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Comparative study of host response to chytridiomycosis in a susceptible and a resistant toad species

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Abstract

In the past century, recently emerged infectious diseases have become major drivers of species decline and extinction. The fungal disease chytridiomycosis has devastated many amphibian populations and exacerbated the amphibian conservation crisis. Biologists are beginning to understand what host traits contribute to disease susceptibility, but more work is needed to determine why some species succumb to chytridiomycosis while others do not. We conducted an integrative laboratory experiment to examine how two toad species respond to infection with the pathogen *Batrachochytrium dendrobatidis* in a controlled environment. We selected two toad species thought to differ in susceptibility – *Bufo marinus* (an invasive and putatively resistant species) and *Bufo boreas* (an endangered and putatively susceptible species). We measured infection intensity, body weight, histological changes and genomewide gene expression using a custom assay developed from transcriptome sequencing. Our results confirmed that the two species differ in susceptibility with the more susceptible species, *B. boreas*, showing higher infection intensities, loss in body weight, more dramatic histological changes and larger perturbations in gene expression. We found key differences in skin expression responses in multiple pathways including upregulation of skin integrity-related genes in the resistant *B. marinus*. Together, our results show intrinsic differences in host response between related species, which are likely to be important in explaining variation in response to a deadly emerging pathogen in wild populations. Our study also underscores the importance of understanding differences among host species to better predict disease outcomes and reveal generalities about host response to emerging infectious diseases of wildlife.

Keywords: amphibian, *Batrachochytrium dendrobatidis*, chytridiomycosis, comparative transcriptomics, disease biology, emerging infectious disease, host response

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Introduction

Emerging infectious disease (EID) events have increased in incidence and severity over the past several decades (Daszak *et al.* 2000; Jones *et al.* 2008; Fisher *et al.* 2012). The emergence and spread of novel pathogens into naïve host species has led to dramatic population losses in a number of natural systems (e.g. Blehert *et al.* 2009; Wilfert *et al.* 2016). Variation in host response among species can have important impacts on host–pathogen interactions at both individual and population scales. At the individual scale, host response affects susceptibility to infection, pathogen-related pathology and disease outcome (e.g. Hawley & Altizer 2011; James *et al.* 2015). Susceptible hosts are prone to high infection rates, loss in fitness and even mortality, while less susceptible hosts may avoid infection or carry infection but with lower fitness costs (e.g. Scott 1988). Variation in susceptibility can in turn have population-level effects. For example, differences in host response affect epidemic dynamics by influencing pathogen transmission

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rates within and among host species, ultimately driving disease emergence and spread in populations and communities (Dobson 2004; Keesing *et al.* 2006).

Elucidating variation in host response is particularly important for emerging infectious diseases of threatened wildlife species because designing effective interventions (e.g. enhancing host immunity, captive breeding) requires understanding mechanisms of host response in both resistant and susceptible species (Voyles *et al.* 2011; James *et al.* 2015). Chytridiomycosis is a recently emerged disease that affects hundreds of amphibian species around the world (Skerratt *et al.* 2007; Wake & Vredenburg 2008). Caused by the chytrid fungus *Batrachochytrium dendrobatidis* (Bd), chytridiomycosis has led to devastating population crashes in numerous amphibian species (Berger *et al.* 1998; Skerratt *et al.* 2007; Wake & Vredenburg 2008). Chytridiomycosis is a transmissible skin infection and can spread rapidly in host populations, causing high mortality rates (Berger *et al.* 1998; Skerratt *et al.* 2007; Wake & Vredenburg 2008). However, previous work has shown that disease outcome following Bd infection is variable among species. Field and laboratory studies show that species vary in infection rate and intensity, behavioural and physiological effects of infection, survival rate and time to death (Blaustein *et al.* 2005; Woodhams *et al.* 2007; Searle *et al.* 2011; Gervasi *et al.* 2013). For example, North American frog species show differences in infection intensity and mortality dynamics in natural populations (Gervasi *et al.* 2013), and similarly Australian frog species show differences in susceptibility correlated with skin peptide defence effectiveness (Woodhams *et al.* 2007). Determining how different hosts respond to infection is essential for understanding – and ultimately limiting – the devastating effects of Bd.

Variation in susceptibility to Bd among amphibian species has been associated with multiple host traits in previous studies. For example, ecological traits including water dependence and population density can modulate pathogen exposure and transmission risks (Briggs *et al.* 2010; Murray *et al.* 2011). In addition, individual traits (e.g. physiological and immunological responses) can influence tolerance and susceptibility to infection (reviewed in James *et al.* 2015). In fact, variation in intrinsic host traits such as skin structure and immune response has been proposed to play a key role at the interface of host and pathogen processes (van Rooij *et al.* 2012; Gervasi *et al.* 2013; James *et al.* 2015). However, few studies have focused on understanding mechanisms of host response across species that differ in susceptibility under common conditions.

We performed an integrative study of host response in two toad species that differ in susceptibility. Using related species enables a comparative analysis of host response in orthologous genetic and physiological pathways. For our experiment, we selected two bufonid species thought to vary in susceptibility: *Bufo marinus* (Cane Toad) and *Bufo boreas* (Boreal Toad). Note that some authors have suggested alternate nomenclature for these species (i.e. *Rhinella marinus* and *Anaxyrus boreas*, Frost *et al.* 2008), but we retain use of *Bufo* given taxonomic debate over nomenclature and phylogenetic relationships in this group (Pauly *et al.* 2009). Divergence time estimates place the split between these species at ~24.6 million years ago (Garcia-Porta *et al.* 2012). *Bufo marinus* is reported to be resistant to chytridiomycosis as infected individuals exhibit only minor symptoms and suffer a low mortality rate (Fisher & Garner 2007). Further study on *B. marinus* is warranted given its status as an invasive species in parts of Australia (Shine 2010), which may contribute to spreading Bd. In contrast, *B. boreas* typically displays very high susceptibility with a high mortality rate (Muths *et al.* 2003; Carey *et al.* 2006; Pilliod *et al.* 2010). Previous studies on wild *B. boreas*, a native species of grave conservation concern in the Western North America, have documented population crashes that resulted from Bd epidemics (Muths *et al.* 2003; Pilliod *et al.* 2010).

We conducted a controlled laboratory infection experiment in common garden conditions and sequenced transcriptomes to generate resources for a genome-scale study of response to infection. During the course of the experiment, we tracked pathogen infection intensity and body weight and used histology and custom gene expression microarrays to characterize host response. We characterized and compared host response to Bd in the two toad species, and sought to determine potential mechanisms underlying differences in susceptibility.

Materials and methods

Infection experiment procedure

We performed an experimental infection of Bd with *Bufo marinus* and *Bufo boreas* in a controlled laboratory setting. We obtained 20 captive-bred adult *B. marinus* from Carolina Biological Supply (Burlington, NC, USA) and 20 captive-bred adult *B. boreas* from Colorado Division of Parks and Wildlife (CDPW). The mass of *B. marinus* ranged from 53.94 g to 122.20 g with a mean ± SE of 81.72 ± 4.92, and the mass of *B. boreas* ranged from 35.47 g to 56.53 g with a mean ± SE of 45.29 ± 1.24. The *B. marinus* sample included nine male subjects and eleven female subjects, and the *B. boreas* sample included twenty male subjects (only male *B. boreas* were available from the CDPW as 'surplus' subjects). The exact ages of individuals were unknown, but were classified as adults based on mass and the presence of

nuptial pads on male subjects (Scherff-Norris *et al.* 2002). We obtained adult subjects rather than subadults for both species to test host response at a stage where the immune system was fully developed (Rollins-Smith *et al.* 2011). Time and resource constraints precluded us from raising experimental subjects from eggs given the relatively long time of development from egg to adult (sexual maturation) in *B. boreas* (4–5 years) and *B. marinus* (1–2 years) (Lannoo 2005). We received a pathogen-free testing certification for the *B. boreas*, which included a panel of diseases described in the Boreal Toad Husbandry Manual (e.g. red leg syndrome, mycobacteriosis, chytridiomycosis, saprolegniasis) prepared by Colorado Division of Wildlife (Scherff-Norris *et al.* 2002), but we performed a swab-qPCR assay on all toads to ensure that they were all Bd negative prior to the experiment.

The toads were maintained in separate tanks in a dedicated room with temperature at 20 °C, humidity at 50% and 12:12-h light:dark cycle. We used polycarbonate tanks that measured 30 cm L × 20 cm W × 13 cm H with high profile filter top lids that measured 9.5 cm in height (Alternative Design, Siloam Springs, AR, USA). The tanks contained unbleached folded paper towels as substrate and 100 mL Holtfreter's solution to maintain the osmolarity and pH during the experiment (Carey *et al.* 2006). One end of each tank was elevated by 4 cm with a rubber stopper to create an area of dry substrate. We fed the toads vitamin-dusted crickets *ad libitum* twice per week. We cleaned the tanks and changed the water after each feeding. We weighed each toad to an accuracy of 0.01 g prior to starting the experiment to determine baseline body mass. The toads in each species were randomly and evenly divided into control and treatment groups.

Our inoculation protocol consisted of a single dose of Bd zoospores followed by a 24-h incubation period. To inoculate the toads, we pipetted one million Bd zoospores per 40 g of body mass onto the flanks of each toad. We adjusted the inoculation dose by host mass to account for the size difference between the two species. On average, *B. marinus* were inoculated with $2.04 \times 10^6 \pm 0.12$ zoospores (mean ± SE), and *B. boreas* were inoculated with $1.13 \times 10^6 \pm 0.03$ zoospores (mean ± SE), which was comparable to the daily dosage in previous studies on *B. boreas* (Carey *et al.* 2006; Murphy *et al.* 2009). For 24 h, we kept the toads in 950-mL Ziploc polypropylene plastic containers with 10 mL of Holtfreter's solution (pH 6.5) and topped with perforated lids (SC Johnson, Racine, WI, USA). The selected size of the container limited movement for the toads and ensured that the toads were in close contact with the inoculum. After 24 h, we returned the toads to clean, individual tanks.

We used Bd isolate 'JEL275', which was collected from a *B. boreas* individual in Clear Creek, Colorado (Annis *et al.* 2004). No Bd isolates collected from *B. marinus* were available at the time of the experiment. We selected JEL275 as a representative of the 'global pandemic lineage' of Bd, which is a lineage that has a genetic signature of recent spread around the world characterized by low genetic diversity and worldwide distribution (Rosenblum *et al.* 2013). JEL275 was also used in a previous experimental exposure study on *B. boreas* (Carey *et al.* 2006). The closely related isolate JEL274 [collected from the same location as JEL275 (Annis *et al.* 2004) and shown to be closely related to JEL275 based on whole-genome sequencing (Rosenblum *et al.* 2013)] was used in several previous multispecies experiments (e.g. Searle *et al.* 2011; Gervasi *et al.* 2013). The Bd isolate was grown on 1% tryptone agar 100-mm plates for 1 week to generate the inoculum. We prepared the inoculum by first flooding plates with 1 mL of sterilized Holtfreter's solution, then determining zoospore concentration using a haemocytometer and finally adjusting the zoospore concentration to 4×10^6 zoospores per mL. We weighed and swabbed each toad on days 7, 14 and 18 following the inoculation. We used an established qPCR method to estimate pathogen infection intensity (Boyle *et al.* 2004).

We let the experimental infection run until the first *B. boreas* host died on day 18 postinoculation. The *B. boreas* that died attained the highest Bd infection intensity of any toad during the experiment (3.02×10^5 zoospore genomic equivalents). We stopped the experiment at this time point for three reasons: (i) we intended to compare host response between species at a single time point postinoculation; (ii) the increasing infection intensities suggested that additional *B. boreas* would succumb to chytridiomycosis, and downstream gene expression analyses could not be conducted on dead toads; and (iii) enough time had elapsed since inoculation for immune responses to develop. Previous studies on *B. marinus* found that antibody responses to bacterial and viral antigens developed within 14–16 days of immunization (Diener & Nossal 1966; Diener & Marchalonis 1970; Zupanovic *et al.* 1998). At this time point, all toads were sacrificed by decapitation and immediately dissected for tissue preparation of histological and RNA samples.

Infection experiment data analysis

We implemented a linear mixed model to test for differences in infection intensity between species using the R (R Core Team 2015) package 'lme4' (Bates *et al.* 2015). We used the R package 'lmerTest' (Kuznetsova *et al.* 2016) to test for significant effects of the candidate fixed

effect parameters: species, time (number of days since inoculation), sex and respective interaction terms, and one random effect parameter: subject identifier (given that each individual was swabbed at multiple time points). We utilized the 'step' function in the 'lmerTest' R package to perform backward elimination of non-significant candidate parameters using the *F*-test with Satterthwaite's approximation for fixed effects and the likelihood ratio test (LRT) for random effects (Kuznetsova *et al*. 2016). We also used the parametric bootstrap approach with 10 000 iterations to test for the significance of the random effect given the tendency of LRT to be conservative (Faraway 2006). The response variable was infection intensity in units of $\log_{10}$ zoospore equivalents, which was approximately normally distributed after log scaling as determined by the Shapiro–Wilks normality test implemented in R (R Core Team 2015). Control groups were excluded from this model as all unexposed individuals tested negative for Bd infection throughout the experiment.

We used ANOVA to test for differences in weight gain or loss between species with the 'aov' function in R (R Core Team 2015). The model included three predictor variables: species, experimental group (Bd exposure or control) and the interaction term. The response variable was change in body weight over the course of the experiment in units expressed as $\log_2(\text{weight}_{\text{day18}} / \text{weight}_{\text{day0}})$, which provided a comparable metric for between-species comparisons and was approximately normally distributed after log scaling as determined by the Shapiro–Wilks normality test implemented in R (R Core Team 2015). We used Tukey's *post hoc* test to compare weight change between pairs of experimental groups.

Histology

We conducted histological examination of frog ventral integument in control and experimentally infected individuals. To prepare the samples, we fixed freshly dissected skin samples in 4% paraformaldehyde for 24 h and then sunk the samples in 30% sucrose overnight. Two ~10-mm^2 pieces of skin were histologically examined per individual. Sectioning and staining with haematoxylin and eosin was performed by University of California, Davis Comparative Pathology Laboratory. A pathologist examined 10–15 histological sections per piece of skin with 20× magnification light microscopy to identify secondary changes including hyperkeratosis (thickening of the top layer of the epidermis), hyperplasia (increased number of cells), dermatitis (inflammation) and spongiosis (fluid accumulation). The secondary changes were recorded as either focal or multifocal and intensity was given on a nominal scale: minimal, mild, moderate and severe. The presence or absence of Bd in histological sections was recorded along with an approximate infection score ranging from zero to three on an ordinal scale where zero represents no observed Bd thalli and three represents the highest observed Bd thalli infection burden. We calculated the sensitivity and specificity of disease detection via histology by assuming that the qPCR-based results at the final time point represented the true disease status. For each species, histological sections were examined for five control and five Bd-exposed toads.

Transcriptome sequencing and microarray design

We performed transcriptome sequencing of the study species, *B. marinus* and *B. boreas*, because no genomic resources were available and then developed a custom Nimblegen (Madison, WI, USA) gene expression microarray platform. We extracted total RNA from several tissues – liver, skin and spleen – of four individuals in order to maximize transcript discovery. Tissues were preserved by flash freezing in liquid nitrogen immediately after dissection. We used the TRIzol reagent (Invitrogen, Carlsbad, CA, USA) for RNA extraction according to the manufacturer's protocol, and then generated cDNA from each RNA sample using Invitrogen's SuperScript cDNA Synthesis Kit (Invitrogen). For each species, we pooled cDNA samples from two uninfected and two infected individuals for all three tissue types and added MID barcodes to each species. We used the Roche (Basel, Switzerland) 454 sequencing platform to generate sequence data from the barcoded cDNA pool. We used custom scripts to preprocess the raw sequence data. We used the barcodes to separate the sequence data for each species into separate data sets. Then, we cleaned the reads by removing adapters and filtering low-quality reads using lucy with default parameters (Chou & Holmes 2001). We performed *de novo* transcriptome assembly for each species using Newbler (Margulies *et al*. 2006), which yielded 8520 contigs for *B. marinus* and 6095 contigs for *B. boreas*. For the microarray design, we designed probes for contigs in three categories: *B. marinus* -specific contigs, *B. boreas* -specific contigs and *Xenopus tropicalis* transcripts (19 312 transcripts). We used this design strategy to maximize the diversity of contigs on the microarray and leverage the entire available probe area. The *X. tropicalis* transcripts were downloaded from Ensembl database (XTROP v4.2), which also curate gene annotation, and processed to reduce paralogous transcripts by clustering with CD-HIT (parameters: cd-hit-est -T 8 -c .95 -s .95 -n 8 -d 200 -r 1 -g 1) (Li & Godzik 2006). The custom Nimblegen microarray design included 135 200 60-base pair probes (excluding control probes) targeting 33 822 transcript

contigs. Most probesets included four probes (33 780/33 822). We used the 12-plex microarray platform (12 arrays per glass slide) to cost-effectively increase sample size in the experimental design.

Annotation was performed using the blat program to identify *Bufo* contigs with high sequence identity to *X. tropicalis* transcripts (ENSEMBL ASSEMBLY v4.2). We ran blat with translated *Bufo* contigs and translated *X. tropicalis* transcripts (parameters: -q = dnax –t = dnax) in order to find orthologous sequences at the protein level, given that protein sequences are more conserved than nucleotide sequences for divergent species. We parsed blat hits to remove short hits (<100 bp aligned to query) and low scoring hits (Expectation value >1.0e-12). We used the Ensembl Gene Identifier (e.g. ENSXETG00000XXXXXX) for the blat hit with longest alignment to the query as the final annotation call. We then imported annotation metadata with the biomart.org pipeline: Ensembl gene name and description, Gene Ontology (GO) terms, Ensembl protein families, Interpro protein domains. For both species combined, 65.4% (9565/14615; *B. marinus*: 64.4% – 5485/8520; *B. boreas* 66.9% – 4080/6095) contigs were annotated with a *X. tropicalis* Gene Identifier. Of the annotated contigs, 86.7% (8287/9565) contained at least one GO term. Next, we used the blast2go pipeline to annotate *Bufo* contigs without GO term metadata (6328 contigs). We used the blastx method with the NCBI nonredundant protein database to identify blast hits, and proceeded with blast2go pipeline with default settings (Conesa *et al.* 2005).

Gene expression microarray preprocessing

We processed 72 tissue samples in total: six biological replicates, three tissue types, two treatment groups and two host species. Briefly, we extracted total RNA from flash-frozen tissues samples with a standard TRIzol protocol and assessed RNA quality with a Bioanalyzer 2100 Total RNA Pico assay (minimum RIN score = 8.0) (Agilent, Santa Clara, CA, USA). Downstream processing was performed by University of Idaho Genomic Resources Core and followed Nimblegen standard protocols: double-stranded cDNA synthesis, Cy3 fluorescent dye labelling, sample array hybridization and data collection via fluorescence imaging. We visually assessed array images for fluorescence artefacts. Raw fluorescence intensities were extracted with NIMBLESCAN v2.5. Downstream analyses were performed using Bioconductor microarray analysis packages in R. We used the R package 'pdInfoBuilder' to construct a microarray design package for our custom microarray (Falcon *et al.* 2015). We assessed raw data quality for each array by graphing intensity distributions, boxplots and hierarchical clustering. In addition, we used the quality control pipeline in the R package 'arrayQualityMetrics' (Kauffmann *et al.* 2009). Based on the outlier analyses (i.e. principal component analysis, global expression boxplot), we excluded one control group *B. marinus* individual, which we later determined to have an intradermal nematode infection based on histological analysis. For analyses in each species, we filtered out off-species probesets with redundant annotations as determined by *Xenopus* Ensembl Gene Identifier. For example, *B. marinus* analyses excluded redundant *B. boreas* and *X. tropicalis* probesets, but included uniquely annotated probesets from all three source species in order to maximize functional diversity of probesets in the analyses. We used RMA preprocessing algorithm with default parameters in the 'oligo' R package to perform three steps: background correction and probe-level normalization for each array, and probeset summarization via median polish (Carvalho & Irizarry 2010). To limit the number of probesets in the statistical analyses, we nonspecifically filtered out probesets where interquartile range <0.5, which contain relatively low variability across all samples.

Gene expression microarray analysis

We analysed gene expression responses to infection in three tissue types (ventral skin, liver and spleen) using a custom microarray at the final sampling point – day 18. We conducted separate statistical analyses for each species and for each tissue type. In each statistical analysis, we assessed differential expression between the Bd-exposed and control groups, thus examining the response to infection in the Bd-exposed group. We conducted analyses at the two levels, probeset and gene set, which are analogous to gene and pathway levels, respectively. First, we statistically tested for differential expression with the R package 'limma', which implements a linear model with an empirical Bayes adjustment to the variances (Ritchie *et al.* 2015). We used the microarray slide ID as a blocking factor as samples for each tissue were randomly placed in arrays across two glass slides. We also repeated the analysis for *B. marinus* with added factors: sex and the interaction of sex and treatment. This was not necessary for *B. boreas* as all subjects were male. We controlled for the expected false discovery rate using the Benjamini and Hochberg (BH) method for multiple tests (Benjamini & Hochberg 1995). We initially considered probesets to be significantly differentially expressed with BH-adjusted *P*-value <0.05 for both species. However, few probesets were differentially expressed at this level for *B. marinus*, so we also considered a less stringent cut-off of $P < 0.1$ for this species. Given the increased potential for false

positives with a less stringent cut-off, we are particularly cautious in interpreting the $P < 0.1$ results. It is important to note that results from the probeset analysis were cross-validated with the gene set analysis described below, which increases confidence in the robustness of our findings. We functionally profiled the differentially expressed probesets using GOstats R package to identify overrepresented GO terms in probeset annotations (Falcon & Gentleman 2007), and reduced redundancy and overlap in gene sets using Revigo (Supek *et al.* 2011).

Our second level of differential expression – gene set – examined pathway-level expression changes. We used GO term annotations to define gene sets in the Biological Process category of the GO framework. We estimated the response to infection at the gene set level using the Gene Set Variation Analysis (GSVA) methodology (Hänzelmann *et al.* 2013). GSVA produces gene set expressions scores, which summarize the expression level for each gene set in each sample. Expression scores are then used in the same differential expression framework as above using Bd exposure as the main factor with microarray slide as the blocking factor. For analyses in both species, we defined differential expression as gene sets with BH-adjusted *P*-value <0.05. We reduced redundancy and overlap in differentially expressed gene sets using Revigo (Supek *et al.* 2011). We found concordant results when analysing the data set with the GSVA method as compared to the probeset-level analysis. With the GSVA approach, we found differential expression of the same or related gene sets as those described above in the GOstats enrichment test (Table 1). This observation qualitatively validates our use of a less stringent *P*-value cut-off (BH-corrected *P*-value = 0.1) in the probeset-level analysis for *B. marinus*.

Cross-species comparison

We compared response to infection ($\log_2$ fold change [FC] ratios) between the two species using linear

Table 1 Summary of gene expression results from GO stats enrichment analysis

Group	No. of DE probesets*	No. of enriched GO Terms†	Selected enriched GO Terms
Skin: Upregulated			
Bufo marinus	487	66	Epidermis development; wound healing; cell proliferation; apoptotic signalling pathway; response to stress; metabolic process; biological adhesion; immune system development
Bufo boreas	1108	68	Regulation of complement activation; response to stress; wound healing; cell redox homeostasis; response to external stimulus; response to yeast; haematopoietic or lymphoid organ development; leucocyte migration; apoptotic process; cellular metabolic process; innate immune response; coagulation
Skin: Downregulated			
B. marinus	70	2	Cellular localization; metabolic process
B. boreas	1055	58	Collagen catabolic process; extracellular structure organization; blood vessel development; response to wounding; haemostasis; cell-matrix adhesion; tissue development; epithelium development; actin cytoskeleton organization
Liver: Upregulated			
B. marinus	0	NA	—
B. boreas	1947	70	Vesicle-mediated transport; protein folding; regulation of cell cycle; cellular metabolic process; RNA processing; cellular respiration; cell proliferation; response to stress
Liver: Downregulated			
B. marinus	2	NA	—
B. boreas	951	74	Actin filament organization; wound healing; response to stress; immune system process; complement activation; antigen processing and presentation of peptide antigen; innate immune response; blood coagulation; blood vessel morphogenesis
Spleen: Upregulated			
B. marinus	0	NA	—
B. boreas	0	NA	—
Spleen: Downregulated			
B. marinus	0	NA	—
B. boreas	3	NA	—

*Differential expression (DE) threshold criteria for *B. marinus* was BH-corrected *P*-value < 0.1; and for *B. boreas* was BH-corrected *P*-value < 0.05.

†List of 'Biological Process' category GO terms was reduced using Revigo to remove semantic redundancies.

regression in the three tissue types. We used the Revigo-filtered gene set-level $\log_2$ FC ratios from the GSVA method described above. We then regressed gene set $\log_2$ fold change ratios using a linear model: *B. boreas* $\log_2$ FC ~ *B. marinus* $\log_2$ FC. We repeated this analysis for 1000 iterations using permuted sample labels and recorded the regression slope at each iteration. We calculated the *P*-value as the number of permutation regression slope values greater than the test regression slope value (slope = −0.369) divided by 1000.

Results

Infection intensity and body mass dynamics

Bufo boreas experienced higher infection intensities than *Bufo marinus* through the 18-day course of the experiment, which confirmed differential susceptibility between these species under common garden conditions. All *B. boreas* tested positive for Bd infection at all three sampling points after inoculation, whereas eight of ten *B. marinus* tested positive by the final sampling point (Fig. 1). In the backward elimination process for the mixed model, we found that fixed effect parameters – species and time – yielded significant *P*-values

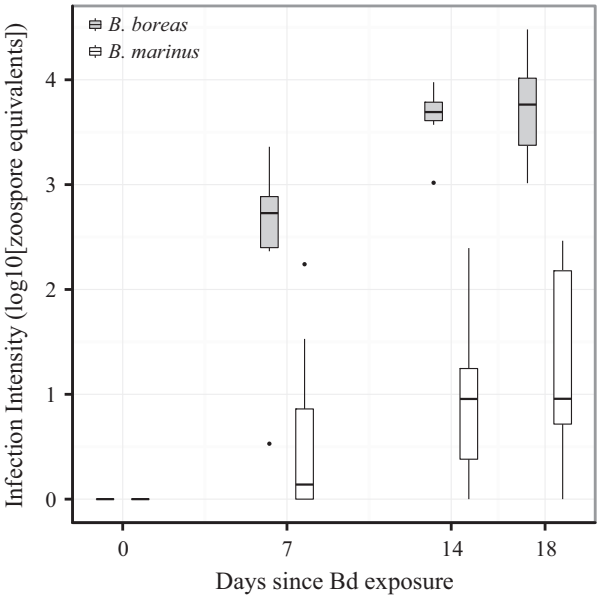

Fig. 1 Time course of Bd infection intensities in *Bufo boreas* and *Bufo marinus*. Boxplots of infection intensities are in units of $\log_{10}$ zoospore equivalents measured via qPCR on days 7, 14 and 18 after Bd exposure. Results are shown for only the Bd-exposed groups. All *B. boreas* tested positive for Bd infection at all three sampling points after Bd exposure, and eight of ten *B. marinus* tested positive at the final sampling point. Black dots represent outliers.

($P < 1 \times 10^{-7}$) from F-tests in model comparisons where each parameter was sequentially removed. The interaction term for species and time was not significant in the *F*-test ($P = 0.156$) and therefore was left out of the final model. The random effect parameter subject identifier had a marginally significant effect from the LRT ($P = 0.065$) and significant effect from the parametric bootstrap test ($P = 0.003$) and therefore was retained. The final model included two fixed effect parameters, species and time, and one random effect parameter, subject identifier. From the final mixed effect model, we found that species identity affected Bd infection intensity ($X^2(1) = 39.510$, $P < 0.0001$), increasing it by 2.38 ± 0.21 $\log_{10}$ zoospore equivalent units (ZE) in *B. boreas* relative to *B. marinus*. At the final time point (day 18), the *B. marinus* infection intensities ranged from 0.00 to 2.46 $\log_{10}$ ZE with a mean ± SE of 1.26 ± 0.30 $\log_{10}$ ZE; while *B. boreas* infection intensities ranged from 3.01 to 4.48 $\log_{10}$ ZE with a mean ± SE of 3.73 ± 0.14 $\log_{10}$ ZE.

The different body weight trajectories experienced between species also indicates differences in susceptibility. From the ANOVA, we found that the interaction of Bd exposure and species affected change in body weight ($F(1, 36) = 20.9$, $P = 5.3 \times 10^{-5}$). Post hoc Tukey tests confirmed that weight change for Bd-exposed *B. boreas* was significantly different than control *B. boreas* and both *B. marinus* experimental groups ($P < 0.0001$). The weight loss effect for Bd-exposed *B. boreas* is illustrated in Fig. 2, where only Bd-exposed *B. boreas* showed a decrease in body weight from day 0 pre-inoculation to day 18 postinoculation with a mean ± SE of -0.22 ± 0.05 $\log_2$ units (or $-13.5 \pm 2.88\%$). Sex did not have a significant effect on Bd infection intensity ($P = 0.25$) nor weight change ($P = 0.45$) in *B. marinus*.

Histological findings

Histological examination of ventral skin sections revealed more dramatic effects of infection on *B. boreas* than *B. marinus* (Fig. 3). Bd-exposed *B. boreas* samples contained a higher incidence of epidermal hyperplasia (increased number of cells) (60%, Fig. 3e) compared with exposed *B. marinus* (0%). While both species experienced parakeratotic hyperkeratosis (thickening of the epidermis), the extent of the change tended to be higher in *B. boreas*. Most *B. marinus* (80%, Fig. 3c) experienced minimal hyperkeratosis, while most *B. boreas* (80%, Fig. 3f) experienced mild hyperkeratosis with one individual achieving moderate hyperkeratosis (Fig. 3e) (Table S1, Supporting information). In addition, spongiosis (intercellular oedema) only occurred in exposed *B. boreas*, at mild to moderate levels (Fig. 3e–f).

Interestingly, both species showed minimal to mild evidence of chronic lymphoplasmacytic dermatitis (i.e. elevated numbers of lymphocytes and plasma cells, Clarke *et al*. 2014) in a subset of exposed and control individuals (Fig. 3a,d). The sensitivity of disease detection via histology was 77.8% (two false negatives occurred for the two lowest qPCR infection intensities), and specificity was 100% (0 false positives). The correlation between the histology disease scores and qPCR infection intensities ($\log_{10}$ scaled) across all individuals was 0.77 (Pearson's correlation coefficient). Overall, we observed sharp differences between species with *B. boreas* experiencing higher intensities of histological changes in Bd-exposed individuals than *B. marinus*.

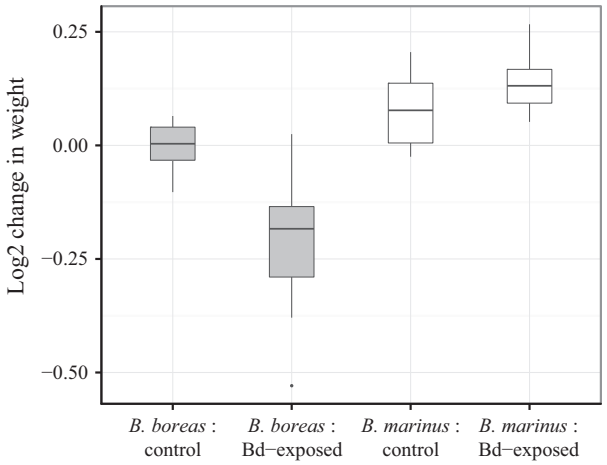

Fig. 2 Effect of Bd exposure on change in body weight. Boxplot of change in body weight in units of $\log_2(\text{weight}_{\text{day18}} / \text{weight}_{\text{day0}})$ for Bd-exposed and control groups for both species. Black dots represent outliers.

Broadscale gene expression patterns

We measured gene expression changes in Bd-exposed individuals using a custom microarray developed from newly sequenced transcriptomes. Our host response analysis revealed a relatively weak transcriptional response to Bd in *B. marinus* and strong response in *B. boreas* (Fig. 4, full results for skin, liver, spleen data sets in Table S2, Supporting information). Bd-exposed *B. boreas* experienced major gene expression perturbations in the skin and liver, while Bd-exposed *B. marinus* experienced fewer and weaker gene expression changes only in the skin. Again, sex did not have a significant effect on gene expression in our analysis (zero genes were differentially expressed between the sexes for control or Bd-exposed individuals).

In the skin data set, we observed contrasting expression responses to infection at the gene set level. From the GSVA analysis comparing expression responses between species, we found a marginally significant negative association in gene set log ratios using a permutation test (regression slope = −0.369, $P = 0.061$, Fig. 5).

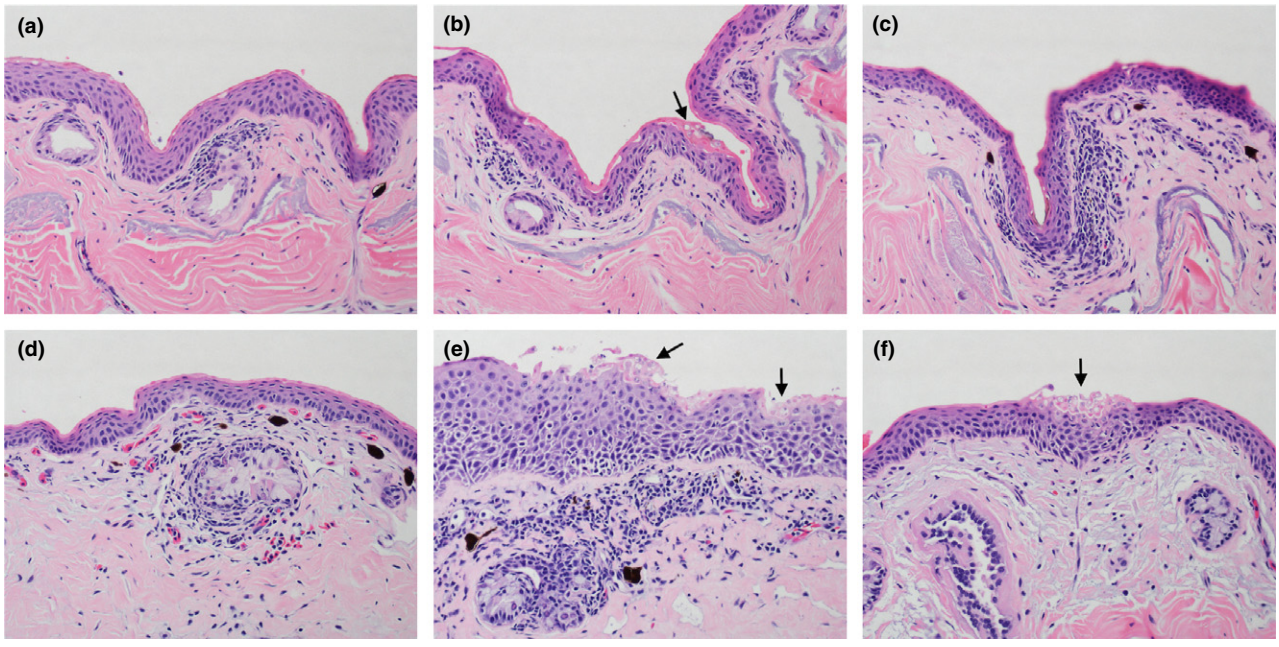

Fig. 3 Histology micrographs at 20× magnification showing stained skin sections. (a) *Bufo marinus* control group, (b, c) *B. marinus* Bd-exposed group, (d) *Bufo boreas* control group and (e, f) *B. boreas* Bd-exposed group. The epidermis is oriented upward in each panel. Arrows indicate sites of Bd infection. [Colour figure can be viewed at wileyonlinelibrary.com].

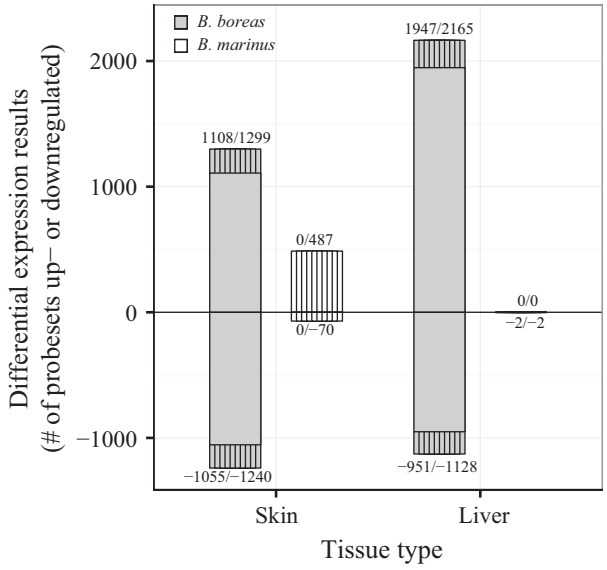

Fig. 4 Summary of gene expression microarray results for skin, liver and spleen in both species. Bars show number of differentially expressed probesets after correcting of multiple tests with Benjamini–Hochberg *P*-value adjustment at alpha = 0.05 (open bar) and alpha = 0.1 (vertical lines within bar). Positive values represent upregulated probesets in the Bd-exposed group relative to the control group, while negative values represent downregulated probesets. Values are also given above and below bars for alpha = 0.05 and alpha = 0.1, respectively. We include spleen results here to highlight the unexpected finding that very few genes were differentially expressed in the spleen.

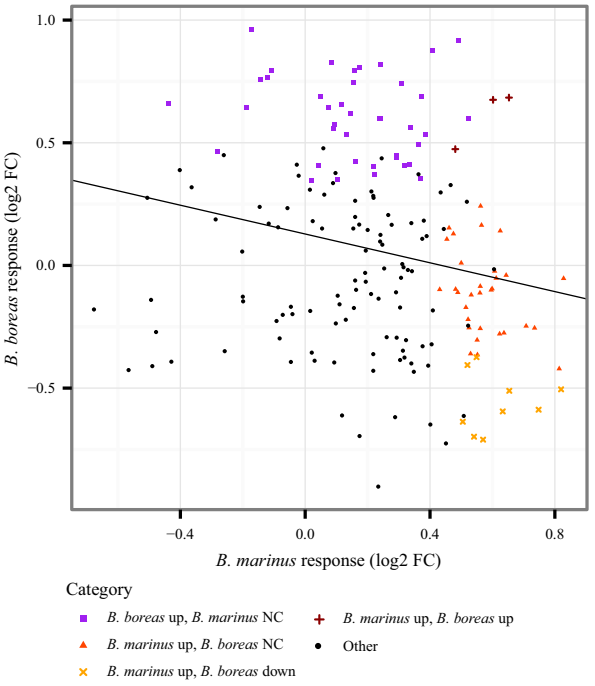

Fig. 5 Comparison of gene set-level differential expression in the skin between species. Points represent $\log_2$ fold change ratios for gene sets and are coloured by differential expression category as defined in the legend. For both species, we defined differential expression (up- or downregulated) as gene sets with BH-adjusted *P*-value <0.05. NC: no change in expression; $\log_2$ FC: $\log_2$ fold change ratios. [Colour figure can be viewed at wileyonlinelibrary.com].

This pattern suggests that gene sets tended to be regulated in discordant directions between the two species in response to infection in the skin. We did not observe any trends for the liver and spleen data sets, which suggests that a general host response was not shared in these tissues either. Below, we detail the gene expression results in the three focal tissues.

Gene expression patterns in the skin

At the site of infection, we found weak expression changes for *B. marinus* and massive perturbation in gene expression for *B. boreas*. In the putatively resistant *B. marinus*, we identified differential expression in pathways that are likely linked to response to low-level infection. Our GOstats tests for gene set enrichment included 487 upregulated and 70 downregulated probesets, respectively. In the set of upregulated probesets, we identified enriched GO terms related to skin structure maintenance and remodelling: 'wound healing', 'epidermis development' and 'biological adhesion' (Table 1). Relevant upregulated probesets include integrin beta 3, tropomyosin 3, alpha-2-macroglobulin, coagulation factor 9 and collagen. We also identified enriched GO term 'immune system development' (Table 1), which contained immunomodulatory genes (e.g. interferon regulatory factor 4 and zinc finger protein 36) that tend to have regulatory effects on immune response. However, in individual probeset tests, we found that three complement pathway probesets (complement c3, c8, c9) were upregulated indicating some evidence of a weak innate inflammatory response, although the entire gene set was not found to be enriched using GOstats. We also found upregulation of stress-related pathways based on enrichment of GO terms 'response to stress' and 'apoptotic signalling pathway'. Finally, the small number of downregulated probesets was enriched for metabolism-related gene sets (Table 1).

In the susceptible *B. boreas*, infected individuals experienced a relatively large degree of gene expression perturbations in the skin. At the probeset level, we found 1108 upregulated and 1055 downregulated probesets in the Bd-exposed group. From the GOstats analysis, a large number of gene sets were significantly enriched in these tissues. Notably, upregulated probesets in the skin were enriched for immune- and stress-related gene sets. We found evidence of an innate immune response

Table 2 Between-species comparative analysis of gene set expression patterns in the skin

Differential expression directions	GO term	GO description
Bufo marinus up; *Bufo boreas* down	GO:0008104	Protein localization
	GO:0006139	Nucleobase-containing compound metabolic process
	GO:0001889	Liver development
	GO:0031290	Retinal ganglion cell axon guidance
	GO:0001501	Skeletal system development
	GO:0060070	Canonical Wnt signalling pathway
	GO:0007160	Cell-matrix adhesion
	GO:0009887	Organ morphogenesis
	GO:0030866	Cortical actin cytoskeleton organization
	GO:0007015	Actin filament organization
	GO:0048699	Generation of neurons
	GO:0001843	Neural tube closure
	GO:0061314	Notch signalling involved in heart development
B. marinus up; *B. boreas* up	GO:0016043	Cellular component organization
	GO:0016570	Histone modification
	GO:0019827	Stem cell maintenance
	GO:0048583	Regulation of response to stimulus
B. marinus down; *B. boreas* up	GO:0022904	Respiratory electron transport chain

supported by enrichment of gene sets: 'regulation of complement activation', 'innate immune response', 'response to external stimulus' and 'response to yeast'. Relevant upregulated probesets included several classical complement pathway genes (components b, 2, 3, 4 and 7), Toll-like receptor 5 and tumour necrosis factor-alpha pathway genes. Other enriched gene sets were associated with stress: 'response to stress' and 'cell redox homeostasis'. This analysis found some parallels with the *B. marinus* response to infection: wound healing, response to stress. With respect to downregulated probesets, we found enrichment in several gene sets related to skin structure maintenance: 'extracellular structure organization', 'blood vessel development', 'cell-matrix adhesion' and 'epithelium development'. This pattern involved downregulation of important structural proteins including 29 collagen probesets and four keratin probesets.

In our cross-species gene expression analysis, described above, we found gene sets with discordant and concordant expression patterns between species (Table 2). Gene sets that were upregulated in *B. marinus* and downregulated in *B. boreas* included skin integrity-related pathways such as 'cell-matrix adhesion' and 'cortical actin cytoskeleton organization'. The between-species comparison also uncovered pathways with concordant expression patterns, which point towards shared responses despite the general negative association. The four gene sets that were upregulated in both species included 'cellular component organization', 'histone modification', 'stem cell maintenance' and 'regulation of response to stimulus'.

Gene expression patterns in the liver

We found that infection status had little detectable impact on gene expression in the liver tissues of Bd-exposed *B. marinus*, and a large impact on gene expression for *B. boreas*. For the resistant *B. marinus*, we observed zero upregulated probesets and only two downregulated probesets (Table 1), which were too few to conduct a GOstats enrichment test, and similarly zero gene sets were differentially expressed based on the GSVA analysis at BH-corrected $P < 0.05$. The two downregulated probesets were annotated as MHC class 2 probesets (part of the antigen processing and presentation process, Du Pasquier & Flajnik 1990).

We detected a large number of differentially expressed probesets in the susceptible *B. boreas* liver samples: 1947 upregulated and 951 downregulated probesets in the Bd-exposed group. We found substantially different expression patterns in liver tissue compared with skin. In the liver analysis, the list of downregulated probesets was enriched for immune- and defence-related gene sets, whereas immune-related gene sets were upregulated in the skin (Table 1). Downregulated gene sets included: 'positive regulation of T-cell-mediated cytotoxicity', 'antigen processing and presentation', 'defence response', 'immune response'. Relevant downregulated probesets from these gene sets

included several complement pathway genes, MHC class 1a and class 2, antibody receptor epsilon and tapasin. Multiple stress-related gene sets were upregulated including 'response to stress' and 'cell redox homeostasis'. We also observed upregulation in several gene sets that typically have coordinated expression profiles with the stress response including 'regulation of cell cycle', 'cellular metabolic process' and 'cellular respiration' (Table 1).

Gene expression patterns in the spleen

Finally, we found no differential expression activity for *B. marinus* and a very low level of differential expression in the *B. boreas* spleen data set (only three upregulated probesets). Notably, one of the upregulated probesets is annotated as an Fc receptor-like 2, which has potential immunomodulatory effects on B-cell development (Jackson *et al.* 2010). Given previous reports on the negative effects of Bd on splenocyte proliferation (Fites *et al.* 2013), we further examined the GSVA results for *B. boreas* at the uncorrected *P*-value level (i.e. without correction for multiple tests). Interestingly, we found that two of four downregulated gene sets were related to immune cell processes: 'positive regulation of T-cell-mediated cytotoxicity' (uncorrected $P = 0.01$) and 'B-cell receptor signalling' (uncorrected $P = 0.02$).

Discussion

Differential susceptibility between species

Studying variation in host response is critical for gaining a better understanding of the epidemiology of emerging infectious diseases. In the amphibian chytridiomycosis system, host species exhibit a range of outcomes following Bd infection (Berger *et al.* 2010; Voyles *et al.* 2011; McMahon *et al.* 2014), and uncovering mechanisms of susceptibility is important for both empirical studies and applied conservation. We used an integrative approach to examine disease progression and host response in two toad species that exhibit different responses to Bd. The focal species are of interest due to their endangered status (*Bufo boreas*) or status as an invasive species (*Bufo marinus*) and their difference in predicted susceptibility. Although previous studies have documented susceptibility of *B. boreas* (Muths *et al.* 2003; Carey *et al.* 2006; Murphy *et al.* 2009; Pilliod *et al.* 2010), the comparative framework used here allowed us to evaluate susceptibility of *B. boreas* and resistance of *B. marinus* simultaneously and more mechanistically. Integrating multiple data sets (e.g. disease load, body condition, skin histology and gene expression) allowed us to characterize the nature of differential host susceptibility in these two species. Below, we discuss the potential mechanisms of resistance in *B. marinus* (which exhibits only a local response at the site of infection) and susceptibility in *B. boreas* (which exhibits both local and systemic effects of Bd infection). We discuss our findings in the context of previous Bd infection studies on phylogenetically divergent species and the broader emerging infectious disease literature.

Our findings of susceptibility to Bd in *B. boreas* and resistance to Bd in *B. marinus* provide a possible explanation of epidemiological patterns for these species in nature. All of the Bd-exposed *B. boreas* in our experiment became infected with a rapid increase in infection intensity over several weeks, whereas only 80% of *B. marinus* became infected at much lower infection intensities (Fig. 1). Carey *et al.* (2006) also documented high susceptibility to chytridiomycosis in *B. boreas* with high mortality of juveniles between 15 and 25 days after inoculation (Carey *et al.* 2006). Previous field studies showed that wild populations of *B. boreas* can harbour high Bd prevalence over multiple years (Pilliod *et al.* 2010) and demonstrated a proximate role of Bd in population declines in this species (Muths *et al.* 2003). For *B. marinus*, there is a relative dearth of published studies on chytridiomycosis effects despite this species potential importance as a 'spreader' of Bd (Fisher & Garner 2007). While one study showed that captive *B. marinus* tadpoles experienced a moderate mortality rate (three of eight) with Bd infection after metamorphosis (Berger *et al.* 1998), there are no published reports on Bd-caused population die-offs of *B. marinus* in the wild.

Body condition deteriorated with Bd infection for *B. boreas* (but not for *B. marinus*). Loss of weight has been documented with infection in other species (*Pseudacris triseriata*, Retallick & Miera 2007; *Rana muscosa*, Harris *et al.* 2009), but not in all susceptible species (Voyles *et al.* 2009) suggesting variation in body condition effects among susceptible species. Our histological results were generally consistent with our qPCR-estimated infection intensities, with high infection intensities in *B. boreas* but not in *B. marinus*. We found several important histological differences between species. First, *B. boreas* samples contained generally higher degrees of epidermal keratosis than *B. marinus* samples. The highest degree of epidermal hyperkeratosis was observed in the *B. boreas* individual with the highest infection intensity (>10 000 zoospore equivalents, Table S1, Supporting information). Second, we documented spongiosis in Bd-exposed *B. boreas* toads, but not in *B. marinus*. Our result that increased infection intensities were associated with increased spongiosis provides increased generality to results from previous

studies, which have found spongiosis and hyperkeratosis to be a common feature of Bd infection (Berger *et al.* 1998, 2005; Carey *et al.* 2006; Voyles *et al.* 2011). Together, the differences in infection intensities, body condition and histological effects provide a robust and multifaceted characterization of the differential susceptibility between *B. marinus* and *B. boreas*.

Gene expression responses in the skin

Our skin expression analysis revealed a trend of contrasting responses between the susceptible and resistant species at the gene set level. The host response in the skin is critically important because the skin is the site of infection and because Bd is known to disrupt skin functions including osmoregulation and electrolyte balance (Voyles *et al.* 2009; Marcum *et al.* 2010). Maintaining skin integrity during infection is important for physiological homeostasis and coping with Bd infection (Voyles *et al.* 2009). We found discordant gene expression patterns between species for skin integrity pathway genes (e.g. 'cell-matrix adhesion' gene set). Our findings provide increased generality when integrated with results from previous studies in other phylogenetically divergent host species. For example, Ellison *et al.* (2015) found that the gene coexpression module enriched for 'cell adhesion' was upregulated in the most resistant species (*Agalychnis callidryas*) in the experiment and downregulated in the three more susceptible species (*Atelopus zeteki*, *Atelopus glyphus* and *Craugastor fitzingeri*). Rosenblum *et al.* (2012) also found the downregulation of skin integrity genes during Bd infection in two highly susceptible species (*R. muscosa* and *Rana sierrae*) including several keratin and collagen genes. Likewise, we observed downregulation of 29 collagen probesets and four keratin probesets in *B. boreas* skin. The regulation of skin integrity pathways appears to be an important marker of infection outcome, and more work is needed to reveal whether the (dys)regulation is a cause or consequence of susceptibility.

Our study also uncovered pathways with concordant expression patterns between species, which may play a shared or general role in host response. We observed notable shared expression changes in both the susceptible and resistant species for gene sets related to response to stimulus and skin repair, specifically wound healing and apoptosis. These pathways were also upregulated in three of four tropical amphibian species (*A. zeteki*, *A. glyphus* and *C. fitzingeri*) in a recent study (Ellison *et al.* 2015). Thus, genes involved in regulating response to stimuli (e.g. alpha-2-macroglobulin, TNF-R12) may represent a common response to Bd at the site of infection. Similarly, the coordinated upregulation of wound healing and apoptosis genes are likely to be linked to coping with infection given the importance of these pathways in skin repair. Finally, both the susceptible and resistant species in our study responded to infection with upregulation of stress-related pathways. Previous studies focused primarily on susceptible species have documented induction of host stress response with Bd infection, with evidence from gene expression studies (Rosenblum *et al.* 2009, 2012) and physiological studies that found increased markers of stress (i.e. corticosterone) during Bd infection (Gabor *et al.* 2013; Peterson *et al.* 2013). Thus, stress is likely a common and important component of host response to Bd and motivates further study given the potential for stress hormones to suppress immune cells in frogs (Rollins-Smith *et al. 1997, 2011*).

Immunogenetic responses across tissues

We found evidence of immune-related gene expression responses in both species, but with different specific pathways in the sampled tissues. In the resistant *B. marinus* skin data set, we observed some expression changes related to immune function (albeit with a relaxed *P*-value cut-off), but we did not detect the robust immune or inflammatory response that we expected given lower observed infection intensities. We did observe upregulation of immune regulatory pathways that typically have modulatory effects on immune response in this species. In contrast, the susceptible *B. boreas* showed stronger upregulation of innate immune-related pathways including complement pathway genes in the skin data set. Recent studies have also documented different patterns of regulation of innate immune pathways in different species. For example, the complement pathway has been shown to be activated in response to Bd infection in some species studied thus far (i.e. *A. zeteki*, *A. glyphus*, and *C. fitzingeri*, Ellison *et al.* 2015), but downregulated in other species (ie. *Xenopus tropicalis*, *R. muscosa*, and *R. sierrae*, Rosenblum *et al.* 2009, 2012). It is not yet clear whether complement pathway activation plays a general role in host response to Bd and more targeted immunological studies are needed.

In contrast to our findings in the skin, we observed a striking pattern of downregulation of immune-related pathways in the *B. boreas* liver data set. This pattern may be due to either host-mediated immune regulatory pathways or pathogen-mediated immunosuppression. In some cases, strong immune regulation can serve to modulate an ongoing immune response to limit collateral immunopathology (Sears *et al.* 2011). Host-mediated immune regulation is conceivable given the high Bd infection intensities and potential for strong inflammatory response. We expected a coordinated

splenic response based on the localization of regulatory T cells in the spleen during immune responses in other vertebrates (Wei *et al.* 2006). Although overall we found weak differential expression in *B. boreas* spleen, the differentially expressed probesets were suggestive of immune suppression. For example, in *B. boreas* spleen, we observed upregulation of a probeset annotated as an Fc receptor-like 2, which has immunomodulatory effects on B-cell development (Davis 2007) and downregulation of immune cell signalling pathways was also suggestive of immunosuppression, albeit with weak statistical support. Pathogen-mediated immunosuppression is also a possible mechanism for observed patterns. Previous work suggests that Bd factors inhibit *in vitro* growth of frog spleen-derived T cells and B cells (Fites *et al.* 2013) and that Bd infection can lead to downregulation of immune response and T-cell activation in the spleens of infected frogs (i.e. *A. zeteki*, *A. glyphus*, and *C. fitzingeri*, Ellison *et al.* 2014, 2015; *X. tropicalis*, Ribas *et al.* 2009). Our *B. marinus* data set also supports the possibility that Bd has immunosuppressive effects. The *B. marinus* liver data set included only two significantly downregulated probesets, which were annotated as MHC class 2. The MHC class 2 gene family produces antigen presentation proteins necessary for T-cell activation (Du Pasquier & Flajnik 1990). MHC downregulation could suggest either a reduction in antigen processing and presentation or a reduction in the number of antigen-presenting cells in the tissue. Our expression results for both *B. boreas* and *B. marinus* should motivate continued exploration of the effects of Bd factors on immune activation and suppression of liver- and spleen-derived cells in additional host species.

The overall weak expression response for *B. marinus* in all sampled tissues was surprising given the striking difference in infection dynamics between species. Two nonmutually exclusive mechanisms may explain the basis of resistance given the weak response we observed. First, *B. marinus* may limit the colonization and proliferation of the pathogen by mounting an immediate innate immune response at the site of infection in a process involving a cellular-mediated response (e.g. macrophage, neutrophils) (Rollins-Smith *et al.* 2011). The infection could then persist at low levels as Bd may continue to grow at a slow rate on the host or on skin sloughs off of the host. As the low-level infection is contained by an innate immune response, the infection may not trigger a strong systemic adaptive immune response. Local responses could maintain skin structural integrity while pathological inflammation could be avoided due to weak or modulated pro-inflammatory signals. In this scenario, sampling gene expression and histological changes at earlier time points postinoculation would be advantageous to documenting a rapid innate immune response. Second, certain structural or molecular features of the epidermis may reduce the ability of Bd to colonize and proliferate. Although Bd has been documented to infect a wide range of species, it has been proposed that infectivity varies among host species due to epidermal differences (Greenspan *et al.* 2012). Several factors may be involved including lower binding capacity of Bd receptors for species-specific epidermal features, effects of mucus composition, and presence of bacterial communities that limit Bd growth (Rollins-Smith *et al.* 2011; Voyles *et al.* 2011). Further work is needed to dissect both the early stage host responses and the potential effects of host integument variation on resistance.

The overall weak expression responses observed in our resistant species, *B. marinus*, should motivate further exploration of the mechanistic basis of resistance at the host level. Few studies have profiled gene expression in Bd-resistant species, but one other study similarly found a generally weak expression response in a resistant frog (*Agalychnis callidryas*, Ellison *et al.* 2015). The commonality is intriguing especially in the light of previous laboratory studies that show evidence of adaptive immune response to Bd infection in some species (Ramsey *et al.* 2010; McMahon *et al.* 2014). For example, a previous study showed innate and adaptive immune defences to Bd in *X. laevis* via experimental depletion of mucus antimicrobial peptides and immunosuppression via sublethal irradiation, respectively (Ramsey *et al.* 2010). In addition, Bd-specific antibodies were detected in the mucus of Bd-exposed *X. laevis* (Ramsey *et al.* 2010). A separate study documented acquired resistance via learned behavioural avoidance in *Bufo quercicus* and adaptive immune activation in *Osteopilus septentrionalis* following repeated exposure to dead Bd (McMahon *et al.* 2014). Applying these experimental approaches in *B. marinus* and other more resistant host species is needed to examine the progression of infection, host response dynamics and ultimately how some species curb Bd infection. In addition, adding more sampling points (e.g. longer interval between inoculation and sampling) and additional tissues (e.g. thymus, kidney) to gene expression studies may reveal more about systemic host response to Bd infection. Finally, future studies should utilize multiple Bd isolates to test for interaction effects between host and pathogen genotypes.

Conservation implications

Given the global threat of Bd, the difference in host response to Bd exposure found in this study is likely to have implications for amphibian communities in the wild. We found striking differences in disease

progression between two congeneric species in multiple assays, suggesting that *B. marinus* and *B. boreas* are at near opposite ends of the susceptibility resistance spectrum. The *B. marinus* hosts carried lower Bd infection intensities in our experiment, and this type of persistent low-level infection may have transmission implications for wild populations. As asymptomatic carriers of Bd infection, *B. marinus* could function as spreaders of the disease to the environment and to other species. The *B. boreas* hosts in contrast experienced high infection intensities and evidence of chytridiomycosis. These results are consistent with the potential for high transmission to lead to rapid population collapse, as has been observed in wild *B. boreas* populations (Pilliod *et al*. 2010).

The results change our understanding of chytridiomycosis by providing taxa-specific response profiles for species of conservation relevance and a new level of generality about host response to Bd. Mechanistic studies have not previously been conducted for our focal susceptible and resistant species, nor for close relatives in the species-rich Bufonidae family (aged at ~88.2 Ma Pramuk *et al.* 2008) whose c. 481 species (http://amphibiaweb.org) span a broad geographic distribution around the world). Thus, our study contributes an important phylogenetic and geographic perspective. Integrating results from our study with previous work also allows us to make stronger conclusions on the general nature of host response to Bd. For example, our finding on the positive correlation of skin structural integrity pathways (i.e. cell adhesion gene set) with disease resistance gains general importance given concordance with previous work. Similarly, our evidence of immunosuppression contributes to a growing body of literature, suggesting that Bd may actively suppress host response during infection. Overall, our results suggest the importance of *intrinsic* differences between host species. Our results also demonstrate that an integrative comparative approach – leveraging multiple data types – is a powerful way to isolate distinctive features of host responses in resistant and susceptible hosts. By analysing the mechanistic basis of the interaction between two bufonid species and Bd in a single framework, we show contrasting host response in the context of varying disease progression. Future studies incorporating broader temporal sampling and additional host species will lead to a better understanding of the mechanisms involved in amphibian defences and contribute to the design of mitigation strategies for diseased-caused population declines.

With the increasing threat of emerging infectious diseases on biodiversity (Daszak *et al.* 2000; Fisher *et al.* 2012), studies on host response variation are increasingly needed to understand and predict the epidemic outcomes. For example, research on white-nose syndrome (WNS), which is an emerging threat to North American bats, is also demonstrating that host response varies among species (Frank *et al.* 2014; Johnson *et al.* 2015). Interestingly, work on WNS also indicates that canonical adaptive immune responses (i.e. antibody production) are not playing a major role in disease resistance (Johnson *et al.* 2015). Thus, it is possible that some vertebrate responses to fungal diseases are quite general, and integrative approaches applying multiple assays of host response are needed to reveal these generalities. Despite the considerable challenges of studying and mitigating emerging infectious diseases in wildlife populations, researchers can now feasibly conduct comparative analyses across multiple host species. Moreover, with broader access to genomic resources in recent years, it is increasingly possible to conduct studies on genomics of host response in almost any species. Research in other emerging infectious disease systems is also revealing how resistance can evolve following disease outbreaks. Case studies in birds (Bonneaud *et al*. 2012) and rabbits (Best & Kerr 2000) show the importance of tracking both intraspecific variation and temporal dynamics in resistance for understanding host–pathogen interactions in the wild. Further work linking variation in host response to epidemic outcome is important to understand general mechanisms of host resistance and develop appropriate management interventions. While accelerating such studies is needed to further characterize individual-level host response, it will also be critical to extend genomics methods into field-based studies in order to verify laboratory-based results and to investigate host–pathogen interactions in natural conditions.

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T.J.P. and E.B.R. designed the experiment and wrote the manuscript. T.J.P. performed laboratory work and analysed data.

Data accessibility

Transcriptome sequencing data have been deposited to NCBI SRA (Accession nos SRP065907, SRP065908). Gene expression microarray data have been deposited to NCBI GEO (Accession no. GSE74788).

Supporting information

Additional supporting information may be found in the online version of this article.

Table S1 Combined experimental data table including Bd infection intensities, body weights, and histological results from stained skin sections.

Table S2 Gene expression microarray results tables for each species and tissue with probeset-level information: $\log_2$ fold change values, statistical test results, and annotation information.