components, such as lysozyme and antibodies (Soltis et al., 1979; Vogel & Fritzinger, 2010). These methods would allow investigators to isolate and appropriately evaluate the relative role that the complement system plays in bacteria-killing activity.

If BKAs provide evidence that the complement system decreases infection intensity and Bd-induced mortality, we suggest investigating the involvement of the terminal complement processes underlying decreases in Bd load. For example, lysis by the membrane attack complex (MAC) may not be effective against fungal pathogens because the MAC cannot form through a chitinious cell wall (Speth et al., 2008). Because Bd zoosporangia have a cell wall (Friesen & Kuhn, 2012; Kozel, 1996; Speth et al., 2008), zoosporangia may not be susceptible to cell lysis by this mechanism. In contrast, Bd zoospores may be susceptible to MAC formation because they may not possess a chitinious cell wall (Berger et al., 2005). Experimental work using a MAC inhibitory protein (e.g., CD59) could help address this question. This protein disables the formation of the MAC, providing a tool to discriminate between MAC formation and other terminal processes in the complement cascade (e.g., chemotaxis, opsonization, inflammation, etc.) that could be effective against Bd zoospores (Rollins & Sims, 1990; Speth et al., 2008).

#### 8.2 Evaluation of different complement pathways

Previous studies using molecular methods discussed the involvement of the alternative and classical pathways in Bd infection (Grogan, Robert, et al., 2018; Rosenblum et al., 2009), but we currently have little information regarding the lectin pathway. The lectin pathway is triggered by other fungal pathogens such as Candida and Aspergillus
species (Speth et al., 2008) and similar mechanisms may be at play during a Bd infection. Bd possesses various glycoproteins on the extracellular surface (Dillon et al., 2017). Because the lectin pathway is triggered by monosaccharides (Cunha et al., 2012), these glycoproteins may interact with mannose-binding lectin to trigger a complement response to Bd (Patin et al., 2019). Furthermore, it is possible that Bd may activate multiple complement pathways, potentially at different stages of infection (Speth et al., 2008). Therefore, experimental approaches that investigate molecules specific to each pathway will allow us to understand which complement pathways are responding to Bd and at which stages of infection complement is activated.

8.3 Timing of complement activation

We lack critical information on the timing of complement activation in response to Bd colonization. Complement proteins are assembled and exist in the blood ubiquitously as inactive proteins until they are cleaved after complement activation (Janeway et al., 2001). The activity of these existing proteins could influence the timeline during which transcription of new complement proteins is activated. The variation in time points used in gene expression studies is based on what is understood about chytridiomycosis progression, observation of clinical signs of disease, and what is logistically achievable (Grogan, Cashins, et al., 2018; Grogan, Robert, et al., 2018). However, this sampling regime may overlook important lag effects in complement gene expression. In addition, multiple studies have shown that increases in infection intensity and the onset of clinical signs of chytridiomycosis varies with respect to many factors (e.g., species, population, temperature, etc.; Grogan, Cashins, et al., 2018; Poorten & Rosenblum, 2016; Raffel et al., 2006). Therefore, our understanding of amphibian complement responses to Bd will be considerably improved by modifying the methods for the most appropriate timelines for species-specific Bd infection development and complement activation.

8.4 Environmental influences on complement efficacy

In contrast with the physiology of endotherms, ectotherms rely on their environment to regulate physiological processes, including immunity (Huey & Kingsolver, 1989). As such, many abiotic factors could mediate seasonal fluctuations in immune function, including the complement system (Raffel et al., 2006). Indeed, a seasonal fluctuation in complement activity has been observed in fish (Brown et al., 2016; Collazos et al., 1994; Hayman et al., 1992), tortoises (Sandmeier et al., 2019), turtles (Zimmerman et al., 2010), snakes (Graham et al., 2017), alligators (Merchant et al., 2005), caimans (Siroski et al., 2010), and amphibians (Raffel et al., 2006; Ruben et al., 1977). Additionally, BKAs performed on North American salamanders (Cryptobranchus alleganiensis; Terrell et al., 2013), the Northern Leopard frog (Lithobates (Rana) pipiens; Maniero & Carey, 1997), and three toad species from the genus Rhinella (Moretti et al., 2019) have demonstrated that bacteria-killing ability is temperature-dependent, suggesting that temperature could be a main factor influencing seasonal fluctuation in the efficacy of the complement system (Maniero & Carey, 1997; Moretti et al., 2019; Raffel et al., 2006; Ruben et al., 1977; Terrell et al., 2013). As such, the thermal sensitivity of amphibian immune responses, and the complement system, in particular, may be key to understanding seasonal fluctuations in chytridiomycosis disease dynamics (Berger et al., 2004; Kriger & Hero, 2007; Phillott et al., 2013; Woodhams, 2005).

9 CONCLUSIONS

We have made considerable advances in understanding amphibian immune responses to chytridiomycosis in recent years (Reviewed in Grogan, Robert, et al., 2018; Kohli et al., 2019; Rollins-Smith, 2020), but there is still much to learn about amphibian complement responses to chytridiomycosis (Grogan, Robert, et al., 2018). An early activation of the complement system could provide a key mechanism for amphibian resistance to chytridiomycosis by controlling and possibly eliminating Bd infection (Grogan, Cashins, et al., 2018), but this remains to be investigated.

We can advance research on the amphibian complement system by standardizing experimental procedures to obtain comparable data across taxa. Additionally, to better characterize complement response to Bd, researchers can (1) deactivate the complement system and evaluate disease outcomes, (2) examine the three complement pathways in depth, and (3) determine the species-specific and environmental factors that could mediate complement responses to Bd. Furthermore, since the complement system is generally known for facilitating immune responses to fungal pathogens, additional research on this aspect of amphibian immunity may prove useful in extrapolating our understanding of host defenses against other lethal fungal diseases in addition to chytridiomycosis.

A better understanding of amphibian immune responses to chytridiomycosis will provide a cornerstone for conservation and management efforts (Grogan, Robert, et al., 2018; Venesky et al., 2012; Woodhams et al., 2011). Understanding existing variation in immune responses among individuals, populations, and species will enable science-based conservation planning with a wide range of potentially beneficial outcomes. Conservation programs consistently operate with finite resources, which makes triage and decision making an ongoing challenge for management practitioners (Woodhams et al., 2011). The ability to discriminate among amphibian species that are likely to be more vulnerable to disease-induced declines, or less likely to recover following Bd emergence, will allow conservation programs to direct limited resources towards species most in need of intervention (Phillips et al., 2008; Voyles et al., 2014). In addition, immunological research may inform ex situ breeding programs that aim to reintroduce captive-bred amphibians back into the wild (Venesky et al., 2012). In response to the emergence of
chytridiomycosis, multiple captive breeding programs have been established globally with the intention of reintroducing amphibians to their original habitat (Cagliardo et al., 2008; Garner et al., 2016; Gascon et al., 2007). Some have speculated that reintroduction plans could be bolstered with the ability to select for certain immune traits that protect against Bd (Venesky et al., 2012). While the implementation of this idea faces multiple challenges and remains to be tested, it is nevertheless expected that understanding amphibian immunity can lead to more efficient and optimized management practices (Garner et al., 2016).

The recent and unprecedented emergence of deadly EIDs in wildlife have underscored the importance of ecomimmunological research (Hawley & Altizer, 2011). Because ecomimmunology integrates a mechanistic understanding of organismal immunity with host ecology and evolutionary biology, this nascent subdiscipline can offer insights spanning multiple biological scales (Hawley & Altizer, 2011). This multi-level approach will be key to resolving infectious EID dynamics and understanding infectious disease ecology (Hawley & Altizer, 2011), which may prove to be especially true for ectotherms because immune function is directly affected by environmental conditions (Huey & Kingsolver, 1989). As emerging pathogens continue to threaten ectotherm populations (e.g., snakes, Lorch et al., 2016; sea turtles, Sarmiento-Ramirez et al., 2010; and salamanders, Stegen et al., 2017), research that focuses on host immunity and ecomimmunology can inform conservation and management programs and will be increasingly valuable for protecting the world’s biodiversity.

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CONFLICT OF INTERESTS
The authors declare that there are no conflict of interests.

DATA AVAILABILITY STATEMENT
Data sharing is not applicable to this article as no new data were created or analyzed in this study.

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