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Sustained immune activation is associated with susceptibility to the amphibian chytrid fungus

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Abstract

The disease chytridiomycosis caused by the fungus *Bd* has devastated amphibian populations worldwide. Functional genomic contributions to host susceptibility remain enigmatic and vary between species and populations. We conducted experimental *Bd* infections in *Rana yavapaiensis*, a species with intraspecific variation in chytridiomycosis susceptibility, to assess the skin and spleen transcriptomic response to infection over time. We predicted that increased immune gene expression would be associated with a positive disease outcome, but we instead found that surviving frogs had significantly reduced immune gene expression compared to susceptible frogs and to uninfected controls. MHC class IIβ gene expression was also significantly higher in susceptible frogs compared to surviving frogs. Furthermore, susceptible frogs expressed a significantly larger number of distinct class IIβ alleles, demonstrating a negative correlation between class IIβ expression, functional diversity, and survival. Expression of the MHC class IIβ locus previously associated with *Bd* disease outcomes was a significant predictor of *Bd* infection intensity at early infection stages but not at late infection stages, suggesting initial MHC-linked immune processes are important for ultimate disease outcomes. We infer through disease association and phylogenetic analysis that certain MHC variants are linked to the immune expression that was negatively associated with survival, and we hypothesize that frogs that did not express these alleles could better survive infections. Our study finds that MHC expression at early and late infection stages predicts *Bd* infection intensity, and suggests that generating a sustained immune response against *Bd* may be counterproductive for surviving chytridiomycosis in this partially susceptible species.

KEYWORDS

adaptation, amphibians, disease biology, host parasite interactions, transcriptomics

1 | INTRODUCTION

Emerging infectious diseases are increasing globally in wildlife populations, a phenomenon that is largely driven by anthropogenic factors (Cunningham, Daszak, & Wood, 2017; Daszak, Cunningham, & Hyatt, 2000; Tompkins, Carver, Jones, Krkošek, & Skerratt, 2015). Understanding the role of host immunity in driving disease epizootics is critical for mitigating the impact of pathogens in wildlife populations to conserve biodiversity (Hawley & Altizer, 2011; Martin, Hopkins, Mydlarz, & Rohr, 2010). The pathogenic chytrid fungus *Batrachochytrium dendrobatidis* (*Bd*) (Longcore, Pessier, & Nichols, 1999) is a leading cause of worldwide amphibian declines

(Berger et al., 1998; Kilpatrick, Briggs, & Daszak, 2010; Scheele et al., 2019; Skerratt et al., 2007). Previous work on immune function and immunogenetics indicate that innate and acquired immune mechanisms play a role in driving susceptibility, tolerance or resistance to *Bd*. Immune activation is traditionally considered to be an indication of pathogen resistance, because resistance is defined as the ability to reduce a pathogen burden, whereas tolerance is a process of limiting the damage caused by a specific parasite burden (Råberg et al., 2007; McCarville & Ayres, 2018). However, more recent studies suggest that both innate and acquired immune processes are activated and lead to regulatory and tissue repair responses as well as pathogen destruction, suggesting that tolerance is more complex than the original paradigm (McCarville & Ayres, 2018), and that tolerance and resistance can be complimentary co-occurring processes that determine a species' susceptibility to disease (Bonneaud et al., 2019; McCarville & Ayres, 2018). Because we know very little about amphibian immune processes outside of *Xenopus* model systems (e.g., Ramsey, Reinert, Harper, Woodhams, & Rollins-Smith, 2010), understanding the role of tolerance and resistance mechanisms in partially susceptible species will help us to understand how they persist in the face of this globally distributed pathogen.

Genetic and physiologic studies of *Bd* susceptibility suggest that functional immune variation is linked to survival. Genetic variation at immune loci correlates with differential *Bd* susceptibility across diverse anuran taxa, both within (Kosch et al., 2017; Savage & Zamudio, 2011, 2016) and between (Woodhams, Bigler, & Marschang, 2007; Bataille et al., 2015; Savage, Mulder, Torres, & Wells, 2018) species. Functional differences in the magnitude and type of immune response also contribute to *Bd* susceptibility, although patterns vary between infection studies and host species. In one highly susceptible species, *Bd* infection caused strong innate and acquired immune responses, but did not enhance survival (Ellison, Savage, et al., 2014). In contrast, a second highly susceptible species reduced immune processes among *Bd*-challenged animals (Rosenblum, Poorten, Settles, & Murdoch, 2012). For other species with intermediate *Bd* susceptibility, improved survival rates were associated with increased innate immunity measured from bacterial killing assays and white blood cell ratios (Gervasi, Hunt, Lowry, & Blaustein, 2014; Savage et al., 2016). Whether these variable patterns reflect adaptive evolution to *Bd* in some species, rather than purely ecophysiological differences, remains to be tested. Further observations and experimental studies are therefore needed to clarify the role of genetic adaptation to chytridiomycosis.

In addition to host responses to disease, variation in *Bd* pathogenicity may cause different disease outcomes. Pathogenic strains of *Bd* actively suppress host immune responses in vitro by producing metabolites that induces lymphocyte apoptosis (Fites et al., 2013; Rollins-Smith et al., 2015). In at least some species, acquired immune responses in the skin are inhibited by *Bd* metabolites, but innate leucocyte proliferation remains intact (Fites, Reinert, Chappell, & Rollins-Smith, 2014; Young et al., 2014), suggesting that only the acquired branch of the immune system is targeted. Because genetic variation among *Bd* strains is significantly correlated with pathogenicity (Fisher et al., 2009), the ability of a given *Bd* strain to effectively inhibit host immunity may depend on the particular combination of host and pathogen genotypes. Indeed, in vivo *Bd* transcriptome sequencing reveals that gene expression patterns are significantly different when the same *Bd* strain infects different host species (Ellison, DiRenzo, McDonald, Lips, & Zamudio, 2017).

Hyperdiverse immune genes of the major histocompatibility complex (MHC) are likely to mediate acquired immune activation and suppression responses among individual *Bd*-host combinations. In particular, MHC class IIβ genes encode central regulators of vertebrate acquired immune function associated with susceptibility and resistance to pathogens in humans and other animals (Cooke & Hill, 2001; Cresswell, 1994; Sommer, 2005). Amphibian species vary in numbers of class IIβ gene copies and levels of polymorphism (Lillie, Cui, Shine, & Belov, 2016). One orthologous locus appears to be constitutively expressed (Kiemnec-Tyburczy, Richmond, Savage, & Zamudio, 2010; Richmond, Savage, Zamudio, & Rosenblum, 2009) and exhibits high levels of polymorphism and significant signatures of positive selection across a wide variety of species (Carey, Cohen, & Rollins-Smith, 1999; Flajnik & Kasahara, 2001; Lillie et al., 2015; Mulder et al., 2017). At this locus, class IIβ alleles are significantly associated with infection outcomes, both generally (Hu, Dong, Kong, Mao, & Zheng, 2017) and specifically in response to *Bd* infection (Bataille et al., 2015; Kosch et al., 2016; Savage & Zamudio, 2011, 2016). However, we lack a mechanistic understanding of the immunological processes underlying allele-specific *Bd* infection outcomes.

While much transcriptome work has focused on the MHC complex, whole transcriptome expression studies of amphibian chytridiomycosis expand the suite of genes examined and could improve our understanding of a wider array of host responses to the disease. For example, gene expression in immune tissues of tolerant or partially tolerant species suggest survival derives from innate immune activation (Ribas et al., 2009), increased expression of skin integrity genes (Poorten & Rosenblum, 2016), downregulation of T-cell activation in the spleen (Ellison, Tunstall, et al., 2014), and/or an overall decrease in immune gene expression (Rosenblum et al., 2009). In contrast, susceptible species show a wide range of transcriptomic responses to *Bd* infection. In the spleen, some species suppress expression of splenic T-cell genes (Ellison, Tunstall, et al., 2014) or mount robust but ineffective innate and acquired immune responses that do not prevent mortality (Ellison, Savage, et al., 2014). At the site of *Bd* infection, a number of susceptible species have significantly reduced expression of skin integrity genes when compared to tolerant species (Ellison, Tunstall, et al., 2014; Poorten & Rosenblum, 2016). At the intraspecific level, populations with a longer history of exposure to *Bd* showed significantly higher upregulation of innate and acquired immune genes at very early infection stages compared to populations where *Bd* was only recently introduced (Grogan et al., 2018), suggesting that evolution of *Bd* tolerance is mediated by shifts towards higher immune activation. Given the host breadth of chytridiomycosis and the observed differences in susceptibility both among and within species, further studies across multiple scales of host and pathogen genetic diversity

may help us to determine whether tolerance derives from concordant or from idiosyncratic evolutionary mechanisms.

Here, we use the lowland leopard frog (*Rana yavapaiensis*), a species that exhibits substantial intraspecific variation in *Bd* susceptibility (Savage & Zamudio, 2011, 2016), to characterize the functional genomic response to *Bd* infection over time among susceptible and surviving individuals following experimental exposure to *Bd*. Some *R. yavapaiensis* populations and individuals demonstrate *Bd* tolerance, maintaining heavy pathogen burdens but showing no disease signs (Savage, Sredl, & Zamudio, 2011; Savage & Zamudio, 2016). Pathogen burden in these tolerant individuals is more correlated with climate (Savage, Becker, & Zamudio, 2015). In contrast, susceptibility to infection is linked to overall genetic polymorphism (Savage et al., 2015) and specific immune gene variants (Savage & Zamudio, 2016). Specifically, *R. yavapaiensis* individuals exhibited individual-level variation in survival based on acquired immune system (MHC class IIβ) genotype in the laboratory (Savage & Zamudio, 2011) and in natural populations (Savage & Zamudio, 2016). Surprisingly, the previous laboratory study found that some MHC alleles promoted *Bd* resistance (complete clearance of the pathogen; Savage & Zamudio, 2011), whereas in the field study, the same alleles were linked to pathogen tolerance (surviving while maintaining heavy pathogen burdens; Savage & Zamudio, 2016). Thus, there is evidence for both resistance and tolerance mechanisms associated with *Bd* survival, but the precise role of different immune mechanisms is unclear.

In this study, we hypothesized that under infection-promoting conditions, frogs surviving *Bd* infection would show an earlier and larger magnitude of acquired immune gene expression compared to susceptible frogs because their MHC molecules would be better able to initiate some mechanism of either tolerance or resistance to *Bd*. To test this hypothesis, we reared multiple clutches of *R. yavapaiensis* eggs through metamorphosis in a common garden experiment and exposed them to a virulent strain of *Bd* or to a control sham inoculation. We used RNAseq (Wang, Gerstein, & Snyder, 2009) at early and late infection timepoints to characterize total gene expression in spleen and the skin among sham-exposed and *Bd*-exposed individuals. We then compared gene expression, gene enrichment, and *Bd* infection dynamics over time to determine characteristics of immune responses leading to differential disease outcomes. Although previous RNAseq-based investigations of chytridiomycosis have found contradictory immune responses to *Bd*, these studies were conducted within and between species with fixed rather than variable patterns of *Bd* susceptibility. We examined a single species with known variation in *Bd* susceptibility and our goal was to identify heritable and physiological differences associated with differential disease outcomes within a single species.

2 | MATERIALS AND METHODS

2.1 | Animal husbandry

We collected partial egg masses (50–75 eggs per clutch) from the Muleshoe Ranch Cooperative Management Area in Graham County, Arizona, USA in September 2012 and shipped them overnight to the National Zoological Park in Washington, DC. Clutch 2 was collected from the Secret Spring Pond (UTMs: 571093E, 3578368N), clutches 4 and 5 were collected from the upper thermal spring pools (UTMs: 571502E, 3570135N), and clutch 7 was collected from the main thermal spring pond (UTMs: 571681E, 3578002N). Larvae hatched from eggs within two days of arrival, and were confirmed to be free of *Bd* via swabbing their mouthparts (Retallick, Miera, Richards, Field, & Collins, 2006) and testing swabs using a qPCR assay (see below). We reared individuals through metamorphosis in an animal holding room at the National Zoological Park's Reptile Discovery Center at 24°C ± 3°C with a 12 hr:12 hr light:dark regime, as described in detail previously (Savage et al., 2016). When all frogs had reached or exceeded Gosner stage 41, we transferred them to a climate chamber held at 20°C ± 2°C with a 12 hr:12 hr light:dark regime during lighted hours. We placed metamorphosed frogs individually in polycarbonate mouse cages held at a slight angle with a water pooled at the lower end and an opaque plastic shelter with wet paper towels at the other end. We fed the juvenile frogs with vitamin-dusted crickets, mealworms, and wingless flies daily ad libitum.

2.2 | Experimental *Bd* exposures

As described in detail previously (Savage et al., 2016), we swabbed all animals for *Bd* one day prior to the start of the experiment, then exposed all frogs to an estimated 100,000 zoospores of *Bd* strain JEL423 a global pandemic lineage strain isolated from *Hylomantis lemur* in Panama ($N = 52$ infected frogs; 10 from clutch 2, 11 from clutch 4, 11 from clutch 5, and 20 from clutch 7) or sham-exposed with 1 ml of reconstituted water ($N = 30$ sham-infected frogs; seven from clutch 2, five from clutch 4, seven from clutch 5, and 11 from clutch 7). Animals were 3–4 weeks post-metamorphosis, which is sufficient time for immunocompetence to return based on studies of the model frog *Xenopus laevis* (Barlow & Cohen, 1983), and ranged in mass from 1.6–3.9 g (mean = 2.3 g) at the start of the experiment. We euthanized at least eight frogs (at least five infected and three control) on one, four, eight, 15, 29, and 42 days post-infection (DPI; Table S1). We selected individuals for euthanasia using a random number generator to choose individuals within each clutch, with infected frogs selected from all four clutches and control frogs selected from three clutches per euthanasia day (chosen based on total number of individuals available per clutch, and rotating out clutches evenly across sampling dates). We monitored frogs daily for disease signs associated with chytridiomycosis: lethargy, loss of righting ability, and ventral redness. We euthanized frogs when these three clinical signs of chytridiomycosis first appeared.

We swabbed all animals weekly on the abdomen, drink patch, hands, and feet with a sterile swab to quantify the *Bd* infections (Hyatt et al., 2007). All individuals were swabbed weekly, and additionally swabbed at the time of euthanasia when transcriptome samples were collected. We quantified *Bd* infection intensity from all swabs using DNeasy Blood and Tissue kits for extraction (Qiagen),

and running samples in duplicate Taqman quantitative (q)PCR (Boyle, Boyle, Olsen, Morgan, & Hyatt, 2004) with JEL423 standards of 0.1, 1, 10, 100, 1,000, 10,000, and 1,000,000 *Bd* genome equivalents (GEs) to determine *Bd* presence and infection intensity. The setup of the *Bd* exposure experiment is illustrated in the (Figure S1). Throughout the experiment, we prevented cross-contamination by using a fresh pair of gloves when handling each individual or the inside surface of a cage and cleaned cages biweekly rinsing for a minimum of one minute using a solution of one part bleach to nine parts water. This exposure time and concentration exceeds the minimum required to kill this strain of *Bd* (Becker & Gratwicke, 2017). Challenge experiments were performed with approval from and in accordance with the ethical standards of the US Institutional Animal Care and Use Committee under Smithsonian protocol #13–15.

To evaluate whether clutch or *Bd* infection intensity were predictors of chytridiomycosis susceptibility, we used a generalized linear model (GLM) for binomial response data and Wald Z test in R (Bolker et al., 2008). We only included *Bd* infection intensity values measured at seven, 14, and 28 DPI because after 28 DPI most individuals had been euthanized and we therefore had insufficient data points.

2.3 | Sample collection

We euthanized all individuals via injection with ~ 200 μl of MS-222 (10 g/L in sterile water buffered to a pH of 7 with sodium bicarbonate). Each frog was injected intracoelomically on the right flank using a 1 cc syringe and a 25 gauge 19 mm needle. After approximately one minute, we confirmed lack of cardiac movement using trans-illumination, and then proceeded with blood and tissue harvest. Although this euthanasia method may have slightly altered gene expression in the brief time between injection and tissue harvest, our precise standardization of implementing this method across all individuals meant that any biases would be consistent and not alter our comparison of gene expression across treatment groups. We used sterile equipment for dissection and collected an approximately 1 cm^2 piece of skin from the drink patch and the entire spleen and placed them in separate 0.5 ml tubes with 5× volume of RNAlater buffer. We selected these tissues because skin is the primary site of *Bd* infection (Longcore et al., 1999) and the spleen is the major lymphoid organ in amphibians (Tischendorf, 1985). Tissues were stored at −80°C until RNA extractions were performed.

2.4 | Transcriptome sequencing

We extracted messenger RNA from each tissue sample using the Dynabeads mRNA Direct kit (Invitrogen), a poly-A tail binding bead-based approach that captures only mRNA. We converted messenger RNA to first and second strand cDNA using SuperScript III reverse transcriptase (Invitrogen) with random hexamer primers, and NEBNext mRNA Second strand Synthesis Module (New England Biolabs). We generated Nextera-style dual-indexed cDNA libraries for Illumina sequencing using the following steps: We sheared cDNA to appropriate size for 2 × 100 bp sequencing. Next, we blunted ends with DNA Polymerase I, Large (Klenow) Fragment (New England Biolabs), dC-tailing fragments with Klenow Fragment (3′–5′ exo⁻) (New England Biolabs); and ligating the Stubby adapter (Operon) with Quick T4 DNA Ligase (New England Biolabs). We performed an 18 cycle dual-indexing PCR with High-Fidelty Taq (Kapa) and Nextera-style i5 and i7 adapters with unique 8-mer molecular identifiers (Operon) in a unique combination for each sample. We assessed equimolar quantities of each library using Illumina qPCR DNA quantification kits (Kapa) and BioAnalyser High Sensitivity DNA chips (Agilent) pooled into groups of 20–25 tissue libraries per 2 × 150bp HiSeq2000 lane (Illumina). Because RNAseq samples failed from 4 *Bd*-exposed individuals (two from clutch 4 and two from clutch 7) and one control individual from clutch 7, the final number of individuals with gene expression analysis was 48 *Bd*-exposed frogs and 29 control frogs.

2.5 | Gene ontology annotation

After Illumina standard quality control filtering, we visualized read quality for each sample using FastQC version 0.10.0 (Andrews, 2010). We used PRINSEQ (PReprocessing and INformation of SEQuences) v. 0.20.3 (Schmieder & Edwards, 2011) to trim low quality reads or bases. Trimming included any Illumina adapter sequence, the 5′ and/or 3′ end of reads where quality score dropped below Q20, anywhere within each read where a 5 bp window drops below Q20, and any trimmed reads less than 30 bp long. All reads from every individual and tissue were pooled to assemble a consensus reference transcriptome using Trinity (Grabherr et al., 2011) with default parameter settings on the Smithsonian HYDRA cluster, a high-performance cluster with 1800 central processing units and 1 TB random access memory. To avoid low-level expression noise, we filtered out transcripts that did not have at least two reads per million mappable reads in at least two samples (Harrison, Mank, & Wedell, 2012; Moghadam, Harrison, Zachar, Székely, & Mank, 2013). Each assembled Trinity contig (roughly equivalent to a single gene or gene isoform) was aligned via a local batch BLAST+ (Camacho et al., 2009) to the National Center for Biotechnology Information (NCBI) nonredundant (nr) protein database, retaining up to 20 hits with a minimum *E*-value of 1×10^{-6} and minimum bit score of 55. We removed any transcript aligning to the *Bd* transcriptome (*Bd* Sequencing Project, www.broadinstitute.org) from downstream analyses. BLAST2GO v. 3.3 (Conesa et al., 2005) was used to functionally annotate the assembled transcriptomes. Gene ontology (GO; Ashburner et al., 2000) mapping was performed, extracting the GO terms associated with homologies identified by BLASTX, and producing a list of GO annotations represented as hierarchical categories of increasing specificity. We retained annotations with a minimum *E*-value of 1×10^{-6} and a minimum annotation cutoff of 55. GO annotations were enhanced using the annotation augmentation tool ANNEX (Myhre, Tveit, Mollestad, & Lægreid, 2006).

2.6 | Gene expression analyses

Gene expression was measured as fragments per kilobase per million mapped reads (FPKM) following the Trinity pipeline with BWA read mapping and RSEM read count normalization (Li & Dewey, 2011). We analysed differential gene expression (DGE) between the following four infection groups: (a) "control" (uninfected) ($N = 33$); (b) "early infection" (1–15 DPI, $N = 20$); (c) late-infection "surviving" (29–55 DPI, disease signs were absent, but disease outcome is unknown, $N = 19$); and (d) late-infection "susceptible" (29–55 DPI, all classic chytridiomycosis disease signs present, and animal was predicted to die from infection, $N = 9$, Figure S1). Using these categories was the best method of inferring gene expression relationships to *Bd* responses, given that *R. yavapaiensis* individuals can exhibit tolerance while maintaining high *Bd* loads, and therefore disease signs are essential for assessing susceptibility (Savage et al., 2015; Savage & Zamudio, 2016). Our study faced design limitations, because in order to collect material required to evaluate transcriptomic responses we had to euthanize frogs prior to directly observing mortality. We therefore have a clear understanding of the disease outcome of the susceptible frogs, but for the surviving *Bd*-infected frogs the eventual disease outcome remains uncertain. We do know that surviving frogs tolerated infection longer than the susceptible group, and displayed no external disease symptoms.

DGE among the four treatment groups (control, early infection, surviving, and susceptible) was assessed using the edgeR (Robinson, McCarthy, & Smyth, 2010) R package (R version 2.15.2; R Core Team, 2012), which estimates sample dispersion and normalization factors and determines significance with an exact test. A false discovery rate (FDR)-corrected *p*-value less than 0.01 was considered evidence of significant DGE.

We used discriminant analysis of principle components (DAPC) to define transcriptome-wide expression clusters including all contigs with significant DGE. Immunome-wide expression clusters included only contigs with significant DGE and GO mapping to the "immune system process" GO:0002376. We performed DAPC analysis separately for skin and spleen using the adegenet 1.4-0 package (Jombart, Devillard, & Balloux, 2010) in R, which implements a *k*-means clustering algorithm using the Bayesian Information Criterion (BIC). The optimal number of clusters was determined using the criterion of $\Delta BIC \leq 2$.

We tested for enrichment of biological process GO terms among significant DGE contigs in skin and spleen using Fisher's exact tests with FDR correction implemented in BLAST2GO v. 3.3. This analysis determined whether DGE genes were biased towards immune-related functions compared to the complete reference transcriptome. To quantify the overlap of significant immune DGE between *Bd*-exposed and unexposed individuals across genetic groups, we also tested for enrichment of biological process GO terms among significant DGE contigs for skin and spleen of infected and uninfected individuals at early (1–15 DPI) and late (29–55 DPI) time points with the entire transcriptome as background reference. We selected "immune function" GO terms with significant over- or underrepresentation and constructed four-way Venn diagrams comparing *Bd*-infected and uninfected individuals within each clutch, tissue, and time point (1–15 DPI and 29–55 DPI) using VENNY (Oliveros, 2007).

2.7 | MHC class IIβ allelic diversity

To measure MHC class IIβ sequence polymorphism among individuals, we extracted all transcriptome contigs with significant homology to MHC class IIβ loci via BLASTx alignment to the NCBI nr protein database ($N = 5$). We then mapped skin and spleen reads from each individual to those contigs using Geneious v. 9.1.2 (Kearse et al., 2012). We determined the minimum number of alleles per locus based on the maximum number of single nucleotide polymorphisms (SNPs) per site for each individual. Only SNPs sequenced at a depth of at least three reads per sequence were considered to be real polymorphisms. For the previously characterized locus with known associations to *Bd* susceptibility (Savage & Zamudio, 2011, 2016) that is constitutively expressed (Kiemnec-Tyburczy et al., 2010), hereafter the "classic" locus, we extracted consensus allele sequences for exon 2, which encodes the majority of peptide-binding residues for class II molecules (Jones, Fugger, Strominger, & Siebold, 2006). For individuals expressing two alleles, alleles were only extracted when overlapping reads were sufficient to infer allelic identify for all SNPs; in rare cases where polymorphisms could not be assigned to a particular allele, alleles were not extracted.

2.8 | MHC class IIβ genealogical relationships

We used ClustalW (Larkin et al., 2007) implemented in Geneious v. 9.1.2 and manual adjustment to generate an alignment of all extracted classic locus transcriptome-derived exon 2 alleles. We also included previously sequenced *L. yavapaiensis* exon 2 alleles with known *Bd* associations (Savage & Zamudio, 2011), and *Xenopus laevis* and *Silurana tropicalis* exon 2 alleles (GenBank accession numbers NM_001114771 and NM_001045794) as outgroup sequences. Using this alignment, we reconstructed genealogical relationships among alleles via a Bayesian analysis with model parameters determined using the Akaike information criteria (AIC) in jModeltest (Posada, 2008). We used the best-fit model (GTR + I + G) to estimate a 95% credible set of rooted MHC genealogies in the software MrBayes 3.1 (Ronquist & Huelsenbeck, 2003). We ran two separate analyses in MrBayes for 1×10^7 generations and sampled every 500th generation of the Markov chain. We used Tracer v1.4 to assess stationarity of model parameters, convergence of model parameters between runs, the number of burnin samples, and the effective sample sizes for each parameter.

2.9 | MHC class IIβ supertyping and disease associations

To collapse MHC class IIβ classic locus exon 2 alleles into functional supertypes, we extracted the 13 codon positions in our alignment

known to determine peptide-binding capabilities of human class II alleles (Jones et al., 2006). We then characterized each site based on five physiochemical descriptor variables: z1 (hydrophobicity), z2 (steric bulk), z3 (polarity), z4 and z5 (electronic effects; Sandberg, Eriksson, Jonsson, Sjostrom, & Wold, 1998). We used DAPC (Jombart et al., 2010) to define functional exon 2 supertype clusters. Exon 2 alleles within clusters were collapsed into a single MHC exon 2 supertype.

Differences in MHC supertype frequencies across late-stage (29–55 DPI) infection groups that were susceptible to the disease were determined by calculating the Relative Risk (Sistrom & Garvan, 2004) for each MHC supertype. Significance of each Relative Risk value was assessed using Fisher's exact test, with sequential Bonferroni correction for multiple comparisons (Rice, 1989).

2.10 | GLMM analyses of *Bd* infection intensity, disease, and gene expression relationships

We further analysed the relationship between *Bd* infection intensity, susceptibility, and immune gene expression data using generalized linear mixed model (GLMM) Penalized Quasi-Likelihood (PQL) analyses using the MASS package in R (Ripley et al., 2019). This was particularly important for early infection individuals that were sampled through 15 DPI, which is prior to when chytridiomycosis disease signs appear, meaning that susceptibility could not be inferred from any data other than *Bd* infection intensity. Thus, we ran one set of GLMM analyses on the early infection individuals examining the effects of (a) mean immune gene expression across all spleen DGE immune genes; (b) total spleen MHC class IIβ gene expression; and (c) whether or not the "classic" class IIβ locus was expressed, on log-transformed *Bd* infection intensity at the time the individual was sampled for RNAseq, with clutch as a random factor. We ran the same set of GLMM analyses on all of the late infection individuals, but also added an analysis examining the effects of susceptibility (i.e., presence or absence of chytridiomycosis disease signs) on *Bd* infection intensity.

3 | RESULTS

We compared *Bd*-exposed ($N = 48$) and unexposed ($N = 29$) individuals across four clutches at early (1–15 DPI) and late (29–55 DPI) infection stages. Because *Bd* susceptibility could only be assessed for individuals sampled at least 21 DPI when disease signs begin to manifest in *R. yavapaiensis* (Savage et al., 2016; Savage & Zamudio, 2011), susceptibility of early infection stage frogs could not be determined, whereas late infection stage frogs were compared between susceptible (chytridiomycosis disease signs) versus surviving (*Bd* positive but no disease signs throughout the 55 day experiment) individuals. *Bd* infection intensity among all exposed individuals rose between seven to 28 DPI, remained high thereafter (Figure 1b), and the majority of frogs that were diagnosed as susceptible with chytridiomycosis signs occurred within two weeks of this peak *Bd* infection intensity (Figure 1a). Our GLM found that clutch was not a significant predictor of chytridiomycosis susceptibility ($Z = 0.66$, $p = .51$), nor was *Bd* infection intensity at seven DPI ($Z = 0.34$, $p = .73$) or 14 DPI ($Z = 0.29$, $p = .77$). However, *Bd* infection intensity at 28 DPI was a significant predictor of chytridiomycosis susceptibility ($Z = 1.95$, $p = .05$), with susceptible individuals displaying higher infection intensities at this timepoint compared to all other individuals. Furthermore, GLMM analysis of all late stage infected individuals found that disease signs were a significant predictor of *Bd* infection intensity ($t = 2.46$, $p = .02$). Specifically, individuals with disease signs harbored significantly more *Bd* in their skin than individuals without disease signs (Figure 1c), consistent with our phenotypic disease categories of susceptible and surviving, respectively.

Our final de novo assembled transcriptome, including all skin- and spleen-derived cDNA fragments (averaging 6,536,908 reads/tissue; Table S2), consisted of 143,189 contigs, 1,888 of which (1.3%) mapped to the *Bd* genome, had predominantly binding and catalytic activity (Figure S2; Table S3), and were excluded from all subsequent analyses because they were fungal-derived. 875 additional contigs were bacterial-derived and also removed. Of the remaining 140,426 contigs, 20,537 were identified and characterized via homology to known genes using BLAST and GO (Figure S3). A total of 3,340 skin (Table S4) and 7,387 spleen (Table S5) genes had significant differential gene expression (DGE) among at least two of the four infection groups (control, early infection, susceptible, and surviving; FDR p-value $< .01$). Global gene expression clustering patterns based on DAPC of DGE genes showed a high degree of individual-level variation, with little differentiation corresponding to *Bd* infection groups (Figure S4). One exception was that susceptible frogs trended towards differentiated clustering in the spleen when considering immune genes only, but not when clustering based on all DGE genes (Figure S3, A versus C).

Genes with significant DGE among infection groups were significantly enriched for 33 immune function GO terms in the skin (Table S6) and 107 immune function GO terms in the spleen (Table S7) compared to non-DGE genes, including an overrepresentation of MHC protein complex in both tissues and a range of innate, inflammatory and acquired immune functions. Clutch 2, which had the lowest incidence of chytridiomycosis ($N = 1/7$ infected individuals), had significant under-representation of immune DGE genes in *Bd*-exposed ($N = 6$) versus unexposed ($N = 5$) frogs at late infection stages in both spleen and skin (Figure 1c; Table S8). In contrast, clutch 7, which had the highest incidence of chytridiomycosis ($N = 7/12$ infected individuals), had significant over-representation of immune DGE genes in *Bd*-exposed versus unexposed frogs at early infection stages ($N = 4$ control and 8 exposed frogs) in both spleen and skin, and at late infection stages ($N = 4$ control and 10 exposed frogs) in spleen (Figure 1d; Table S8). At late infection stages in the spleen, clutch 2 and 7 were significantly enriched for five of the same immune functions, but clutch 2 individuals were significantly underenriched whereas clutch 7 individuals were significantly over-enriched (Figure 1d; Table S5).

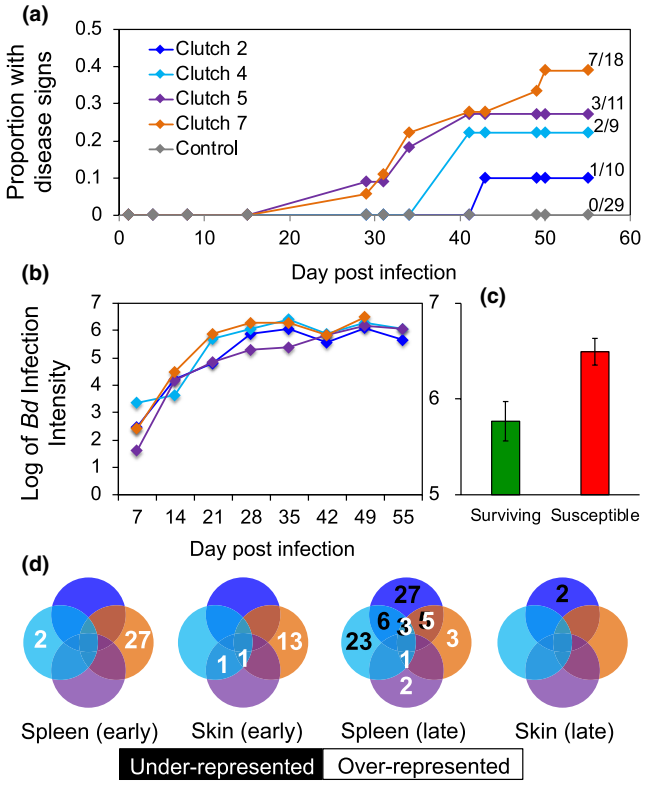

FIGURE 1 Experimental exposure of *Rana yavapaiensis* to *Bd*. (a) Sham-exposed control frogs from all four clutches (grey line) did not develop disease signs, but some *Bd*-exposed individuals from all four clutches (2, 4, 5 and 7; coloured lines) developed disease signs. (b) *Bd* infection intensity measured using quantitative PCR increased among all *Bd*-exposed individuals in all four clutches until 28 DPI and remained high through 55 DPI. (c) At late infection stages (>28 DPI), *Bd* infection intensity (± standard error) at the time of sampling for transcriptomics was significantly higher in susceptible (red; individuals with chytridiomycosis disease signs) compared to surviving (green; no disease signs) individuals across all four clutches. (d) Significantly over- and underrepresented immune functions based on gene ontology (GO) annotations for *Bd*-exposed versus control frogs across clutches and time points. Numbers represent the number of immune functions that are significantly overrepresented (white) or under-represented (black) for infected frogs compared to control frogs from the same clutch during early infection (left diagrams) and late infection (right diagrams) in skin and spleen transcriptomes. Sample sizes for each clutch and timepoint (control vs. exposed) were as follows: clutch 2 early, $N = 2$ versus 4; clutch 2 late, $N = 5$ versus 6; clutch 4 early, $N = 2$ versus 4; clutch 4 late, $N = 4$ versus 5; clutch 5 early, $N = 2$ versus 4; clutch 5 late, $N = 6$ versus 7; clutch 7 early, $N = 4$ versus 8; clutch 7 late, $N = 4$ versus 10

Numerous immune function genes showed significant DGE across the four infection groups (Figure 2). In contrast, DGE analysis of all nonimmune function genes did not show any overall clustering based on infection groups (Figure S5). Significant DGE of individual immune genes among infection groups (FDR p-value < .01) suggests an inverse relationship between sustained immune activation and *Bd* survival (Figure 2). Acquired, innate, and inflammatory immune genes showed an overall pattern of significant upregulation in the spleen of susceptible frogs compared to surviving frogs (Figure 2a). Indeed, frogs that survived *Bd* infection for the entire 55 day experiment significantly downregulated spleen immune gene expression across categories, showing significantly lower expression compared to susceptible frogs and to uninfected control frogs for most genes. Certain Grb2 associated binding, chitin-binding and heat shock proteins were upregulated in the spleen of late infection surviving frogs (Figure 2a). However, a majority of spleen DGE genes, including hallmarks of acquired immune activation (MHC and T-cell related genes) and inflammation (interleukins, interferons and cytokines) are significantly upregulated in susceptible frogs and downregulated in surviving frogs, demonstrating that sustained transcriptomic immune activation in a primary amphibian immune tissue (the spleen) is associated with *Bd* susceptibility. Spleen immune gene expression patterns in early infection individuals were positively associated with a functional metric of immunity, bacterial killing ability (BKA; Figure S6), that was measured in a previous study of these frogs (Savage et al., 2016),

In contrast to spleen, and consistent with enrichment results, few skin immune genes had significant DGE, and those that did show significant expression changes had an overall pattern of downregulation among all *Bd*-infected frogs compared to control frogs (Figure 2b). Innate immune function shows the largest deviation from these patterns; skin of early infection frogs showed significant upregulation of several innate immune genes including lysozyme and antimicrobial peptides, and skin of surviving frogs showed upregulation of leptin receptors, which are regulators of immunity and inflammation (Naylor and Petri, 2016).

We identified five distinct MHC class IIβ loci in our reference transcriptome (Table S9), including a previously characterized single-copy "classic" locus that appears to be constitutively expressed (Kiemnec-Tyburczy et al., 2010) and four additional loci that may each be multicopy based on the presence of > 2 alleles per individual per locus (Figure S7). Inferring distinct MHC loci from RNAseq data is limited due to ambiguity in read mapping to multiple similar reference transcripts (Carapito, Radosavljevic, & Bahram, 2016), therefore we focused our analyses on the classic locus and on the total number of alleles present within an individual (Figure 3). Susceptible frogs had significantly higher total MHC class IIβ expression compared to surviving frogs (Figure 3a), but comparisons to control and early infection frogs were not significant, highlighting that class IIβ expression specifically at late infection stages is an important predictor of susceptibility Susceptible frogs also expressed a significantly higher number of distinct class IIβ alleles compared to other treatment groups (Figure 3b), consistent with previous wildlife disease studies finding that optimal MHC diversity is lower than maximal MHC diversity (Nowak, Tarczy-Hornoch, & Austyn, 1992). Expressed MHC class IIβ alleles from the classic locus, which are associated with *Bd* infection outcomes (Bataille et al., 2015; Savage & Zamudio, 2011), fell into six functional supertypes (STs; Table S10; Figure 3c; Figure S8). Individuals bearing ST3, which was previously associated with susceptibility in *R. yavapaiensis* (Savage & Zamudio, 2011), had a significantly increased risk of susceptibility at

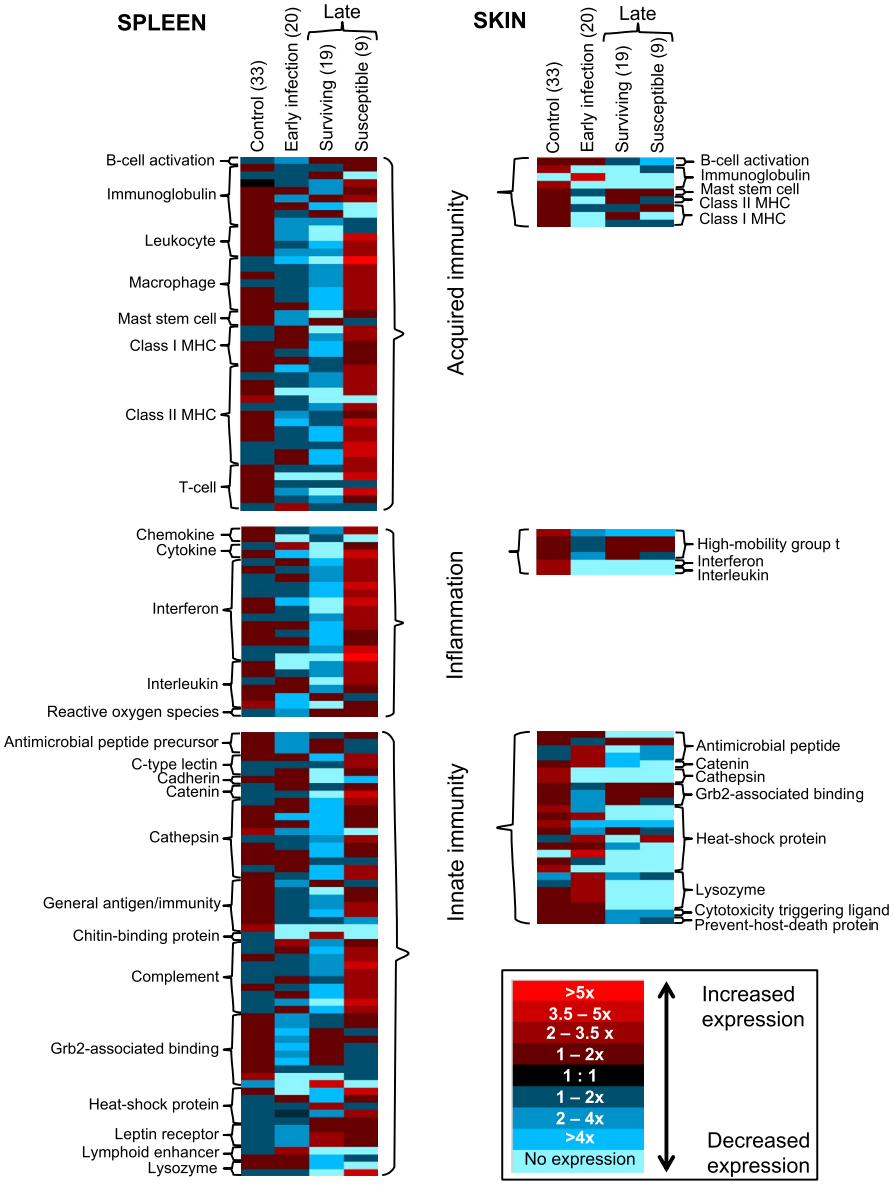

FIGURE 2 Differential expression of immune genes among *Bd*-exposed and *Bd*-unexposed *R. yavapaiensis* individuals. Relative expression in spleen (a) and skin (b) transcriptomes for genes that function in acquired, inflammatory, or innate immune pathways showing significant differential gene expression (DGE) among ≥ 2 groups (FDR *p*-value < .01). Numbers indicate transcriptome sample sizes per group. *Bd*-exposed individuals were grouped over time and response group into Early (sampled 1–15 DPI), Late Healthy (sampled 29–55 DPI without disease signs) and Late Dying (sampled 29–55 DPI with signs of severe chytridiomycosis). Control frogs did not show significant immune DGE or physiological differences over time and were grouped together

late infection stages (Rel. Risk = 3.3, $z = 3.77$, $p = .0002$; Figure 3d). We only recovered ST5 alleles from surviving frogs, suggesting links to survival, but sample size was insufficient for statistical analysis. ST4 alleles, which were significantly associated with *Bd* survival in a previous field study (Savage & Zamudio, 2016), were expressed in control frogs but not infected frogs (Figure 3c), suggesting that individuals bearing ST4 may downregulate class IIβ expression upon *Bd* infection. Phylogenetic analysis (Figure 3d) placed the alleles linked to survival in these previous laboratory and field studies into a moderately well-supported clade (74%; Figure 3d) that only contained alleles we recovered from control frogs and surviving frogs. In contrast, susceptible frogs were distributed throughout the remainder of genealogy (Figure 3d).

GLMM analysis of early infection individuals found no relationship between *Bd* infection intensity and mean immune gene expression across all DGE genes ($t = -0.59$, $p = .56$), or total MHC class IIβ gene expression ($t = -0.20$, $p = .84$). In contrast, whether or not the classic class IIβ locus was expressed was a significant predictor of *Bd* infection intensity ($t = -2.47$, $p = .03$). Specifically, individuals expressing the classic locus had significantly lower log-transformed *Bd* infection intensity ($N = 8$, mean = 2.91 ± 0.26 SE) compared to individuals that did not express the classic locus ($N = 12$, mean = 3.83 ± 0.29 SE) at early infection stages. This pattern disappeared at late infection stages: neither expression of the classic class IIβ locus ($t = 0.67$, $p = .51$) or mean immune gene expression across all DGE genes ($t = -0.24$, $p = .81$) were significant predictors of *Bd* infection intensity at late infection stages, and total MHC class IIβ gene expression only trended towards a positive association with *Bd* infection intensity ($t = 1.88$, $p\beta = 0.07$).

4 | DISCUSSION

In this study, we observed an overall pattern of significantly decreased immune gene expression in surviving frogs relative to both uninfected control frogs and to susceptible frogs. We designed this

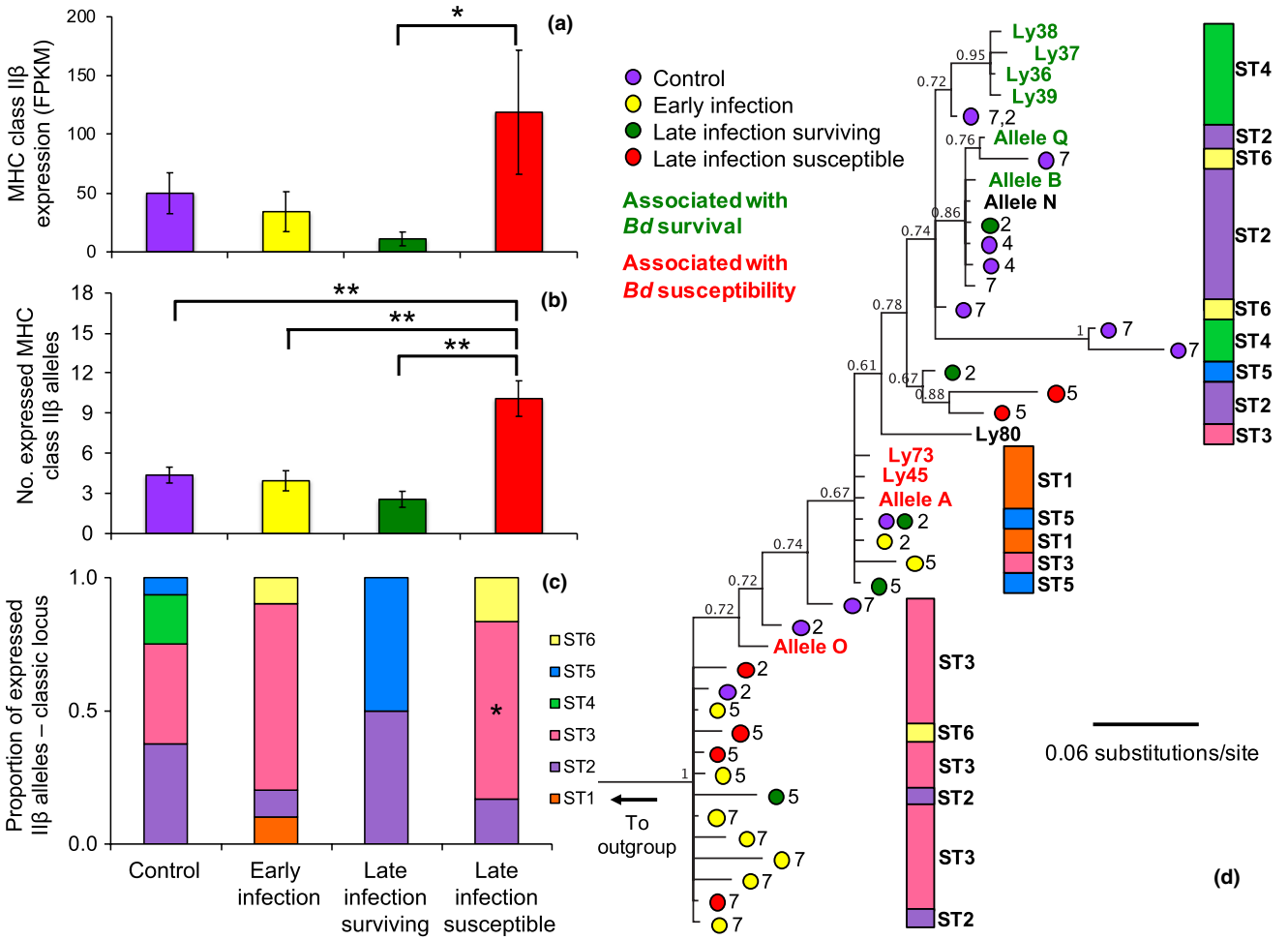

FIGURE 3 MHC class IIβ expression variation and genetic variation linked to *Bd* exposure outcomes among *R. yavapaiensis* individuals. Expression (fragments per kilobase per million mapped reads) and diversity of five transcriptome-derived class IIβ loci for sham-exposed individuals (control), *Bd*-exposed individuals sampled at early infection stages (1–15 DPI; early infection), and *Bd*-exposed individuals sampled at late infection stages (29–55 DPI) without disease signs (late infection surviving) or with disease signs (late infection susceptible). Total class IIβ gene expression was significantly higher in individuals with disease signs compared to without disease signs (a), and individuals with disease signs expressed a significantly larger number of MHC class IIβ alleles compared to individuals without disease signs, individuals sampled at early infection stages, or controls (b). Error bars show standard error; $^{*}p < .01$ $^{**}p < .0001$ (ANOVA with post hoc test). (c) The proportion of expressed functional allelic supertypes (STs) varies among infection groups for the classic class IIβ locus previously associated with *Bd* susceptibility (11–12), and individuals bearing ST3 (pink) have a significantly elevated odds ratio of developing disease signs, $^{*}p < .01$ (Fisher exact test). (d) Bayesian analysis of classic class IIβ locus expressed allelic variation among *Bd* exposure groups (colours) and clutches (numbers). Sample sizes are not shown (see Table S10). ST4 alleles are only expressed in controls and fall within the clade containing alleles associated to *Bd* survival, whereas ST3 is excluded from this clade

experiment expecting to recover the opposite pattern of increased immune gene expression in surviving relative to susceptible frogs, and we predicted that these upregulated immune genes would provide important clues about the immune pathways responsible for *Bd* survival. Instead, we observed significant upregulation of immune genes in susceptible frogs (Figure 2) that had disease signs and were maintaining significantly higher *Bd* burdens (Figure 1), suggesting that sustained immune responses are associated with *Bd* susceptibility rather than survival. Significant gene expression increases in susceptible frogs was specific to certain immune genes, and was not a generalized overexpression across all pathways (Figure S4), thus this finding cannot be explained by a classic death-signal response (Pozhitkov et al., 2017). Furthermore, surviving frogs had lower spleen immune gene expression levels even compared to uninfected control frogs (Figure 2), implying that decreased expression of these types of genes was specifically associated with survival. Although the four clutches showed significant differences in the type of immune genes that were enriched at early and late infection stages (Figure 1c), *Bd* infection and susceptibility were not influenced by clutch. In contrast, early expression of the classic MHC class IIβ locus was predictive of *Bd* burdens. Thus, the observed association between chytridiomycosis and elevated transcriptome-derived immune function at late infection stages in susceptible individuals suggests a counterproductive or dysregulated immune function across frogs of different genetic backgrounds that may be linked to early MHC-linked responses and contributes to *Bd* susceptibility.

Because we observed that transcriptome-based intraspecific variation in *Bd* susceptibility is associated with differences in acquired immune activation (Figure 2), we hypothesize that these acquired immunity differences may be caused by MHC immunogenetic variation among individuals (Figure 3). We observed that MHC class IIβ expression was higher in susceptible individuals compared to surviving individuals. *Bd* susceptibility was associated with a significantly higher magnitude of inflammation and acquired immune activation at late infection stages. *Bd*-surviving frogs had significantly lower inflammation and acquired immune activation compared to both susceptible frogs and uninfected controls. At late infection stages, BKA was significantly lower in susceptible compared to surviving frogs (Savage et al., 2016), which potentially indicates metabolic breakdown due to advanced disease progression. However, the significantly higher acquired and inflammatory immune function in spleen RNAseq profiles of susceptible frogs suggests they were still able to generate considerable, although potentially dysregulated, acquired immune responses. Thus, the higher BKA values previously measured in the surviving frogs compared to susceptible frogs may instead represent an effective innate immune response contributing to *Bd* tolerance.

Based on these results, we suggest that *Bd* susceptibility in *R. yavapaiensis* may arise from over-activation of immune responses in the spleen leading to inflammation but a lack of effective acquired immunity, potentially homologous to the cytokine storm in human infectious disease (Tisoncik et al., 2012). This may not be observable on the skin, which is the site of infection (Berger et al., 1998), due to immunosuppressive properties of *Bd* (Fites et al., 2013). The significantly reduced immune gene expression in late-stage surviving frogs compared to susceptible frogs and to uninfected controls suggests that downregulation of immune molecules below background levels may prevent activation of acquired immune pathways and allow some individuals to tolerate *Bd*. However, further immunological studies are necessary to validate this hypothesis, and it remains uncertain whether survival is a form of host-immune deviation initiated because lymphocyte-killing abilities of *Bd* render acquired immune responses counterproductive (Parish, 1996). Susceptibility to human fungal diseases often arises from overactivation of the inflammatory response (Romani, 2011) and a similar process may be occurring here.

Our study highlights the variation in pathogen tolerance and resistance that can occur in the chytridiomycosis disease system. All infected frogs maintained heavy pathogen burdens throughout the experiment, regardless of susceptibility (Figure 1d), but susceptible frogs showed significantly higher *Bd* infection intensity at 28 DPI, and surviving frogs maintained significantly lower *Bd* infection intensity compared to susceptible frogs at the time of transcriptome sampling (Figure 1c), suggesting both pathogen resistance as well as tolerance in surviving frogs. The ability of frogs to remain aclinical while harbouring millions of *Bd* organisms in the epidermis has been detected in other studies (e.g., Venesky, Mendelson, Sears, Stiling, & Rohr, 2012; Venesky, Raffel, McMahon, & Rohr, 2014; Woodhams, Bigler, & Marschang, 2012) and the presence of surviving "supershedder" individuals in aquatic systems has been suggested as a mechanism maintaining *Bd* zoospores within the water column (DiRenzo, Langhammer, Zamudio, & Lips, 2014). The specific mechanisms allowing surviving frogs to tolerate these heavy pathogen burdens remains unclear, but regular skin sloughing is an effective method contributing to *Bd* tolerance in at least some host species (Ohmer, Cramp, Russo, White, & Franklin, 2017).

The negative impact of host-acquired immune responses on survival potentially explains how this fungus can be so lethal to amphibians despite evidence that memory responses can be generated (Ellison, Savage, et al., 2014; McMahon et al., 2014). It could also explain why amphibian species showing largely innate and not acquired transcriptomic responses against *Bd* (Ribas et al., 2009; Rosenblum et al., 2009) persist in the wild with infections and no pathology (Soto-Azat, Clarke, Poynton, & Cunningham, 2010). There is also evidence that anti-*Bd* skin secretions (an innate defence) have evolved in amphibian populations persisting and recovering from *Bd* (Voyles et al., 2018). Induction of immune deviation from a proinflammatory to a more regulatory immune phenotype can lead to effective antigen-induced tolerance in human disease (Parish, 1996; Prakken et al., 2004). A similar mechanism may therefore be operating in *Bd*-tolerant amphibians, and this hypothesis should be tested with more detailed and extensive immunological assessments in field and laboratory studies.

The link between *Bd* survival and lower acquired and inflammatory immune gene expression that we observed in *R. yavapaiensis* is consistent with other recently characterized inter- and intraspecific host responses to *Bd*. In the tree frog *Litoria verreauxii alpine*, immune gene expression became significantly elevated over time in frogs with higher compared to lower *Bd* susceptibility (Grogan et al., 2018). Our finding that susceptible frogs significantly increased acquired immune gene expression is also consistent with an experimental RNAseq study of *Bd*-susceptible wood frogs (*Rana sylvatica*) compared to *Bd*-resistant bullfrogs (*Rana catesbiana*) (Eskew et al., 2018). In this study, the *Bd*-infected wood frogs significantly increased acquired immune gene expression but had high mortality, whereas *Bd*-infected bullfrogs had no mortality and did not increase acquired immune gene expression (Eskew et al., 2018). From these limited studies, a general pattern of immune responses driving susceptibility may be starting to emerge. However, additional infection experiments across a wider range of host species utilizing consistent *Bd* strains and infection time points are critical to robustly assess whether mechanisms of survival derive from the same types and temporal patterns of immune function.

Our study reinforces the importance of MHC class IIβ genotypes in determining *Bd* susceptibility, but suggests an inverse mechanism to our original hypothesis scenario in which MHC binding of *Bd* epitopes activates acquired immunity and leads to survival (Bataille et al., 2015; Savage & Zamudio 2011). Instead, our study suggests that class IIβ alleles expressed in susceptible individuals may be the alleles that have strong *Bd*-binding efficacy, initiating immune pathways that produce host-damaging effects. This damaging effect of MHC expression appears particularly important at late infection

stages, as our GLMM analysis found that early infection individuals that expressed the classic class IIβ locus had significantly lower *Bd* infection intensity. Although we do not know the ultimate susceptibility of these individuals sampled < 15 DPI, this early pattern of MHC expression associated with managing *Bd* loads, but late pattern of MHC expression corresponding to susceptibility, suggests that initial immune responses based on specific MHC allelic variation may be critical for regulating immunity and determining ultimate disease progression. We hypothesize that damage to the host via sustained immune activation could occur via two mechanisms. First, generating acquired immune responses involves large metabolic costs and produces host-damaging inflammatory byproducts (McDade, Georgiev, & Kuzawa, 2016), limiting the host's ability to direct resources towards innate immunity (Savage et al., 2016; Woodhams et al., 2007) skin sloughing (Ohmer et al., 2017), or other important metabolic processes. Second, the direct destruction of amphibian lymphocytes via immunosuppressive properties of *Bd* cells (Ellison et al., 2017; Fites et al., 2013) exacerbates the energetic toll on the host, which is now producing a costly response rendered ineffective by the pathogen. These two factors combined may contribute to *Bd* susceptibility, whereas class IIβ alleles in *Bd*-tolerant individuals may have conformations that are either unable to bind *Bd* epitopes or that bind *Bd* but only induce regulatory immune pathways (Unanue, Turk, & Neefjes, 2016). Under this scenario, class IIβ alleles that do not promote acquired immunity protect the host from producing wasteful and ineffective responses, leading to a higher chance of surviving *Bd* infection through other behavioral or physiological processes. Our data suggest a high number of putative class IIβ loci that were not detected in previous cDNA analysis (Kiemnec-Tyburczy et al., 2010). Given the likely importance of class IIβ alleles in contributing to *Bd* susceptibility, but the lack of knowledge on typical MHC expression patterns within and among loci in non-model frogs (Rollins-Smith & Woodhams, 2012), future studies should validate the number of MHC loci and their expression patterns using qPCR and more robust sequence capture approaches (e.g., Reed, Mendoza, & Settlage, 2016).

We conclude that the generation of acquired and inflammatory immune responses in the spleen has detrimental consequences for *Bd* survival in *R. yavapaiensis* because individuals with immune gene expression below baseline levels tolerated *Bd* infections while individuals that died from chytridiomycosis had elevated immune gene expression. We hypothesize that anuran immune responses to *Bd* can be characterized by early innate immune responses that are followed by apparently ineffective generation of acquired immune responses in susceptible individuals. These findings prompt numerous unanswered questions, including whether these patterns from *R. yavapaiensis* are consistent across species, *Bd* strains, regions within tissues, and individual immune cells. Future single-cell analyses may be a powerful approach to reveal unique processes among cells, particularly in the heterogeneous landscape of the amphibian skin where *Bd* infections occur. Exploring whether acquired immune responses are also detrimental for *Bd* survival across a wide range of host taxa is an important next step towards understanding how broadly we can generalize about the nature of amphibian *Bd* immune responses. *Bd* immunizations have been proposed and attempted in a number of host anurans, and have successfully produced faster and larger acquired responses (Ellison, Savage, et al., 2014; McMahon et al., 2014), but in most cases these acquired responses have not improved *Bd* survival outcomes (Cashins et al., 2013; Ellison, Savage, et al., 2014; Stice & Briggs, 2010). Our observations suggest that an acquired response in *R. yavapaiensis* is likely to be generally ineffective for promoting *Bd* survival, and further mechanistic studies of genomic, proteomic and immunological responses across susceptible and tolerant species and individuals exposed to *Bd* will further clarify these complex frog-fungal interactions.

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AUTHOR CONTRIBUTIONS

A.E.S., B.G., and R.C.F. conceived and designed the study. A.E.S. and K.H. performed the experiments. A.E.S. analysed the data. A.E.S., B.G., K.H., E.B., and R.C.F. wrote the manuscript.

DATA AVAILABILITY STATEMENT

Illumina RNA-seq raw and assembled sequence data are available in NCBI SRA and TSA archives under BioProject PRJNA636076. *Bd* infection intensity data for experimental infections are available in Dryad, data set https://doi.org/10.5061/dryad.g1jwstqnt.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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