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Research article

Temperature-mediated shifts in salamander transcriptomic responses to the amphibian-killing fungus

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Abstract

Life processes of ectothermic vertebrates are intimately linked to the temperature of their environment, influencing their metabolism, reproduction, behaviour, and immune responses. In amphibians infected by the generalist chytrid pathogen *Batrachochytrium dendrobatidis* (*Bd*), host survival, infection prevalence, and infection intensity are often temperature- and/or seasonally-dependent. However, the transcriptional underpinnings of thermal differences in infection responses are still unknown. Measuring the impact of temperature on host responses to infection is a key component for understanding climatic influences on chytrid disease dynamics. *Bd*-responsive gene pathways in frogs are well documented, but our understanding of salamander immune expression profiles during infection with chytrids remains limited. We characterize the transcriptomic responses of *Plethodon cinereus* using RNAseq by comparing skin and splenic gene expression of individuals uninfected, succumbing to *Bd* infection, and naturally cleared of *Bd* infection at three temperatures. We propose amphibian temperature-dependant susceptibility to *Bd* is likely driven by shifts in expression of innate and adaptive immune axes. Our study shows increased expression of transcripts associated with inflammation at cooler temperatures and a shift towards increased expression of adaptive immune genes, including MHC, at higher temperatures. In the face of climate change, and as concerns for the spread of emergent chytrid pathogens increase, our results provide important functional genomic resources to help understand how these pathogenic fungi may continue to affect amphibian communities globally in the future.

Introduction

Temperature influences the metabolism, reproduction, behaviour, and immune responses of ectothermic vertebrates, and this has implications for disease susceptibility. Temperatures at the low end of thermal tolerances are generally considered immunosuppressive, reducing lymphocyte numbers (Raffel, Rohr, Kiesecker, & Hudson, 2006), T cell activity (Maniero & Carey, 1997), rates of antibody production (Mikkelsen, Lindenstrøm, & Nielsen, 2006), and serum complement activity (Maniero & Carey, 1997). In some species, underlying individual thermal preferences may be a predictor of infection susceptibility (Sauer et al., 2018), while in others ectotherm behavioural fever (active preference for warmer environments) appears to be important in enhancing survival during infections (Boltana et al., 2013). Widespread thermal-dependence of parasite and pathogen life-history traits (Bakke, Cable, & Harris, 2007, Voyles et al., 2012, Muletz-Wolz et al., 2019) indicate that temperature is key to understanding infection processes and disease dynamics in ectotherm hosts.

Temperature has profound effects on the transcriptional activity of organisms; from prokaryotes (Smoot et al., 2001), plants (Winfield, Lu, Wilson, Coghill, & Edwards, 2010), fungi (Steen et al., 2002), invertebrates (Wang, Espinosa, Tanguy. & Allam, 2016) to vertebrates (Gracey et al., 2004). In multicellular organisms, temperature dependent gene expression responses are often tissue specific (Gracey et al., 2004). In vertebrates, temperature effects on gene expression are well known in terms of temperature-dependent sex determination (Shen & Wang, 2014) and acclimation to thermal stress (Quinn, McGowan, Cooper, Koope, & Davidson, 2011). However, we know far less about the effects of temperature on gene expression during infection, despite its likely impact on immune function. To fully appreciate the significance of temperature on infection processes and disease dynamics of vertebrate hosts, studies of transcriptional infection responses under different thermal regimes are needed.

The aquatic fungal pathogen *Batrachochytrium dendrobatidis* (*Bd*) is one the most devastating emergent pathogens of ectotherms, widely implicated in global amphibian population declines and extinctions (Bellard, Genovesi, & Jeschke, 2016, Scheele et al., 2019). Among frogs, hundreds of species are known hosts and *Bd* has a wide range of disease outcomes, ranging from high susceptibility to tolerance and resistance (Scheele et al., 2019). Salamanders, particularly terrestrial species, are typically at less risk from *Bd* (Lips, Reeve, & Witters, 2003, Bancroft et al., 2011) often exhibiting low natural prevalence (Hossack et al., 2010, Muletz, Caruso, Fleischer, McDiarmid, & Lips, 2014), relatively low susceptibility, and higher rates of infection clearance in laboratory studies (Vazquez, Rothermel, & Pessier, 2009, Pasmans et al., 2013). Despite this, salamander declines have been linked to *Bd* emergence (Cheng, Rovito, Wake, & Vrendenburg, 2011) and infections can induce high mortality rates in some species (Weinstein, 2009).

Temperature is also key to *Bd* physiology. In culture, optimal growth of *Bd* is in the range of 17 to 25 °C, with substantially reduced growth rates below 10 °C or above 28 °C (Piotrowski, Annis, & Longcore, 2004). Moreover, environmental temperature is an important predictor of geographic distribution of *Bd* infections and amphibian mortality rates (Kriger, Pereoglou & Hero, 2007, Longo, Burrowes, & Joglar, 2010, Savage, Sredl, & Zamudio, 2011). Elevated body temperature in frogs can clear infections (Woodhams, Alford, & Marantelli, 2003), and individual preference for warmer temperatures has been linked to increased resistance to *Bd* (Rowley & Alford 2013, Sauer et al., 2018). Yet, even within the optimal thermal range of *Bd*, anuran mortality rates vary considerably (Andre, Parker, & Briggs, 2008), suggesting that temperature-dependent host responses contribute to disease outcome. Indeed, expression profiling of *Xenopus tropicalis* revealed differential activation of innate immune genes in response to infection at two temperatures (Ribas et al., 2009). However, despite evidence of temperature-dependent survival in salamanders (Vazquez et al., 2009, Muletz-Wolz et al., 2019), we do not yet have any study of temperature-

dependent gene expression responses of salamanders to *Bd*. More broadly, we have limited knowledge of how salamander immune expression responses to *Bd* infection – regardless of temperature - compare to those in anurans (Farrer et al., 2017). While frog diversity far exceeds that of salamanders, a substantial proportion of salamander species studied have experienced severe declines (>90% reductions) due to chytrid pathogens (Scheele et al., 2019), and therefore it is critical *Bd* immunity and pathogenesis is understood across all amphibian groups. Frog and salamander immune systems are broadly similar, however there are sufficient differences in immunological tissues (e.g. spleen structure, lymphomyeloid organs, immunoglobulin types) (Zapata & Amemiya 2000, Miller & Fowler 2014) to suggest distinct infection responses.

Many species of *Plethodon* salamanders have experienced widespread declines in the eastern United States (Highton, 2005). *Plethodon cinereus* is well studied with respect to chytrid infections, typically exhibiting low *Bd* infection prevalence in the wild (Muletz, Caruso, Fleischer, McDiarmid, & Lips, 2014), and capable of clearing moderate laboratory experimental infections (Muletz et al., 2012). This species is a popular model for studying the protective role of commensal skin microbiota against *Bd* infection (e.g. Harris et al., 2009, Loudon et al., 2014, Muletz-Wolz et al., 2018, Muletz-Wolz et al., 2019). However, we do not know their functional genetic responses to infection that control disease outcomes, especially under variable temperatures. In this study we use this temperate species to capture transcriptional responses of individuals succumbing to chytridiomycosis and those cleared of infection, under relevant seasonal temperatures. We characterized the transcriptomic responses of *P. cinereus* to infection with a novel (non-North American) *Bd* strain. By comparing gene expression of individuals uninfected, succumbing, or naturally cleared of *Bd* infection at three different temperatures, we address the hypothesis that amphibian temperature-dependent variation in survival to *Bd* infection is due to underlying differences in expressed genes.

Materials & Methods

Experimental infections

The salamanders used in this study represent a subset of a larger study investigating temperature-dependent mortality in response to Bd infection (Muletz-Wolz et al., 2019). Briefly, adult *P. cinereus* (> 35 mm snout-vent length) were acclimatised for 47 days to either 13 (n = 29), 17 (n = 29), or 21 °C (n = 29). These temperatures represent average body temperature for *Plethodon* in spring (13 °C) and summer (17 °C), and a higher temperature (21 °C) within their natural range (Caruso, Sears, Adams, & Lips, 2014). In addition, the three treatment temperatures are within the range that *Bd* grows and reproduces (Piotrowski, Annis, & Longcore, 2004).

Salamanders were individually exposed for 24 hours to a *Bd* inoculum (*Bd*-exposed: 5 ml of 5.3 x 10^6 zoospores/ml solution of strain JEL423, 15 per temperature) or sham exposed (*Bd*-control: 5 ml sterile water, 14 per temperature). JEL423, a Panamanian strain, was chosen because wild salamanders from our collecting site should all be naïve to this chytrid lineage (Muletz, Caruso, Fleischer, McDiarmid, & Lips, 2014). Salamanders were monitored for morbidity (abnormal posture, excess skin sloughing, loss of appetite, lethargy, and loss of righting reflex) daily for 42 days and individuals were euthanized if they lost their right ability, or displayed all four of the other clinical signs, by applying 20% benzocaine to their dorsal side. *Bd* infection status and infection intensity (number of zoospore genomic equivalents, ZGE) were measured at 5, 11, 25, and 42 days post inoculation using skin swabs as described in Muletz-Wolz et al. (2019). Moribund salamanders were also swabbed immediately prior to euthanasia. All salamanders surviving the length of the experiment were euthanized 42 days post-inoculation.

Immediately after euthanasia, we dissected salamanders using sterilized instruments and harvested skin and spleen tissues from each individual. Tissue samples were immediately placed

in RNAlater (Invitrogen), stored at 4 °C for 24 hours, and then stored at −80 °C until RNA extraction and library preparation. All animal use was approved by IACUC protocol UMD # R-14-04. We used qPCR to confirm that individuals euthanized due to clinical signs of chytridiomycosis were "infected", control individuals were "uninfected", and *Bd*-exposed individuals that survived had "cleared" their infections. Because the individuals used in this experiment were part of a larger *Bd*-survival study (Muletz-Wolz et al., 2019) and because progression of chytridiomycosis varies widely among individuals even under controlled inoculation doses (Carey et al., 2006), we opted for a tissue sampling regime that maximized the opportunity to capture expression responses in a broadly comparable "mature" stage of infection, i.e. when hosts were actively shedding zoospores. Thus we sampled hosts over a range of days post-inoculation (Figure 1, Supplementary File 1). This sampling strategy – allowing resolution of disease outcome, either morbidity or clearance - provides opportunity to compare critical late-stage responses to *Bd* (Grogan et al., 2018a) across a temperature range. However, we recognise that one shortcoming of this design is that we cannot detect early-stage responses in the first few days post-inoculation, which can also have important consequences for disease outcome (Grogan et al., 2018b).

Transcriptome sequencing

We performed RNAseq on four to five randomly selected salamanders from those that were sham-infected ("uninfected"), *Bd*-challenged succumbing to infection ("infected"), and *Bd*-challenged cleared infection ("cleared") at each of the three temperatures (Figure 1, Supplementary File 1). Because only one salamander survived infection at 13 °C, we excluded this animal from sequencing, as no statistical tests could be applied. We followed RNA extraction and transcriptome sequencing methods of Ellison et al. (2015). Briefly, total RNA was extracted from each tissue sample separately using RNAdvance tissue kit (Beckman Coulter, Inc.). Libraries were generated using the Illumina TruSeq RNA sample preparation kit v2 (low throughput protocol)

according to the manufacturer's instructions (Illumina, San Diego, CA). Randomly pooled equimolar samples were run on 8 lanes of the Illumina HiSeq flowcell (8 samples per lane). All sequencing runs were 100-bp single-end reads. After read quality controls (Ellison et al., 2015), reads from all individuals and tissues were pooled to assemble a consensus transcriptome. Assemblies were performed using Trinity (Grabherr et al., 2011) with default parameter settings on a high-performance cluster with 64 central processing units and 512 GB random access memory. We filtered out transcripts with expression support of less than two reads per million mappable reads in at least five samples, to eliminate low-level expression noise (Harrison, Mank, & Wedell, 2012; Moghadam, Harrison, Zachar, Székely, & Mank, 2013). Genes were annotated using the BLASTX, BLAST2GO, and InterPro pipelines described in Ellison et al (2015). Any transcript aligning to the *Bd* transcriptome (*Bd* Sequencing Project, Broad Institute of Harvard and MIT, www.broadinstitute.org, accessed January 2, 2015) was removed from downstream analyses. Only salamander genes that had significant BLASTX alignments (E-value of 1 × 10−6 and minimum bit score of 55) were used for subsequent gene expression analyses.

Differential expression and gene network analyses

Gene expression was determined using the Trinity pipeline, using BWA read mapping (Li & Durbin 2009) and RSEM read count normalization (Li & Dewey 2011). We analysed differential gene expression (DGE) of control (uninfected), infected, and cleared individuals at each temperature separately using the edgeR (Robinson, McCarthy, & Smyth, 2010) R package (R version 2.15.2, R Development Core Team). This consisted of estimating tagwise dispersion and normalization factors and differentially expressed (DE) testing using an exact test. A false discovery rate (FDR)–corrected P value of less than 0.05 was considered to be evidence of DGE. To quantify the overlap of differentially expressed genes between temperatures, we constructed Venn diagrams for each tissue using VENNY (Oliveros 2007) for significantly increased and decreased expressed genes

separately. We tested for enrichment of biological process GO terms in each group of DE genes (e.g., specific to one temperature or shared among two or more temperatures) using BLAST2GO. To compare gene expression between infected salamanders and those cleared of infection at day 42, we excluded genes found to be differentially expressed between controls and infected samples. This method excludes genes that may have returned to baseline (i.e. non-infected) levels since clearing infection.

Differential gene expression analyses consist of exact tests on each gene separately and thus necessitate multiple test correction methods (e.g., FDR), and typically only genes with the largest differences in expression are identified. An alternative for quantifying systematic transcriptional responses of salamanders to temperature and infection challenge by *Bd* is weighted gene coexpression network analysis (WGCNA), which identifies networks (modules) of coexpressed genes (i.e., genes that show consistent expression profiles across samples), and thus potentially identifies functionally important genes with only subtle changes in expression that may not be detected in typical DGE analyses. First, read counts were TMM normalized using a Trinity-provided Perl script to produce fragments per kilobase per million mapped expression values. Next, the R package WGCNA was used for network constructions (Langfelder & Horvath 2008). Our modules were defined using the dynamicCutTree function and TOMType "signed" with a minimum module size of 100. A module eigengene distance threshold of 0.25 was also used to merge highly similar modules. Modules were then correlated with log-transformed *Bd* infection intensity (ZGE), days post-inoculation (DPI), and temperature to identify gene networks significantly involved in temperature-dependent responses to *Bd* infection. GO term enrichment tests of each gene module that significantly correlated with *Bd* load were performed using BLAST2GO as described above. Each gene within a module was ranked by its module membership (kME), calculated by WGCNA. Network hub genes were defined as those ranked in

the top 100 module membership values and with the highest 150 network connection weights. Hub gene network connections were exported to Cytoscape (Shannon et al., 2003) for visualization. Gene modules were labelled numerically with the prefix “SK” for skin networks and “SP” for spleen networks (Table 2).

Results

Bd infection challenge

All uninfected control salamanders remained *Bd*-negative for the duration of the experiment. In the larger survival study, from which we sampled individuals for this study, survival of uninfected salamanders was 100%, 100% and 86% at 13 °C, 17 °C, and 21 °C, respectively. Survival of *Bd*-challenged salamanders was 6.6%, 26.7%, and 33.3% at 13 °C, 17 °C, and 21 °C respectively (Muletz-Wolz et al., 2019). We found a significant effect of *Bd* exposure on survival rate for *Bd*-exposed vs. *Bd*-control; exposed individuals had lower survival rates. *Bd*-exposed salamanders at 13°C had a higher mortality rate compared to other temperature treatments, although this difference was not statistically significant (post-hoc $p > 0.33$, Muletz-Wolz et al., 2019). However, at 11 days post-exposure *Bd*-exposed salamanders at 13 °C had significantly higher loads than at 17 °C and 21 °C (Supplementary Figure 1). We found a significant negative correlation between *Bd* load and sampling day; salamanders with higher loads had shorter survival (Pearson’s $R = -0.762$, $P = 0.004$). The studied individuals succumbing to infection were sampled between 5 and 21 days post-inoculation (Figure 1, Supplementary File 1), with surviving salamanders and non-exposed controls sampled at day 42.

Transcriptome assembly

Skin and spleen tissue samples were sequenced on eight lanes of Illumina HiSeq, resulting in more than 2,099 million 100 bp single-end reads after quality controls and trimming, with an

average of 33.51 million reads per sample. Sequences are deposited in the NCBI Short Read Archive under submission accession number PRJNA559247 (https://www.ncbi.nlm.nih.gov/bioproject/PRJNA559247). Our *de novo* assembly of the transcriptome, after filtering out low expression transcripts (threshold of at least two reads per million mappable reads in at least five samples), yielded 117,812 transcripts, with a mean length of 1084 bp and N50 length of 2345 bp. We expect that the vast number of minimally expressed contigs that fall below our threshold to largely represent transcriptional errors such as intron expression, exon chimaeras, and sequencing and assembly errors common to current *de novo* assembly techniques (Moghadam et al., 2013). Of the assembled genes, 38,266 (32.48%) had at least one significant hit against the nonredundant NCBI protein database, and of these, 19,975 (52.20%) were successfully annotated with GO terms.

Responses to infection at all temperatures

Infected moribund salamanders had distinct transcriptome-wide gene expression profiles compared to both uninfected and cleared groups in both tissues (Figure 2). At this broad scale, separation of cleared and uninfected samples, and by temperature (in any treatment group) was not apparent (Figure 2). Yet, there were many genes that showed differential expression among infection status and temperature levels. Moreover, by excluding genes showing expression differences between infected and control individuals, we could separate key genes with ongoing responses to *Bd*-challenge in the cleared groups from those returned to a “healthy” state. Full lists of differentially expressed genes and enrichment of gene groups and modules are provided as supplementary material (Supplementary Files 2-4).

Skin responses

For skin samples, we found 3,318 genes at 13 °C, 4,538 genes at 17 °C, and 3,305 genes at 21 °C with significant expression differences between uninfected and infected individuals. Of these, 1,071 exhibited increased expression in infected salamanders compared to uninfected controls at all temperatures. For the purposes of this study, we focus the presentation of our results on genes of known immune function in vertebrates and/or highlighted in previous amphibian *Bd* infection studies. We found significant gene ontology (GO) term enrichment of these genes to include numerous immune-related terms including “metalloendopeptidase activity” (the top molecular function, Table 1), “cytokine production”, “inflammatory response”, “neutrophil migration”, “lysozyme activity”, “T cell activation”, “macrophage activation”, and “detection of fungus”. The 397 genes sharing significant decreases in expression in infected skin samples (compared to uninfected) were enriched for GO terms related to skin integrity such as “intermediate filament organization”, “skin development”, and “keratinization”.

We found 19 gene modules in the skin significantly correlated with *Bd* load and not temperature (Table 2). Eight modules were positively correlated with *Bd* load (increased expression with higher infection intensity) and negatively with days post-inoculation (DPI, lower expression with increasing days), of which three were enriched for immune-related GO terms. The top molecular function enrichment of module SK15 was threonine-type peptidase activity and included the GO terms antigen processing & presentation via MHCI and MHCII receptor activity. SK16 was enriched for several cytokine terms including regulation of INF-γ production, interleukin-2 production, and regulation of TLR3 signalling pathway. Four of the ten modules negatively correlated with *Bd* load (decreased expression with increased infection intensity) and positively with DPI (increased expression in later samples) were enriched for immune-related GO terms (Table 2). These included cytokine responses (SK4; IL5 and IL13 secretion, SK8; cytokine production), chitinase activity (SK5), NF-kB responses (SK6; NF-kB signaling and FC-γ complex

binding), lymphocyte signaling (SK8; T cell aggregation and B cell activation), and MHC activity (SK8; antigen presentation via MHC I). We also found a gene module (SK6), enriched for skin integrity terms (e.g. extracellular matrix organization, collagen binding, and skin morphogenesis), to be negatively correlated with *Bd* load and positively with DPI.

Spleen responses

For spleen samples, we found 2,958 genes at 13 °C, 2,204 genes at 17 °C, and 2,587 genes at 21 °C with significant expression differences between uninfected and infected individuals. We found 887 of these genes had higher expression in infected salamanders compared to uninfected controls at all temperatures. These transcripts were enriched for several immune-related GO terms including leukocyte migration, IL2 production, and acute inflammatory response (Table 1). In contrast, only 75 genes shared significant decreases in expression in infected spleen samples (compared to uninfected). These genes were also enriched for GO terms related to immune functions, notably activation of immune response, cytokine secretion, toll-like receptor signaling pathway, inflammatory response, B cell receptor signaling pathway, and T-helper 1 type immune response (Table 1).

We found 15 gene modules in the spleen significantly correlated with *Bd* load and not temperature (Table 2). Five modules were positively correlated with *Bd* load and negatively with DPI, all of which were enriched for immune-related GO terms including negative regulation of B cell activity (SP13), regulation of wound healing (SP14), establishment of T cell polarity (SP15), response to cytokine (SP16), and MHC II receptor activity (SP17). Six of the ten modules negatively correlated with *Bd* load and positively with DPI were enriched for immune-related GO terms (Table 2). These were predominantly related to B and T cell responses such as positive regulation of T cell receptor signaling (SP1), positive regulation of T cell cytokine production (SP2), immature B cell differentiation (SP3), T cell cytotoxicity (SP4), and B cell signaling pathway (SP6).

Temperature-dependent responses to infection

Skin

To determine temperature-dependent differential gene expression responses to infection, we compared gene expression between uninfected (control) and infected salamanders within each temperature treatment separately. Then, differentially expressed gene lists were compared across temperatures. We found 482 genes with higher expression in infected salamanders compared to uninfected controls at only 17 °C and 21 °C. GO term enrichment testing revealed threonine-type peptidase activity as the most significant molecular function and also included antigen processing and presentation via MHC I (Table 1, Figure 3). The 357 genes sharing increases in expression in infected salamanders at 13 °C and 17 °C were enriched for a number of immune-related terms including response to INF-γ, B cell apoptotic process, innate immune response, and mast cell proliferation (Table 1). The 156 genes with higher expression in infected salamanders at 13 °C and 21 °C were enriched for serine-type peptidase activity. Genes exhibiting higher expression at only 17 °C were enriched for negative regulation of activation-induced cell death of T cells.

Further interrogation of specific immune genes showing temperature-dependent responses in the skin revealed a number involved in MHC presentation (Figure 3). MHC II beta chain (TR517114|c0_g5) was only significantly increased in expression in infected salamanders at 13 °C, whereas four MHC I antigens (20% of those in our transcriptome assembly) had higher expression at 17 °C or 21 °C. Furthermore, expression of heat-shock proteins (HSPs) and proteasome genes involved in MHC antigen presentation were temperature-dependant (Figure 3). Of note, 26S proteasome subunits were consistently only significantly higher at 17 °C and 21 °C. In addition, components of immunoglobulins exhibited significantly higher expression in infected samples (compared to controls) at one of the three temperatures (Figure 3). Innate immune genes

influenced by temperature included anti-microbial peptides (cathelecidin), chitinase, and lysozymes (Figure 3).

We found one skin gene module significantly correlated with both temperature and *Bd* infection (SK3, Table 2). This module, negatively correlated with *Bd* load yet positively correlated with temperature and days post-inoculation, was found to be enriched for the GO terms MHC II protein binding and type I interferon signaling.

Spleen

We examined gene expression patterns that were unique to specific temperatures and found 361 genes sharing increases in expression in infected salamanders at 13 °C and 17 °C, which were enriched for regulation of I-kappaB kinase/NF-kappaB signalling and negative regulation of interleukin-8 biosynthetic process (Table 1). In contrast, we found the genes sharing increases in expression of infected spleen samples at 13 °C and 17 °C (n = 204) and 17 °C and 21 °C (n = 185) were not enriched for any known immune function (Table 1). However, the genes with significantly lower expression in infected salamanders at 13 °C and 17 °C (n = 44) were enriched for a number of immune related GO terms including MyD88-dependent toll-like receptor signalling pathway and negative regulation of lymphocyte differentiation (Table 1). We also found a number of immune GO terms enriched in infected/uninfected comparisons at only one of the three experimental temperatures. At 21 °C, genes with higher expression in infected salamanders (n = 497) were enriched for chitinase activity, whilst genes with lower expression (n = 445) included alpha-beta T cell activation, interleukin-4 production, and NK T cell differentiation. At 13 °C, genes with higher expression in infected salamanders (n = 411) were enriched for T cell homeostasis, negative regulation of activation-induced cell death of T cells, and T cell apoptotic process. No immune-related GO term enrichment was found in genes only differentially expressed at 17 °C.

We found two spleen gene modules significantly correlated with both temperature and *Bd* infection (Table 2), yet neither showed significant enrichment of any immune related GO terms. However, the "hub" genes (genes with strongest co-expression connections) of module SP12 (top GO enrichment: "cAMP binding") included interleukin 1β and a number of matrix metalloproteases (Figure 4). The genes in this module, while predominantly significantly upregulated in infected salamanders in all temperature groups, show stronger over-expression at lower temperatures.

Survivors of infection

Broad-scale skin expression profiles of control salamanders and salamanders that had cleared infection 42 days post-inoculation were very similar (Figure 2). Therefore, we excluded genes found to be differentially expressed between controls and infected samples to identify differences between salamanders clearing infection from those succumbing to infection. This method excludes genes that may have returned to baseline (i.e. non-infected) levels since clearing infection, and reveals genes with ongoing expression changes post-infection. Only a single individual survived *Bd*-challenge at 13 °C, and so was excluded from these analyses.

We found 1,456 and 511 skin genes differentially expressed between infected and cleared salamanders at 21 °C and 17 °C respectively, of which 120 were shared between temperatures. We found MHC II receptor activity GO term enrichment only in genes with significantly higher expression in infected compared to cleared skin samples at 21 °C (Table 1). In contrast, several MHC I antigens were more highly expressed by cleared salamanders in either 17 °C or 21 °C (Figure 5), though none were found to show significant differences in both temperatures. Immune genes with higher expression only in cleared salamanders at 21 °C included lymphocyte markers and attractants (lymphotactin and CXCR3), cathepsins, and immunoglobulins (Supplementary File 2). At 17 °C, we also found immunoglobulins and cathepsins with higher expression in cleared salamanders, in addition to lysozyme G and chitinase (Supplementary File 2). Chemokine 19

(CCL19) was found to have higher expression in the skin of cleared salamanders at both 17 °C and 21 °C.

We found 519 and 580 spleen genes differentially expressed between infected and cleared salamanders at 21 °C and 17 °C respectively, of which 88 were shared between temperatures. The B cell marker CD72 and immunoglobulin light chains had significantly higher expression in cleared salamanders at both temperatures (Supplementary File 2). Similar to the skin, several MHC I antigens were more highly expressed by cleared salamanders in either 17 °C or 21 °C, though none were found to show significant differences in both temperatures. Temperature-specific increases in expression in cleared spleen samples also included CCR10, CD40L, and TBX21 at 21 °C (Supplementary File 2).

Discussion

In amphibians infected by *Batrachochytrium dendrobatidis*, host survival, infection prevalence, and infection intensity are often temperature- and/or seasonally-dependent (Kriger, Pereoglou & Hero, 2007, Longo, Burrowes, & Joglar, 2010, Savage, Sredl, & Zamudio, 2011), which we hypothesized is related to temperature-dependant transcriptional responses to the fungal pathogen. Here, we characterize the transcriptomic profiles of *P. cinereus,* demonstrating substantial differences in expression of several thousand genes in two infection-relevant tissues - the skin and spleen – between infected and non-infected salamanders. We find key gene functional groups, particularly those related to inflammation and adaptive immunity, to have a temperature-dependent response to infection that likely contribute to observed variation in survival.

In this study, to measure transcriptomic responses of salamanders carrying *Bd* (contrasted with unchallenged controls), salamanders were sampled at a late stage of infection once they showed

391 clinical signs of chytridiomycosis. Our aim was to capture gene expression of infected individuals
392 at a broadly comparable point in chytridiomycosis disease progression. The rate of *Bd* infection
393 progression within species varies considerably (Carey et al. 2006), so this approach (sampling
394 over a small range of days post-inoculation, Figure 1) maximises opportunity to achieve this aim.
395 In addition, as infection loads were not ascertained until after the end of the experiment,
396 salamanders cleared of infection (surviving and *Bd*-negative at 42 days) were sampled at a
397 different time point to the "infected" group. Therefore, a degree of caution must be used when
398 interpreting these contrasts. First, susceptible amphibians at late stages of infection appear to
399 have ineffective constitutive and innate defenses, and a late-stage response characterized by
400 immunopathology and *Bd*-induced suppression of lymphocyte responses (Grogan et al 2018a).
401 Here, we discuss specifically all responses and do not attempt to disentangle differential
402 expression due an active fight against the pathogen versus late stage immunopathology.
403 Nonetheless, data on late stage responses are important to improve our understanding of the
404 impact of chytridiomycosis under different thermal regimes. Second, although "cleared"
405 salamanders were sampled at a different time to "infected" groups, by using a highly conservative
406 subtractive expression approach (see Methods) we are able to show ongoing responses to
407 infection (and importantly how this differs with temperature) and propose key pathways that may
408 contribute to successful clearance of *Bd*. Our data provide new information towards understanding
409 the commonly observed thermal and/or seasonal impact of disease outcome across amphibians.
410 Future studies that characterise infection time-courses (specifically using earlier sampling points)
411 under different temperature regimes will be an important complement to our study.

412 The core *Bd*-response genes of infected *P. cinereus* – genes with differential expression
413 compared to non-infected animals at all temperatures – were enriched for the key functional
414 classes metallopeptidase activity, inflammation, and cytokine production (Table 1). These immune

pathways have been consistently highlighted as responsive to *Bd* in frogs (Rosenblum, Poorten, Settles, & Murdoch, 2012, Ellison et al., 2014, Ellison et al., 2015) and suggests these are markers deeply conserved in *Bd* responses of amphibia. Yet, comparison of expression profiles of salamanders infected with either *Bsal* or *Bd* show substantial differences in immune activation (Farrer et al., 2017). *Bsal* elicits no substantial immune response in salamanders (Farrer et al., 2017), indicating the transcriptional responses observed here and in previous anuran studies are not necessarily shared across all chytrid infections.

We show that *Bd* influences the expression of genes involved in skin integrity and spleen lymphocyte production in salamanders. Disruption of skin integrity and spleen lymphocyte suppression are considered key factors in the pathogenicity of *Bd* and have been demonstrated in a number of susceptible anuran species (Voyles et al., 2009, Fites et al., 2013, Ellison et al., 2014, Ellison et al., 2015, Grogan et al., 2018b). Conversely, more effective responses to *Bd* include generally lower levels of gene dysregulation, robust early innate and adaptive immune responses (Grogan et al., 2018a), and increased skin structural protein and splenic lymphocyte production during infection (Ellison et al., 2015). We found that skin genes in infected individuals sharing decreased expression at all temperatures were rich in functions related to collagen and keratin production (Table 1, Supplementary File 3). Moreover, we found a skin gene module – negatively correlated with *Bd* load – to be associated with skin development and structure indicating that as infection load increases skin integrity decreases (Table 2).This module was also positively correlated with days post-inoculation (DPI), suggesting individuals surviving longer (even if eventually succumbing) had higher expression of skin integrity genes. In addition, comparison of salamanders that cleared infection to those that succumbed, indicated higher expression levels of keratins and collagens in the cleared groups (Supplementary File 2) suggesting maintenance of skin integrity may be crucial to intraspecific differences in survival in

salamanders. We found evidence for *Bd*-induced immunosuppression; spleen genes associated with Th_1 responses and B cell signalling were lower in infected salamanders at all temperatures (Table 1). We also found five of the splenic gene modules (negatively correlated with *Bd* load) enriched for various lymphocyte development, activation, and signalling functions (Table 2). These modules also were positively associated with DPI; individuals surviving longer had higher expression. Taken together, these results indicate that the immunosuppressive ability of *Bd* is widespread throughout its host species range and effective induction of skin repair and lymphocyte responses may be key resistance mechanisms.

The temperature dependence of chytridiomycosis within the thermal range of *Bd*, leads to the hypothesis that the effect of temperature on amphibian host immune responses influences disease outcome. In the skin, we found key components of anti-fungal activity to be differentially expressed with temperature, including chitinases (greater upregulation at high temperature), lysozymes and anti-microbial peptides (greater upregulation at low temperatures, Figure 3). Spleen expression profiles of *Bd*-infected *Xenopus tropicalis* indicate that temperature-dependant induction of innate immunity – particularly anti-microbial peptides and inflammatory responses – but not adaptive immune responses, are responsible for greater host survival at warmer temperatures (Ribas et al., 2009). Similarly, salamander interleukin expression increases with temperature in the spleen (Figure 4). In contrast, we observe greater expression changes in these cytokines at the lower temperatures in the skin (Figure 3), the primary site of infection. This is potentially in response to shifts of pathogen life history traits with temperature (Muletz-Wolz et al., 2019).

Shifts in immune expression from adaptive to innate pathways is observed in wild ectothermic vertebrates in winter (Brown et al., 2016). Here we find, in response to *Bd* infection, generally increased activation of innate immune pathways (e.g. increased anti-fungal, anti-microbial, and

interleukin expression) in the skin with cooler temperatures, and yet significantly higher expression of adaptive immune genes at 17 °C and 21 °C (Figure 3). Genes involved in MHC-mediated antigen processing and presentation, including MHC I antigens and MHCII-related heat-shock proteins and proteasome subunits, show the most pronounced response to temperature, with increase in expression as temperature increases. Although these findings are from individuals succumbing to infection, these results still suggest a greater ability to activate adaptive immune responses to *Bd* at higher temperatures, where more salamanders survived infection. In contrast, there appears to be a greater reliance on innate and inflammatory pathways when temperatures are cooler, where the greatest number of mortalities were observed. We now have evidence for the importance of the major histocompatibility complex (MHC) genotypes in amphibian-chytrid resistance (Savage & Zamudio, 2011, Bataille et al., 2015, Savage & Zamudio, 2016, Kosch et al., 2018). However, our results indicate that environmental temperature and MHC genotypes must be considered together to fully explain population differences in *Bd* susceptibility. Temperature-dependence of MHC activity is likely key to observed patterns of seasonal trends in chytridiomycosis prevalence and intensity (Kriger & Hero, 2007, Longo et al., 2010, Savage et al., 2011, Grogan et al., 2016).

At the two highest experimental temperatures (17 °C and 21 °C) more *Bd*-challenged salamanders cleared infection (Muletz-Wolz et al., 2019). As survivors of infection had broadly similar transcriptomic profiles to unchallenged individuals at time of sampling (Figure 2), to assess specifically the ongoing expression responses of *Bd*-surviving salamanders we excluded any genes differentially expressed between control and infected groups (i.e. those that have returned to a normal/healthy state). This approach detected key differences in their gene expression compared to salamanders that succumbed to the infection challenge. We found higher levels of immunoglobulin markers in cleared salamanders compared to infected samples. The animals

used in this study were sampled from a wild population in an area known to harbour *Bd* (Muletz et al., 2014). The increased antibody expression found here in survivors of infection may indicate their prior exposure to *Bd*, which could prime antibody production responses and provide greater protection under our experimental challenge to a novel strain. However, as the infection history of the studied salamanders could not be determined (all salamanders were *Bd*-negative at the start of the study), further controlled comparisons of *Bd*-naïve and *Bd*-exposed individuals are required to support this hypothesis.

Interestingly, in both skin and spleen samples we found higher expression of MHC I antigens in individuals that had survived infection (Figure 5). Previous amphibian MHC-*Bd* studies have been primarily focussed on MHC class II genotype-survival associations (Savage & Zamudio, 2011, Bataille et al., 2015, Savage & Zamudio, 2016). Though more recently, specific MHC I alleles have been linked with increased *Bd* susceptibility (Kosch et al., 2018). Both classes of MHC genes have been found upregulated in the skin during late-stage infections in frogs (Rosenblum et al., 2012, Ellison et al., 2014, Grogan et al., 2018b). We propose that MHC pathways expression is temperature dependant, with upregulation at warmer temperatures, but also high levels of late-stage MHC I expression are likely important for resolution of *Bd* infection. This class of immune genes clearly requires further scrutiny with respect to chytridiomycosis, particularly studies considering both MHC genotypes and their expression levels across thermal gradients simultaneously.

The host range of *Bd* is extraordinarily diverse; capable of infecting hundreds of amphibian species including frogs, salamanders, and caecilians worldwide (Scheele et al., 2019). We present the transcriptomic responses of the salamander *P. cinereus* to *Bd* challenge, across the natural thermal range of both host and fungus. We also show that, in this host species, temperature-dependant susceptibility is apparently underpinned by differences in activation of innate and

adaptive immune pathway gene expression. As the evidence of temperature and seasonal effects on *Bd* prevalence and infection intensity in amphibian communities continues to grow (Lenker, Savage, Becker, Rodriguez, & Zamudio, 2014, Blooi et al., 2015, Sapsford, Alford, & Schwarzkopf, 2018), it is essential that the underlying mechanisms for the observed trends are uncovered. Our study suggests that gene expression responses to *Bd* are thermally-dependant and may be a key component in seasonality of chytridiomycosis. Intriguingly, our data indicate that adaptive immunity, particularly MHC-related pathways, are thermally sensitive. Given the recent findings for the importance of MHC genotypes in survival against *Bd* (Savage & Zamudio, 2011, Bataille et al., 2015, Savage & Zamudio, 2016, Kosch et al., 2018), it would be valuable to follow this up with an expanded population MHC genotyping and expression study of *P. cinereus*. Furthermore, in the face of climate change, and as concerns for the spread of the newly discovered chytrid *Bsal* increase (Stegen et al., 2017), comparison of salamander functional genomic responses to both chytrid pathogens incorporating thermal variation, will be vital to understand how these emergent pathogens may continue to effect amphibian communities globally in the future.

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The authors declare no conflicts of interest.

Author Contributions

All authors designed the study. KRL, CMW and KRZ obtained funding for study. CMW performed animal experiments. ARE carried out molecular work. ARE and CMW analysed the data. All authors contributed to writing and editing of manuscript.

Data Availability

All raw sequence data are available at NCBI Short Read Archive (SRA) under accession PRJNA559247 (https://www.ncbi.nlm.nih.gov/bioproject/PRJNA559247). All other data are available in supplemental materials.

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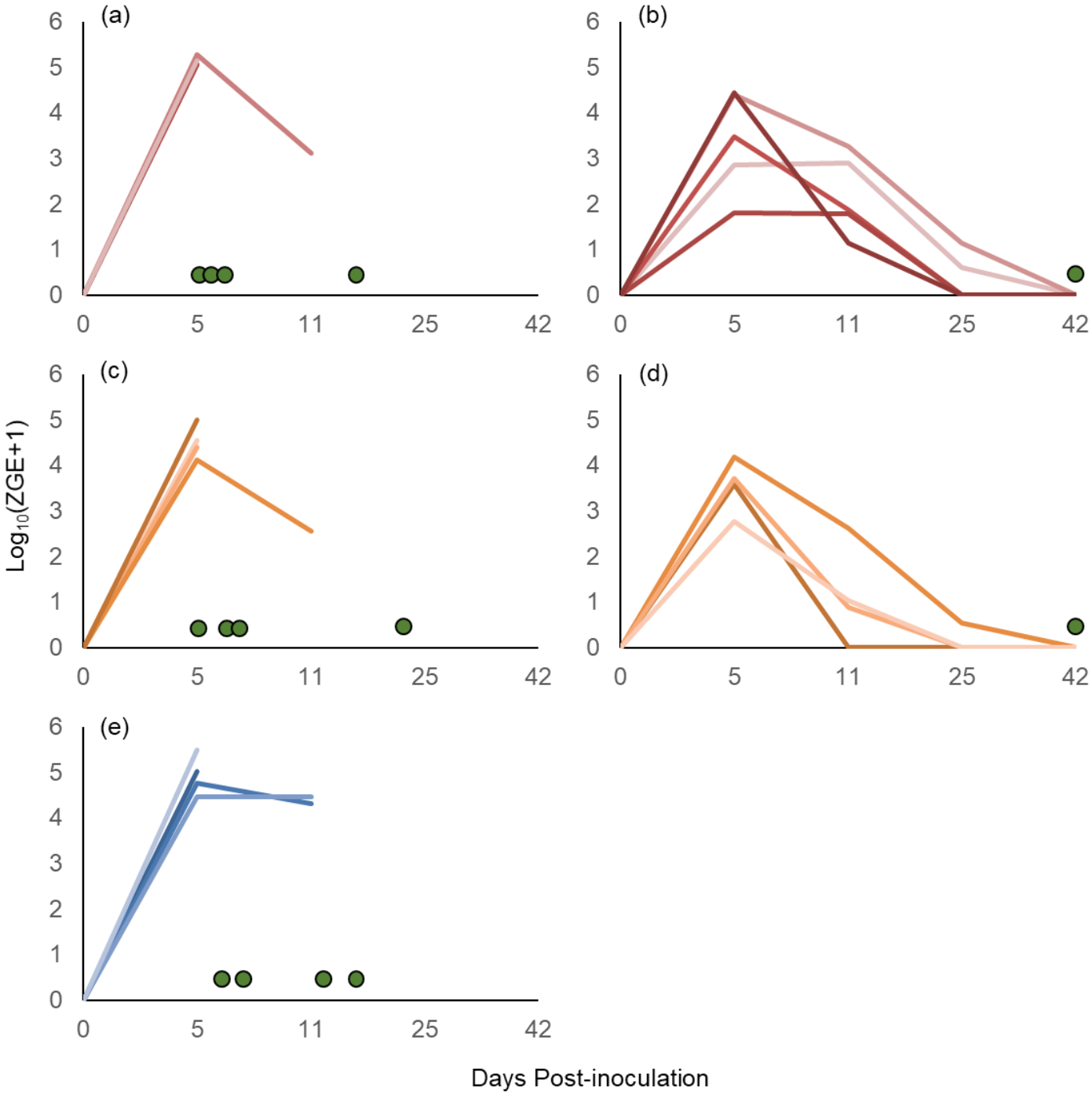


Figure 1. Individual infection trajectories of *Bd*-exposed *Plethodon cinereus* at 21 °C (a, b), 17 °C (c, d), and 13 °C (e). Green circles indicate day of tissue sampling for "infected" group succumbing to infection (left column) and "cleared" group surviving infection (right column). At day 11, individuals at 13 °C had significantly higher *Bd* loads. All *Bd*-exposed individuals that survived infection to the end of the experiment at day 42 were negative for *Bd*.

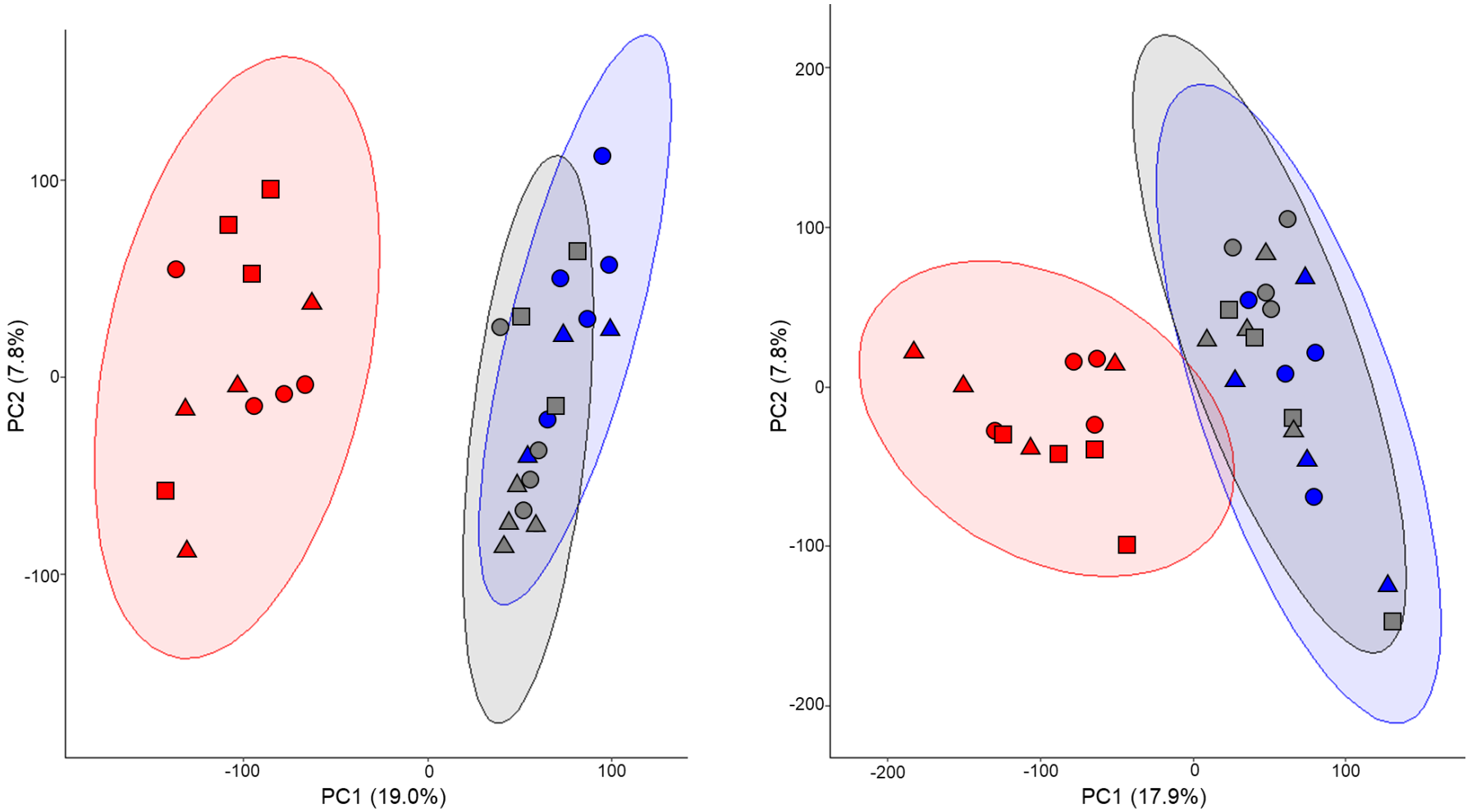
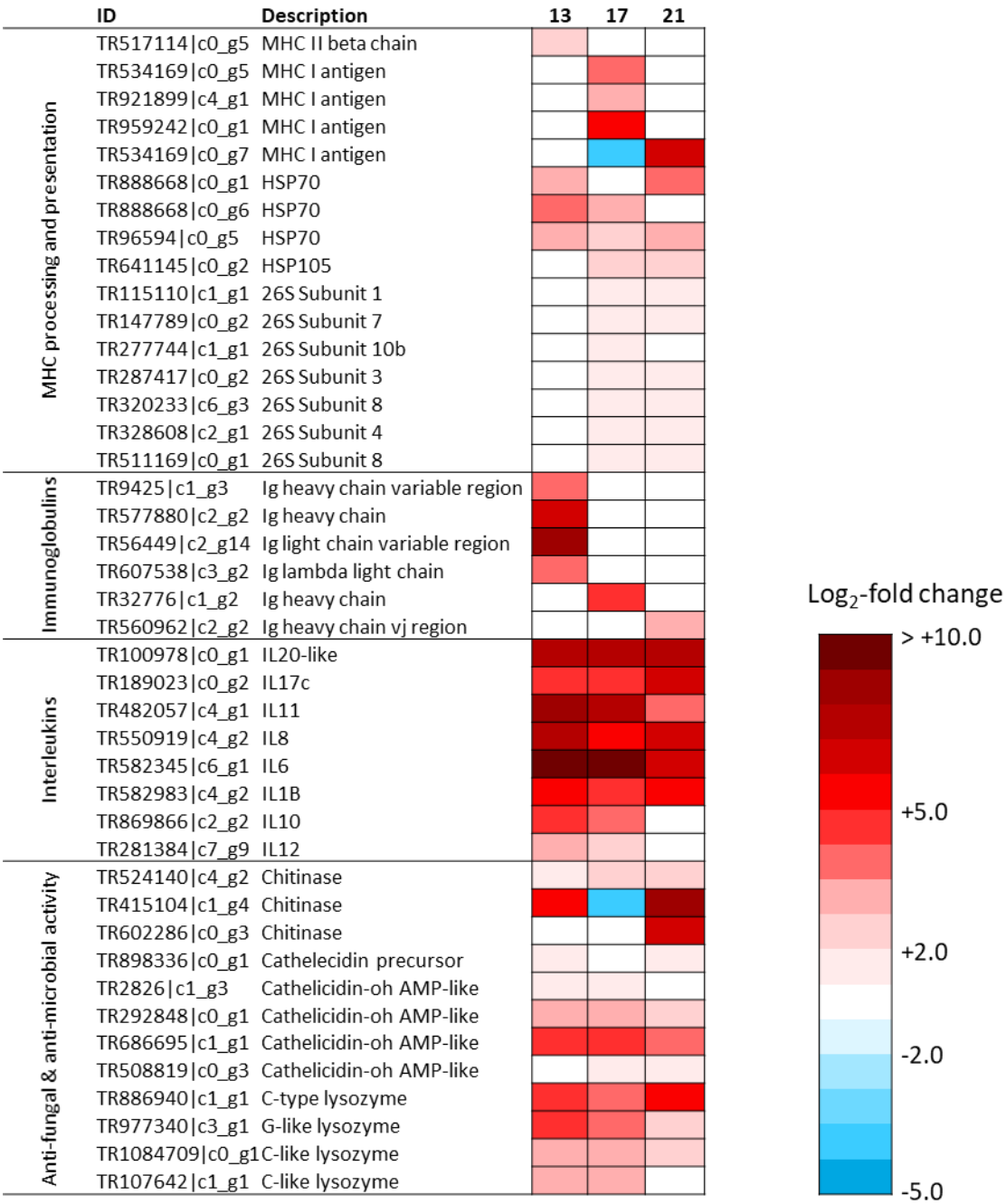


Figure 2. Principal component analysis of FPKM (Fragments Per Kilobase of transcript per Million mapped reads) normalized gene expression profiles of skin (left), and spleen (right) of *Bd*-infected (red), *Bd*-cleared (blue), and uninfected (black) *Plethodon cinereus* at 13 °C (squares), 17 °C (triangles), and 21 °C (circles). 95% confidence ellipses of infection status.



762

763 **Figure 3.** Heatmap of skin differentially expressed contigs (adjusted P <0.05) related to immune
764 responses against *Bd*, comparing infected to control (uninfected) individuals at each temperature.

765

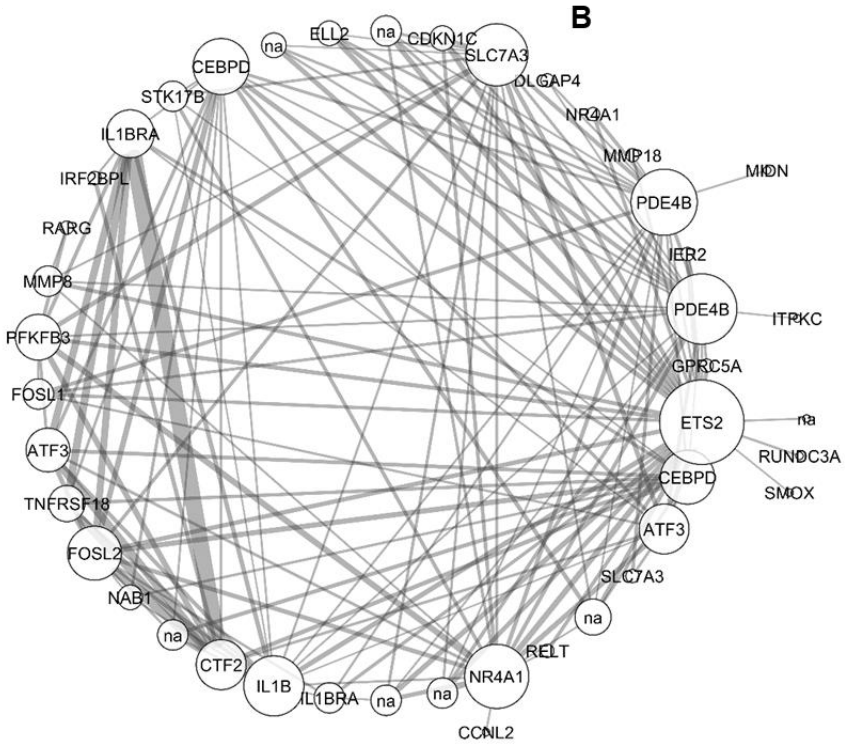
A

Contig ID	Gene symbol	Description	Relative expression 17 v 21 °C	Relative expression 17 v 13 °C	Differential expression 13 °C	Differential expression 17 °C	Differential expression 21 °C
TR1086309|c0_g1	RUNDC3A	run domain-containing protein 3a			↑	↑	↑
TR1109877|c3_g2	CTF2	cardiotrophin-2-like			↑	↑	↑
TR12160|c1_g1	PDE4B	camp-specific 3 -cyclic phosphodiesterase 4b			↑	↑	↑
TR16625|c2_g2	NR4A1	nuclear receptor subfamily 4 group a member 1			↑	↑	↑
TR170257|c0_g2	*na*	reverse transcriptase			↑	↑	↑
TR183407|c2_g3	*na*	gram domain-containing protein 3-like			↑	↑	↑
TR187259|c1_g1	SMOX	spermine oxidase			↑	↑	↑
TR229093|c0_g2	SLC7A3	cationic amino acid transporter 3			↑	↑	↑
TR229093|c0_g3	SLC7A3	cationic amino acid transporter 3			↑	↑	↑
TR257031|c0_g1	GPRC5A	retinoic acid-induced protein 3			↑	↑	↑
TR372697|c1_g1	FOSL1	fos-related antigen 1			↑	↑	↑
TR388925|c7_g3	ETS2	protein c-ets-2			↑	↑	↑
TR542|c3_g3	CEBPD	ccaat enhancer-binding protein delta			↑	↑	↑
TR542|c3_g6	CEBPD	ccaat enhancer-binding protein delta			↑	↑	↑
TR576317|c3_g4	PDE4B	camp-specific 3 -cyclic phosphodiesterase 4b			↑	↑	↑
TR582983|c4_g1	IL1BRA	interleukin-1 receptor antagonist			↑	↑	↑
TR582983|c4_g2	IL1B	interleukin-1 beta			↑	↑	↑
TR615049|c0_g1	*na*	reverse transcriptase			↑	↑	↑
TR624399|c4_g2	MIDN	midnolin			↑	↑	↑
TR654481|c2_g3	PFKFB3	6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 3			↑	↑	↑
TR658744|c0_g1	FOSL2	fos-related antigen 2			↑	↑	↑
TR658744|c0_g2	*na*	fos-related antigen partial			↑	↑	↑
TR756203|c2_g1	MMP18	mmp18 protein			↑	↑	↑
TR775068|c0_g1	MMP8	neutrophil collagenase			↑	↑	↑
TR95467|c4_g3	ELL2	ma polymerase ii elongation factor ell2			↑	↑	↑
TR245419|c7_g2	TNFRSF18	tumor necrosis factor receptor superfamily member 18			↑	○	↑
TR316127|c1_g1	ATF3	cyclic amp-dependent transcription factor atf-3			↑	○	↑
TR10109|c0_g1	ITPKC	inositol-trisphosphate 3-kinase c			○	↑	↑
TR144335|c1_g2	RELT	tumor necrosis factor receptor superfamily member 19l			○	↑	↑
TR16625|c2_g4	NR4A1	nuclear receptor subfamily 4 group a member 1			○	↑	↑
TR582983|c4_g3	IL1BRA	interleukin-1 receptor antagonist			○	↑	↑
TR1078938|c9_g1	*na*	reverse transcriptase-like protein			○	○	↑
TR251166|c2_g2	DLGAP4	disks large-associated protein 4			○	○	↑
TR311464|c1_g1	CDKN1C	cyclin-dependent kinase inhibitor 1c			○	○	↑
TR316127|c1_g3	ATF3	cyclic amp-dependent transcription factor atf-3			○	○	↑
TR624171|c2_g1	IRF2BPL	interferon regulatory factor 2-binding			○	○	↑
TR637870|c0_g1	CCNL2	cyclin-l2 isoform x2			○	○	↑
TR793608|c1_g2	STK17B	serine threonine-protein kinase 17b			○	○	↑
TR801514|c13_g3	*na*	uncharacterized protein			○	○	↑
TR863392|c0_g1	IER2	immediate early response gene 2 protein			○	○	↑
TR968926|c0_g1	RARG	retinoic acid receptor gamma			○	○	↑
TR596414|c0_g1	NAB1	ngfi-a-binding protein 1			○	○	○
TR981806|c6_g2	*na*	cat eye syndrome critical region protein			↓	↑	↑

Log2 Fold-change: 1.6, 1.2, 0.8, 0.4, 0, -0.4, -0.8, -1.2, -1.6

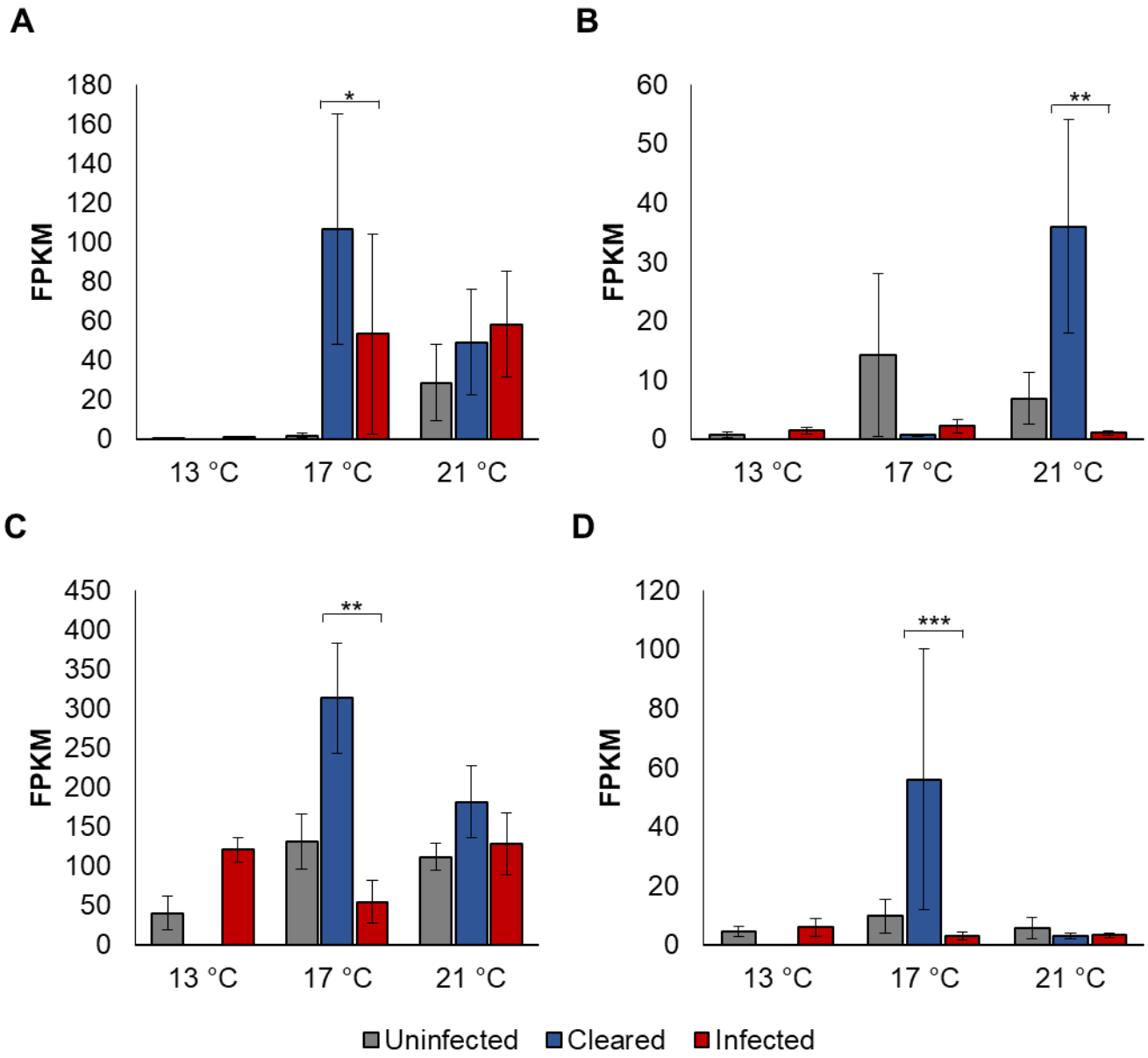
↑ Increased
○ Non-significant
↓ Decreased

B



766

Figure 4. Temperature dependent responses of SP12 spleen module (“cAMP binding”) hub genes. A) Heatmap showing *Bd*-infected salamander expression relative to 17 °C. Symbols indicate significance of differential expression tests. B) Network connection overview. Nodes are labeled with official gene symbols when available. Edge line width represents connection strength (weight); thicker lines denote stronger connections. Node size is proportional to number of connections.



772

773 **Figure 5.** MHC I antigen contigs A) TR769668|c9_g1, B) TR534169|c0_g4, C) TR281472|c2_g2,
774 and D) TR769668|c8_g1, found to be differentially expressed in the skin by temperature and
775 infection status. Expression levels represented as Fragments Per Kilobase of transcript per Million
776 mapped reads (FPKM). Asterisks indicate significance levels after FDR p-value correction.

Table 1. Gene ontology (GO) term enrichment of differentially expressed genes. U = uninfected control, I = died infected, C = cleared infection. H = high (21 °C), M = medium (17 °C), L = low (13 °C).

Comparison	T°	Direction	No. of terms	Top biological process	Top molecular function	Infection-related terms
Skin						
U v I	All	Up	722	response to stimulus	metalloendopeptidase activity	response to bacterium, INF-γ production, IL2 production, cytokine production, inflammatory response, TNF production, neutrophil migration, lysozyme activity, T cell activation, detection of fungus, macrophage activation
U v I	All	Down	58	intermediate filament organization	structural molecule activity	skin development, keratinization
U v I	H & M	Up	145	protein catabolic process	threonine-type peptidase activity	antigen processing and presentation via MHC I
U v I	H & M	Down	106	collagen fibril organization	extracellular matrix structural constituent	collagen biosynthetic process, skin morphogenesis
U v I	M & L	Up	84	response to external stimulus	GTP binding	response to INF-γ, B cell apoptotic process, innate immune response, mast cell proliferation, reg. of TLR4 pathway, reg. of inflammatory response, monocyte chemotaxis
U v I	H & L	Up	15	digestion	serine hydrolase activity	serine-type peptidase activity

U v I	H	Up	4	-	endopeptidase inhibitor activity	peptidase regulator activity
U v I	M	Up	79	glycosphingolipid metabolic process	carbohydrate derivative binding	negative regulation of activation-induced cell death of T cells
U v I	M	Down	48	digestion	serine-type endopeptidase activity	-
C v I	H	Up	181	muscle contraction	motor activity	MHC class II receptor activity, keratinization,
C v I	M	Up	24	skeletal muscle thin filament assembly	myosin binding	-
U v C	H	Up	37	sulfate transmembrane transport	secondary active sulfate transmembrane transporter activity	-
U v C	M	Down	327	actin-myosin filament sliding	cytoskeletal protein binding	-
Spleen						
U v I	All	Up	513	response to stimulus	metalloendopeptidase activity	defense response to bacterium, leukocyte migration, IL2 production, acute inflammatory response, regulation of macrophage derived foam cell differentiation, IL1 receptor activity

U v I	All	Down	94	immune response-activating signal transduction	transmembrane signaling receptor activity	activation of immune response, cytokine secretion, toll-like receptor signaling pathway, inflammatory response, B cell receptor signaling pathway, T-helper 1 type immune response
U v I	H & M	Up	17	retinoid metabolic process	-	-
U v I	H & L	Up	67	digestion	serine-type endopeptidase activity	regulation of I-kappaB kinase/NF-kappaB signaling, negative regulation of interleukin-8 biosynthetic process
U v I	M & L	Up	65	regulation of multicellular organismal process	vascular endothelial growth factor-activated receptor activity	-
U v I	M & L	Down	57	MyD88-dependent toll-like receptor signaling pathway	signaling receptor activity	negative regulation of lymphocyte differentiation, innate immune response-activating signal transduction, negative regulation of T cell differentiation
U v I	H	Up	30	chitin catabolic process	chitinase activity	-
U v I	H	Down	23	ethanol catabolic process	alcohol dehydrogenase (NAD) activity	alpha-beta T cell activation, interleukin-4 production, NK T cell differentiation
U v I	M	Up		cell differentiation	receptor activity	-
U v I	L	Up	88	single-organism process	GTP binding	T cell homeostasis, negative regulation of activation-induced cell death of T cells, T cell apoptotic process

U v I	L	Down	19	tetrapyrrole biosynthetic process	oxygen transporter activity	-
C v I	H	Up	12	histidine catabolic process	-	-
C v I	M	Up	9	single-organism cellular process	-	-
U v C	H	Up	8	pyruvate biosynthetic process	GTP binding	-
U v C	H	Down	54	oxygen transport	oxygen transporter activity	-

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782 **Table 2.** Gene ontology (GO) term enrichment of gene co-expression modules defined by WGCNA.

Module	N genes	Temperature		Infection load		Days P.I.		Top molecular funtion GO	Top biological process GO	Infection-related GOs
		R	P value	R	P value	R	P value			
Skin										
SK1	512	-0.36	4.45E-02						DNA metabolic process	
SK2	475	+0.36	4.55E-02			+0.41	2.08E-02	Chromatin binding	Cell cycle	
SK3	399	+0.81	2.48E-08	-0.41	2.37E-02	+0.45	1.21E-02	RNA binding	Translation	MHC II protein binding, type I interferon signalling
SK4	2113			-0.51	3.05E-03	+0.45	1.06E-02	Heme binding	Single organism process	Interleukin-5 secretion, interleukin-13 secretion
SK5	622			-0.43	1.64E-02	+0.39	3.18E-02	Serine-type endopeptidase activity	Cell wall macromolecule catabolic process	Chitinase activity
SK6	702			-0.79	1.58E-07	+0.71	6.21E-06	Extracellular matrix structural constituent	Extracellular matrix organization	Collagen binding, skin morphogenesis
SK7	297			-0.60	3.88E-04	+0.57	9.14E-04	Structural constituent of ribosome	Ribonucleoprotein complex biogenesis	FC-γ complex binding, NF-kB signalling
SK8	2069			-0.52	2.51E-03	+0.40	2.62E-02	NAD+ ADP-ribosyltransferase activity	Immune system process	Lymphocyte activation, T cell aggregation, response to virus, cytokine production, B cell activation, antigen presentation via MHC I
SK9	759			-0.52	3.00E-03	+0.47	7.20E-03		Embryionic hemopoeisis	
SK10	7061			-0.94	1.27E-14	+0.87	2.12E-10	Metal ion binding	Regulation of transcription, DNA templated	
SK11	891			-0.49	4.73E-03	+0.45	1.13E-02		DNA integration	
SK12	1405			-0.89	2.32E-11	+0.81	3.69E-08	Nucleic acid binding	Nucleic acid metabolic process	
SK13	724			-0.55	1.45E-03	+0.51	3.24E-03	Nucleic acid binding		
SK14	791			-0.77	5.03E-07	+0.51	3.74E-03	Nucleic acid binding		

SK15	1280	+0.75	1.20E-06	-0.61	3.07E-04	Threonine-type peptidase activity	Protein catabolic process	Antigen processing & presentation via MHCI, MHCII receptor activity
SK16	2857	+0.75	1.45E-06	-0.79	1.01E-07	2-methylcitrate dehydralase activity	Response to stimulus	Regulation of INF-γ production, interleukin-2 production, regulation of TLR3 signalling pathway
SK17	655	+0.65	8.22E-05	-0.71	7.78E-06	GTP binding	Immune response	Response to bacterium, negative regulation of TLR4 signalling, response to INF-γ, myeloid leukocyte activation
SK18	714	+0.80	4.80E-08	-0.52	2.86E-03	Lipoprotein particle receptor activity	Lipid biosynthetic process	
SK19	352	+0.40	2.72E-02	-0.37	4.03E-02	Unfolded protein binding	Protein refolding	
SK20	1844	+0.72	4.43E-06	-0.59	4.22E-04	Protein binding	Cellular protein modification process	
SK21	206	+0.40	2.62E-02			RNA binding	Ribonucleoprotein complex biogenesis	
SK22	1110	+0.66	4.51E-05	-0.58	6.70E-04	Cytoskeletal protein binding	Muscle contraction	
Spleen								
SP1	261	-0.34	5.40E-02			Hydrolase activity	Porphyrin-containing compound metabolic process	Positive regulation of T cell receptor signalling, germinal B cell differentiation, regulation of leukocyte differentiation
SP2	379	-0.58	5.40E-04	+0.55	1.04E-03	ATP binding	Cell cycle	Positive regulation of T cell cytokine production
SP3	153	-0.55	9.95E-04	+0.44	1.24E-02	Oxidoreductase activity	1,2-dichloroethane metabolic process	Immature B cell differentiation, V(D)J recombination
SP4	655	-0.56	9.46E-04	+0.46	7.59E-03	GTP binding	Antigen processing and presentation	MHC protein complex, T cell cytotoxicity, INF-γ signalling
SP5	607	-0.57	5.97E-04	+0.48	5.09E-03	GTP binding	Response to virus	Defense response to virus, activation of innate immue response
SP6	1356	-0.80	5.33E-08	+0.71	4.45E-06	Protein kinase C activity	Regulation of cellular process	B cell receptor signalling pathway

SP7	205			-0.74	1.64E-06	+0.63	1.28E-04	Protein-arginine demaination activity	Protein citrullination	
SP8	5898			-0.56	9.03E-04	+0.54	1.28E-03	Endonuclease activity	DNA metabolic process	
SP9	185			-0.35	4.78E-02	+0.40	2.15E-02	Scavenger receptor activity	RNA phosphodiester hydrolysis	
SP10	341			-0.41	2.08E-02	+0.38	3.37E-02		Postive regulation of execution phase apotosis	
SP11	2758	+0.37	3.74E-02	-0.88	2.69E-11	+0.80	4.13E-08	Nucleic acid binding	RNA metabolic process	
SP12	2012	-0.39	2.62E-02	+0.69	1.43E-05	-0.62	1.68E-04	cAMP binding	Single organism cellular process	
SP13	3112			+0.79	6.30E-08	-0.78	1.21E-07	Substrate-specific transporter activity	Localization	Negative regulation of B cell activity, TGF-β production
SP14	1449			+0.57	6.98E-04	-0.42	1.64E-02	Calcium ion binding	Single multicellular organism process	Regulation of wound healing
SP15	986			+0.60	2.47E-04	-0.61	1.94E-04	RNA binding	RNA processing	FC-γ receptor, establishment of T cell polarity
SP16	328			+0.35	4.74E-02	-0.35	4.78E-02	NAD(P)H oxidase activity	Thyroid hormone generation	Response to cytokine
SP17	499			+0.54	1.30E-03	-0.60	2.65E-04	MHC II receptor activity	Intermediate filament organization	MHC II receptor activity

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