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RNA-Seq analysis of the guppy immune response against *Gyrodactylus bullatarudis* infection

Running title: RNA-Seq of the guppy immune response

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Author contribution: KPP and JR designed the experiment. KPP and RSM performed experiment and sampled fish. MK and JR organized RNA extraction and sequencing. MK and AE performed data analyses. MC, JR and JC interpreted data and discussed principle findings. JR, MC and MK wrote manuscript with contributions and edits from all authors. All authors read and approved the final manuscript.

36 ## Abstract

37 Gyrodactylids are ubiquitous ectoparasites of teleost fish, but our understanding of
38 the host immune response against them is fragmentary. Here, we used RNA-Seq to
39 investigate genes involved in the primary response to infection with *Gyrodactylus*
40 *bullatarudis* on the skin of guppies, *Poecilia reticulata,* an important evolutionary
41 model, but also one of the most common fish in the global ornamental trade. Analysis
42 of differentially expressed genes identified several immune-related categories,
43 including IL-17 signalling pathway and Th17 cell differentiation, cytokine-cytokine
44 receptor interaction, chemokine signalling pathway, NOD-like receptor signalling
45 pathway, natural killer cell-mediated cytotoxicity, and pathways involved in antigen
46 recognition, processing and presentation. Components of both the innate and
47 adaptive immune responses, play a role in response to gyrodactylid infection. Genes
48 involved in IL-17/Th17 response were particularly enriched among differentially
49 expressed genes, suggesting a significant role for this pathway in fish responses to
50 ectoparasites. Our results revealed a sizable list of genes potentially involved in the
51 teleost-gyrodactylid immune response. .

52

53 ## Keywords

54 Gyrodactylus, guppy, fish, transcriptome, RNA-Seq, Th17 response, immunity,
55 ectoparasites

56

57 # 1. Introduction

58 Fish ectoparasites are an important selective agent in natural fish populations
59 (1–4) and a major pest in commercial aquaculture (5). Monogenean worms of the
60 genus *Gyrodactylus* cause skin and/or gill damage that can result in severe
61 pathology and host death (6). This causes major problems in aquaculture and the
62 ornamental fish trade (reviewed by Bakke et al. (7)). Gyrodactylids have also served
63 as a model for host-parasite dynamics in ecological, evolutionary and epidemiological
64 research, in laboratory, mesocosm and natural scenarios (1, 8–11).

65 Despite the commercial importance and research value of gyrodactylids,
66 understanding of the associated host immune responses is fragmentary. Previously,
67 Buchmann (12) demonstrated that the complement system in rainbow trout
68 (*Oncorhynchus mykiss*) can have a lethal effect on *G. derjavini*. The results
69 suggested that the response is mediated by binding of complement C3 factor to
70 carbohydrate-rich structures of the parasite. In contrast, Zhou et al. (13) observed
71 down-regulation of C3 and IFN-γ gene expression in the skin of infected goldfish
72 (*Carassius auratus*). In the same studies, up-regulation of gene expression of pro-
73 inflammatory cytokines IL-1β and TNF-α was detected. Similarly, in rainbow trout,
74 primary infection with *G. derjavini* led to increased gene expression of pro-
75 inflammatory mediators IL-1β (14), TNF-α, COX-2 and iNOS (15). These findings
76 stress the importance of pro-inflammatory cytokines/innate immune response in the
77 initiation of immune reactions against gyrodactylid infection.

78 Adaptive immunity also plays a role in the host response to gyrodactylids.
79 Rainbow trout, for example, re-challenged with gyrodactylids showed a stronger
80 response one month after full recovery from the primary infection compared to
81 previously unchallenged fish (14). Furthermore, lower infection levels were observed
82 within fish carrying a secondary infection, and, in these primed hosts, clearance
83 began earlier compared to that seen in naïve fish, although no clear parasite-related
84 changes in transcript levels were detected from two candidate markers of adaptive
85 immune response (TCRβ and MHCIIβ) (14). Similarly, Cable and van Oosterhout (16)
86 demonstrated that guppies (*Poecilia reticulata*) that have recovered from gyrodactylid
87 infections possess a highly efficient acquired immunity that may minimize detrimental
88 effects associated with subsequent gyrodactylid infections. In gene expression terms,
89 increased expression of INFγ, Mx and CD8α and MHC I genes was detected in

90 *Salmo salar* (Baltic salmon from River Ume Älv in Sweden) resistant to *G. salaris*
91 relative to a susceptible strain (East Atlantic salmon from River Skjernå in Denmark)
92 (17).
93 In contrast, recent studies of goldfish immune responses against *G. kobayashii*
94 showed no significant differences in parasite load and no changes in the transcript
95 levels of genes involved in adaptive immunity, such as MHCIIβ and TCRβ1, between
96 primary and secondary infection (13). Similarly, Jørgensen et al. (18), when studying
97 genes encoding the inflammation-involved cytokines (IL-1β, TNF-α, IFN-γ, IL-10) and
98 markers for adaptive immune response (CD4, CD8, TCRα, IgM, IgT and MHC II) in
99 the skin of rainbow trout infected with *G. salaris*, did not find significant changes in
100 expression. Moreover, no histological differences between infected and non-infected
101 skin and fin tissue were detected, implying that infection did not cause skin infiltration
102 with T- and B-cells and neutrophilic granulocytes.
103 Studies based on panels of candidate genes, though valuable, are likely to
104 overlook important pathways. A more comprehensive approach is now offered by
105 RNA-Seq, which allows large-scale screening of genes changing expression in
106 response to infection without the limitations of using predefined probes (19, 20).
107 Here, we used RNA-Seq to investigate the response of guppies (*Poecilia reticulata*)
108 to infection with its common ectoparasite, *G. bullatarudis*. Guppies are tropical fish
109 that, owing to their high natural polymorphism, rapid generation time and amenability
110 to lab rearing have long served as a model species in behavioural and evolutionary
111 biology (21–23). Guppy research has included numerous studies of host-parasite
112 coevolution (1, 10, 24, 25), which have investigated the widespread and common
113 gyrodactylid infections in both wild and ornamental guppies. Both innate and adaptive
114 immune responses have been inferred as playing roles in guppy response to
115 G*yrodactylus* infection (24) but this is the first RNA-Seq-based screening of the
116 immune response to gyrodactylids in any fish species.
117

118 ## 2. Materials and methods

119 ### 2.1. Host maintenance

120 To obtain fish naïve with respect to exposure to species of *Gyrodactylus,* 38
121 pregnant guppy females were collected from a tributary of the Bacolet River near
122 Scarborough Health Centre on Tobago in March 2016 ('HC' population henceforth).

123 Guppies were transported to our field station and screened for gyrodactylids.
124 Screening involved briefly anaesthetising fish in 0.02% tricaine methane sulfonate
125 (MS-222, Sigma-Aldrich) and examining them under a dissecting microscope (as
126 detailed by Schelkle et al. (26)). After observing gyrodactylids among the sample of
127 fish, we treated all fish with 20 ‰ sodium chloride solution for 1 minute (27). This
128 treatment was deemed effective for this fish population after observing no
129 gyrodactylids when re-screening all fish 1, 3 and 5 days post treatment. Fish were
130 then reared for 3.5 months in a 100 litre aquarium, with a daily feed of either *Artemia*
131 nauplii or generic fish flakes. Because guppies are cannibalistic, plastic grids
132 excluding access of adult fish to ca. 1/3 of the aquarium were used to enhance fry
133 survival. The adult and subadult offspring of these females were used for the gene
134 expression analyses described below.
135 Fish sampling and maintenance were conducted according to national
136 guidelines and with the permission from the Tobago House of Assembly (permission
137 number: 004/2014).
138
139 **2.2. Gyrodactylus worm isolation, characterization and culture**
140 Adult wild guppies from the Roxborough River, Tobago, were collected and
141 screened for gyrodactylids in June 2016. Infected fish were separated and served as
142 donors. As infection intensities among the infected fish were low (1-3 parasites per
143 fish), worms were cultured to obtain sufficient numbers for the experiments (see
144 Stewart et al. (28)). In brief, to establish cultures, a donor fish and a gyrodactylid-
145 naïve recipient 'farm' fish were anaesthetised and 1-2 worms were allowed to move
146 from donor to recipient. The farm fish were from a mesocosm population at our field
147 station, which was founded in November 2014 by crossing gyrodactylid-free male
148 guppies from a Trinidad population with gyrodactylid-free females from a Tobago
149 population (different populations from those used in the present study) and which
150 were maintained free of exposure to gyrodactylids. These cultures also provided us
151 with a quick means of assessing whether we had collected *G. bullatarudis* rather than
152 *G. turnbulli*, which is also widespread in Trinidad and Tobago guppies. *G. bullatarudis*
153 infections show a pronounced rostral bias with worms aggregated on the head,
154 opercula and pectoral fins of the host (29). Species identification was later confirmed
155 by mtDNA COII sequencing: we extracted DNA from 4 worms, and Sanger-
156 sequenced a 262 bp section (primers and PCR conditions as in Xavier et al. (30)).

157 BLAST searches of the resulting sequences showed their strongest matches (97-
158 100% identity) to published *G. bullatarudis.*
159
160 **2.3. Infection model**
161 For experimental infections, we selected at random 28 adult and juvenile fish
162 (>10 mm) from our captive gyrodactylid-naïve HC population. Twenty-one fish were
163 infected in July 2016 using the controlled infection procedure described above; the
164 remaining seven were handled in the same manner but not exposed to parasites, to
165 serve as uninfected controls. A single donor fish was used to initiate all infections on
166 the same day. Each recipient received two gyrodactylids; any additional worms that
167 accidently transferred were removed using watchmaker's forceps. Recipients were
168 then revived and housed individually in 400 ml isolation containers at ambient shade
169 temperature (mean = 27.1°C; mean daily min. = 25.9°C; mean daily max. = 28.4°C),
170 receiving feed followed by a water change every other day. Control fish were kept
171 under the same isolation conditions. Fish were anesthetised for worm counting two
172 days post-infection.
173 All national guidelines for the care and use of animals were followed.
174 Procedures and protocols were conducted under UK Home Office license (PPL
175 302876) with approval by the Cardiff University Animal Ethics Committee.
176
177 **2.4. Skin sampling**
178 Four days post-infection, we screened all fish again and selected eight infected
179 fish to euthanize (Tricaine Methanesulfonate [MS-222, 500 mg L^{-1}] overdose) for
180 tissue sample collection, along with three uninfected control fish. To choose which
181 infected fish to sample, we ranked infections by intensity and then made random
182 selections within blocks of fish with similar intensities. Our aim was to have
183 representation from a range of infection intensities while still leaving sufficient fish
184 from across the susceptibility range to progress in their infections. We repeated this
185 at infection day 8, euthanizing seven infected fish and two control fish. At infection
186 day 12, we ended the experiment by euthanizing all remaining fish for tissue
187 sampling. All fish were euthanized with an overdose of MS-222 (500 mg L^{-1}). From
188 each fish, we collected two tissue samples: 1) caudal fin and pectoral fins, being sure
189 to take only skin and fin ray, and no muscle or scales; and 2) head skin, usually

190 collected by inserting forceps at the base of the cranium and ‘peeling’ forward, taking
191 the lips and gill opercula. Due to the small sizes of the fish, contamination of the head
192 skin sample with muscle tissue, gill fragments and scales could not be completely
193 avoided. Between each euthanized fish, tools and the work station were cleaned with
194 RNaseZap (Sigma-Aldrich). Each tissue sample was placed in 1 ml RNAlater in a 1.5
195 ml RNAse-free Eppendorf tube. Samples were refrigerated (+4°C) for one week and
196 then frozen at -20°C.
197
198 **2.5. RNA sequencing and expression analyses**
199 RNA from fins and head skin was extracted with RNAzol (Sigma-Aldrich), followed by
200 quality control assessment on a Tape Station. We used the 19 samples with the
201 highest RNA Integrity Numbers for library preparation and sequencing (Table 1 for
202 sample overview). A poly-A stranded library was prepared from each sample at the
203 CRG Barcelona Genomic Unit and sequenced to generate 50bp single-end reads
204 using the Illumina 2500 platform. All sequence data have been submitted to the NCBI
205 Sequence Read Archive (Accession: PRJNA526802).
206 Read quality was assessed with FASTQC, and low quality reads were filtered
207 with Trimmomatic, version 0.35, (31) with the following settings:
208 ILLUMINACLIP:TruSeq3-SE:2:30:10,LEADING:3,TRAILING:3,
209 SLIDINGWINDOW:4:15, and MINLEN:36. Cleaned reads were mapped to the guppy
210 reference genome, version GCF_000633615.1, (32) with STAR software, version
211 2.5.3a and default parameters (33).
212
213 **2.6. Differential Gene Expression (DGE) Analysis**
214 Gene expression analyses were performed following the Bioconductor RNA-
215 Seq workflow (34). Briefly, we downloaded the guppy genome annotation from NCBI
216 and used it for defining gene models. After counting the numbers of reads mapped to
217 the gene models, we used DESeq2 library (35) to create *DESeqDataSet* object, and
218 included only genes with at least 10 reads mapped to the gene model. Transformed
219 counts (rlog) were used for calculating sample distances, visualising samples with
220 heatmaps, and principal component analyses (PCA). Because heat map visualisation
221 suggested that gene expression profiles grouped by body location (head vs fins) but
222 not by day of sampling (Supplementary Figure S1), we decided to analyse each
223 tissue separately to determine infection-related changes in gene expression (head

infected vs head non-infected and fins infected vs fins non-infected). Equivalent analyses performed separately for days 4 and 8 (not reported) showed similar patterns but with fewer infection-related genes identified, likely due to smaller numbers of samples per group. To confirm our findings, we used another software, edgeR, to analyse gene expression (36). We then compared p–values estimated with DESeq2 and edgeR for fin samples.

To assign genes to molecular pathways, we used the Kyoto Encyclopedia of Genes and Genomes (KEGG) Automatic Annotation Server. KEGG Orthology assignments were then used to search and colour pathways in the KEGG database. All protein coding genes were blasted against Swiss-Prot databases, and Gene Ontology (GO) terms were annotated with blast2GO (37) and interproscan (38). GO terms predicted by both software were merged and used for enrichment tests, calculated using the topGO package in R and summarized with REViGO (39).

2.7. Weighted Gene Co-expression Network Analyses

Differential gene expression is usually identified using exact tests carried out on each gene separately; however, due to the need of correcting P-values with stringent multiple testing methods, only genes with the largest differences in expression are typically identified. An alternative for quantifying transcriptional responses is weighted gene co-expression network analysis (WGCNA) which can reveal more subtle but biologically-relevant systematic changes in expression (40). We used this method to quantify transcriptional responses of fish to infection, enabling the identification of networks (modules) of co-expressed genes (genes that show consistent expression profiles across samples), and thus potentially identifying functionally important genes with only subtle changes in expression that may otherwise not have been detected. Read counts, normalized using a variance stabilizing transformation (VST) in DESeq2, were analysed using the R package WGCNA. Our gene modules were defined using the dynamic Cut Tree function and TOM Type “signed” with a minimum module size of 100. A module eigengene distance threshold of 0.25 was used to merge highly similar modules. Gene module preservation (by tissue or sample day) was determined using Z-summary statistics in the WGCNA package (40). Modules were then correlated with tissue type or sampling day, plus infection status and worm burden, to identify gene networks significantly associated with factors of interest.

257 Biological Process GO term enrichment tests of each significant gene module were
258 performed using topGO as described above.
259

260 3. Results

261 *Differential expression analyses*
262 We obtained a total of 19 samples from head skin (10) and fins samples (9) taken
263 from control (9 samples) and infected fish (10 samples). Among fish that were
264 infected, there was no significant bias in age class (juvenile or female) against day of
265 killing (4, 8 or 12; χ^2 = 2.48, bootstrap P = 0.39). Across all fish, including those not
266 infected, there was no significant effect of day of killing on size (Kruskal-Wallis test:
267 χ^2 = 1.28, df = 2, P = 0.53). There was no significant size difference between infected
268 and uninfected fish (Mann-Whitney test: U = 22, P = 0.82), and there was no
269 significant age/sex bias among infected v. uninfected fish (Fisher's exact test: P =
270 0.23).
271 Comparison of transcriptomic profiles revealed that samples clustered by tissue,
272 (Figure 1), and therefore head skin and fin samples were analysed separately. We
273 found very few differentially expressed genes in head skin samples (n = 8;
274 Supplementary Table S1), possibly a consequence of higher heterogeneity of tissues
275 collected during sampling (skin, scales, muscle tissue) compared to fins. However, in
276 the fin tissue we found 342 differentially expressed genes (P-values adjusted for false
277 discovery rate = 0.1, Supplementary Table S2). Results were the same, regardless of
278 the software used (Supplementary Figure S2, S3). Gene ontology analysis of these
279 genes revealed enrichment for multiple terms (Supplementary Figures S4-S6),
280 including immune function (in 'biological processes' category) and
281 cytokine/chemokine (in 'molecular function' category). Metabolic pathway analysis
282 (KEGG) of differentially expressed genes identified orthologues of several immune-
283 related categories, including cytokine-cytokine receptor interactions (14 genes), IL-17
284 signalling pathway (9) and Th17 cell differentiation (4), chemokine signalling pathway
285 (7), NOD-like receptor signalling pathway (6), natural killer cell-mediated cytotoxicity
286 (4), T cell receptor signalling pathway (3), and B cell receptor signalling pathway (3)
287 (see Supplementary File S1 for full list). More detailed analysis of the cytokine-
288 cytokine receptor interaction category revealed several genes with significantly
289 increased expression in infected fish belonging to CXC and CC chemokine

290 subfamilies, IL3RB family, TNF family and IL17 family (Figure 2). The last family was
291 particularly well represented, with 6/14 genes showing higher expression in infected
292 fish compared to uninfected ones (Figure 3). Most of these genes were upregulated
293 in infected fish (Figure 4).
294

295 *Expression of immune-related genes*

296 Follow-up examination of the list of differentially expressed genes
297 (Supplementary Tables S1 and S2) revealed upregulation in fins of several genes
298 involved in the innate immune response, including: i) receptor for pathogen
299 recognition, ii) molecules directing leukocyte migration, as well as iii) enzymes
300 catalyzing eicosanoid synthesis in arachidonic acid cascade. From the first category,
301 we found upregulation of gene expression of C-type mannose receptor 2 and
302 macrophage mannose receptor 1, as well as NOD-like receptors (NLRs NLRP12
303 (NACHT, LRR and PYD domains-containing protein 12) and NLRP3, NLRC3/NOD3
304 (NOD-like receptor family CARD domain containing 3) and NOD1 (nucleotide-binding
305 oligomerization domain-containing protein 1). From the second category, we found
306 up regulation of arachidonate 15-lipoxygenase B-like (ALOX15B) and ALOX 12 gene
307 expression.

308 Among molecules involved in leukocyte migration, we observed upregulation of
309 gene expression of several chemokines (CXCL1/growth-regulated alpha protein,
310 CXCL13/B cell-attracting chemokine 1, CCL2/monocyte chemoattractant protein 1b,
311 CCL20/macrophage inflammatory protein-3) and chemokine receptors (CCR1,
312 CCR2, CXCR1), permeability factor 2-like and receptor for C3a complement factor
313 (chemokine-like receptor 1). Furthermore, upon infection in fin skin we detected up
314 regulation of lipocalin-2, cathepsin B and matrix metalloproteinase 13/collagenase 3.
315 In the fins of infected fish, we found increased gene expression of several cytokine
316 receptors: interferon-α/β receptor - IFNAR, interleukin-1 receptor 1 - IL-1R1, IL-13R
317 subunit alpha-1, IL-21R, IL-31R subunit alpha, TNFR superfamily member 1A and 4,
318 as well as cytokine receptor common subunit gamma which is common to the
319 receptor complexes for interleukin receptors such as IL-2, IL-4, IL-7, IL-9, IL-15 and
320 IL-21.

321 Several genes involved in the adaptive immune response were upregulated in
322 fins. This included two transcription factors implicated in Th1 (STAT4) and Th2
323 (GATA3) differentiation, and adhesion molecules such as cadherin-like protein 26,

324 cell surface glycoprotein CD9 and expressed on T cells and NK cells adhesion
325 molecule CD2. We also found upregulation of glycosylphosphatidylinositol (GPI)-
326 linked differentiation antigen (lymphocytes antigen 6G, Ly6G) that in mammals is
327 expressed by myeloid-derived cells and T-cell surface glycoprotein CD4-like usually
328 expressed on T helper cells, monocytes, macrophages, and dendritic cells.
329 Finally, during infection we observed elevated expression of genes involved in
330 ubiquitination and antigen processing (E3 ubiquitin/ISG15 ligase TRIM25, TRIM21,
331 TRIM8, E3 ubiquitin-protein ligase Itchy, RNF19A, SMURF2 and proteasome
332 activator complex subunit 4 as well as ubiquitin carboxyl-terminal hydrolase 11, 12,
333 19) and antigen presentation (beta-2-microglobulin, MHC class I related protein).
334 We did not find a significant difference in expression of MHC class II, which has
335 been inferred as influencing the effectiveness of the guppy immune response to
336 gyrodactylids (1, 41). However, we noted its high constitutive expression (e.g.
337 LOC103461570, predicted: DRB1-8 beta chain-like, mean =2959.7 reads, Log2FC=-
338 0.26; LOC103460899, predicted: E-S beta chain-like, mean =5675,97, log2FC=-0,03)
339 compared to the mean (565.1) for 514 genes expressed in our sample which fell into
340 the immune function category.
341
342 *Gene co-expression network analyses*
343 Gene co-expression network analyses revealed 33 and 25 modules in the head
344 and fins respectively. Six head gene modules were significantly correlated with either
345 infection status or worm burden, of which five were significantly preserved in fin
346 tissues (Table 2). This result highlights the power of WGCNA analysis compared to
347 standard DGE analysis, where only a handful of differentially expressed genes were
348 found in the head tissue (in contrast to fins, see above). One of the modules in the
349 head tissues (“head-violet”; Table 2) was negatively correlated with infection status
350 (i.e. lower expression in infected fish) and enriched for several GO terms including
351 mucus secretion. The head gene module (“head-cyan”) positively associated with
352 infection status (higher expression in infected fish) was enriched for GO terms
353 including type I interferon production. Furthermore, all 3 head gene modules (“head-
354 red”, “head-darkred” and “head-pink”) positively correlated with worm burden
355 (increasing expression with higher worm number) were enriched for genes involved
356 in T-cell differentiation and proliferation, as well as antigen processing and
357 presentation (head-red; “regulation of T-cell apoptosis and formation of

358 immunological synapse", head-darkred; "regulation of T-cell differentiation" and
359 "antigen processing and presentation", head-pink; "T-cell proliferation") (Table 2). The
360 gene module ("head-brown") negatively correlated with worm burden (lower
361 expression in more heavily infected fish) included functions related to MHC II and IL-
362 1β biosynthesis (Table 2).
363 In contrast to head gene networks, only a single gene module defined in the fins
364 was significantly associated with infection status ("fin-black") and preserved in head
365 tissue (Table 2). This module included functions related to innate immune response
366 such as macrophage activation and production and secretion of pro-inflammatory
367 cytokines (TNF, IL-6) and chemokine CCL2. In addition, a single fin gene module
368 ("fin-cyan") was positively correlated with worm burden, not preserved in the head,
369 enriched for GO terms including regulation of macrophage chemotaxis and pathogen-
370 recognition (Toll signalling) (Table 2).
371 In both tissue networks, we found several gene modules associated with
372 sampling day (head; 6, fins; 3), suggesting temporal variation in infection responses.
373 Therefore, we re-defined gene modules including both tissue types, separating data
374 instead by sampling day. At day 4, of the 28 gene modules found, four associated
375 with infection status and/or worm burden yet were not preserved by day 8
376 (Supplementary Table S3). At day 8, nine gene modules were significantly
377 associated with either infection status or worm burden, of which only two were
378 significantly preserved in day 4 (Supplementary Table S3). All time-specific modules
379 were enriched for immune response GO terms, particularly for T cell and other
380 leukocyte-related pathways.
381

382 ## 4. Discussion

383 Previous studies based on panels of candidate genes have suggested that the
384 fish immune response against gyrodactylids involved C3 complement factor (12), pro-
385 inflammatory cytokines (13–15) as well as some elements of Tc-mediated reaction
386 (17). In the present study, use of RNA-Seq has allowed us to identify many other
387 genes not previously identified as being part of fish immune responses against
388 gyrodactylid infection. These include genes and gene families with known links to the
389 immune systems of other vertebrates.
390

391 *Resolution of inflammation and wound healing*

392 The most significant upregulated gene (most significant *P*-value for both head
393 and fin tissue and largest absolute fold-change value in the fin; Supplementary
394 Tables S1-S2) was 15-lipoxygenase-2 (ALOX15B). In fins, we also found increased
395 expression of the related arachidonate 12-lipoxygenase (ALOX12). Both enzymes
396 catalyze synthesis of lipoxin A4 (LXA4) from leukotriene A4 (LTA4) and may also
397 convert arachidonic acid to 15-hydroxyeicosatetraenoic acid (15SHETE), which can,
398 in turn, be converted into LXA4 by ALOX5 (42). In mammals, LXA4 has been
399 ascribed an anti-inflammatory function, inhibiting leukocyte-mediated injury,
400 stimulating macrophage clearance of apoptotic neutrophils, and inhibiting pro-
401 inflammatory cytokine production and cell proliferation (43, 44). In fish, however,
402 information about the roles of ALOX and lipoxins in the immune response are limited.

403 First described in rainbow trout, LXA4 was found to be synthesized in trout
404 macrophages when stimulated *in vitro* with either calcium ionophore or opsonized
405 zymosan (45). Knight and Rowley (46) tested the effect of LXA4 on the number of
406 plaque-forming cells (PFC) following *in vitro* challenge of trout splenocytes with
407 sheep erythrocytes and found that LXA4 caused a significant dose-dependent
408 increase in PFC generation. In contrast, however, *in vivo* fin amputation in zebrafish
409 embryos decreased expression of ALOX12 and ALOX15b genes and LXA4
410 concentration (42). Thus, while mammalian data suggest that upregulation of
411 ALOX12 and 15B may indicate their role in preventing inflammation-induced tissue
412 damage, the present study suggests a similar role of these genes in fish. Increased
413 expression of genes involved in wound healing, establishment of skin barrier and
414 keratinocyte proliferation is also supported by increased expression of cathepsin B,
415 which enhances the activity of other proteases, including matrix metalloproteinase, as
416 well as matrix metalloproteinase 13/collagenase, which can be involved in matrix
417 remodelling events by collagen degradation and therefore associated with wound
418 healing response. Similarly, Braden et al. (47) observed elevated expression of tissue
419 repair enzymes (MMP9, MMP13) in the skin of salmonids infected with the
420 ectoparasitic copepod *Lepeophtheirus salmonis*.

421

422 *Th17-driven and innate immune response*

423 Our special interest was drawn to the *G. bullatarudis*-induced changes in the
424 expression of a number of genes involved in the Th17 response in the skin of

425 infected fish. Although we did not find upregulation of the IL-17 gene itself, two types
426 of IL-17 receptors (A and C), IL-17-induced transcription factors, and
427 cytokines/chemokines involved in Th17 differentiation and action, were all
428 upregulated (Fig. 1B). Also of relevance to the Th17 response was the upregulation
429 of CD4 glycoprotein. Previously, Infante-Duarte et al. (2000) observed that CD4+T
430 cells, primed with a synthetic peptide in the presence of spirochete bacteria, may
431 differentiate into distinct T-cell lineage expressing high level of IL-17A (48, 49). To
432 date, six mammalian IL-17-family ligands (IL-17A, IL-17B, IL-17C, IL-17D, IL-17E (IL-
433 25) and IL-17F) and five receptors (IL-17RA, IL-17RB/IL-25R, IL-17RC, IL-17RD/SEF
434 and IL-17RE) have been identified (50). In mammals, these are thought to mediate
435 immunity against extracellular bacteria, particularly those that colonize exposed
436 surfaces such as the airways, skin, and intestinal lumen. Th17 is also involved in T
437 cell polarization in response to infection by extracellular and intracellular bacteria and
438 fungi (51).
439 Genes related to known IL-17 family members and IL-17 receptors have been
440 identified in other teleosts (52, 53). IL-17 homologues, for example, have been found
441 in the genomes of zebra fish (*Danio rerio*), fugu (*Takifugu* spp.), grass carp and
442 salmonids (54–61), while five IL-17Rs were found in the large yellow croaker
443 (*Larimichthy scrocea*) (53). These receptors are constitutively expressed in several
444 tissues and organs, including high constitutive expression in mucosal tissues of the
445 gills and skin (54, 62, 63). Moreover, gill mucosal tissue, along with the lymphoid
446 organs of the head kidney and spleen, showed upregulation of IL-17 receptors in *L.*
447 *crocea* infected with *Aeromonas hydrophila* (see Ding et al. (53)), in line with our
448 observation of increased IL-17 receptor expression in the skin of infected guppies.
449 Upon infection differential expression of genes involved cytokine-cytokine
450 receptor interactions and chemokine signalling pathway was observed. Among
451 others, we found infection-induced changes in the expression of cytokine receptors
452 IL-1, IL-21 and TNF, which, in mammals, are believed to be both drivers of Th17
453 differentiation and release of cytokines from activated Th17 cells (64–67). Previous
454 work on teleosts has shown that IL-21 is a potent stimulator for IL-17A/F1a (68), in
455 line with the increased expression of genes involved Th17 response we found in
456 response to *G. bullatarudis* infection. Moreover, in the fins of *G. bullatarudis*-infected
457 guppies, several IL-17-inducible genes were upregulated (chemokines:
458 CXCL1,CXCL8 and CXCL13, CCL2 and 20, and MMP-13/collagenase 3). This

459 finding also has parallels in mammals, where both homo- and heterodimers of IL-17A
460 and IL-17F induced the expression of pro-inflammatory mediators (e.g. IL-1b, IL-6,
461 GM-CSF, CXCL8, CXCL1, CXCL10 and MMP-13) and, accordingly, mobilize, recruit,
462 and activate neutrophils (49). Our results may imply that members of the IL-17 family
463 also function as potent pro-inflammatory modulators in fish. This interpretation is
464 supported by *in vitro* work in fish: recombinant carp IL-17D upregulated expression of
465 pro-inflammatory IL-1b, TNF-a and CXCL-8 and activated NF-KB signalling (63), and,
466 similarly, trout recombinant IL-17A increased the expression of pro-inflammatory IL-6,
467 CXCL8 and the antimicrobial peptide BD-3 (54). Furthermore, in mammals also IL-23
468 induces a polarization of Th17 cell population with a unique inflammatory gene
469 signature that includes IL17, IL6, TNF, CCL20, CCL22, IL1R1, and IL23R (69).
470 Recently, Yin and co-workers (70) confirmed also for fish that recombinant IL-23 is
471 able to enhance the mRNA levels of IL-17A/F1 and its secretion from head kidney
472 leukocytes. Interestingly, our WGCN analysis indicated existence of the positive
473 correlation between worm burden and expression of genes involved in inflammation
474 and Th17-response such as IL-23 in the samples from head skin of guppies.
475 Previous research has already indicated a role of Th17 in fish immune response
476 and found increased expression of IL-17 genes during viral, bacterial and myxozoan
477 (*Tetracapsuloides bryo salmonae and Enteromyxum leei)* infections (54, 63, 71–73).
478 Enhanced Th17-like immune responses was also found in mucosal and adipose
479 tissue of vaccinated fish (59, 60, 74) and it was involved in vaccine-induced
480 granulomatous reactions (58). Moreover, IL-17 up-regulation was observed in fish
481 leukocytes stimulated in vitro with LPS, poly I:C, PHA and ConA (61, 75). IL-17A was
482 also increased in the head kidney of carp infected with some, but not all, species of
483 *Trypanoplasma* (also known as *Cryptobia*) parasites (76). Our study is the first
484 indication of Th17 involvement in the fish immune response against gyrodactylid
485 ectoparasites.
486 In fin tissue, we found upregulation of several genes involved in pathogen
487 recognition, such as C-type mannose receptor 2, macrophage mannose receptor 1,
488 and a number of NOD-like receptors. These observations agree with Hu et al. (77),
489 they described involvement of a NOD-like receptor signalling pathway in the skin of
490 orange-spotted grouper (*Epinephelus coioides*) infected with the holotrich protozoan
491 *Cryptocaryon irritans*. Moreover, in WGCN analysis we found that expression of the
492 genes involved in Toll-signalling correlates in fin samples with worm burden. Finally,

493 upon infection in fin skin, we noted upregulation of lipocalin-2. Lipocalin (neutrophil
494 gelatinase-associated lipocalin, NGAL) is involved in iron sequestrating which in turn
495 limits infection. These results support the suggestion that innate immunity plays an
496 important role in the response to gyrodactylid skin parasites, supporting and is in
497 corroboration of the fact that Th17 immune response drives neutrophil infiltration to
498 the site of infection (78, 79).
499
500 *Adaptive immune response*
501 In addition, molecules associated with antigen presentation and adaptive
502 immune response were significantly upregulated upon infection. This list includes T
503 and B cell markers (CD4 mentioned before, but also CD2 – both markers of Th cells
504 including Th17, CD9, CD22), genes involved in ubiquitination and antigen processing
505 and presentation (e.g. TRIMs, beta-2-microglobulin, MHC I). Moreover, we found a
506 positive correlation between worm burden and expression of genes involved in
507 antigen processing and presentation (e.g. formation of immunological synapse) and
508 T-cell differentiation, proliferation and apoptosis. These data suggest that
509 lymphocytes infiltrate the infected skin. Similarly, T cell marker tetraspanin CD9, B
510 cell receptor CD22, and MHC class I and class II genes were also significantly
511 upregulated in skin of orange-spotted grouper infected with *C. irritans* (see (77)).
512 Previous studies found associations between the level of infection with
513 *Gyrodactylus* and guppy MHC II, both in the field (41) and in controlled experimental
514 infection (1). Here, although MHC class I genes were significantly upregulated in
515 differential expression analyses (Supplementary Table S1), we did not find
516 significantly increased expression of MHC II genes in the skin of infected fish.
517 However, constitutive expression of MHC II gene in skin of uninfected fish was
518 roughly an order of magnitude higher compared to all other immunity genes, and the
519 lack of differential expression in infected skin is therefore not inconsistent with the
520 role of MHC II in mediating immune response against *Gyrodactylus*. MHC II
521 expression on the surface of antigen presenting cells (in particular dentritic cells) is
522 regulated by ubiqutination (80, 81), and we did find significant changes in expression
523 of several genes involved in ubiquitation and deubiquitation. Finally, we found a gene
524 co-expression module negatively correlated with worm burden and enriched for MHC
525 II biosynthesis, suggesting fish with increased activation of MHC II pathways are
526 more resistant to infection.

527 When defining gene co-expression modules by sampling day (rather than
528 tissue), we found several time-specific gene modules enriched for immune
529 responses, particularly for leukocyte-related pathways, associated with infection
530 status and/or worm burden (Supplementary Table S3). This indicates a broad shift in
531 the immune expression response throughout the course of infection. The greater
532 number of adaptive immune-enriched modules specific to day 8 is consistent with
533 typical guppy-*Gyrodactylus* infection profiles; where worm clearance is usually
534 observed over a week into infection and assumed to be associated with initiation of
535 adaptive immunity (24). However, our sample sizes restricted our ability to interrogate
536 temporal co-expression patterns in each tissue separately. Future work on tissue-
537 specific temporal variation in activation of immune gene expression is required to fully
538 resolve the critical timings of infection responses.

539 *Conclusions*

540 Summarizing, our RNA-seq screen of gene expression changes following *G.*
541 *bullatarudis* infection in guppies resulted in a sizeable list of genes potentially
542 involved in the teleost immune response. Our results are consistent with earlier
543 studies of limited sets of candidate genes in implying the role of both innate and
544 adaptive responses to infection with gyrodactylids. However, many immune-related
545 genes we found differentially expressed in infected and uninfected fish have not been
546 studied before in such context. Of these new genes, those involved in the Th17
547 response were particularly well represented, highlighting Th17 pathway as a strong
548 candidate for further study of immune response to infection with fish ectoparasites.

549

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Ethics approval

The project was conducted with the permission from the Tobago House of Assembly (permission number: 004/2014). All national guidelines for the care and use of animals were followed. Procedures and protocols were conducted under UK Home Office license (PPL 302876) with approval by the Cardiff University Animal Ethics Committee.

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808

809 # Tables

810 **Table 1.** Overview of samples used for RNA-seq analyses

Sample ID	Ind. ID	Date of sampling	Tissue	Inf. day	Inf. status	No of worms	No of reads (mln)	% of uniquely mapped reads
RNA_014	HC_13	13-07-2016	head skin	d4	infected	3	12.4	74.3
RNA_016	HC_15	13-07-2016	fins	d4	infected	7	13.2	86.9
RNA_019	HC_17	13-07-2016	fins	d4	noninfected	0	13.7	92.5
RNA_020	HC_17	13-07-2016	head skin	d4	noninfected	0	12.8	87.1
RNA_022	HC_19	13-07-2016	fins	d4	noninfected	0	16.7	94.0
RNA_023	HC_19	13-07-2016	head skin	d4	noninfected	0	18.1	83.8
RNA_028	HC_21	13-07-2016	fins	d4	infected	25	14.8	92.4
RNA_029	HC_21	13-07-2016	head skin	d4	infected	25	15.4	71.1
RNA_032	HC_24	13-07-2016	head skin	d4	noninfected	0	14.1	88.8
RNA_070	HC_06	17-07-2016	fins	d8	infected	1	14.2	85.1
RNA_076	HC_09	17-07-2016	fins	d8	infected	8	17.0	91.4
RNA_081	HC_11	17-07-2016	head skin	d8	infected	120	17.4	86.7
RNA_082	HC_16	17-07-2016	fins	d8	infected	8	17.7	89.8
RNA_084	HC_16	17-07-2016	head skin	d8	infected	8	17.3	93.8
RNA_087	HC_22	17-07-2016	head skin	d8	infected	13	14.8	85.9
RNA_088	HC_25	17-07-2016	fins	d8	noninfected	0	13.5	91.7
RNA_090	HC_25	17-07-2016	head skin	d8	noninfected	0	16.1	86.3
RNA_091	HC_26	17-07-2016	fins	d8	noninfected	0	13.1	90.1
RNA_093	HC_26	17-07-2016	head skin	d8	noninfected	0	12.1	68.3

811

812

813 **Table 2.** Summary of gene co-expression networks associated with *Gyrodactylus*
814 infection including number of genes per module, significant correlations, module preservation, and
815 gene ontology enrichment. Gene module names denote tissue type in which they were defined (fin
816 or head skin) and given an arbitrary colour label (assigned during WGCNA) to distinguish individual
817 modules.

Module Name	n genes	Infection status correlation	Worm burden correlation	Preserved in other tissue?	Most significant GO	Infection-related GO terms
Fins						
Fin-black	889	+0.67	NS	Yes	peptide biosynthetic proces	macrophage activation, antimicrobial humoral response, response to fungus, tumor necrosis factor production, response to virus, chemokine (C-C motif) ligand 2 secretion, interleukin-6 secretion,

						B cell receptor transport
Fin-cyan	417	NS	+0.93	No	oxaloacetate metabolic process	regulation of macrophage chemotaxis, Toll signaling pathway, viral release from host cell
Head						
Head-violet	116	-0.66	NS	No	calcium import into the mitochondrion	mucus secretion
Head-cyan	544	+0.63	NS	Yes	visual perception	viral transport, type I interferon production
Head-red	1288	NS	+0.63	Yes	RNA processing	suppression of host defences, globlet cell differentiation, immunological synapse formation, regulation of T cell apoptotic process
Head-darkred	272	NS	+0.74	Yes	regulation of DNA damage response	establishment of skin barrier, response to interferon-gamma, wound healing, leukocyte aggregation, immune response, antigen processing and presentation, positive regulation of T cell differentiation
Head-pink	1103	NS	+0.94	Yes	JAK-STAT cascade	regulation of defence response, regulation of immune system process, keratinocyte proliferation, innate immune response, activated T cell proliferation, type I interferon signaling pathway, leukocyte migration, viral latency, regulation of immunoglobulin production, B cell proliferation, inflammatory response, interleukin-23 production, response to interleukin-18

Head-brown	1744	NS	-0.92	Yes	sodium ion export across plasma membrane	mast cell migration, response to chemokine, regulation of platelet aggregation, MHC class II biosynthetic process, interleukin-1 biosynthetic process, lymphocyte mediated immunity

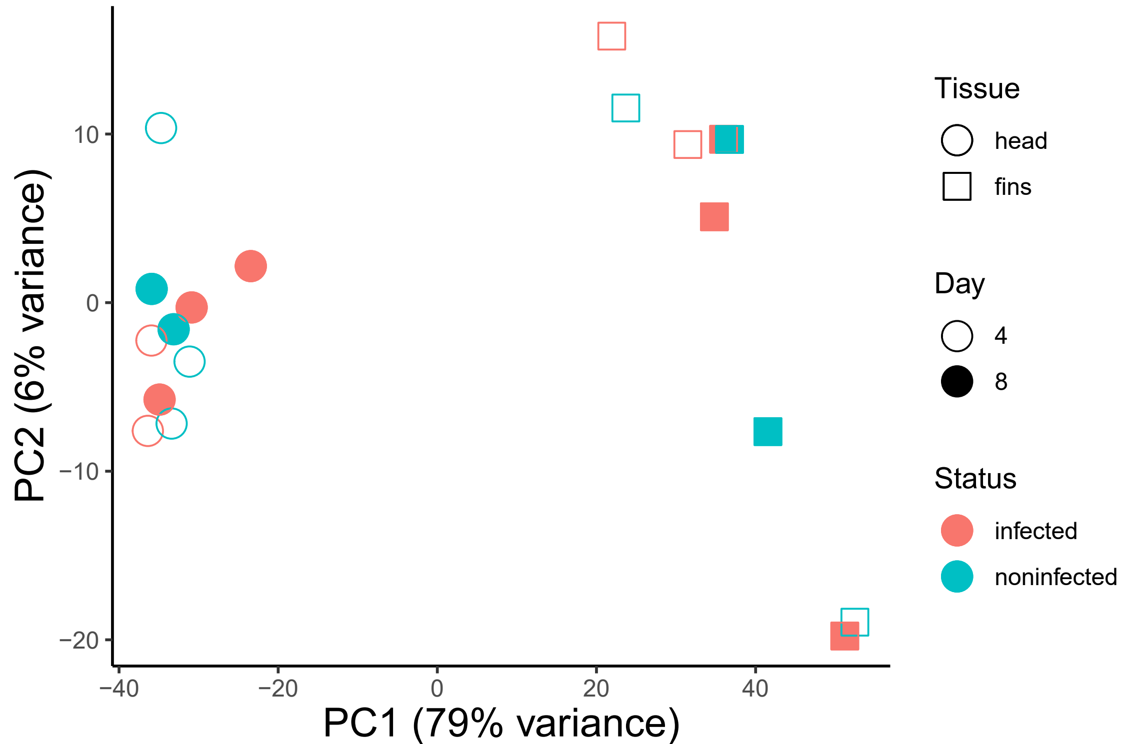
Figure captions

Figure 1. Principal component analysis of RNA-Seq samples, sequenced from fins and head skin of guppies (*Poecilia reticulata*), four and eight days after infection with *Gyrodactylus bullatarudis.*

Figure 2. Cytokine-cytokine receptor interactions identified by metabolic pathway analysis (KEGG) to be differentially expressed in fins of infected and uninfected fish. Significantly differentiated genes are in pink; genes which were missed from automatic annotation, but which were included into the list of differentially expressed genes in Supplementary Table S3 are framed with red (CXCL1, growth-regulated alpha protein, LOC103476162 in Supplementary Table S2; CCL2, monocyte chemotactic protein 1B-like, LOC103466287 in Supplementary Table S3). Blue boxes indicate automatically annotated but not significantly differentiated genes, while white boxes indicate genes which were not annotated in the guppy genome.

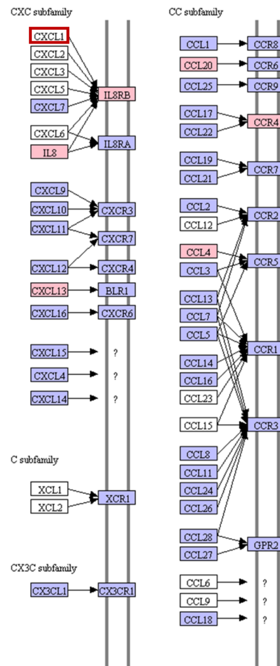
Figure 3. IL-17 signalling pathway identified by metabolic pathway analysis (KEGG) to be differentially expressed in fins of infected and uninfected fish. Significantly differentiated genes are in pink; genes which were missed from automatic annotation, but which were included into the list of differentially expressed genes in Supplementary Table S3 are framed with red (CXCL1, growth-regulated alpha protein, LOC103476162 in Supplementary Table S2; CCL2, monocyte chemotactic protein 1B-like, LOC103466287 in Supplementary Table S3). Blue boxes indicate automatically annotated but not significantly differentiated genes, while white boxes indicate genes which were not annotated in the guppy genome.

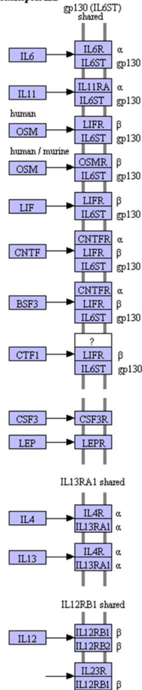
846 **Figure 4.** Heatmap of RNA-Seq expression z-scores computed for genes identified
847 as differentially expressed in fins of infected and uninfected fish. Only genes
848 annotated as belonging to cytokine-cytokine receptor interaction (Figure 1) and IL17
849 (Figure 2) families are shown. Gene names follows KEGG annotation from Figures 1
850 and 2 and genes IDs are given in brackets.
851

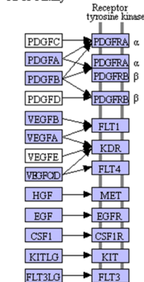


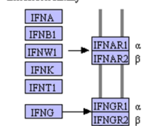
PC2 (6% variance)
10
0
−10
−20
−40
−20
0
20
40
PC1 (79% variance)
Tissue
head
fins
Day
4
8
Status
infected
noninfected

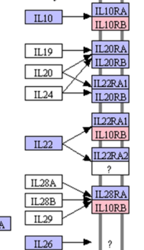
CYTOKINE-CYTOKINE RECEPTOR INTERACTION

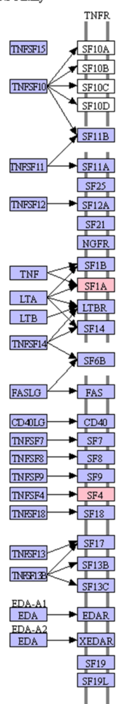


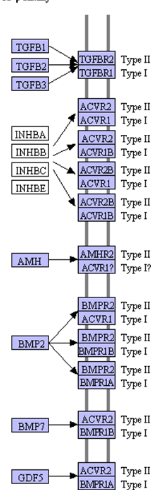


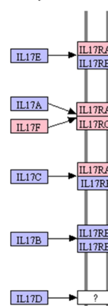


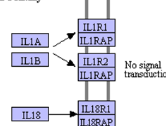


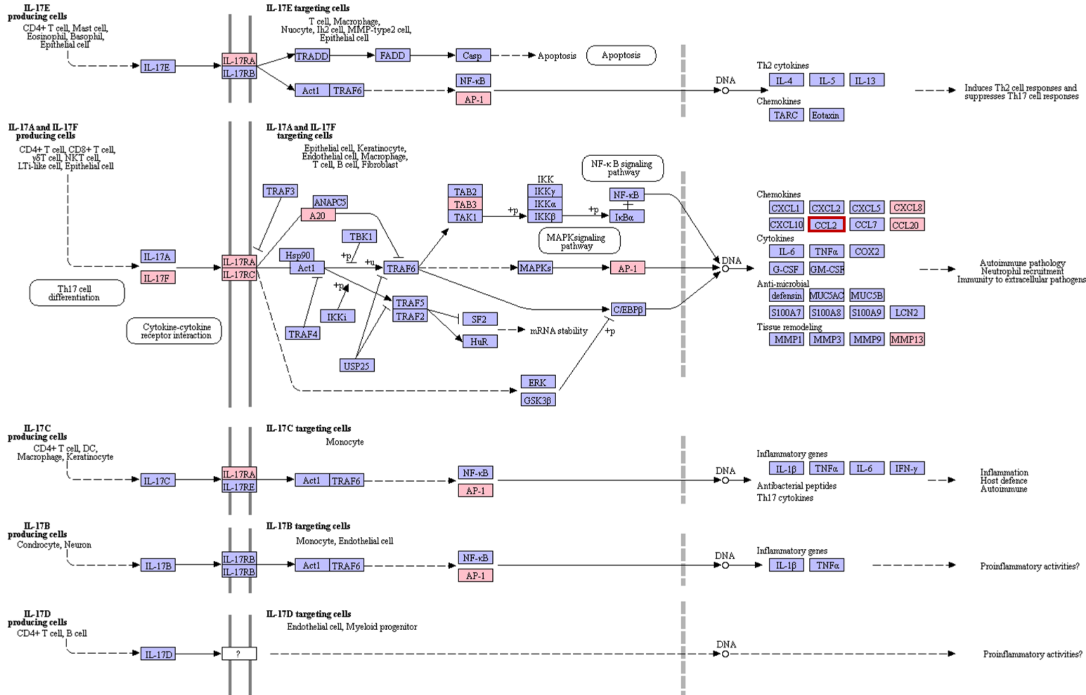




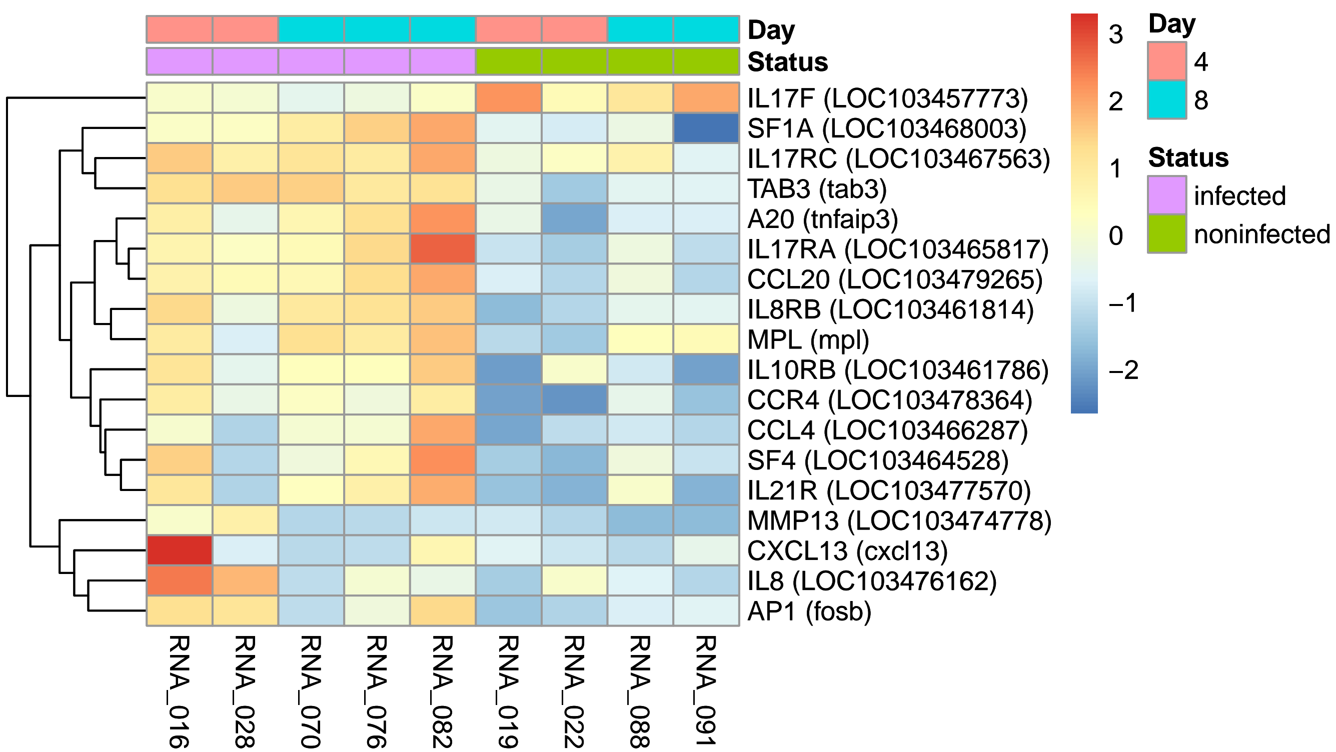








IL-17 SIGNALING PATHWAY
IL-17E producing cells
CD4+ T cell, Mast cell, Eosinophil, Basophil, Epithelial cell
IL-17E targeting cells
T cell, Macrophage, Nuocyte, Ih2 cell, MMP-type2 cell, Epithelial cell
IL-17E
IL-17RA
IL-17RB
TRADD
FADD
Casp
Apoptosis
Act1
TRAF6
NF-κB
AP-1
DNA
Th2 cytokines
IL-4
IL-5
IL-13
Chemokines
TARC
Eotaxin
Induces Th2 cell responses and suppresses Th17 cell responses
IL-17A and IL-17F producing cells
CD4+ T cell, CD8+ T cell, γδT cell, NKT cell, LTi-like cell, Epithelial cell
IL-17A and IL-17F targeting cells
Epithelial cell, Keratinocyte, Endothelial cell, Macrophage, T cell, B cell, Fibroblast
IL-17A
IL-17F
Th17 cell differentiation
Cytokine-cytokine receptor interaction
IL-17RC
TRAF3
ANAPC5
A20
Hsp90
TBK1
TRAF4
IKKi
USP25
TRAF5
TRAF2
SF2
HuR
mRNA stability
TAB2
TAB3
TAK1
IKK
IKKγ
IKKα
IKKβ
IκBα
NF-κB signaling pathway
MAPKsignaling pathway
MAPKs
C/EBPβ
ERK
GSK3β
+p
+u
Chemokines
CXCL1
CXCL2
CXCL5
CXCL8
CXCL10
CCL2
CCL7
CCL20
Cytokines
IL-6
TNFα
COX2
G-CSF
GM-CSF
Anti-microbial
defensin
MUC5AC
MUC5B
S100A7
S100A8
S100A9
LCN2
Tissue remodeling
MMP1
MMP3
MMP9
MMP13
Autoimmune pathology
Neutrophil recruitment
Immunity to extracellular pathogens
IL-17C producing cells
CD4+ T cell, DC, Macrophage, Keratinocyte
IL-17C targeting cells
Monocyte
IL-17C
IL-17RE
Inflammatory genes
IL-1β
IFN-γ
Antibacterial peptides
Th17 cytokines
Inflammation
Host defence
Autoimmune
IL-17B producing cells
Condrocyte, Neuron
IL-17B targeting cells
Monocyte, Endothelial cell
IL-17B
Proinflammatory activities?
IL-17D producing cells
CD4+ T cell, B cell
IL-17D targeting cells
Endothelial cell, Myeloid progenitor
IL-17D
?


Day
Status
IL17F (LOC103457773)
SF1A (LOC103468003)
IL17RC (LOC103467563)
TAB3 (tab3)
A20 (tnfaip3)
IL17RA (LOC103465817)
CCL20 (LOC103479265)
IL8RB (LOC103461814)
MPL (mpl)
IL10RB (LOC103461786)
CCR4 (LOC103478364)
CCL4 (LOC103466287)
SF4 (LOC103464528)
IL21R (LOC103477570)
MMP13 (LOC103474778)
CXCL13 (cxcl13)
IL8 (LOC103476162)
AP1 (fosb)
RNA_016
RNA_028
RNA_070
RNA_076
RNA_082
RNA_019
RNA_022
RNA_088
RNA_091
3
2
1
0
−1
−2
Day
4
8
Status
infected
noninfected