More than Skin Deep: Functional Genomic Basis for Resistance to Amphibian Chytridiomycosis

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Abstract

The amphibian-killing chytrid fungus Batrachochytrium dendrobatidis (Bd) is one of the most generalist pathogens known, capable of infecting hundreds of species globally and causing widespread population declines and extinctions. However, some host species are seemingly unaffected by Bd, tolerating or clearing infection without clinical signs of disease. Variation in host immune responses is commonly evoked for these resistant or tolerant species, yet to date, we have no direct comparison of amphibian species responses to infection at the level of gene expression. In this study, we challenged four Central American frog species that vary in Bd susceptibility, with a sympatric virulent strain of the pathogen. We compared skin and spleen orthologous gene expression using differential expression tests and coexpression gene network analyses. We found that resistant species have reduced skin inflammatory responses and increased expression of genes involved in skin integrity. In contrast, only highly susceptible species exhibited suppression of splenic T-cell genes. We conclude that resistance to chytridiomycosis may be related to a species’ ability to escape the immunosuppressive activity of the fungus. Moreover, our results indicate that within-species differences in splenic proteolytic enzyme gene expression may contribute to intraspecific variation in survival. This first comparison of amphibian functional immunogenomic architecture in response to Bd provides insights into key genetic mechanisms underlying variation in disease outcomes among amphibian species.

Key words: Batrachochytrium dendrobatidis, immunogenomics, comparative transcriptomics, immunosuppression, amphibian immunity.

Introduction

A substantial proportion of pathogens are capable of infecting more than one host species, and of these some are considered “ecological generalists,” which can successfully infect and be transmitted by multiple hosts (Woolhouse et al. 2001). The ability of a pathogen to exploit multiple host species is a key factor in both their epidemiology (Poulin et al. 2011) and evolution (Poulin et al. 2011; Walker et al. 2014). Generalism necessitates functional trade-offs that may limit the fitness of the pathogen in any one particular host (Leggett et al. 2013) and variation in host species abundance, exposure, and susceptibility makes it unlikely that each host species contributes equally to pathogen transmission (Brisson et al. 2008; Cronin et al. 2010; Streicker et al. 2013). Therefore, discovering the mechanisms by which variable host species respond, or not, to infection is a critical step in understanding how generalist emerging pathogens will spread and persist within multihost communities.

Sympatric host species, even within a single community, often exhibit remarkable differences in infection intensity and severity of disease-related clinical signs (Searle, Gervasi, et al. 2011; Johnson et al. 2012). Host life-history traits, such as habitat use and breeding behavior, influence the likelihood of pathogen exposure and undoubtedly play a role in observed species differences in infection (Bancroft et al. 2011; Hoverman et al. 2011). However, exposure alone is unlikely to
account for host species variations in disease outcome, and host functional genomic variation can also be pivotal (Hahn et al. 2013; Sutherland et al. 2014). Likewise, pathogen fitness depends on its ability to evade, exploit, or manipulate host responses (Jiménez-López and Lorenz 2013; Boyett and Hsieh 2014; Cambier et al. 2014). Therefore, if a generalist pathogen differs in its ability to exploit host species, variation in disease outcomes will ultimately result from a complex combination of host traits that mediate exposure, and the functional genetic architecture underlying pathogen and host adaptations.

*Batrachochytrium dendrobatidis* (Bd), a fungus that causes chytridiomycosis, is a remarkable generalist, and has caused widespread amphibian declines and extinctions (Lips 1999; Lips et al. 2006). The fungal pathogen invades the skin of hundreds of amphibian species worldwide and causes death in many hosts (Berger et al. 1998; Longcore et al. 1999; Voyles et al. 2011). Initially, high Bd susceptibility was attributed to a lack of host immune responses to the fungus (Rosenblum et al. 2012), but recent transcriptomic studies revealed that highly susceptible species mount considerable immunogenetic responses, leading to the hypothesis that their immune responses are somehow ineffective against Bd (Ellison et al. 2014). Bd inhibits splenic lymphocyte proliferation and induces apoptosis of T cells in vitro (Fites et al. 2013) and in vivo gene expression supports this mechanism (Ellison et al. 2014). Nonetheless, some species appear to be less affected by Bd, tolerating infections without clinical signs of disease or clearing infections (Lips et al. 2003; Brem and Lips 2008). To date, studies of amphibian immune responses to Bd have focused primarily on highly susceptible species, because those species are of greatest concern in terms of current conservation efforts in the face of chytridiomycosis. However, characterization of immune pathway activation in resistant and tolerant species is essential. By identifying how resistant and susceptible species differ in their response to Bd, we can elucidate the mechanisms underpinning the variation in chytridiomycosis infections and, consequently, population decline among worldwide amphibian fauna.

In this study, we experimentally challenged four Central American frog species with varying Bd susceptibility (*Agalychnis callidryas*, *Atelopus glyphus*, *Atelopus zeteki*, and *Craugastor fitzingeri*) and compared transcriptome-wide gene expression in the skin and spleen of uninfected, infected, and self-cleared frogs. We used a Bd strain, JEL-423, that belongs to the Global Pandemic Lineage (GPL) that swept throughout Central America, devastating amphibian populations (Lips et al. 2008). We chose the four focal species because they are found in Central American tropical upland amphibian communities where Bd-induced declines are most prominent (Lips et al. 2008; Becker and Zamudio 2011) and because these species show marked variation in Bd susceptibility in the field. The Panamanian golden frog, *At. zeteki*, is highly susceptible to Bd, and the species is extinct in the wild (La Marca et al. 2005; Gewin 2008). *Atelopus glyphus* is one of the few species of *Atelopus* (out of 113) that had not experienced dramatic population declines in recent years (La Marca et al. 2005), but is currently listed as critically endangered and at high risk from chytridiomycosis (http://www.iucnredlist.org, last accessed January 2, 2015). *Craugastor fitzingeri* and *A. callidryas* are naturally infected with Bd, generally at low infection intensities (Puschendorf et al. 2006; García-Roa et al. 2014; Rebollar et al. 2014), and have not experienced Bd-induced population declines despite epizootic outbreaks of the pathogen in Central America (Puschendorf et al. 2006; Crawford et al. 2010). Here, for the first time, we use interspecific comparative transcriptomics to characterize and quantify differential gene expression responses to Bd infection in amphibian species with divergent Bd susceptibility. We test the hypothesis that species-specific immunogenomic architecture underlies the broad spectrum of chytridiomycosis susceptibility, tolerance, and resistance in global amphibian populations. To do so, we identify similarities and key differences in host functional genomic profiles and thus define crucial gene expression responses central to amphibians’ successful resistance to the deadly fungal pathogen.

**Materials and Methods**

**Experimental Infections**

The number, source of experimental animals, and Bd challenge assay protocols are summarized in [supplementary table S1, Supplementary Material](https://学术.oup.com/gbe/article-abstract/7/1/286/603062) online. All animals were housed individually throughout the experiment in plastic aquaria, at 18–19 °C, 12:12 light cycle, and fed crickets weekly. Frogs in the infected treatment were challenged with Bd strain JEL-423 (GPL). All animals (controls and Bd challenged) were swabbed once a week to measure the growth rate of Bd (see below). Throughout the experiment, we used a fresh pair of gloves when handling each individual. Frogs were monitored daily for clinical signs of chytridiomycosis and we euthanized those that had lost righting abilities by applying 20% benzocaine to the venter. All other individuals (controls and those showing no clinical signs) were euthanized at the end of the experiments (table 1). Challenge experiments were performed with approval from and in accordance with the ethical standards of the US Institutional Animal Care and Use Committee under protocols 2013-0201-2016 (*At. glyphus*), R-12-98 (*At. zeteki*), and 2013-0401-2016-2 (*A. callidryas, C. fitzingeri*).

For all species, we swabbed the abdomen, drink patch, hands, and feet five times each with a sterile cotton tipped swab weekly, and stored samples in capped tubes (Hyatt et al. 2007). All animals were also swabbed prior to the start of the experiment. We tested swabs for Bd using PrepMan Ultra and DNeasy (Qiagen), running samples in singlicate Taqman quantitative PCR (qPCR) (Boyle et al. 2004). A subset of swabs...
were also run in triplicate to ensure consistency. We ran each plate with JEL-423 standards of 0.1, 1, 10, 100, 1,000, and 10,000 zoospore genomic equivalents (GEs) to determine Bd presence and infection intensity. We categorized individuals as Bd positive when qPCR results showed an infection load greater than or equal to 1 Bd zoospore GEs (Kriger et al. 2006).

Immediately after euthanasia, frogs were dissected using sterilized instruments, and skin (ventral thigh) and spleen tissue samples were harvested from each individual. These tissues were chosen because skin is the primary site of infection for Bd (Longcore et al. 1999) and the spleen is the major lymphoid organ in frogs (Tischendorf 1985). In addition, Bd suppresses splenic leukocytes in susceptible species (Fites et al. 2013; Ellison et al. 2014). Tissue samples were immediately placed in RNA Later (Invitrogen) and stored at −80 °C prior to RNA extraction and library preparation.

Because inoculation doses and timing of tissue harvest differed for our four focal species, we cannot ascertain that all frogs were at the exact same stages of disease progression at the time of sampling. The number of Bd zoospores that infect individuals during inoculations is highly variable, causing natural differences in infection intensities and time to death among individuals and species (Carey et al. 2006). However, the infected frogs used in the transcriptomic analysis in this study had in common that all were at a “mature” infection stage, colonized with mature zoosporangia and shedding zoospores. At the point of tissue sampling, all frogs categorized as infected carried their highest individual Bd load and thus all could be considered still within the disease progression phase (supplementary fig. S1, Supplementary Material online).

Transcriptome Sequencing and Gene Orthology

Total RNA was extracted from each tissue sample separately using RNAAdvance tissue kit (Beckman Coulter, Inc.). We quantified and assessed RNA integrity using a Bioanalyzer 2100 RNA nano assay (Agilent Technologies). Four spleen samples of At. glyphus did not pass RNA integrity quality controls and thus were not sequenced (table 1). All other samples had RNA integrity values above 8.0. Libraries were generated using the Illumina TruSeq RNA sample preparation kit v2 (low throughput protocol) according to the manufacturer’s instructions (Illumina, San Diego, CA). Briefly, 0.4–1.2 μg of RNA was subjected to messenger RNA (mRNA) selection using poly-T oligo-attached magnetic beads followed by chemical fragmentation (6 min, 94 °C). Cleaved RNA fragments were then copied into first strand cDNA using SuperScript II reverse transcriptase (Invitrogen) and Illumina proprietary random hexamer primers. After second strand synthesis using Illumina-supplied consumables, the cDNA was amplified with reagents of the same kit and ligated to barcoded adapters. The final libraries were amplified using 14 PCR cycles. We quantified and assessed library quality on a Bioanalyzer 2100 and, within each species, randomly pooled equimolar samples onto two or three lanes of the Illumina flowcell (8–10 samples per lane). All sequencing runs were 100-bp single-end reads.

After Illumina standard quality control filtering, read quality for each sample was visualized using FastQC version 0.10.0 (Andrews 2010). All samples had higher than average guanine and cytosine content in the first 15 bp due to use of random hexamer primers during library preparation. Therefore, we used Trimmomatic version 0.27 (Lohse et al. 2012) to trim or delete the following: 1) The first 15 bp of each read, 2) any Illumina adapter sequence, 3) the 5’ and/or 3’ end of reads where quality score dropped below Q20, 4) anywhere within each read where a 5-bp window drops below Q20, and 5) any trimmed reads less than 36-bp long. This ensured only the highest quality reads were used for subsequent de novo assembly.

For each species, reads from all individuals and tissues were pooled to assemble a consensus transcriptome. Assemblies were performed using Trinity (Grabherr et al. 2011) with
default parameter settings on a high-performance cluster with 64 central processing units and 512 GB random access memory. We filtered out transcripts with expression support of less than two reads per million mappable reads in at least two samples, to eliminate low-level expression noise (Harrison et al. 2012; Moghadam et al. 2013).

The longest sequence of each Trinity component (roughly equivalent to a single gene) was extracted from the assembly using custom Perl scripts. Next, these sequences (transcripts) were aligned via a local BLASTX to the National Center for Biotechnology Information (NCBI) vertebrate nonredundant (nr) protein database, retaining up to 20 hits with a minimum E-value of 1 × 10⁻⁶ and minimum bit score of 55. Any transcript aligning to the Bd transcriptome (Bd Sequencing Project, Broad Institute of Harvard and MIT, www.broadinstitute.org, last accessed January 2, 2015) was removed from downstream analyses. BLAST2GO version 2.5.0 (www.blast2go.com, last accessed January 2, 2015) was used to functionally annotate the assembled transcriptomes. Gene ontology (GO; www.geneontology.org, last accessed January 2, 2015) 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, a minimum annotation cut-off of 55, and a GO weight of 5. GO annotations were enhanced using the annotation augmentation tool ANNEX (Myhre et al. 2006).

Finally, we performed InterPro (Quevillon et al. 2005) searching the reciprocal best hit between all species pairs were tested, and identifying 1:1 orthologs between the four species. We used BLAST2GO via the InterPro EBI web server and merged InterProScan GOs with the original GO annotations. To assess the completeness of the transcriptomes, we examined the proportion of assembled transcripts that were full length or near full length, based on the NCBI model amphibian (Xenopus tropicalis) reference protein data using Trinity Perl scripts.

We performed a reciprocal best-hit analysis to identify 1:1 orthologs between the four species. We used BLASTN with an E-value threshold of 1 × 10⁻⁶ to search all transcripts from one species against the other three. Only transcripts that were the reciprocal best hit between all species pairs were retained for further analyses.

Gene Expression Analyses

Gene expression levels were determined using the Trinity pipeline; utilizing BWA read mapping (Li and Dubin 2009) and RSEM read count normalization (Li and Dewey 2011). For each species, we analyzed differential gene expression (DGE) of control (uninfected) and infected individual orthologous gene sets using the edgeR (Robinson et al. 2010) R package (R version 2.15.2, R Development Core Team). This consisted of estimating tagwise dispersion and normalization factors and differentially expressed (DE) testing using an exact test. A false discovery rate (FDR)–corrected P value of less than 0.05 was considered to be evidence of DGE. Two species (A. callidryas and At. glyphus) had individuals that were infected during the course of the experiment but cleared infection (swabbed Bd negative) by the end of the trials (table 1, supplementary fig. S1, Supplementary Material online). In these species, DGE tests were performed to compare expression between infected, cleared, and control frogs.

To quantify the overlap of differentially expressed genes between species, we constructed four-way Venn diagrams for each tissue using VENNY (Oliveros 2007), separating significantly increased and decreased expressed genes. We tested for enrichment of biological process GO terms and InterPro protein domains in each group of DE genes (e.g., specific to one species or shared among two or more species) using DAVID (Huang et al. 2008) with all orthologous genes as the background reference.

Weighted Gene Coexpression Network Analyses

Differential gene expression analyses consist of exact tests on each gene separately and thus necessitate stringent multiple test correction methods (e.g., FDR), and typically only genes with the largest differences in expression are identified. An alternative for quantifying systematic transcriptional responses to infection challenge by Bd is weighted gene coexpression network analysis (WGCNA), which identifies networks (modules) of coexpressed genes (i.e., genes that show consistent expression profiles across samples), and thus potentially identifies functionally important genes with only subtle changes in expression that may not be detected in typical DGE analyses. As A. callidryas is the most resistant species in this study, gene modules were defined in this species as the reference.

First, read counts were TMM normalized using a Trinity-provided Perl script to produce fragments per kilobase per million mapped expression values. Next, the R package WGCNA was used for network constructions (Langfelder and Horvath 2008). Briefly, WGCNA constructs networks using the absolute value of the Pearson’s correlation coefficient as the gene coexpression measure, which is raised to a power to create the adjacency matrix. The topological overlap distance calculated from the adjacency matrix is then clustered with the average linkage hierarchical clustering. Our modules were defined using the dynamicCutTree function and TOMType “signed” with a minimum module size of 100. A module eigengene distance threshold of 0.25 was also used to merge highly similar modules. One thousand permutations of randomly sampled modules were generated. Modules were considered robust if average module adjacencies were significantly higher than the randomly generated modules. Modules were then correlated with log-transformed Bd load to identify gene networks significantly involved in responses to Bd infection. GO term and protein domain enrichment tests of each gene module that significantly correlated with Bd load were performed using DAVID as described above.
Each gene within a module was ranked by its module membership (kME), calculated by WGCNA. Network hub genes were defined as those ranked in the top 100 module membership values and with the highest 150 network connection weights. Median log2 fold change of hub genes of each module was calculated for each species. Hub gene network connections were exported to Cytoscape (Shannon et al. 2003) for visualization.

To assess the degree to which *A. callidryas* modules were conserved across the other three species, module preservation statistics were computed using the modulePreservation function (500 permutations) (Langfelder et al. 2011). Network module preservation statistics quantify how density and connectivity patterns of modules defined in a reference data set are preserved in a test data set without the need to define modules in the test data set. A module can be significantly preserved in another species but not necessarily with the same direction of expression.

### Results

#### Experimental Infections

Frogs of all four species were *Bd* negative at the start of the experiments. All control frogs remained *Bd* negative during the course of the experiment except one *A. callidryas* which we removed from subsequent analyses. Sample sizes, time course of experiments, and *Bd* loads are reported in supplementary information and supplementary table S1, Supplementary Material online.

For each species, we followed protocols used previously for *At. zeteki* infections (Ellison et al. 2014). Briefly, all *Bd*-challenged *At. zeteki* (N = 6) were euthanized on exhibiting signs of advanced chytridiomycosis (e.g., loss of righting reflex) between day 22 and 33 postinoculation. The average *Bd* load at death was 6,819,500 zoospore GEs (ZGEs, standard error [SE] = 2,431,929, table 1). Twelve *At. glyptus* were challenged with *Bd*. Two frogs were euthanized prior to the end of the experiment (day 46 and 55) due to severe signs of chytridiomycosis. All other individuals were euthanized on days 60–62 postinoculation. Average *Bd* load of infected *At. glyptus* was 3,353 ZGEs (SE = 1,412). The two individuals that were euthanized early did not possess the highest infection intensities (453 and 8,075, highest = 11,769 ZGEs). Three *Bd*-challenged frogs tested positive during the course of the experiment but had cleared infection by the end of the trial and so were grouped separately for subsequent analyses. Three of the six *A. callidryas* tested positive for *Bd* at the end of the experiment, but with low infection intensities (day 41 postinoculation, average ZGE = 27, SE = 23). The remaining three tested positive during the experiment but had cleared infection by day 41. Infected and cleared frogs were grouped separately for subsequent analyses. All five *Bd*-challenged *C. fitzingeri* tested positive for *Bd* by day 41 (average = 102,998, SE = 57,652).

#### Transcriptome Assemblies and Gene Orthologs

Average Illumina sequencing reads per sample and assembly statistics are summarized in table 1. All raw sequencing reads are deposited in NCBI Sequence Read Archive under submission accession numbers SRP029154 (*At. zeteki*), SRP046003 (*At. glyptus*), SRP045866 (*C. fitzingeri*), and SRP045871 (*A. callidryas*). The *At. zeteki* assembly (40,074 transcripts) had 15,252 sequences (38.1%) with at least one significant hit against the nonredundant NCBI protein database and of these, 12,056 (79.0%) were successfully annotated with GO terms. The *At. glyptus* assembly (34,947 transcripts) had 16,322 with hits (46.7%) and of those 13,409 (82.2%) were annotated. The *C. fitzingeri* assembly (29,257 transcripts) had 14,980 (51.2%) with hits and of those 12,409 (82.8%) were annotated. The *A. callidryas* assembly (36,645 transcripts) had 16,040 (43.8%) with hits and of those 12,929 (80.6%) were annotated. The top BLAST hit for the majority of all sequences (between 72.1% and 76.9%) were to *Xenopus* species. The majority of assembled transcripts (~70% in all species) were considered full length or near full length (~90% coverage) based on matching to *X. tropicalis* reference protein data (supplementary fig. S2, Supplementary Material online). A total of 8,732 annotated sequences had reciprocal best hits in all four species and were deemed gene orthologs.

#### Host Species Overlap of Differentially Expressed Genes

Figure 1A summarizes the number and overlap of genes found to be significantly differentially expressed (DE) between control (uninfected) and infected frogs in each of the four focal species. Figure 1B and C demonstrates overall separation of individual samples based on the entire orthologous gene set. Table 2 summarizes the groups of DE genes that were significantly enriched for GO (classifications of molecular function or biological process) terms. Complete lists of genes with significant differential expression in each species are provided in supplementary materials. For brevity, here we report groups of genes shared or unique to host species deemed as most relevant to *Bd*-infection responses.

We found 251 genes with increased expression in the skin of infected frogs (compared with controls) in both *Atelopus* species. These genes were enriched in 15 GO terms including “response to wounding,” “inflammatory response,” and a number of cell death–related terms. The 277 genes sharing increased expression in the skin of infected *At. zeteki* and *C. fitzingeri* were also enriched for similar inflammatory, wounding response and apoptosis-related terms. These shared genes also included leukocyte-related terms such as “T-cell activation” and “lymphocyte activation.” This gene group was also enriched in immunoglobulin protein domains (supplementary material). A substantial number of genes (259) exhibited significantly increased expression in infected individuals of both *Atelopus* species and *C. fitzingeri*. These were enriched for a wide variety of immune-related terms.
including inflammatory response, “B cell-mediated immunity,” “complement activation,” and “positive regulation of T-cell activation.” The majority of genes exhibiting decreased expression in the skin of infected *A. glyphus* were shared by *A. zeteki* (193) and enriched for terms related to bone development and regionalization.

The small number of genes showing increased expression in infected *A. callidryas* spleen were predominantly unique to the species (44; 80%) and enriched for terms related to bone development and regionalization.

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Cleared Versus Infected Frogs

During the course of *Bd*-challenge experiments, three individuals of *A. callidryas* and *A. glyphus* tested *Bd* positive but...
cleared infection by the end of the experiment. Comparisons of *Bd*-infected and *Bd*-cleared *A. callidryas* skin samples found 49 genes significantly differentially expressed (30 decreased and 19 increased in cleared frogs, relative to infected frogs). Genes with increased expression in the cleared group were enriched for “response to virus” and “defense response.” Cleared versus infected skin comparisons of *A. glyphus* found 69 genes differentially expressed (58 decreased and 11 increased in cleared frogs) but showed no significant GO term enrichment. In the spleen, 133 genes were differentially expressed between *Bd*-cleared and *Bd*-infected *A. callidryas* (30 decreased in cleared frogs, 103 increased). Genes with lower expression in cleared frogs were enriched for 11 GO terms primarily related to T-cell activation and differentiation. Nine genes were differentially expressed between *A. glyphus* cleared and infected frogs (4 increased, 5 decreased) but showed no significant GO term enrichment. No genes with differential expression between cleared and infected groups were shared between the two species. In comparisons of cleared and control frogs, *A. callidryas* showed 44 differentially expressed genes in the skin (cleared; 32 increased, 12 decreased) and six in the spleen (4 increased, 2 decreased). *Atelopus glyphus* cleared-control comparisons found 46 differentially expressed genes in the skin (35 increased, 11 decreased) and 14 in the spleen (10 increased, 4 decreased). No significant enrichment was identified in any cleared-control gene group. Full lists of differentially expressed genes and GO terms are provided in supplementary material.

### Gene Coexpression Module Identification and Preservation

Gene coexpression modules—networks of genes with consistent expression patterns across samples—provide a complementary approach to individual gene DE tests for summarizing large gene expression data sets. In addition, module preservation statistics allow a more quantitative view of how well preserved or divergent changes in gene expression are among species in broad-scale comparative transcriptomic studies such as ours. *Agalychnis callidryas*—the most resistant focal species—was used as a reference. A total of 19 and 7 modules were defined in the skin and spleen gene coexpression networks of *A. callidryas*, respectively. All modules were considered “robust” as permutation testing confirmed that average module adjacency was always greater than the mean of 1,000 random modules of equal size (all modules $P < 0.001$). In addition, one of the skin modules and three of the spleen modules were significantly associated with *Bd* load (table 3).

The skin module SK1 (947 genes, supplementary fig. S4, Supplementary Material online) was positively correlated with *Bd* load ($r = 0.83$, $P = 0.003$) and predominantly enriched for
Table 3
Summary of Gene Coexpression modules defined by WGCNA

| Module | No. of Genes | Bd Load Correlation | P Value | Top Hub Gene | Top 5 Hub Genes<sup>b</sup> | Top 5 Hub Genes<sup>c</sup> |
|-------|--------------|---------------------|---------|--------------|-----------------------------|-----------------------------|
| Skin  | 947          | 0.88                | 9.00 x 10^-4 | +0.08 | Cell adhesion | KTN1, CARM1, DMAP1, COLS1A1, MTMR3 |
|       |              |                     |         |              | 13 (2.17)                  | 6 (6.86)                    |
| Spleen| 1,234        | -0.72               | 0.02    | -0.20        | Immune response | EXO2C, PPP2CA, CNDP2, SECD24D, GCSH |
|       |              |                     |         |              | 2 (2.88)                  | 2 (9.13)                    |
|       | 154          | -0.77               | 0.009   | -0.22        | Immune response | KRT19, FAM82B, LDHA, C1S, HPSE |
|       |              |                     |         |              | 7 (-2.38)                 | 6 (-1.20)                   |
|       | 145          | 0.84                | 0.002   | +1.00        | Regulation of T-cell activation | BACH2, LCK, CCR7, MBP, S1PR4 |
|       |              |                     |         |              | 3 (0.00)                  | 1 (3.59)                    |

Notes.—Full lists of enriched GO terms are provided in supplementary materials. Significant values in italics.

<sup>a</sup>Median log2 fold change of module hub genes (based on top 150 node connections).

<sup>b</sup>Most significantly enriched GO biological process term.

<sup>c</sup>Five most interconnected genes.

<sup>d</sup>Median ranked preservation (Z summary preservation; C21 = weak to moderate preservation, C10 = high preservation).

Discussion

Bd causes chytridiomycosis in hundreds of amphibian species worldwide and is responsible for dramatic population declines and extinctions in many species (Lips et al. 2006; Skerratt et al. 2008). The underlying mechanism(s) for such wide species variation and response to Bd infection is not well understood, although differences in disease outcome are not well characterized in amphibians (Bren et al. 2003; Bren and Lips 2007). Yet some species carry infection with no apparent clinical signs of chytridiomycosis after 41 days postinoculation. Four species present in Central American tropical uplands (A. callidryas, A. glyphus, C. fragilis, and A. callidryas) were challenged with a mildly pathogenic strain (JEL-423) isolated from Panama (Lips et al. 2006). We observed considerable differences in disease expression among species and within a month of inoculation, C. fragilis harbored the next highest infection loads (Table 1) yet did not exhibit the clinical signs of chytridiomycosis after 41 days postinoculation.

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Atelopus glyphus, although cited at high risk of chytridiomycosis (http://www.iucnredlist.org, last accessed January 2, 2015), had relatively low to moderate infections (on the order of tens to thousands of zoospore GE), with only 2 of 12 inoculated exhibiting chytridiomycosis by the end of the experiment. Finally, A. callidryas developed the lowest level infections, with no more than 70 zoospore GE in any infected individual, and no Bd-induced mortality. These findings generally corroborate population changes observed in the field during the wave-like epizootic spread of virulent Bd through Central America (Lips et al. 2008). Atelopus zeteki is the only species in this study to have undergone severe declines (La Marca et al. 2005), while populations of A. callidryas and C. fitzingeri are apparently stable (Puschendorf et al. 2006; Crawford et al. 2010). However, although At. glyphus populations were considered stable (La Marca et al. 2005), Bd has recently been found in this species (unpublished data) and thus considered currently at high risk from chytridiomycosis.

Our aim in this study was to capture gene expression of infected frogs of all species at a broadly comparable point in chytridiomycosis disease progression. However, in addition to species differences in infection intensities (as seen above), the rate of Bd infection progression among and within species may also vary considerably (Carey et al. 2006). Thus, a degree of caution must be used when interpreting comparative gene expression data at a fixed time point such as this.
That said, all infected frogs carried their highest individual load at the time of tissue harvest (supplementary fig. S1, Supplementary Material online) and therefore all species could be considered within the disease progression/growth phase (rather than disease retreat).

The overlap of genes with significantly higher expression in the skin of infected frogs (compared with controls) in both Atelopus species and C. fitzingeri was considerable (fig. 1). The shared increased skin expression gene groups were all enriched for inflammatory response. Inflammatory response genes increased in these species were particularly rich in components of the complement system (e.g., C1QA/B/C, C3, C5AR1, C7, and CFB). Decreased complement activation was observed earlier in infected Rana and Silurana species (Rosenblum et al. 2009, 2012), suggesting complement activity against Bd may be phylogenetically inconsistent. In addition, classic markers of inflammation such as TGF-β, interleukin (IL)-1B, and IL-8 were increased in the skin in C. fitzingeri and Atelopus species (supplementary fig. S7, Supplementary Material online). Curiously, IL-10 and its receptor exhibited increased expression only in the Atelopus species (supplementary fig. S7, Supplementary Material online). Although the anti-inflammatory role of IL-10 is well recognized (Mosser and Zhang 2008), prolonged high concentrations may actually have proinflammatory effects (Lauw et al. 2011). Our results indicate that differences in the relative balance of these cytokines among species in response to Bd infection alters splenic lymphocyte functions (table 3, supplementary fig. S5, Supplementary Material online). This module contains a number of important markers of inflammatory responses, such as complement (C1R, C3, C4A, CFB, CH50), tumor necrosis factor, and interleukins (IL-1RAp, IL-17, IL-10), indicating that, while A. callidryas and C. fitzingeri have a coordinated lowering of expression of certain proinflammatory genes (at least in late stage infections), Atelopus species, in contrast, have increased inflammatory responses in the spleen. The second spleen module (SP2) that negatively correlated with Bd load in A. callidryas was not significantly preserved in C. fitzingeri and At. glyphus, and only weak preservation was observed in At. zeteki; suggesting that genes in this module exhibit decreased coexpression unique to A. callidryas (table 3). Of note, this module includes neutrophil markers (MMP8, CXCR1), proteinase inhibitors (SERPINB6, SERPINF2), genes linked to wound healing processes (HPSE, FAP), and inflammation (CTS, PTAfR) (supplementary fig. S6, Supplementary Material online).

We found remarkable overlap of genes with increased expression in the spleen of A. callidryas individuals that cleared infection in this study and infected At. zeteki from a prior study that had previously been Bd exposed (Ellison et al. 2014). In the earlier study, At. zeteki that survived infection with an attenuated strain of Bd (Langhammer et al. 2013) had highly increased expression of a small set of genes in the spleen when re-exposed to Bd compared with Bd-naïve individuals. A substantial number (38; approximately 37%) of these genes were also more highly expressed in A. callidryas individuals that cleared infection. These were primarily genes of proteolytic enzymes, such as IL-10, which is known to be involved in the regulation of inflammation and immune responses. It is interesting to note that while these genes were expressed at higher levels in infected Atelopus species, they were significantly preserved in all other species; however, both Atelopus species had reversed patterns of expression in infected frogs (compared with controls) in both Atelopus species and C. fitzingeri (supplementary fig. S7, Supplementary Material online). This module contains a number of important markers of inflammatory responses, such as complement (C1R, C3, C4A, CFB, CH50), tumor necrosis factor, and interleukins (IL-1RAp, IL-17, IL-10), indicating that, while A. callidryas and C. fitzingeri have a coordinated lowering of expression of certain proinflammatory genes (at least in late stage infections), Atelopus species, in contrast, have increased inflammatory responses in the spleen. The second spleen module (SP2) that negatively correlated with Bd load in A. callidryas was not significantly preserved in C. fitzingeri and At. glyphus, and only weak preservation was observed in At. zeteki; suggesting that genes in this module exhibit decreased coexpression unique to A. callidryas (table 3). Of note, this module includes neutrophil markers (MMP8, CXCR1), proteinase inhibitors (SERPINB6, SERPINF2), genes linked to wound healing processes (HPSE, FAP), and inflammation (CTS, PTAfR) (supplementary fig. S6, Supplementary Material online).

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enzymes such as chymotrypsins (e.g., CTRL), elastases (e.g., CTRC), and carboxypeptidases (e.g., PCPA1). Chitinase (CHIT1), crucial for the degradation of the main constituent of fungal cell walls, also showed highly elevated expression in both species. These genes could provide important markers of either 1) innate individual differences in ability to cope with Bd infections and/or 2) prior exposure to Bd. Unfortunately, the A. calidryas in this study was captured from wild populations in an area that has been infected with Bd for several years (Woodhams et al. 2008), with unknown infection histories; thus, our data do not allow us to discriminate between the two hypotheses. However, this set of genes clearly warrant rigorous investigation in additional species as they are undoubtedly a key part of the amphibian response to repeated Bd exposure and likely integral to general amphibian defense mechanisms against fungal or other pathogens.

The most striking result of our study was that of the third spleen gene coexpression module that significantly correlated with Bd infections (SP3, table 3). Genes in this module had increased expression in infected A. calidryas, many of which were also detected in single gene DE tests (fig. 2). Coexpression was not well preserved in C. fitzingeri but was significant in both Atelopus species, albeit with substantial decreased gene expression patterns. Crucially, this module is rich in genes implicated in the regulation of T cells, including receptors (e.g., TCRB, CD3E), transcription factors (e.g., RUNX2, BACH2), inducers of activation and/or proliferation (e.g., CCR7, MBP, PRKCQ, IKZF1, CARD11), and signaling/migration pathways (e.g., LCK, S1PR4). Bd can inhibit amphibian splenic T-cell proliferation and induce apoptosis in culture (Fites et al. 2013) and in vivo (Ellison et al. 2014). However, this appears not to be the case for all species; our results indicate that Bd resistance of some hosts may be due in part to their ability to mount T-cell-mediated responses to infection, escaping the immunosuppressive action of the fungal pathogen. Moreover, in susceptible species, suppression appears not to be limited only to T cells, but include a deeper inhibition of T cell activation and signaling pathways.

Comparisons of skin defenses such as anti-microbial peptides (Rollins-Smith, Carey, et al. 2002; Rollins-Smith, Doersam, et al. 2002) and more recently skin microbiota (Harris et al. 2006; Lam et al. 2010; Woodhams et al. 2014) have shown promising correlations with resistance to chytridiomycosis. Undoubtedly, these defense mechanisms play an important role in the likelihood of Bd colonizing amphibian skin. However, on establishment of Bd within epidermal layers, our results indicate that chytridiomycosis resistance runs deeper than skin responses. We show that the spectrum of susceptibility to this fungal pathogen is mediated by a combination of both innate and acquired arms of the amphibian immune system, including the severity of inflammatory response to infection, the ability to maintain skin integrity during infection, and whether splenic lymphocyte activity is suppressed. More resistant species not only have less severe skin inflammation and can boost skin integrity maintenance during infection, but also apparently escape the immunosuppressive actions of Bd and increase splenic lymphocyte activity. Additionally, individual variation in expression of important proteolytic enzymes in the spleen may contribute to within-species differences in ability to reduce Bd infections.

This first comparison of immunogenetic responses of amphibian species with highly divergent susceptibility to chytridiomycosis provides a wealth of data to guide future studies and raises many important hypotheses about immunogenomic differences in amphibians responsible for variation in disease outcomes in nature. Our results also demonstrate the utility of broad-scale comparative functional genomics, providing promising functional gene categories, and even specific candidate genes that merit in-depth study at both species and population level. For example, further targeted studies of T-cell responses in more resistant species may help define a general mechanism of chytridiomycosis resistance. Furthermore, direct quantification of intraspecific splenic proteolytic enzyme activity against Bd is required to provide a causal link to the within-species patterns found in our data. Our results underscore that despite the importance of abiotic environmental factors (Becker and Zamudio 2011) and external skin defenses (Rollins-Smith, Doersam, et al. 2002; Woodhams et al. 2007, 2014) in shaping host response to the amphibian-killing fungus, the functional genetic architecture of a species provides the basis for chytridiomycosis resistance.

Our study has important implications for amphibian conservation efforts. Recent management efforts have focused on controlling exposure (Kriger and Hero 2007; Searle, Biga, et al. 2011), understanding abiotic control of fungal proliferation (Berger et al. 2004; Voyles et al. 2012), and manipulating commensal microbiota (Harris et al. 2008; Muletz et al. 2012; Bletz et al. 2013). We found that resistance to Bd infections varies naturally among species and within species, and that this variation has genomic underpinnings, not exclusively due to abiotic conditions and pathogen exposure levels. These results improve understanding of the functional genomics underlying individual and species differences in response to Bd infection and provides conservation biologists with potential mechanisms that explain variation in population or species declines. Our results offer new possibilities for conservation action, specifically we endorse practices that select for traits that provide resistance against Bd, which would improve the success rates of captive breeding programs and reintroduction programs in areas where Bd is endemic. Adaptive variation in hosts could arise at the molecular level, in genes and their expression, influenced by selection, and by variation in the way genes interact with the environment to produce phenotypes of varying plasticity. Therefore, we recommend that conservation efforts incorporate an evolutionary approach and be aimed at maintaining conditions necessary for natural selection to operate most efficiently on the components of resistance that are inherited.
More than Skin Deep

Supplementary Material
Supplementary figures S1–S7, table S1, and materials are available at Genome Biology and Evolution online (http://www.gbe.oxfordjournals.org).

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