



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

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Molecular Ecology

DOI:
[10.1111/mec.15327](https://doi.org/10.1111/mec.15327)

Published: 31/01/2020

Peer reviewed version

[Cyswllt i'r cyhoeddiad / Link to publication](#)

Dyfyniad o'r fersiwn a gyhoeddwyd / Citation for published version (APA):
Ellison, A., Zamudio, K. R., Lips, K. R., & Muletz-Wolz, C. (2020). Temperature-mediated shifts in salamander transcriptomic responses to the amphibian-killing fungus. *Molecular Ecology*, 29(2), 325-343. <https://doi.org/10.1111/mec.15327>

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1 *Research article*

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

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14 **Keywords:** *Batrachochytrium dendrobatidis*, MHC, salamanders, temperature-dependent
15 immunity, transcriptomics

16 **Running title:** Temperature alters chytridiomycosis responses

17 **Abstract**

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

37

38 **Introduction**

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

51 Temperature has profound effects on the transcriptional activity of organisms; from prokaryotes
52 (Smoot et al., 2001), plants (Winfield, Lu, Wilson, Coghill, & Edwards, 2010), fungi (Steen et al.,
53 2002), invertebrates (Wang, Espinosa, Tanguy, & Allam, 2016) to vertebrates (Gracey et al.,
54 2004). In multicellular organisms, temperature dependent gene expression responses are often
55 tissue specific (Gracey et al., 2004). In vertebrates, temperature effects on gene expression are
56 well known in terms of temperature-dependent sex determination (Shen & Wang, 2014) and
57 acclimation to thermal stress (Quinn, McGowan, Cooper, Koope, & Davidson, 2011). However,
58 we know far less about the effects of temperature on gene expression during infection, despite its
59 likely impact on immune function. To fully appreciate the significance of temperature on infection
60 processes and disease dynamics of vertebrate hosts, studies of transcriptional infection
61 responses under different thermal regimes are needed.

62 The aquatic fungal pathogen *Batrachochytrium dendrobatidis* (*Bd*) is one the most devastating
63 emergent pathogens of ectotherms, widely implicated in global amphibian population declines and
64 extinctions (Bellard, Genovesi, & Jeschke, 2016, Scheele et al., 2019). Among frogs, hundreds of
65 species are known hosts and *Bd* has a wide range of disease outcomes, ranging from high
66 susceptibility to tolerance and resistance (Scheele et al., 2019). Salamanders, particularly
67 terrestrial species, are typically at less risk from *Bd* (Lips, Reeve, & Witters, 2003, Bancroft et al.,
68 2011) often exhibiting low natural prevalence (Hossack et al., 2010, Muletz, Caruso, Fleischer,
69 McDiarmid, & Lips, 2014), relatively low susceptibility, and higher rates of infection clearance in
70 laboratory studies (Vazquez, Rothermel, & Pessier, 2009, Pasmans et al., 2013). Despite this,
71 salamander declines have been linked to *Bd* emergence (Cheng, Rovito, Wake, & Vrendenburg,
72 2011) and infections can induce high mortality rates in some species (Weinstein, 2009).

73 Temperature is also key to *Bd* physiology. In culture, optimal growth of *Bd* is in the range of 17 to
74 25 °C, with substantially reduced growth rates below 10 °C or above 28 °C (Piotrowski, Annis, &
75 Longcore, 2004). Moreover, environmental temperature is an important predictor of geographic
76 distribution of *Bd* infections and amphibian mortality rates (Kriger, Pereoglou & Hero, 2007, Longo,
77 Burrowes, & Joglar, 2010, Savage, Sredl, & Zamudio, 2011). Elevated body temperature in frogs
78 can clear infections (Woodhams, Alford, & Marantelli, 2003), and individual preference for warmer
79 temperatures has been linked to increased resistance to *Bd* (Rowley & Alford 2013, Sauer et al.,
80 2018). Yet, even within the optimal thermal range of *Bd*, anuran mortality rates vary considerably
81 (Andre, Parker, & Briggs, 2008), suggesting that temperature-dependent host responses
82 contribute to disease outcome. Indeed, expression profiling of *Xenopus tropicalis* revealed
83 differential activation of innate immune genes in response to infection at two temperatures (Ribas
84 et al., 2009). However, despite evidence of temperature-dependent survival in salamanders
85 (Vazquez et al., 2009, Muletz-Wolz et al., 2019), we do not yet have any study of temperature-

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

95 Many species of *Plethodon* salamanders have experienced widespread declines in the eastern
96 United States (Highton, 2005). *Plethodon cinereus* is well studied with respect to chytrid infections,
97 typically exhibiting low *Bd* infection prevalence in the wild (Muletz, Caruso, Fleischer, McDiarmid,
98 & Lips, 2014), and capable of clearing moderate laboratory experimental infections (Muletz et al.,
99 2012). This species is a popular model for studying the protective role of commensal skin
100 microbiota against *Bd* infection (e.g. Harris et al., 2009, Loudon et al., 2014, Muletz-Wolz et al.,
101 2018, Muletz-Wolz et al., 2019). However, we do not know their functional genetic responses to
102 infection that control disease outcomes, especially under variable temperatures. In this study we
103 use this temperate species to capture transcriptional responses of individuals succumbing to
104 chytridiomycosis and those cleared of infection, under relevant seasonal temperatures. We
105 characterized the transcriptomic responses of *P. cinereus* to infection with a novel (non-North
106 American) *Bd* strain. By comparing gene expression of individuals uninfected, succumbing, or
107 naturally cleared of *Bd* infection at three different temperatures, we address the hypothesis that
108 amphibian temperature-dependent variation in survival to *Bd* infection is due to underlying
109 differences in expressed genes.

110 **Materials & Methods**

111 ***Experimental infections***

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

119 Salamanders were individually exposed for 24 hours to a *Bd* inoculum (*Bd*-exposed: 5 ml of 5.3 x
120 10⁶ zoospores/ml solution of strain JEL423, 15 per temperature) or sham exposed (*Bd*-control: 5
121 ml sterile water, 14 per temperature). JEL423, a Panamanian strain, was chosen because wild
122 salamanders from our collecting site should all be naïve to this chytrid lineage (Muletz, Caruso,
123 Fleischer, McDiarmid, & Lips, 2014). Salamanders were monitored for morbidity (abnormal
124 posture, excess skin sloughing, loss of appetite, lethargy, and loss of righting reflex) daily for 42
125 days and individuals were euthanized if they lost their right ability, or displayed all four of the other
126 clinical signs, by applying 20% benzocaine to their dorsal side. *Bd* infection status and infection
127 intensity (number of zoospore genomic equivalents, ZGE) were measured at 5, 11, 25, and 42
128 days post inoculation using skin swabs as described in Muletz-Wolz et al. (2019). Moribund
129 salamanders were also swabbed immediately prior to euthanasia. All salamanders surviving the
130 length of the experiment were euthanized 42 days post-inoculation.

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

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

148 ***Transcriptome sequencing***

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

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

171 *Differential expression and gene network analyses*

172 Gene expression was determined using the Trinity pipeline, using BWA read mapping (Li & Durbin
173 2009) and RSEM read count normalization (Li & Dewey 2011). We analysed differential gene
174 expression (DGE) of control (uninfected), infected, and cleared individuals at each temperature
175 separately using the edgeR (Robinson, McCarthy, & Smyth, 2010) R package (R version 2.15.2,
176 R Development Core Team). This consisted of estimating tagwise dispersion and normalization
177 factors and differentially expressed (DE) testing using an exact test. A false discovery rate (FDR)–
178 corrected P value of less than 0.05 was considered to be evidence of DGE. To quantify the overlap
179 of differentially expressed genes between temperatures, we constructed Venn diagrams for each
180 tissue using VENNY (Oliveros 2007) for significantly increased and decreased expressed genes

181 separately. We tested for enrichment of biological process GO terms in each group of DE genes
182 (e.g., specific to one temperature or shared among two or more temperatures) using BLAST2GO.
183 To compare gene expression between infected salamanders and those cleared of infection at day
184 42, we excluded genes found to be differentially expressed between controls and infected
185 samples. This method excludes genes that may have returned to baseline (i.e. non-infected) levels
186 since clearing infection.

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

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

209 **Results**

210 ***Bd infection challenge***

211 All uninfected control salamanders remained *Bd*-negative for the duration of the experiment. In
212 the larger survival study, from which we sampled individuals for this study, survival of uninfected
213 salamanders was 100%, 100% and 86% at 13 °C, 17 °C, and 21 °C, respectively. Survival of *Bd*-
214 challenged salamanders was 6.6%, 26.7%, and 33.3% at 13 °C, 17 °C, and 21 °C respectively
215 (Muletz-Wolz et al., 2019). We found a significant effect of *Bd* exposure on survival rate for *Bd*-
216 exposed vs. *Bd*-control; exposed individuals had lower survival rates. *Bd*-exposed salamanders
217 at 13°C had a higher mortality rate compared to other temperature treatments, although this
218 difference was not statistically significant (post-hoc $p > 0.33$, Muletz-Wolz et al., 2019). However,
219 at 11 days post-exposure *Bd*-exposed salamanders at 13 °C had significantly higher loads than
220 at 17 °C and 21 °C (Supplementary Figure 1). We found a significant negative correlation between
221 *Bd* load and sampling day; salamanders with higher loads had shorter survival (Pearson’s $R = -$
222 0.762 , $P = 0.004$). The studied individuals succumbing to infection were sampled between 5 and
223 21 days post-inoculation (Figure 1, Supplementary File 1), with surviving salamanders and non-
224 exposed controls sampled at day 42.

225 ***Transcriptome assembly***

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

228 average of 33.51 million reads per sample. Sequences are deposited in the NCBI Short Read
229 Archive under submission accession number PRJNA559247
230 (<https://www.ncbi.nlm.nih.gov/bioproject/PRJNA559247>). Our *de novo* assembly of the
231 transcriptome, after filtering out low expression transcripts (threshold of at least two reads per
232 million mappable reads in at least five samples), yielded 117,812 transcripts, with a mean length
233 of 1084 bp and N50 length of 2345 bp. We expect that the vast number of minimally expressed
234 contigs that fall below our threshold to largely represent transcriptional errors such as intron
235 expression, exon chimaeras, and sequencing and assembly errors common to current *de*
236 *nov*o assembly techniques (Moghadam et al., 2013). Of the assembled genes, 38,266 (32.48%)
237 had at least one significant hit against the nonredundant NCBI protein database, and of these,
238 19,975 (52.20%) were successfully annotated with GO terms.

239 ***Responses to infection at all temperatures***

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

249 *Skin responses*

250 For skin samples, we found 3,318 genes at 13 °C, 4,538 genes at 17 °C, and 3,305 genes at 21
251 °C with significant expression differences between uninfected and infected individuals. Of these,
252 1,071 exhibited increased expression in infected salamanders compared to uninfected controls at
253 all temperatures. For the purposes of this study, we focus the presentation of our results on genes
254 of known immune function in vertebrates and/or highlighted in previous amphibian *Bd* infection
255 studies. We found significant gene ontology (GO) term enrichment of these genes to include
256 numerous immune-related terms including “metalloendopeptidase activity” (the top molecular
257 function, Table 1), “cytokine production”, “inflammatory response”, “neutrophil migration”,
258 “lysozyme activity”, “T cell activation”, “macrophage activation”, and “detection of fungus”. The 397
259 genes sharing significant decreases in expression in infected skin samples (compared to
260 uninfected) were enriched for GO terms related to skin integrity such as “intermediate filament
261 organization”, “skin development”, and “keratinization”.

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

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

278 *Spleen responses*

279 For spleen samples, we found 2,958 genes at 13 °C, 2,204 genes at 17 °C, and 2,587 genes at
280 21 °C with significant expression differences between uninfected and infected individuals. We
281 found 887 of these genes had higher expression in infected salamanders compared to uninfected
282 controls at all temperatures. These transcripts were enriched for several immune-related GO
283 terms including leukocyte migration, IL2 production, and acute inflammatory response (Table 1).
284 In contrast, only 75 genes shared significant decreases in expression in infected spleen samples
285 (compared to uninfected). These genes were also enriched for GO terms related to immune
286 functions, notably activation of immune response, cytokine secretion, toll-like receptor signaling
287 pathway, inflammatory response, B cell receptor signaling pathway, and T-helper 1 type immune
288 response (Table 1).

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

298 ***Temperature-dependent responses to infection***

299 *Skin*

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

312 Further interrogation of specific immune genes showing temperature-dependent responses in the
313 skin revealed a number involved in MHC presentation (Figure 3). MHC II beta chain
314 (TR517114|c0_g5) was only significantly increased in expression in infected salamanders at 13
315 °C, whereas four MHC I antigens (20% of those in our transcriptome assembly) had higher
316 expression at 17 °C or 21 °C. Furthermore, expression of heat-shock proteins (HSPs) and
317 proteasome genes involved in MHC antigen presentation were temperature-dependant (Figure 3).
318 Of note, 26S proteasome subunits were consistently only significantly higher at 17 °C and 21 °C.
319 In addition, components of immunoglobulins exhibited significantly higher expression in infected
320 samples (compared to controls) at one of the three temperatures (Figure 3). Innate immune genes

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

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

327 *Spleen*

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

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

350 ***Survivors of infection***

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

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

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

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

378 **Discussion**

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

389 In this study, to measure transcriptomic responses of salamanders carrying *Bd* (contrasted with
390 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

415 pathways have been consistently highlighted as responsive to *Bd* in frogs (Rosenblum, Poorten,
416 Settles, & Murdoch, 2012, Ellison et al., 2014, Ellison et al., 2015) and suggests these are markers
417 deeply conserved in *Bd* responses of amphibia. Yet, comparison of expression profiles of
418 salamanders infected with either *Bsal* or *Bd* show substantial differences in immune activation
419 (Farrer et al., 2017). *Bsal* elicits no substantial immune response in salamanders (Farrer et al.,
420 2017), indicating the transcriptional responses observed here and in previous anuran studies are
421 not necessarily shared across all chytrid infections.

422 We show that *Bd* influences the expression of genes involved in skin integrity and spleen
423 lymphocyte production in salamanders. Disruption of skin integrity and spleen lymphocyte
424 suppression are considered key factors in the pathogenicity of *Bd* and have been demonstrated
425 in a number of susceptible anuran species (Voyles et al., 2009, Fites et al., 2013, Ellison et al.,
426 2014, Ellison et al., 2015, Grogan et al., 2018b). Conversely, more effective responses to *Bd*
427 include generally lower levels of gene dysregulation, robust early innate and adaptive immune
428 responses (Grogan et al., 2018a), and increased skin structural protein and splenic lymphocyte
429 production during infection (Ellison et al., 2015). We found that skin genes in infected individuals
430 sharing decreased expression at all temperatures were rich in functions related to collagen and
431 keratin production (Table 1, Supplementary File 3). Moreover, we found a skin gene module –
432 negatively correlated with *Bd* load – to be associated with skin development and structure
433 indicating that as infection load increases skin integrity decreases (Table 2). This module was also
434 positively correlated with days post-inoculation (DPI), suggesting individuals surviving longer
435 (even if eventually succumbing) had higher expression of skin integrity genes. In addition,
436 comparison of salamanders that cleared infection to those that succumbed, indicated higher
437 expression levels of keratins and collagens in the cleared groups (Supplementary File 2)
438 suggesting maintenance of skin integrity may be crucial to intraspecific differences in survival in

439 salamanders. We found evidence for *Bd*-induced immunosuppression; spleen genes associated
440 with Th₁ responses and B cell signalling were lower in infected salamanders at all temperatures
441 (Table 1). We also found five of the splenic gene modules (negatively correlated with *Bd* load)
442 enriched for various lymphocyte development, activation, and signalling functions (Table 2). These
443 modules also were positively associated with DPI; individuals surviving longer had higher
444 expression. Taken together, these results indicate that the immunosuppressive ability of *Bd* is
445 widespread throughout its host species range and effective induction of skin repair and lymphocyte
446 responses may be key resistance mechanisms.

447 The temperature dependence of chytridiomycosis within the thermal range of *Bd*, leads to the
448 hypothesis that the effect of temperature on amphibian host immune responses influences
449 disease outcome. In the skin, we found key components of anti-fungal activity to be differentially
450 expressed with temperature, including chitinases (greater upregulation at high temperature),
451 lysozymes and anti-microbial peptides (greater upregulation at low temperatures, Figure 3).
452 Spleen expression profiles of *Bd*-infected *Xenopus tropicalis* indicate that temperature-dependant
453 induction of innate immunity – particularly anti-microbial peptides and inflammatory responses –
454 but not adaptive immune responses, are responsible for greater host survival at warmer
455 temperatures (Ribas et al., 2009). Similarly, salamander interleukin expression increases with
456 temperature in the spleen (Figure 4). In contrast, we observe greater expression changes in these
457 cytokines at the lower temperatures in the skin (Figure 3), the primary site of infection. This is
458 potentially in response to shifts of pathogen life history traits with temperature (Muletz-Wolz et al.,
459 2019).

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

463 interleukin expression) in the skin with cooler temperatures, and yet significantly higher expression
464 of adaptive immune genes at 17 °C and 21 °C (Figure 3). Genes involved in MHC-mediated
465 antigen processing and presentation, including MHC I antigens and MHCII-related heat-shock
466 proteins and proteasome subunits, show the most pronounced response to temperature, with
467 increase in expression as temperature increases. Although these findings are from individuals
468 succumbing to infection, these results still suggest a greater ability to activate adaptive immune
469 responses to *Bd* at higher temperatures, where more salamanders survived infection. In contrast,
470 there appears to be a greater reliance on innate and inflammatory pathways when temperatures
471 are cooler, where the greatest number of mortalities were observed. We now have evidence for
472 the importance of the major histocompatibility complex (MHC) genotypes in amphibian-chytrid
473 resistance (Savage & Zamudio, 2011, Bataille et al., 2015, Savage & Zamudio, 2016, Kosch et
474 al., 2018). However, our results indicate that environmental temperature and MHC genotypes
475 must be considered together to fully explain population differences in *Bd* susceptibility.
476 Temperature-dependence of MHC activity is likely key to observed patterns of seasonal trends in
477 chytridiomycosis prevalence and intensity (Kriger & Hero, 2007, Longo et al., 2010, Savage et al.,
478 2011, Grogan et al., 2016).

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

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

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

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

511 adaptive immune pathway gene expression. As the evidence of temperature and seasonal effects
512 on *Bd* prevalence and infection intensity in amphibian communities continues to grow (Lenker,
513 Savage, Becker, Rodriguez, & Zamudio, 2014, Blooi et al., 2015, Sapsford, Alford, & Schwarzkopf,
514 2018), it is essential that the underlying mechanisms for the observed trends are uncovered. Our
515 study suggests that gene expression responses to *Bd* are thermally-dependant and may be a key
516 component in seasonality of chytridiomycosis. Intriguingly, our data indicate that adaptive
517 immunity, particularly MHC-related pathways, are thermally sensitive. Given the recent findings
518 for the importance of MHC genotypes in survival against *Bd* (Savage & Zamudio, 2011, Bataille
519 et al., 2015, Savage & Zamudio, 2016, Kosch et al., 2018), it would be valuable to follow this up
520 with an expanded population MHC genotyping and expression study of *P. cinereus*. Furthermore,
521 in the face of climate change, and as concerns for the spread of the newly discovered chytrid *Bsal*
522 increase (Stegen et al., 2017), comparison of salamander functional genomic responses to both
523 chytrid pathogens incorporating thermal variation, will be vital to understand how these emergent
524 pathogens may continue to effect amphibian communities globally in the future.

525 **Acknowledgments**

526 This study was supported by grants from the National Science Foundation (DEB-0815315 and
527 DEB-1120249). Additional funding for the project was provided by National Park Service George
528 M. Wright Climate Change Fellowship and an Environmental Protection Agency STAR Fellowship
529 (no. F13B20412) awarded to C.M.W. We thank Miranda Gray for assistance with sequencing
530 library preparations. We thank Jeremy Jacobs, Sam Barnett, Lindsay Powell, Grace DiRenzo,
531 Luke Brown and Anna Savage for field assistance. We thank Chelsea Maguire and Lane Jackson
532 for assistance with animal husbandry and sampling during the experiment. Red-backed
533 salamander collections were carried out under DNR Permit No. 55850 and all animal
534 experimentation was covered by University of Maryland IACUC #R-14-04.

535 The authors declare no conflicts of interest.

536 **Author Contributions**

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

540 **Data Availability**

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

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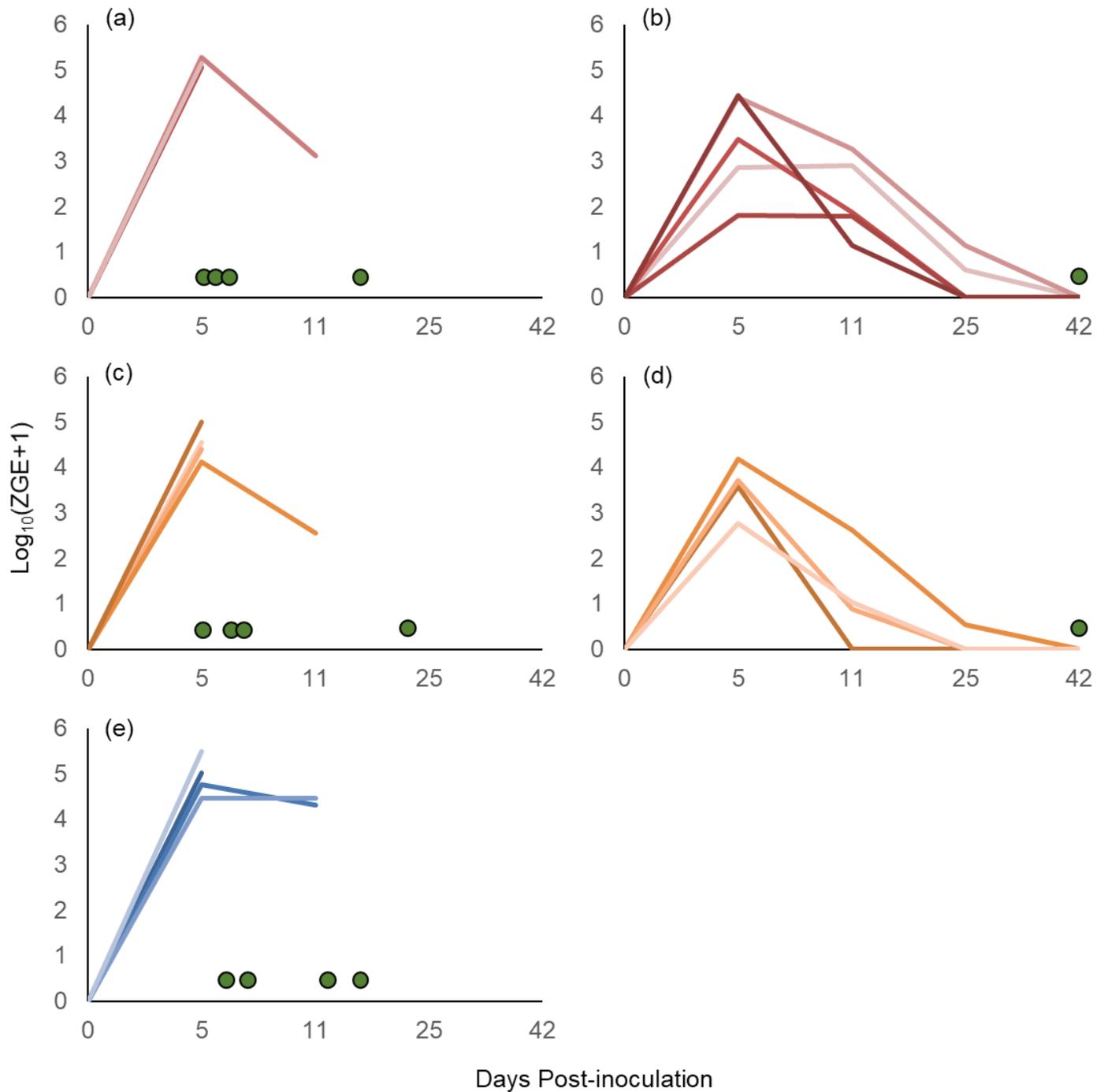
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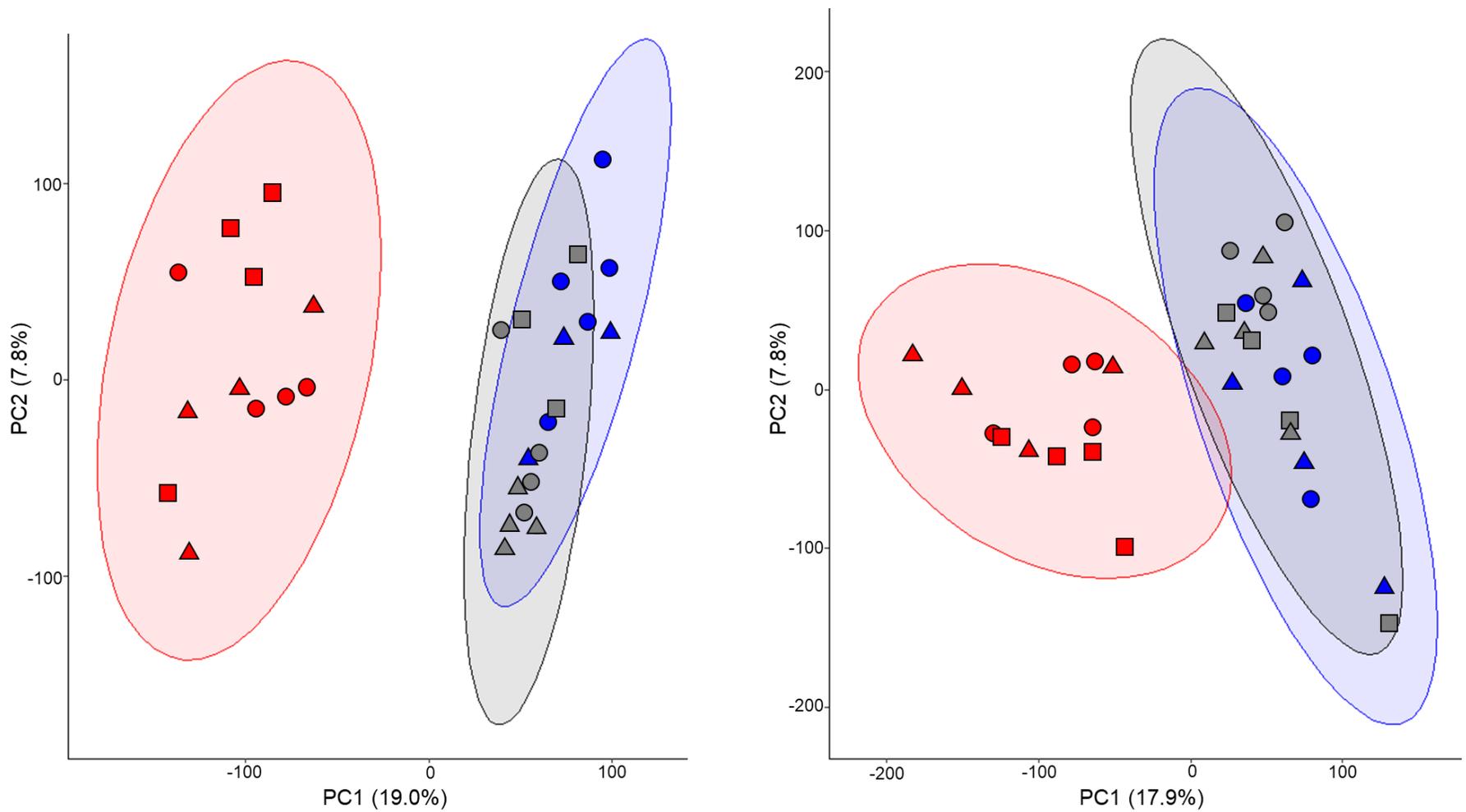
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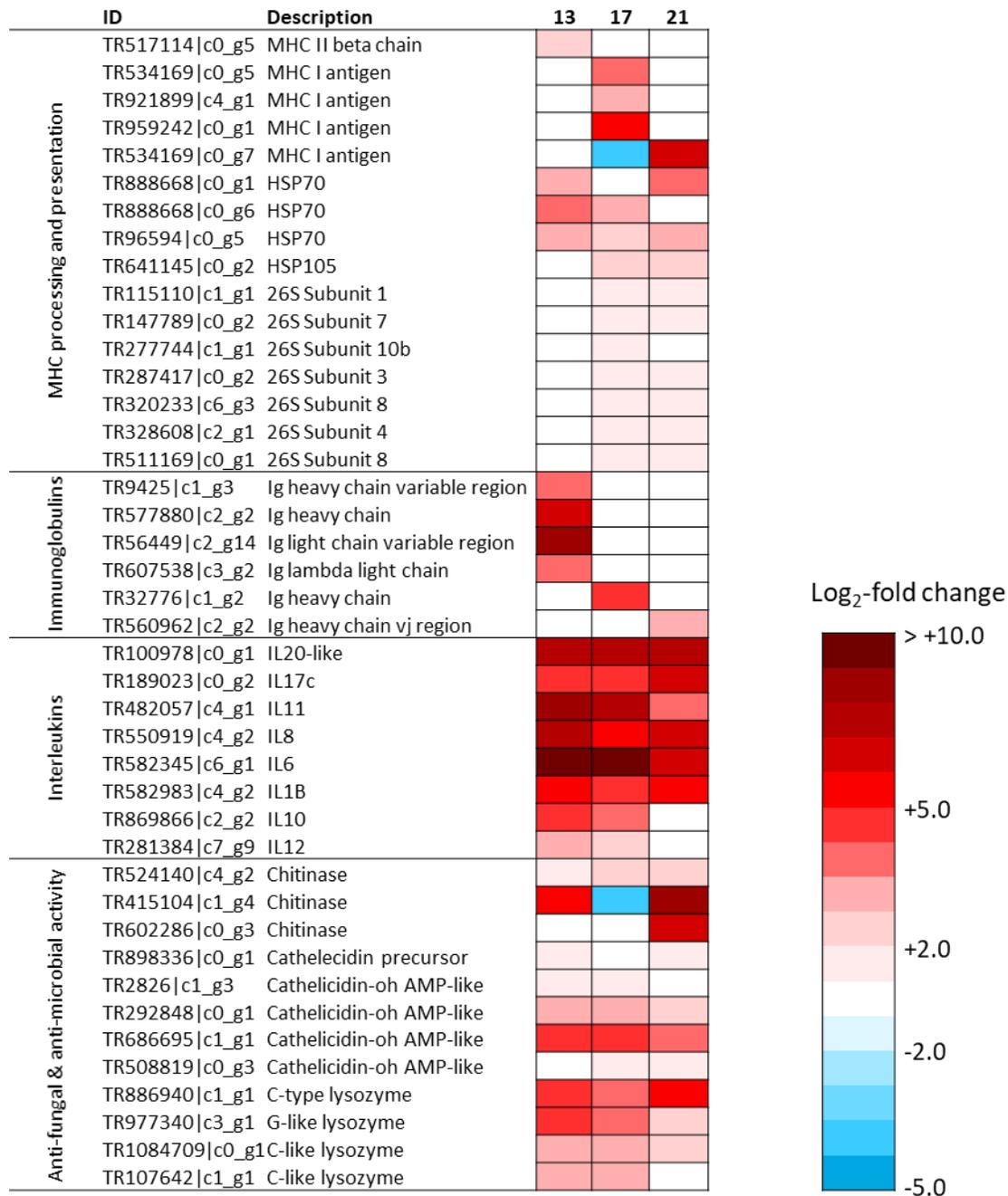
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753 **Figure 1.** Individual infection trajectories of *Bd*-exposed *Plethodon cinereus* at 21 °C (a, b), 17 °C
 754 (c, d), and 13 °C (e). Green circles indicate day of tissue sampling for “infected” group succumbing
 755 to infection (left column)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



758

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



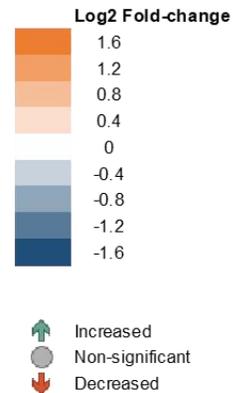
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763 **Figure 3.** Heatmap of skin differentially expressed contigs (adjusted P <0.05) related to immune
 764 responses against *Bd*, comparing infected to control (uninfected) individuals at each temperature.

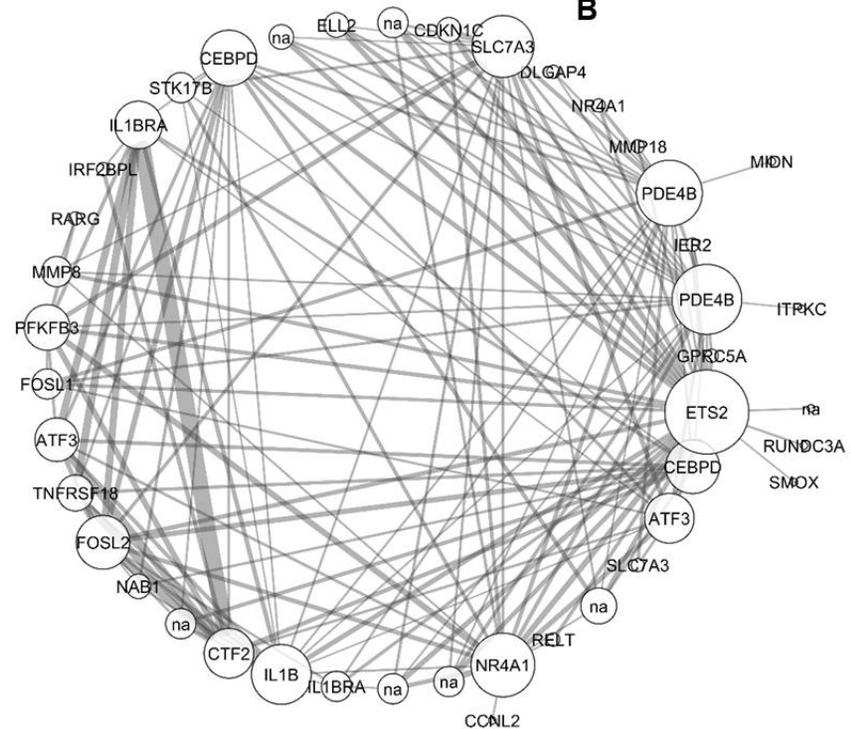
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A

Contig ID	Gene symbol	Description	Relative expression			Differential expression		
			17 v 21 °C	17 v 13 °C	13 °C	17 °C	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	ccaa enhancer-binding protein delta			↑	↑	↑	
TR542 c3_g6	CEBPD	ccaa 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-h2 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			↓	↑	↑	

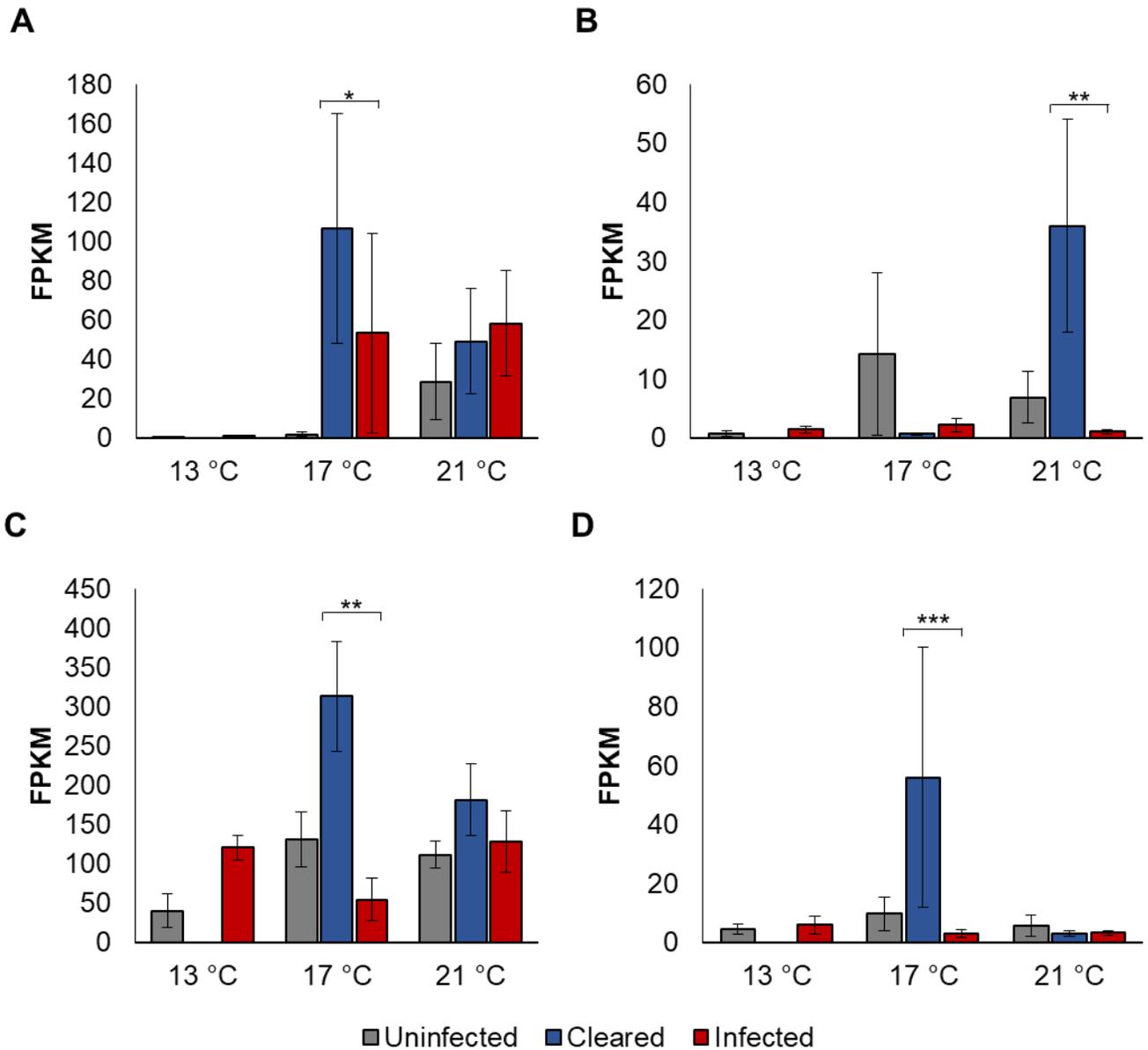


B



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

771



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.

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

779

Comparison	T°	Direction	No. of terms	Top biological process	Top molecular function	Infection-related terms
<i>Skin</i>						
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
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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	-

780

781

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 function GO	Top biological process GO	Infection-related GOs
		R	P value	R	P value	R	P value			
<i>Skin</i>										
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- κ B 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		Embryonic hemopoiesis	
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 MHC I, MHC II receptor activity
SK16	2857	+0.75	1.45E-06	-0.79	1.01E-07	2-methylcitrate dehydratase 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	
<i>Spleen</i>								
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 immune 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 demination 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 apoptosis	
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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784