MOLECULAR ECOLOGY

Molecular Ecology (2012) **21**, 3110–3120 doi: 10.1111/j.1365-294X.2012.05481.x

FROM THE COVER

Only skin deep: shared genetic response to the deadly chytrid fungus in susceptible frog species

ERICA BREE ROSENBLUM,*†§ THOMAS J. POORTEN,*† MATTHEW SETTLES† and GORDON K. MURDOCH†‡

*Department of Biological Sciences, University of Idaho, Moscow, ID 83844, USA, †Initiative for Bioinformatics and Evolutionary Studies (IBEST), University of Idaho, Moscow, ID 83844, USA, ‡Department of Animal and Veterinary Science, University of Idaho, Moscow, ID 83844, USA

Abstract

Amphibian populations around the world are threatened by an emerging infectious pathogen, the chytrid fungus *Batrachochytrium dendrobatidis* (Bd). How can a fungal skin infection kill such a broad range of amphibian hosts? And do different host species have a similar response to Bd infection? Here, we use a genomics approach to understand the genetic response of multiple susceptible frog species to Bd infection. We characterize the transcriptomes of two closely related endangered frog species (*Rana muscosa* and *Rana sierrae*) and analyse whole genome expression profiles from frogs in controlled Bd infection experiments. We integrate the *Rana* results with a comparable data set from a more distantly related susceptible species (*Silurana tropicalis*). We demonstrate that Bd-infected frogs show massive disruption of skin function and show no evidence of a robust immune response. The genetic response to infection is shared across the focal susceptible species, suggesting a common effect of Bd on susceptible frogs.

Keywords: amphibians, disease biology, host-parasite interactions, transcriptomics

Received 3 June 2011; revision received 8 December 2011; accepted 30 December 2011

Introduction

Amphibians around the world are disappearing, highlighting a biodiversity crisis dubbed the 'sixth mass extinction' (Wake & Vredenburg 2008). The chytrid fungus *Batrachochytrium dendrobatidis* (Bd) infects hundreds of amphibian species (Berger *et al.* 1998; Lips *et al.* 2006) and has lead to 'the most spectacular loss of biodiversity due to disease in recorded history' (Skerratt *et al.* 2007). The dramatic impacts of Bd highlight the need to better understand the dynamics of emerging infectious disease in wildlife populations (Smith *et al.* 2009) and the ecological consequences of amphibian declines (Mohneke & Roedel 2009). The devastating effects of chytridiomycosis also underscore the importance of mechanistic studies of amphibian response to Bd. More than a decade after the description of Bd (Longcore *et al.* 1999), the mystery of how a fungal skin infection can be lethal to so many amphibian species still remains (Kilpatrick *et al.* 2010).

Discovering the determinants of susceptibility to Bd is critical for understanding and mitigating disease-related amphibian declines. Identifying whether susceptible species exhibit a shared response to Bd is particularly important when considering whether general solutions to the chytrid crisis may exist. For example, shared responses can be used to identify targets for intervention and to determine whether Bd-mitigation strategies should focus on bolstering or inhibiting particular host responses. Ecological and physiological studies on Bd–host interactions have been conducted on a wide range of amphibian species (e.g. Lips *et al.* 2006; Skerratt *et al.* 2007). However, detailed immunological and genetic studies of Bd–amphibian interactions have been conducted solely with laboratory models of the genus *Silurana* [formerly *Xenopus* (e.g. Rosenblum *et al.* 2009; Ribas *et al.* 2009; Ramsey *et al.* 2010)], which are

Correspondence: Erica Bree Rosenblum, Fax: 510-643-5438; E-mail: rosenblum@berkeley.edu

§Present address: Department of Environmental Science, Policy, and Management, University of California, Berkeley, CA 94720, USA.

not experiencing Bd-related declines and are only distantly related to most species that are being impacted by Bd (Soto-Azat *et al.* 2010). Therefore, replicated studies of nonmodel species are necessary to understand the responses of amphibian species threatened by Bd in nature. Genetic and genomic studies, in particular, can provide an important window into the details of host response to Bd.

Here, we evaluate the genomic response to Bd infection in two species of Ranid frogs that are critically endangered. The mountain yellow-legged frogs, *Rana muscosa* and *Rana sierrae* (Vredenburg *et al.* 2007), inhabit high elevation alpine lakes in the Sierra Nevada and Transverse Ranges of California. Historically, these frogs were abundant, but recently, populations have disappeared from more than 90% of their historic range due primarily to introduced fish predators and disease (Briggs *et al.* 2005; Vredenburg *et al.* 2005). *Rana muscosa* and *R. sierrae* are highly susceptible to Bd and have experienced catastrophic Bd-related declines (Briggs *et al.* 2005, 2010).

We use pyrosequencing and microarrays to characterize the transcriptomes of the endangered *Rana* species and to understand their responses to Bd infection. No genomic resources were previously available for Ranid frogs; therefore, we used Roche 454 sequencing to characterize >20 000 transcripts from *R. muscosa* and *R. sierrae* and developed a custom NimbleGen (Madison, WI) microarray. We then conducted a laboratory infection experiment with *R. muscosa* and *R. sierrae* to compare global gene expression in multiple tissues (skin, liver and spleen) for control and infected frogs at two time points after Bd exposure. Clinical signs of infection and Bd loads in the experiment were comparable to disease symptoms and Bd infection intensities in natural populations during population declines (Vredenburg *et al.* 2010). Here, we implicate the disruption of host skin integrity and a lack of robust immunogenetic response to Bd as important shared features of the genetic response to Bd infection in susceptible frogs.

Methods

Transcriptome sequencing

No genomic resources were previously available for Ranid frogs, so we first characterized the *Rana muscosa* and *Rana sierrae* transcriptomes. To maximize transcript discovery, we used samples from multiple individuals of both species, multiple tissues (liver, spleen and skin) and multiple infection histories (Bd-infected and Bd-naive).

We generated cDNA from total RNA for each tissue separately. We then pooled the cDNA and normalized to ensure representation of transcripts of varying abundances. We sequenced cDNA libraries on two different Roche-454 platforms. For the GS FLX Standard run, cDNA were normalized at Evrogen and sequenced at EnGenCore. For the GS FLX XLR Titanium run, cDNA normalization and sequencing were conducted at Eurofins. We obtained >700 Mb of sequence. We combined reads from both sequencing runs into a single Roche 454 SFF file, screened raw unclipped sequences, assembled reads into ESTs and joined assembled ESTs. We obtained 21 703 transcripts with an average coverage of 25 reads per base. We could confidently annotate more than half of the transcripts by BLASTing (Altschul *et al.* 1990) to the JGI *Silurana tropicalis* (XenTrop4.1) and the Swiss-Prot databases, defining a match as better than *E*-value of 0.001. The robustness of our normalization procedure was evidenced by the large proportion of transcripts represented in our experimental groups and by the fact that *a priori* genes of interest were well represented. Details on molecular protocols, bioinformatics tools and raw sequence data are provided in Supporting Information.

Infection experiment

We conducted a laboratory infection experiment with adult *R. muscosa* from 60 Lakes Basin and *R. sierrae* from Ebbets Pass, California. Frogs in these natural populations are infected with Bd. However, experimental animals were reared in the laboratory from wild-caught egg masses, so development and metamorphosis occurred without Bd exposure. A standardized qPCR method (Boyle *et al.* 2004) was used to confirm from noninvasive swabs that all experimental animals were Bd-free prior to inoculations.

Experimental infections were conducted at 18 °C, the preferred temperature for *Rana muscosa/sierrae* and the temperature at which they were reared. One week prior to laboratory exposures, frogs were moved to individual housing and were housed individually for the duration of the experiment. Twice per week, frogs were fed *ad libitum* and tank water was changed.

For each species, half of the experimental frogs (randomly chosen) were designated as controls and half were inoculated with Bd. The Bd isolate used was maintained in the laboratory for approximately 1 year (approximately four passages) prior to the experiment. Inoculum was prepared by growing Sierra-Nevada Bd on 1% agar, 1% tryptone plates for 2 weeks. Plates were flooded with water and agitated for zoospore release. Each frog in the exposed group was held overnight in a 100-mL water-bath with $>10^6$ zoospores. Exposures were repeated for 3 days. Frogs in the control group were handled identically but received sham

inoculations (no Bd exposure). Frogs were swabbed prior to euthanasia to confirm infection status and quantify Bd load.

Frogs were euthanized by decapitation at two time points. The 'early' group was euthanized 3 days after first Bd exposure. The 'late' group was euthanized at 16 days after first Bd exposure (by which time, many treatment frogs exhibited clinical signs of chytridiomycosis). Three biological replicates were euthanized for each species, each treatment group and each time point for a total of 24 experimental frogs. Dorsal skin, liver and spleen tissues were flash frozen and stored at −80 °C.

All unexposed frogs remained uninfected for the duration of the experiment. Exposed frogs became infected with Bd with increasing loads through time. At the early and late time points, frogs had an average of 10 567 and 39 768 zoospore-equivalents, respectively. These relatively high Bd loads are comparable to infection intensities in natural *R. muscosa/sierrae* populations during population declines (Vredenburg *et al.* 2010).

Gene expression analysis

We developed a custom NimbleGen microarray for *R. muscosa/sierrae* based on our 454 data. The microarray contained 127 867 60-mer probes representing a possible 28 995 expressed transcripts. Probesets were defined as all probes that mapped to a single transcript, which in turn mapped to a single gene. We designed a 12-plex array to minimize experimental noise and allow for increased sample sizes.

We processed samples from 24 total frogs (three biological replicates, two species, two treatment groups and two time points). Skin and liver samples were available for all 24 samples. However, spleens could not be collected for several frogs late in infection because body condition was poor, so only 12 spleen samples were available (four early control, four early infected, two late control and two late infected). All samples from each tissue type were processed simultaneously. Because our arrays were 12-plexes, spleen samples were all processed on a single array, and skin and liver samples were processed simultaneously on two arrays for each tissue. Standard protocols (see Supporting information) were used to isolate RNA, synthesize double-stranded cDNA and fluorescently label cDNA with Cy5. An 18-h hybridization was conducted at 42 °C. Chips were then washed in an automated MAUI System and scanned on an Axon 4000B Scanner using GenePix Pro software with PMT gain adjusted for optimal saturation. We did not conduct separate qPCR analyses because we previously validated expression patterns for many of the focal genes (Rosenblum *et al.* 2009) and because our criteria for significance were even more stringent than recommended in studies that obtain extremely high correlations between microarray and qPCR results (e.g. Morey *et al.* 2006).

Microarray data analysis

NimbleScan v2.5 software was used to align a chip-specific grid and extract raw intensity data. Chip images were visually verified to be free of significant spatial artifacts. Raw intensity data were read into R (R Development Core Team 2011), and chip intensity distributions, boxplots and hierarchical clusters were evaluated for any unusual global patterns. Each array was background corrected and normalized using the quantile normalization procedure. Each probeset was then summarized to a single expression value using the median polish and robust multichip average (RMA) procedures (Bolstad *et al.* 2003; Irizarry *et al.* 2003; Rafael *et al.* 2003). Probesets with low levels of expression variation across all samples (IQR < 0.5) were removed from further analysis, reducing the overall number of statistical tests to be performed. The IQR metric was also used to confirm that the vast majority of transcripts on our array were represented across all experimental groups and thus to confirm that our arrays provided a robust assessment of gene expression (e.g. of the 28 995 transcripts 66%, 81% and 80% were expressed in skin, liver, and spleen, respectively [Fig. S1, Supporting information]).

We assessed differential expression between infected and control frogs using a linear model with an empirical Bayesian adjustment to the variances (LIMMA, Smyth 2005), and comparisons of interest were extracted using contrasts. We used general linear models separately for each tissue with treatment (infected vs. control) and time point (early vs. late) as main effects. Species identity was used as a blocking factor; thus, the model controlled for the effects of species-specific expression and was not simply a pooled analysis. Chip assignment was also used as a blocking factor because liver and skin samples each required two 12-plex chips. The Benjamini and Hochberg (BH) method was used to control for the expected false discovery rate given multiple tests (Benjamini & Hochberg 1995). Probesets were considered statistically differentially expressed with a BH-adjusted P-value of <0.05. For the spleen, no probesets showed differential expression following BH correction (Fig. 1, Table S1, Supporting information), so we evaluated probesets with an uncorrected P-value of <0.05 and provide a more cautious interpretation of patterns in the spleen given the less stringent cut-off.

We also explicitly tested for a parallel response in gene expression to infection between species. We used

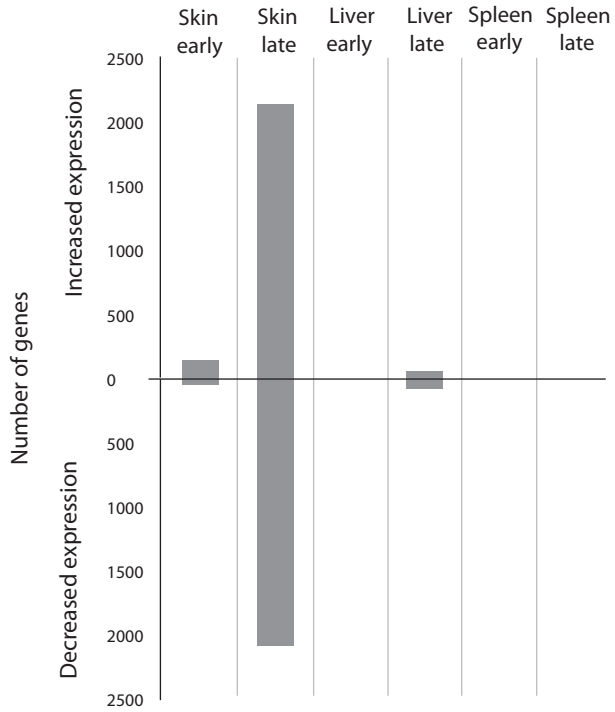

Fig. 1 The number of genes with significantly increased or decreased expression in Bd-infected frogs (compared to control frogs). Data are presented for three tissues (skin, liver and spleen) and two time points (early and late in infection). Genes with significant differences in expression were identified from a model that accounted for species differences and included a correction for multiple testing.

permutation tests to compare the log2 fold change values between *R. muscosa* and *R. sierrae* for the skin late data set, the group with the most differentially expressed genes. We performed differential expression analysis permuting the sample labels 1000 times and at each permutation calculated the correlation between the log2 fold change values in the two species. We calculated a permuted *P*-value as the number of permutation-based correlations greater than the test correlation divided by the number of permutations performed. We also computed correlation coefficients of log2 fold change values for each of the species relative to the global analysis with species as a blocking factor. Finally, to test for a parallel response between *R. muscosa*, *R. sierrae* and *S. tropicalis*, we conducted a complete reanalysis of our *S. tropicalis* data (Rosenblum *et al.* 2009). We re-annotated the *S. tropicalis* genes, mapped orthologs between *Rana* and *Silurana* using BLAST (as described above) and reanalysed the *S. tropicalis* data set using linear models (as described above). We then performed a contingency analysis of orthologs that showed significant differences in expression in both *Rana* and *Silurana* data sets, tallying genes with the same vs. opposite responses.

Results and discussion

The number of genes differentially expressed between control and infected frogs varied strongly by tissue type; the strongest genetic response to infection was in the skin (contingency test, $P < 0.01$). Figure 1 and Table S1 (Supporting information) show the number of genes with statistically significant differences in expression between control and exposed frogs at the two time points (early and late) and the three tissues assayed (skin, liver and spleen). Below, we describe the genetic response of *Rana muscosa* and *Rana sierrae* to infection with Bd. First, we conducted analyses using species as a blocking factor to understand the general response to Bd, and we highlight gene expression changes in the skin and the immune system from this analysis. Second, we conducted analyses to directly compare the responses from the two species, and we highlight aspects of the shared response to Bd in susceptible frogs.

Disruption of skin integrity

The most dramatic response we observed in our experiment was the massive perturbation of transcript abundance from genes involved in maintaining skin integrity. Transcripts related to collagen, fibrinogen, elastin and keratin pathways all showed significant differences in expression between control and infected frogs (Table 1). The collagen matrix is particularly important for maintaining integrity of the five strata of the amphibian epidermis, so reduced transcript for the two predominant collagen types (type I and type III) would disrupt the structural integrity of the epidermal layer. In fact, we found consistent decreased expression of a large number of collagen genes (Table 1). Similarly, the changes in keratin expression indicate that the predominant cells of the frog integument, the keratinocytes, are functionally aberrant. We found a number of keratin 1 genes to have decreased expression in sick frogs (Table 1). Interestingly, we observed increased expression in infected frog skin of an ortholog of human keratin 1 (JGI#178963). This gene exhibited a similar increase in expression in our *Silurana tropicalis* experiment and is also involved in some human skin ailments (Smith 2003; Rosenblum *et al.* 2009), suggesting that it may play a more general role in vertebrate skin diseases.

The precise mechanism of amphibian death by Bd has been a persistent mystery, and our data support the assertion that Bd kills frogs by disrupting the integrity of their skin (Voyles *et al.* 2009). Because amphibian skin is physiologically active, disrupting skin structure compromises a number of critical skin functions. For

Table 1 Selected gene classes with differential expression in the skin of Bd-infected frogs

Category	Description	Direction	Time point	Coefficient	*P*-value	Ensembl ID	JGI ID
Skin integrity							
Keratins	Keratin, type I	Down	Late	−1.8	0.0078	ENSXETG00000005611	179116
	Keratin, type I	Down	Late	−0.9	0.033	ENSXETG00000020417	275213
	Keratin, type I	Down	Late	−1.8	0.025	ENSXETG00000025339	NA
	Keratin, type I	Down	Late	−0.8	0.040	ENSXETG00000021500	NA
Collagens	Collagen, type I	Down	Late	−4.4	1.8 E-7	ENSXETG00000020318	392816
	Collagen, type III	Down	Late	−2.7	7.1 E-7	ENSXETG00000010783	302233
	Collagen, type V	Down	Late	−3.0	3.6 E-6	ENSXETG00000010784	153909
	Collagen, type VI	Down	Late	−1.9	0.0078	ENSXETG00000011490	NA
	Collagen, type VIII	Down	Late	−1.3	0.048	ENSXETG00000005782	286764
	Collagen, type XIV	Down	Late	−1.0	0.022	ENSXETG00000005761	364658
	Collagen, type XXI	Down	Late	−1.4	0.0092	ENSXETG00000022756	165187
Immune response							
Complement	Complement C3a/C4a/C5a (alternative pathway)	Down	Late	−0.6	0.049	ENSXETG00000005203	NA
	Complement component c1q (classical pathway)	Down	Late	−0.9	0.012	ENSXETG00000001223	427112
	Complement component c1q (classical pathway)	Down	Late	−1.4	0.013	ENSXETG00000005727	296730
	Complement component c1q (classical pathway)	Down	Late	−1.5	0.01	NA	136324
	Complement factor H precursor (alternative pathway)	Up	Late	1.0	0.010	ENSXETG00000005261	308546
Interferon-related	Interferon gamma receptor 2	Down	Late	−1.4	0.026	ENSXETG00000000044	461189
	Interferon alpha/beta receptor	Up	Late	0.8	0.029	ENSXETG00000000046	461632
	Interferon regulatory factor	Up	Late	1.9	0.00092	NA	375197
	Interferon regulatory factor 1	Up	Early and Late	1.8 and 1.8	0.013 and 0.0017	ENSXETG00000016586	148456
	Interferon regulatory factor 4	Up	Late	1.5	0.0017	ENSXETG00000011413	349993
	Interferon regulatory factor 8	Up	Late	1.1	0.011	ENSXETG00000003561	334257
	Interferon-induced protein	Up	Late	1.7	0.0032	ENSXETG00000002265	NA
	Interferon-induced transmembrane protein	Up	Late	0.9	0.012	NA	362016
	Interferon-induced transmembrane protein	Up	Late	1.5	0.036	ENSXETG00000020495	NA
	Interferon-related developmental regulator 1	Down	Late	−0.9	0.014	ENSXETG00000009255	158475
	Cytokine receptor	Down	Late	−1.3	0.0031	ENSXETG00000018039	424452
	Cytokine, down-regulator of HLA II	Down	Late	−1.0	0.023	ENSXETG00000016509	NA
Major histocompatibility complex	MHC, class I alpha	Up	Late	0.9	0.046	ENSXETG00000017065	295817
	MHC, class I alpha	Up	Late	1.1	0.025	ENSXETG00000015263	461942
	MHC, class I alpha	Up	Late	1.0	0.039	ENSXETG00000016461	178629
	MHC, class II beta	Up	Late	0.8	0.011	ENSXETG00000025586	151191
Interleukin	Interleukin-1 beta	Up	Early and Late	2.5 and 2.1	0.027 and 0.015	ENSXETG00000001276	449640
	Interleukin-8 receptor	Down	Late	−1.2	0.035	ENSXETG00000008478	NA
	Interleukin-1 receptor	Up	Late	0.8	0.032	ENSXETG00000018434	294414
	Interleukin-3 induced	Up	Late	1.2	0.019	ENSXETG00000000804	172589
Antimicrobial-related	Cathelicidin	Up	Late	1.9	1.8 E-5	NA	193370

Table 1 *Continued*

Category	Description	Direction	Time point	Coefficient	*P*-value	Ensembl ID	JGI ID
Cellular stress							
Heat shock protein	Heat shock protein (acute response)	Down	Late	−2.5	1.0 E-5	ENSXETG00000025255	150887
	Heat shock protein (acute response)	Down	Late	−1.4	0.01	ENSXETG00000010251	NA
	Heat shock protein (constitutive response)	Up	Early	1.5	0.011	ENSXETG00000016838	287936
	Heat shock protein	Down	Late	−0.9	0.028	ENSXETG00000024144	297593
	Heat shock protein	Down	Late	−1.7	0.017	ENSXETG00000009112	453140
	Heat shock transcription factor	Down	Late	−1.0	0.034	ENSXETG00000023290	173321
Cytochrome p450	Cytochrome p450	Down	Late	−2.9	0.0022	ENSXETG00000021140	360545
	Cytochrome p450	Down	Late	−1.2	0.026	ENSXETG00000004753	270281
	Cytochrome p450	Down	Late	−1.4	0.012	ENSXETG00000008983	161782
	Cytochrome p450	Down	Late	−1.5	0.00034	ENSXETG00000027563	NA
	Cytochrome p450	Down	Late	−1.2	0.0026	ENSXETG00000005360	NA
	Cytochrome p450	Down	Late	−0.8	0.045	ENSXETG00000026927	321869
	Cytochrome p450	Down	Late	−1.5	7.1 E-5	ENSXETG00000027566	458107
	Cytochrome p450	Down	Late	−1.1	0.014	NA	379027
	Cytochrome p450	Down	Late	−1.2	0.001	NA	468052
	Cytochrome p450	Up	Late	0.9	0.027	ENSXETG00000020758	170248
NF-kappa inhibitor	NF-kappa inhibitor	Up	Early and Late	1.9 and 1.5	0.0071 and 0.0073	NA	309572

The coefficient shows the log2 fold change between infected and control frogs and the *P*-values are Benjamini and Hochberg corrected for multiple testing.

example, organism-level physiology studies in two other susceptible frog species (*Litoria caerulea* and *Bufo boreas*) found changes in electrolyte ion transport across the skin of diseased frogs (Voyles *et al.* 2009; Marcum *et al.* 2010). Our genetic data corroborate the role of electrolyte imbalances in host mortality. We found indications of systemic ion imbalance in Bd-infected frogs. Specifically, we observed decreased expression of ion channel genes and increased expression of potassium/chloride channel genes in the spleens of Bd-infected frogs (Table 2). In addition, our data suggest that Bd does not directly target ion transport pathways in the skin (Table 1), but rather that ion imbalances are caused by the physical disruption of the epidermis (i.e. indirect leakage of ions). The physical disruption of the skin appears to have cascading effects on other organs, for example, we observed significant changes in expression of genes involved in cellular stress and detoxification in the liver (Table 2).

In addition to interfering with ion balance, epidermal disruption probably leads to myriad additional challenges to amphibian health. Other direct effects of epidermal disruption include a compromised ability of frogs to osmoregulate or rehydrate (Carver *et al.* 2010). Indirect, downstream effects of epidermal disruption could include increased exposure to environmental toxins and increased susceptibility to invasion by additional pathogens. Of particular interest is the potential for Bd infection to facilitate colonization by other skin pathogens (e.g. *Mycobacterium* spp.), and secondary infections by other deadly pathogens should be more strongly considered.

Lack of evidence for a robust immunogenetic response

We found no evidence of a robust or systemic immune response to Bd in *R. muscosa* or *R. sierrae*. In the skin, there was little evidence for the normal activation of localized immune responses that would be expected following challenge by a skin pathogen. For example, we did not observe activation of typical proinflammatory mediators (e.g. no expression change in IL17, TLR2, inconsistent pattern for TNFs) nor did we observe increase in CD4 expression [which would be expected for pathogen recognition and response (Rivera *et al.* 2006)]. In fact, we observed increased expression of notable anti-inflammatory markers in the skin of infected frogs [e.g. NF-κ inhibitors, which would impede the NF-κB-mediated cellular response to infection (Baeuerle & Henkel 1994)]. Additionally, many classes of immune function genes in the skin showed predominantly decreased expression (e.g. complement pathway) or lacked a consistent direction of change (e.g. cytokines, Table 1). In the liver, very few immune

Table 2 Selected gene classes with differential expression in the livers and spleens of Bd-infected frogs

Category	Description	Tissue	Direction	Time point	Coefficient	*P*-value	Ensembl ID	JGI ID
Immune response								
Complement	Complement component c1q (classical pathway)	Spleen	Up	Early	2.45	0.036	ENSXETG00000001613	300050
Immunoglobulin	Immunoglobulin C1 type	Spleen	Down	Early	−1.18	0.032	NA	203881
	Immunoglobulin C1 type	Spleen	Down	Early	−1.16	0.033	NA	443405
	Immunoglobulin C2 type	Spleen	Down	Late	−2.08	0.033	ENSXETG00000008045	171791
	Immunoglobulin V type	Spleen	Down	Late	−2.36	0.04	NA	89604
	Immunoglobulin C2 type	Spleen	Up	Early	1.82	0.037	ENSXETG00000023970	424298
	Immunoglobulin C2 type	Spleen	Up	Early	1.14	0.046	NA	456292
	Immunoglobulin C2 type	Spleen	Up	Late	1.82	0.01	NA	122826
Interferon-related	Interferon regulatory factor 4	Spleen	Up	Early	1.19	0.034	ENSXETG00000011413	349993
Interleukin	Interleukin-6 signal transducer	Spleen	Up	Late	1.75	0.048	ENSXETG00000015503	388215
Major histocompatility complex	MHC, class I alpha	Spleen	Down	Early	−1.36	0.022	ENSXETG00000009089	386310
	MHC, class II	Spleen	Down	Late	−3.11	0.0046	ENSXETG00000005162	154793
Stress response								
Oxidative stress	Oxidative stress	Liver	Down	Late	−2.6	0.02	ENSXETG00000012651	336465
Heat shock proteins	Heat shock protein	Liver	Up	Late	5.0	0.00087	NA	354033
	Heat shock protein	Liver	Up	Late	4.8	0.002	ENSXETG00000021559	NA
	Heat shock protein	Spleen	Up	Late	1.89	0.02	ENSXETG00000004097	280140
Cytochrome p450s	Cytochrome p450	Liver	Down	Late	−3.3	0.0036	ENSXETG00000013846	325065
	Cytochrome p450	Spleen	Down	Early	−1.04	0.037	ENSXETG00000027605	297805
	Cytochrome p450	Spleen	Down	Early	−1.51	0.049	ENSXETG00000027556	379069
	Cytochrome p450	Spleen	Down	Early and Late	−1.56 and −1.75	0.0056 and 0.021	NA	112834
	Cytochrome p450	Spleen	Up	Early	1.31	0.022	ENSXETG00000004753	270281
	Cytochrome p450	Spleen	Up	Late	1.48	0.039	ENSXETG00000018658	458115
Corticosteroid	Corticosteroid-binding globulin precursor	Spleen	Down	Early	−2.30	0.034	ENSXETG00000023329	NA
NF-kappa inhibitor	NF-kappa B repressing factor	Spleen	Down	Early	−1.38	0.024	ENSXETG00000007438	NA
	NF-kappa enhancer	Spleen	Up	Early	1.73	0.029	ENSXETG00000021325	332759
Homeostasis								
Ion imbalance	Sodium channel, nonvoltage-gated 1	Spleen	Down	Early	−1.41	0.02	ENSXETG00000027567	155784
	Sodium-dependent phosphate transporter	Spleen	Down	Late	−1.93	0.043	ENSXETG00000018957	NA
	Potassium/chloride transporter	Spleen	Up	Early	1.045	0.041	ENSXETG00000005430	472209

The coefficient shows the log2 fold change between infected and control frogs. *P*-values for the liver are Benjamini and Hochberg corrected and for the spleen are raw values.

gene families showed changes in expression and the few that did failed to show a consistent direction of change (Table 2). In the spleen, there were indications of cytokine signal-mediated immune response, but this response was neither substantial nor sustained (Table 2). Neither the liver nor spleen had elevated CD4 expression profiles suggesting a lack of systemic T-cell recruitment. Overall, and consistent with what we observed in *S. tropicalis* (Rosenblum *et al.* 2009), there was no activation of many specific immune function genes expected to be involved in the vertebrate response to invasion by a fungal pathogen (e.g. Myrkov *et al.* 2010).

Although the general pattern from our data suggests a lack of robust immunogenetic response, some exceptions in the skin data set bear mention. First, in the skin of infected frogs, we observed increased expression in IL1, which is a marker of an inflammatory response and can be involved in the activation of cellular- and humoral-mediated immunity (Sims & Smith 2010). However, our observations above (e.g. no increase in CD4 and NF-κB) suggest that any inflammatory response was extremely limited. Second, there were several classes of immune genes that showed predominantly increased expression in the skin of infected frogs (e.g. MHC). Third, although five of the seven differentially expressed genes with putative antimicrobial properties showed decreased expression in infected frogs, we found increased expression of a cathelicidin gene (JGI# 193370). Cathelicidins are of particular interest because they are antimicrobial peptides known to play an important role in vertebrate defence against fungal pathogens (e.g. Owais *et al.* 2010). These exceptions suggest that there may be some specific responses that bear further study but do not alter the overall picture of very weak immune activation.

It is important to recognize that our ability to make strong conclusions from the immunogenetic data is limited by incomplete knowledge of amphibian immune responses to fungal pathogens and incomplete characterization of frog genomes. Three specific caveats are important to consider in this regard. First, our analysis of the immune response is necessarily limited by our ability to annotate the *R. muscosa* and *R. sierrae* transcriptomes. Our microarray contained at least 475 genes with immune function annotations, and many of the key players in vertebrate immunogenetic response were well represented. However, some genes that play a role in vertebrate inflammatory response were not annotated on our array (e.g. some interleukins, Toll-like receptors and C-reactive proteins). Therefore, it will be worthwhile to reanalyse our data as new functional annotations become available. Second, we had lower power in our spleen data set and no genes with significant differential expression following correct for multiple testing. However, we still observe very little immunogenetic activation even with a less stringent significance cut-off for the spleen data set (Table 2). Third, it is possible that adaptive immunity could develop over a longer time period than considered in this experiment. However, frogs at our late sampling point already exhibited clinical signs of chytridiomycosis, so whether absent, weak or simply delayed, the adaptive immune response was not mounted adequately to prevent disease development.

Our suggestion that Bd-susceptible frogs lack a robust immune response is consistent with recent immunological data. For example, immunization against Bd is not effective for *R. muscosa* (Stice & Briggs 2010), and Bd-specific antibodies have not been detected in serum from *R. muscosa* after immunization with or exposure to Bd (TJ Poorten, MJ Stice, CJ Briggs and EB Rosenblum, unpublished data). A weak or absent adaptive immune response may help explain why Bd is lethal to *R. muscosa*, *R. sierrae* and many other frog species. The lack of evidence for robust immunogenetic activation in three susceptible species (*R. muscosa*, *R. sierrae* and *S. tropicalis*) is especially interesting when contrasted with recent results that suggest both innate and adaptive defences against Bd in resistant frogs such as *S. laevis* (Ramsey *et al.* 2010). Therefore, it is particularly important to broaden the taxonomic focus of immunological studies of amphibians to determine whether activation of adaptive immune defences is a hallmark of Bd-resistant frogs.

If susceptible frogs do lack a robust immunogenetic response, it is important to consider whether Bd colonization may proceed unrecognized by the adaptive immune system and/or that immune activation is suppressed. We observed a weaker than predicted immune response in three susceptible frog species, highlighting the importance of determining whether Bd employs any mechanisms for immune evasion. Specifically, molecular and cellular studies in the pathogen will be necessary to evaluate the hypothesis of pathogen immune evasion or suppression.

Response to Bd is shared across susceptible species

The direction and magnitude of the *R. muscosa* and *R. sierrae* responses to Bd were strikingly similar (Fig. 2). We explicitly tested for a shared response between control and infected frogs. We calculated the correlation coefficient for gene expression response in the skin of *R. muscosa* vs. *R. sierrae* by comparing between the two species the difference in expression between control and infected frogs for each gene. We saw a significantly shared response with a correlation

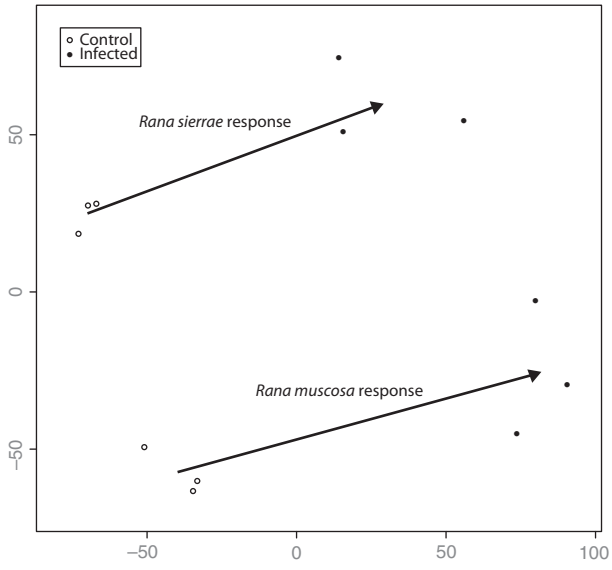

Fig. 2 Shared response to Bd infection in the skin of *Rana muscosa* and *Rana sierrae* late in infection. Multidimensional scaling plot where each point represents the genome-wide expression profile of a single individual. Open and closed circles represent control and infected individuals, respectively. Lines connect mean gene expression profiles. Baseline gene expression profiles in control animals differ between species. However, the response to infection is shared in both direction and magnitude.

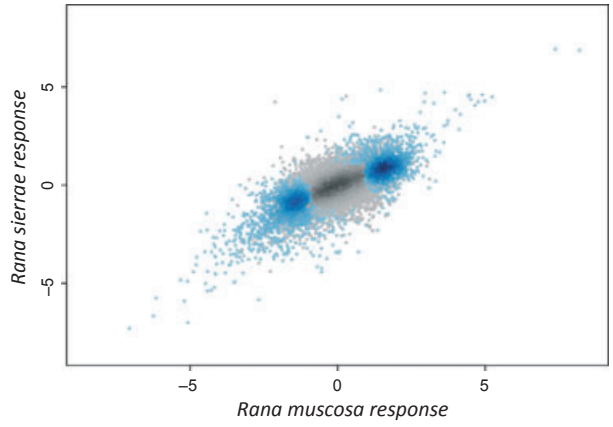

Fig. 3 Explicit test of shared response to Bd infection in the skin late in infection for all genes in the analysis. Correlation between responses (i.e. difference between control and infected animals) of *Rana muscosa* vs. *Rana sierrae* ($r = 0.69$). Axes represent log base 2 fold change in expression between control and infected frogs. Each of the 19 188 points represents expression pattern of a single transcript computed across biological replicates. Blue points represent differentially expressed genes, and grey points represent all other genes. Point density increases with colour darkness.

of 0.69, significantly higher than randomly permuted data sets (randomization test, $P = 0.001$; Fig. 3). We also calculated the correlation coefficient for gene expression response in the skin between each species and the global analysis presented above (with species as a blocking factor) and again saw a dramatically shared signal with correlations of 0.91 and 0.93 for *R. muscosa* and *R. sierra*, respectively). *Rana muscosa* and *R. sierrae* are closely related, so a shared response to Bd may seem intuitive. However, the shared genetic response to infection is particularly noteworthy given differences between the species in baseline gene expression. *Rana muscosa* and *R. sierrae* differ in gene expression profiles for both the control and the treatment groups; thus, it is truly their *response* to Bd that is shared (Fig. 2).

We also found evidence for a shared gene expression response in the skin of infected frogs at a broader taxonomic scale. We compared our *Rana* data with a complete reanalysis of our *S. tropicalis* data from a comparable experiment (Rosenblum *et al.* 2009). *Silurana tropicalis* is susceptible to Bd and can die from chytridiomycosis in laboratory settings (unlike its more resistant relative *S. laevis*). We examined the genes that had clear orthologs between *Rana* and *Silurana* and that exhibited significant changes in expression in the skin between infected and control frogs in both experiments. Seventy-eight percentage (72/92) of these genes exhibited gene expression changes in the same direction (contingency test, $P < 0.0001$). A number of the genes that showed a common response at a broader taxonomic scale are functionally important and have been highlighted above. For example, shared patterns included decreased expression of key keratin genes (ENSXET ID # 10783, 25339), increased expression of the antimicrobial peptide cathelicidin (JGI ID # 193370), increased expression of several interferon and interleukin-related genes (JGI ID$ 148456, 294414, 172589, 449640) and increased expression of NF-kappa inhibitor (ENSXET # 6998). These results indicate that some key genetic responses to Bd may be shared across susceptible frog species.

Our results suggest that future studies of Bd-related amphibian declines should focus in three areas. First, it is important to understand the downstream consequences of disrupted skin function on frog physiology and secondary skin infections. Second, future work should investigate the possibility for Bd immune evasion. Third, it is crucial to complement studies of susceptible frog species with detailed characterizations of Bd-resistant species. Our study provides insight into the mechanisms of mortality in susceptible frog species. But not all frog species are losing the battle with Bd; different species, and sometimes even populations of the same species, vary in their response to Bd (e.g. Daszak *et al.* 2004; Retallick *et al.* 2004; Berger *et al.* 2005; Retallick & Miera 2007; Briggs *et al.* 2010). Future studies should endeavour to characterize and compare the 'core

responses' of susceptible and resistant species to Bd under multiple environmental conditions. Our genomics resources can also be applied to ecological studies of natural populations in an effort to understand and mitigate the impact of emerging infectious disease on threatened amphibians.

Acknowledgements

We thank Mike Eisen, Cherie Briggs, Roland Knapp, Vance Vredenburg, Craig Moritz, Nicole Maddox, Mary Stice, Jamie Voyles, Alan Lemmon, Emily Moriarty Lemmon and Luke Harmon for input on the project. We also thank the Institute for Bioinformatics and Evolutionary Studies at the University of Idaho and the BEACON Center for Evolution in Action at Michigan State University. All live animal work was conducted with approval of the University of California and University of Idaho Animal Care and Use Committees.

Financial disclosure

This work was supported by NSF (EF-0723871) and NIH (#P20 RR0116454). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Author contributions

EBR was involved in research design and direction, data collection and interpretation, and manuscript preparation. TJP was involved in data collection, bioinformatics and manuscript preparation. MS was involved in bioinformatics and manuscript preparation. GKM was involved in data interpretation and manuscript preparation.

References

Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ (1990) Basic local alignment search tool. *Journal of Molecular Biology*, **215**, 403–410.

Baeuerle PA, Henkel T (1994) Function and activation of NF-kappa B in the immune system. *Annual Review of Immunology*, **12**, 141–179.

Benjamini Y, Hochberg Y (1995) Controlling the false discovery rate – a practical and powerful approach to multiple testing. *Journal of the Royal Statistical Society Series B*, **57**, 289–300.

Berger L, Speare R, Daszak P *et al*. (1998) Chytridiomycosis causes amphibian mortality associated with population declines in the rain forests of Australia and Central America. *Proceedings of the National Academy of Sciences of the United States of America*, **95**, 9031–9036.

Berger L, Marantelli G, Skerratt LL, Speare R (2005) Virulence of the amphibian chytrid fungus *Batrachochytrium dendrobatidis* varies with the strain. *Diseases of Aquatic Organisms*, **68**, 47–50.

Bolstad BM, Irizarry RA, Astrand M, Speed TP (2003) A comparison of normalization methods for high density oligonucleotide array data based on variance and bias. *Bioinformatics*, **19**, 185–193.

Boyle DG, Boyle DB, Olsen V, Morgan JAT, Hyatt AD (2004) Rapid quantitative detection of chytridiomycosis (*Batrachochytrium dendrobatidis*) in amphibian samples using real-time Taqman PCR assay. *Diseases of Aquatic Organisms*, **60**, 141–148.

Briggs CJ, Vredenburg VT, Knapp RA, Rachowicz LJ (2005) Investigating the population-level effects of chytridiomycosis, a fungal disease of amphibians. *Ecology*, **86**, 3149–3159.

Briggs CJ, Knapp RA, Vredenburg VT (2010) Enzootic and epizootic dynamics of the chytrid fungal pathogen of amphibians. *Proceedings of the National Academy of Sciences of the United States of America*, **107**, 9695–9970.

Carver S, Bell BD, Waldman B (2010) Does chytridiomycosis disrupt amphibian skin function? *Copeia*, **2010**, 487–495.

Daszak P, Strieby A, Cunningham AA, Longcore JE, Brown CC, Porter D (2004) Experimental evidence that the bullfrog (*Rana catesbeiana*) is a potential carrier of chytridiomycosis, an emerging fungal disease of amphibians. *Herpetological Journal*, **14**, 201–207.

Irizarry RA, Hobbs B, Collin F, Beazer-Barclay YE, Antonellis KJ, Scherf U, Speed TP (2003) Exploration, normalization, and summaries of high density oligonucleotide array probe level data. *Biostatistics*, **4**, 249–264.

Kilpatrick AM, Briggs CJ, Daszak P (2010) The ecology and impact of chytridiomycosis: an emerging disease of amphibians. *Trends in Ecology and Evolution*, **25**, 109–118.

Lips KR, Brem F, Brenes R *et al*. (2006) Emerging infectious disease and the loss of biodiversity in a Neotropical amphibian community. *Proceedings of the National Academy of Sciences of the United States of America*, **103**, 3165–3170.

Longcore JE, Pessier AP, Nichols DK (1999) *Batrachochytrium dendrobatidis* gen et sp nov, a chytrid pathogenic to amphibians. *Mycologia*, **91**, 219–227.

Marcum RD, St-Hilaire S, Murphy PJ, Rodnick KJ (2010) Effects of *Batrachochytrium dendrobatidis* infection on ion concentrations in the boreal toad *Anaxyrus (Bufo) boreas boreas*. *Diseases of Aquatic Organisms*, **91**, 17–21.

Mohneke M, Roedel MO (2009) Declining amphibian populations and possible ecological consequences – a review. *Salamandra*, **45**, 203–210.

Morey JS, Ryan JC, Van Dolah FM (2006) Microarray validation: factors influencing correlation between oligonucleotide microarrays and real-time PCR. *Biological Procedures Online*, **8**, 175–193.

Myrkov I, Stojanovic I, Stosic-Grujicic S, Glamoclija J, Zolotarevski L, Kataranovski D, Kataranovski M (2010) Splenic and lung response to nonlethal systemic *Aspergillus fumigatus* infection in C57BL/6 mice. *Medical Mycology*, **48**, 735–743.

Owais M, Ansari MA, Ahmad I, Zia Q, Pierard G, Chauhan A (2010) Innate immunity in pathogenesis and treatment of dermatomycosis. In: *Combating Fungal Infections: Problems and Remedy*, editors: Ahmad I, Owais M, Shahid M. Springer.

R Development Core Team (2011) *R: A Language and Environment for Statistical Computing*. R Foundation for Statistical Computing, Vienna, Austria. ISBN 3-900051-07-0, URL http://www.R-project.org/.

Rafael A, Bolstad BM, Collin F, Cope LM, Hobbs B, Speed TP (2003) Summaries of affymetrix genechip probe level data. *Nucleic Acids Research*, **31**, e15.

Ramsey JP, Reinert LK, Woodhams DC, Rollins-Smith LA (2010) Immune defenses against *Batrachochytrium dendrobatidis*, a fungus linked to global amphibian declines, in the South African clawed frog, *Xenopus laevis*. *Infection and Immunity*, **78**, 3981–3992.

Retallick RWR, Miera V (2007) Strain differences in the amphibian chytrid *Batrachochytrium dendrobatidis* and non-permanent, sub-lethal effects of infection. *Diseases of Aquatic Organisms*, **75**, 201–207.

Retallick RWR, McCallum H, Speare R (2004) Endemic infection of the amphibian chytrid fungus in a frog community post-decline. *PLoS Biology*, **2**, 5–1971.

Ribas L, Li MS, Doddington BJ (2009) Expression profiling the temperature-dependent amphibian response to infection by *Batrachochytrium dendrobatidis*. *PLoS ONE*, **4**, e8408.

Rivera A, Ro G, Van Epps HL, Simpson T, Leiner I, Sant'Angelo DB, Pamer EG (2006) Innate immune activation and CD4+ T cell priming during respiratory fungal infection. *Immunity*, **25**, 665–675.

Rosenblum EB, Poorten TJ, Settles M, Murdoch GK, Robert J, Maddox N, Eisen MB (2009) Genome-wide transcriptional response of *Silurana* (*Xenopus*) *tropicalis* to infection with the deadly chytrid fungus. *PLoS ONE*, **4**, e6494.

Sims JE, Smith DE (2010) The IL-1 family: regulators of immunity. *Nature Reviews Immunology*, **10**, 89–102.

Skerratt LF, Berger L, Speare R *et al.* (2007) Spread of chytridiomycosis has caused the rapid global decline and extinction of frogs. *EcoHealth*, **4**, 125–134.

Smith FF (2003) The molecular genetics of keratin disorders. *American Journal of Clinical Dermatology*, **4**, 347–364.

Smith KF, Acevedo-Whitehouse K, Pedersen AB (2009) The role of infectious diseases in biological conservation. *Animal Conservation*, **12**, 1–12.

Smyth GK (2005) *Limma: Linear Models for Microarray Data. Bioinformatics and Computational Biology Solutions Using R and Bioconductor*. Springer, New York City, New York. pp. 397–420.

Soto-Azat C, Clarke BT, Poynton JC, Cunningham AA (2010) Widespread historical presence of *Batrachochytrium dendrobatidis* in African pipid frogs. *Diversity and Distributions*, **16**, 126–131.

Stice MJ, Briggs CJ (2010) Immunization is ineffective at preventing infection and mortality due to the amphibian chytrid fungus *Batrachochytrium dendrobatidis*. *Journal of Wildlife Diseases*, **46**, 70–77.

Voyles J, Young S, Berger L *et al.* (2009) Pathogenesis of chytridiomycosis, a cause of catastrophic amphibian declines. *Science*, **326**, 582–585.

Vredenburg VT, Fellers G, Davidson C (2005) The mountain yellow-legged frog *Rana muscosa* (Camp 1917). In: *Status and Conservation of US Amphibians* (ed. Lanoo M), pp. 563–566. University of California Press, Berkeley, California.

Vredenburg VT, Bingham R, Knapp R, Morgan JAT, Moritz C, Wake D (2007) Concordant molecular and phenotypic data delineate new taxonomy and conservation priorities for the endangered mountain yellow-legged frog (Ranidae: *Rana muscosa*). *Journal of Zoology*, **271**, 361–374.

Vredenburg VT, Knapp RA, Tunstall T, Briggs CJ (2010) Large-scale amphibian die-offs driven by the dynamics of an emerging infectious disease. *Proceedings of the National Academy of Sciences of the United States of America*, **107**, 9689–9694.

Wake DB, Vredenburg VT (2008) Are we in the midst of the sixth mass extinction? A view from the world of amphibians. *Proceedings of the National Academy of Sciences of the United States of America*, **105**, 11466–11473.

E.B.R. is interested in the processes that generate and impact biological diversity with a focus on mechanisms of speciation and extinction. T.J.P. is interested in understanding how genomic evolution affects host-pathogen interactions. M.S. is interested in the applied analysis of high-throughput data, including microarray data, genotype data, and high-throughput sequencing data. G.K.M. is interested in the molecular, cellular, and systemic physiology of growth, development, and adaptation to the environment.

Data accessibility

All sequence data are publicly available (SRA project ID SRP010163), and all microarray data are publicly available in accordance with MIAME (GEO accession number GSE34235). Infection experiment data are provided in Table S3.

Supporting information

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

Table S1 Number of differentially expressed genes for each tissue and time point.

Table S2 Genes showing shared expression patterns in the *Rana muscosa/sierrae* and the *Silurana tropicalis* datasets.

Table S3 Sample data for frogs individually housed during infection experiment including Bd load at point of sacrifice (# zoospore equivalents as determined using qPCR).

Fig. S1 Distribution of expressed transcripts in the three tissues (probesets with IQR > 0.5).

Data S1 Methods.

Please note: Wiley-Blackwell are not responsible for the content or functionality of any supporting information supplied by the authors. Any queries (other than missing material) should be directed to the corresponding author for the article.