

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

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4	Running title: RNA-Seq of the guppy immune response
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- and edits from all authors. All authors read and approved the final manuscript.

### 36 **Abstract**

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

52

### 53 Keywords

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

#### 57 **1. Introduction**

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

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

Adaptive immunity also plays a role in the host response to gyrodactylids. 78 Rainbow trout, for example, re-challenged with gyrodactylids showed a stronger 79 response one month after full recovery from the primary infection compared to 80 previously unchallenged fish (14). Furthermore, lower infection levels were observed 81 within fish carrying a secondary infection, and, in these primed hosts, clearance 82 began earlier compared to that seen in naïve fish, although no clear parasite-related 83 changes in transcript levels were detected from two candidate markers of adaptive 84 immune response (TCR<sup>B</sup> and MHCII<sup>B</sup>) (14). Similarly, Cable and van Oosterhout (16) 85 demonstrated that guppies (Poecilia reticulata) that have recovered from gyrodactylid 86 infections possess a highly efficient acquired immunity that may minimize detrimental 87 effects associated with subsequent gyrodactylid infections. In gene expression terms, 88 89 increased expression of INF $\gamma$ , Mx and CD8 $\alpha$  and MHC I genes was detected in

Salmo salar (Baltic salmon from River Ume Älv in Sweden) resistant to *G. salaris*relative to a susceptible strain (East Atlantic salmon from River Skjernå in Denmark)
(17).

In contrast, recent studies of goldfish immune responses against G. kobayashii 93 showed no significant differences in parasite load and no changes in the transcript 94 levels of genes involved in adaptive immunity, such as MHCIIB and TCRB1, between 95 primary and secondary infection (13). Similarly, Jørgensen et al. (18), when studying 96 genes encoding the inflammation-involved cytokines (IL-1 $\beta$ , TNF- $\alpha$ , IFN- $\gamma$ , IL-10) and 97 markers for adaptive immune response (CD4, CD8, TCRa, IgM, IgT and MHC II) in 98 the skin of rainbow trout infected with G. salaris, did not find significant changes in 99 expression. Moreover, no histological differences between infected and non-infected 100 skin and fin tissue were detected, implying that infection did not cause skin infiltration 101 with T- and B-cells and neutrophilic granulocytes. 102

Studies based on panels of candidate genes, though valuable, are likely to 103 overlook important pathways. A more comprehensive approach is now offered by 104 RNA-Seq, which allows large-scale screening of genes changing expression in 105 response to infection without the limitations of using predefined probes (19, 20). 106 107 Here, we used RNA-Seq to investigate the response of guppies (*Poecilia reticulata*) to infection with its common ectoparasite, G. bullatarudis. Guppies are tropical fish 108 that, owing to their high natural polymorphism, rapid generation time and amenability 109 to lab rearing have long served as a model species in behavioural and evolutionary 110 biology (21-23). Guppy research has included numerous studies of host-parasite 111 coevolution (1, 10, 24, 25), which have investigated the widespread and common 112 gyrodactylid infections in both wild and ornamental guppies. Both innate and adaptive 113 immune responses have been inferred as playing roles in guppy response to 114 Gyrodactylus infection (24) but this is the first RNA-Seq-based screening of the 115 immune response to gyrodactylids in any fish species. 116

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#### **118 2. Materials and methods**

#### 119 2.1. Host maintenance

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

Guppies were transported to our field station and screened for gyrodactylids. 123 Screening involved briefly anaesthetising fish in 0.02% tricaine methane sulfonate 124 (MS-222, Sigma-Aldrich) and examining them under a dissecting microscope (as 125 detailed by Schelkle et al. (26)). After observing gyrodactylids among the sample of 126 fish, we treated all fish with 20 ‰ sodium chloride solution for 1 minute (27). This 127 treatment was deemed effective for this fish population after observing no 128 gyrodactylids when re-screening all fish 1, 3 and 5 days post treatment. Fish were 129 then reared for 3.5 months in a 100 litre aquarium, with a daily feed of either Artemia 130 nauplii or generic fish flakes. Because guppies are cannibalistic, plastic grids 131 excluding access of adult fish to ca. 1/3 of the aquarium were used to enhance fry 132 survival. The adult and subadult offspring of these females were used for the gene 133 expression analyses described below. 134

Fish sampling and maintenance were conducted according to national guidelines and with the permission from the Tobago House of Assembly (permission number: 004/2014).

138

#### 139 **2.2.** Gyrodactylus worm isolation, characterization and culture

Adult wild guppies from the Roxborough River, Tobago, were collected and 140 screened for gyrodactylids in June 2016. Infected fish were separated and served as 141 donors. As infection intensities among the infected fish were low (1-3 parasites per 142 fish), worms were cultured to obtain sufficient numbers for the experiments (see 143 Stewart et al. (28)). In brief, to establish cultures, a donor fish and a gyrodactylid-144 naïve recipient 'farm' fish were anaesthetised and 1-2 worms were allowed to move 145 from donor to recipient. The farm fish were from a mesocosm population at our field 146 station, which was founded in November 2014 by crossing gyrodactylid-free male 147 guppies from a Trinidad population with gyrodactylid-free females from a Tobago 148 population (different populations from those used in the present study) and which 149 150 were maintained free of exposure to gyrodactylids. These cultures also provided us with a quick means of assessing whether we had collected *G. bullatarudis* rather than 151 152 G. turnbulli, which is also widespread in Trinidad and Tobago guppies. G. bullatarudis infections show a pronounced rostral bias with worms aggregated on the head, 153 154 opercula and pectoral fins of the host (29). Species identification was later confirmed by mtDNA COII sequencing: we extracted DNA from 4 worms, and Sanger-155 156 sequenced a 262 bp section (primers and PCR conditions as in Xavier et al. (30)).

BLAST searches of the resulting sequences showed their strongest matches (97100% identity) to published *G. bullatarudis.*

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#### 160 **2.3. Infection model**

For experimental infections, we selected at random 28 adult and juvenile fish 161 (>10 mm) from our captive gyrodactylid-naïve HC population. Twenty-one fish were 162 infected in July 2016 using the controlled infection procedure described above; the 163 remaining seven were handled in the same manner but not exposed to parasites, to 164 serve as uninfected controls. A single donor fish was used to initiate all infections on 165 the same day. Each recipient received two gyrodactylids; any additional worms that 166 accidently transferred were removed using watchmaker's forceps. Recipients were 167 then revived and housed individually in 400 ml isolation containers at ambient shade 168 169 temperature (mean = 27.1°C; mean daily min. = 25.9°C; mean daily max. = 28.4°C), receiving feed followed by a water change every other day. Control fish were kept 170 171 under the same isolation conditions. Fish were anesthetised for worm counting two days post-infection. 172

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.

176

#### 177 2.4. Skin sampling

Four days post-infection, we screened all fish again and selected eight infected 178 fish to euthanize (Tricaine Methanesulfonate [MS-222, 500 mg L<sup>-1</sup>] overdose) for 179 tissue sample collection, along with three uninfected control fish. To choose which 180 infected fish to sample, we ranked infections by intensity and then made random 181 selections within blocks of fish with similar intensities. Our aim was to have 182 representation from a range of infection intensities while still leaving sufficient fish 183 from across the susceptibility range to progress in their infections. We repeated this 184 at infection day 8, euthanizing seven infected fish and two control fish. At infection 185 day 12, we ended the experiment by euthanizing all remaining fish for tissue 186 sampling. All fish were euthanized with an overdose of MS-222 (500 mg L<sup>-1</sup>). From 187 each fish, we collected two tissue samples: 1) caudal fin and pectoral fins, being sure 188 to take only skin and fin ray, and no muscle or scales; and 2) head skin, usually 189

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**

RNA from fins and head skin was extracted with RNAzol (Sigma-Aldrich), followed by quality control assessment on a Tape Station. We used the 19 samples with the highest RNA Integrity Numbers for library preparation and sequencing (Table 1 for sample overview). A poly-A stranded library was prepared from each sample at the CRG Barcelona Genomic Unit and sequenced to generate 50bp single-end reads using the Illumina 2500 platform. All sequence data have been submitted to the NCBI 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,

SLIDINGWINDOW:4:15, and MINLEN:36. Cleaned reads were mapped to the guppy
reference genome, version GCF\_000633615.1, (32) with STAR software, version
2.5.3a and default parameters (33).

212

#### 213 **2.6. Differential Gene Expression (DGE) Analysis**

Gene expression analyses were performed following the Bioconductor RNA-214 Seq workflow (34). Briefly, we downloaded the guppy genome annotation from NCBI 215 and used it for defining gene models. After counting the numbers of reads mapped to 216 217 the gene models, we used DESeq2 library (35) to create DESeqDataSet object, and included only genes with at least 10 reads mapped to the gene model. Transformed 218 counts (rlog) were used for calculating sample distances, visualising samples with 219 heatmaps, and principal component analyses (PCA). Because heat map visualisation 220 221 suggested that gene expression profiles grouped by body location (head vs fins) but not by day of sampling (Supplementary Figure S1), we decided to analyse each 222 223 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).

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#### 238 2.7. Weighted Gene Co-expression Network Analyses

Differential gene expression is usually identified using exact tests carried out on each 239 240 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 241 typically identified. An alternative for quantifying transcriptional responses is weighted 242 gene co-expression network analysis (WGCNA) which can reveal more subtle but 243 biologically-relevant systematic changes in expression (40). We used this method to 244 quantify transcriptional responses of fish to infection, enabling the identification of 245 networks (modules) of co-expressed genes (genes that show consistent expression 246 247 profiles across samples), and thus potentially identifying functionally important genes with only subtle changes in expression that may otherwise not have been detected. 248 Read counts, normalized using a variance stabilizing transformation (VST) in 249 DESeq2, were analysed using the R package WGCNA. Our gene modules were 250 251 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 252 merge highly similar modules. Gene module preservation (by tissue or sample day) 253 was determined using Z-summary statistics in the WGCNA package (40). Modules 254 255 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. 256

Biological Process GO term enrichment tests of each significant gene module wereperformed using topGO as described above.

259

#### 260 **3. Results**

#### 261 Differential expression analyses

We obtained a total of 19 samples from head skin (10) and fins samples (9) taken 262 from control (9 samples) and infected fish (10 samples). Among fish that were 263 infected, there was no significant bias in age class (juvenile or female) against day of 264 killing (4, 8 or 12;  $\chi^2 = 2.48$ , bootstrap P = 0.39). Across all fish, including those not 265 infected, there was no significant effect of day of killing on size (Kruskal-Wallis test: 266  $x^2 = 1.28$ , df = 2, P = 0.53). There was no significant size difference between infected 267 and uninfected fish (Mann-Whitney test: U = 22, P = 0.82), and there was no 268 significant age/sex bias among infected v. uninfected fish (Fisher's exact test: P = 269 0.23). 270

Comparison of transcriptomic profiles revealed that samples clustered by tissue, 271 (Figure 1), and therefore head skin and fin samples were analysed separately. We 272 found very few differentially expressed genes in head skin samples (n = 8; 273 Supplementary Table S1), possibly a consequence of higher heterogeneity of tissues 274 275 collected during sampling (skin, scales, muscle tissue) compared to fins. However, in the fin tissue we found 342 differentially expressed genes (P-values adjusted for false 276 discovery rate = 0.1, Supplementary Table S2). Results were the same, regardless of 277 the software used (Supplementary Figure S2, S3). Gene ontology analysis of these 278 279 genes revealed enrichment for multiple terms (Supplementary Figures S4-S6), (in 'biological including immune function processes' category) and 280 cytokine/chemokine (in 'molecular function' category). Metabolic pathway analysis 281 (KEGG) of differentially expressed genes identified orthologues of several immune-282 related categories, including cytokine-cytokine receptor interactions (14 genes), IL-17 283 signalling pathway (9) and Th17 cell differentiation (4), chemokine signalling pathway 284 (7), NOD-like receptor signalling pathway (6), natural killer cell-mediated cytotoxicity 285 (4), T cell receptor signalling pathway (3), and B cell receptor signalling pathway (3) 286 (see Supplementary File S1 for full list). More detailed analysis of the cytokine-287 cytokine receptor interaction category revealed several genes with significantly 288 increased expression in infected fish belonging to CXC and CC chemokine 289

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

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#### 295 Expression of immune-related genes

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

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

Several genes involved in the adaptive immune response were upregulated in fins. This included two transcription factors implicated in Th1 (STAT4) and Th2 (GATA3) differentiation, and adhesion molecules such as cadherin-like protein 26, cell surface glycoprotein CD9 and expressed on T cells and NK cells adhesion molecule CD2. We also found upregulation of glycosylphosphatidylinositol (GPI)linked differentiation antigen (lymphocytes antigen 6G, Ly6G) that in mammals is expressed by myeloid-derived cells and T-cell surface glycoprotein CD4-like usually expressed on T helper cells, monocytes, macrophages, and dendritic cells.

Finally, during infection we observed elevated expression of genes involved in ubiquitination and antigen processing (E3 ubiquitin/ISG15 ligase TRIM25, TRIM21, TRIM8, E3 ubiquitin-protein ligase Itchy, RNF19A, SMURF2 and proteasome activator complex subunit 4 as well as ubiquitin carboxyl-terminal hydrolase 11, 12, 19) and antigen presentation (beta-2-microglobulin, MHC class I related protein).

We did not find a significant difference in expression of MHC class II, which has been inferred as influencing the effectiveness of the guppy immune response to gyrodactylids (1, 41). However, we noted its high constitutive expression (e.g. LOC103461570, predicted: DRB1-8 beta chain-like, mean =2959.7 reads, Log2FC=-0.26; LOC103460899, predicted: E-S beta chain-like, mean =5675,97, log2FC=-0,03) compared to the mean (565.1) for 514 genes expressed in our sample which fell into the immune function category.

341

#### 342 Gene co-expression network analyses

Gene co-expression network analyses revealed 33 and 25 modules in the head 343 and fins respectively. Six head gene modules were significantly correlated with either 344 infection status or worm burden, of which five were significantly preserved in fin 345 tissues (Table 2). This result highlights the power of WGCNA analysis compared to 346 standard DGE analysis, where only a handful of differentially expressed genes were 347 found in the head tissue (in contrast to fins, see above). One of the modules in the 348 head tissues ("head-violet"; Table 2) was negatively correlated with infection status 349 (i.e. lower expression in infected fish) and enriched for several GO terms including 350 351 mucus secretion. The head gene module ("head-cyan") positively associated with infection status (higher expression in infected fish) was enriched for GO terms 352 including type I interferon production. Furthermore, all 3 head gene modules ("head-353 red", "head-darkred" and "head-pink") positively correlated with worm burden 354 (increasing expression with higher worm number) were enriched for genes involved 355 in T-cell differentiation and proliferation, as well as antigen processing and 356 presentation (head-red; "regulation of T-cell apoptosis and formation of 357

immunological synapse", head-darkred; "regulation of T-cell differentiation" and
"antigen processing and presentation", head-pink; "T-cell proliferation") (Table 2). The
gene module ("head-brown") negatively correlated with worm burden (lower
expression in more heavily infected fish) included functions related to MHC II and IL1β biosynthesis (Table 2).

In contrast to head gene networks, only a single gene module defined in the fins 363 was significantly associated with infection status ("fin-black") and preserved in head 364 tissue (Table 2). This module included functions related to innate immune response 365 such as macrophage activation and production and secretion of pro-inflammatory 366 cytokines (TNF, IL-6) and chemokine CCL2. In addition, a single fin gene module 367 ("fin-cyan") was positively correlated with worm burden, not preserved in the head, 368 enriched for GO terms including regulation of macrophage chemotaxis and pathogen-369 370 recognition (Toll signalling) (Table 2).

In both tissue networks, we found several gene modules associated with 371 372 sampling day (head; 6, fins; 3), suggesting temporal variation in infection responses. Therefore, we re-defined gene modules including both tissue types, separating data 373 374 instead by sampling day. At day 4, of the 28 gene modules found, four associated with infection status and/or worm burden yet were not preserved by day 8 375 (Supplementary Table S3). At day 8, nine gene modules were significantly 376 associated with either infection status or worm burden, of which only two were 377 significantly preserved in day 4 (Supplementary Table S3). All time-specific modules 378 were enriched for immune response GO terms, particularly for T cell and other 379 380 leukocyte-related pathways.

381

#### 382 **4. Discussion**

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

#### 391 Resolution of inflammation and wound healing

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

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

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

infected fish. Although we did not find upregulation of the IL-17 gene itself, two types 425 of IL-17 receptors (A and C), IL-17-induced transcription factors, and 426 cytokines/chemokines involved in Th17 differentiation and action, were all 427 upregulated (Fig. 1B). Also of relevance to the Th17 response was the upregulation 428 of CD4 glycoprotein. Previously, Infante-Duarte et al. (2000) observed that CD4+T 429 cells, primed with a synthetic peptide in the presence of spirochete bacteria, may 430 differentiate into distinct T-cell lineage expressing high level of IL-17A (48, 49). To 431 date, six mammalian IL-17-family ligands (IL-17A, IL-17B, IL-17C, IL-17D, IL-17E (IL-432 25) and IL-17F) and five receptors (IL-17RA, IL-17RB/IL-25R, IL-17RC, IL-17RD/SEF 433 and IL-17RE) have been identified (50). In mammals, these are thought to mediate 434 435 immunity against extracellular bacteria, particularly those that colonize exposed surfaces such as the airways, skin, and intestinal lumen. Th17 is also involved in T 436 437 cell polarization in response to infection by extracellular and intracellular bacteria and fungi (51). 438

439 Genes related to known IL-17 family members and IL-17 receptors have been identified in other teleosts (52, 53). IL-17 homologues, for example, have been found 440 441 in the genomes of zebra fish (Danio rerio), fugu (Takifugu spp.), grass carp and salmonids (54-61), while five IL-17Rs were found in the large yellow croaker 442 (Larimichthy scrocea) (53). These receptors are constitutively expressed in several 443 tissues and organs, including high constitutive expression in mucosal tissues of the 444 gills and skin (54, 62, 63). Moreover, gill mucosal tissue, along with the lymphoid 445 organs of the head kidney and spleen, showed upregulation of IL-17 receptors in L. 446 crocea infected with Aeromonas hydrophila (see Ding et al. (53)), in line with our 447 observation of increased IL-17 receptor expression in the skin of infected guppies. 448

Upon infection differential expression of genes involved cytokine-cytokine 449 receptor interactions and chemokine signalling pathway was observed. Among 450 others, we found infection-induced changes in the expression of cytokine receptors 451 452 IL-1, IL-21 and TNF, which, in mammals, are believed to be both drivers of Th17 differentiation and release of cytokines from activated Th17 cells (64-67). Previous 453 work on teleosts has shown that IL-21 is a potent stimulator for IL-17A/F1a (68), in 454 line with the increased expression of genes involved Th17 response we found in 455 response to G. bullatarudis infection. Moreover, in the fins of G. bullatarudis-infected 456 quppies, several IL-17-inducible genes were upregulated (chemokines: 457 CXCL1,CXCL8 and CXCL13, CCL2 and 20, and MMP-13/collagenase 3). This 458

finding also has parallels in mammals, where both homo- and heterodimers of IL-17A 459 and IL-17F induced the expression of pro-inflammatory mediators (e.g. IL-1b, IL-6, 460 GM-CSF, CXCL8, CXCL1, CXCL10 and MMP-13) and, accordingly, mobilize, recruit, 461 and activate neutrophils (49). Our results may imply that members of the IL-17 family 462 also function as potent pro-inflammatory modulators in fish. This interpretation is 463 supported by in vitro work in fish: recombinant carp IL-17D upregulated expression of 464 pro-inflammatory IL-1b, TNF-a and CXCL-8 and activated NF-KB signalling (63), and, 465 similarly, trout recombinant IL-17A increased the expression of pro-inflammatory IL-6, 466 CXCL8 and the antimicrobial peptide BD-3 (54). Furthermore, in mammals also IL-23 467 induces a polarization of Th17 cell population with a unique inflammatory gene 468 469 signature that includes IL17, IL6, TNF, CCL20, CCL22, IL1R1, and IL23R (69). Recently, Yin and co-workers (70) confirmed also for fish that recombinant IL-23 is 470 471 able to enhance the mRNA levels of IL-17A/F1 and its secretion from head kidney leukocytes. Interestingly, our WGCN analysis indicated existence of the positive 472 473 correlation between worm burden and expression of genes involved in inflammation and Th17-response such as IL-23 in the samples from head skin of guppies. 474

475 Previous research has already indicated a role of Th17 in fish immune response and found increased expression of IL-17 genes during viral, bacterial and myxozoan 476 (Tetracapsuloides bryo salmonae and Enteromyxum leei) infections (54, 63, 71–73). 477 Enhanced Th17-like immune responses was also found in mucosal and adipose 478 tissue of vaccinated fish (59, 60, 74) and it was involved in vaccine-induced 479 granulomatous reactions (58). Moreover, IL-17 up-regulation was observed in fish 480 leukocytes stimulated in vitro with LPS, poly I:C, PHA and ConA (61, 75). IL-17A was 481 also increased in the head kidney of carp infected with some, but not all, species of 482 Trypanoplasma (also known as Cryptobia) parasites (76). Our study is the first 483 indication of Th17 involvement in the fish immune response against gyrodactylid 484 ectoparasites. 485

In fin tissue, we found upregulation of several genes involved in pathogen recognition, such as C-type mannose receptor 2, macrophage mannose receptor 1, and a number of NOD-like receptors. These observations agree with Hu et al. (77), they described involvement of a NOD-like receptor signalling pathway in the skin of orange-spotted grouper (*Epinephelus coioides*) infected with the holotrich protozoan *Cryptocaryon irritans*. Moreover, in WGCN analysis we found that expression of the genes involved in Toll-signalling correlates in fin samples with worm burden. Finally, upon infection in fin skin, we noted upregulation of lipocalin-2. Lipocalin (neutrophil
gelatinase-associated lipocalin, NGAL) is involved in iron sequestrating which in turn
limits infection. These results support the suggestion that innate immunity plays an
important role in the response to gyrodactylid skin parasites, supporting and is in
corroboration of the fact that Th17 immune response drives neutrophil infiltration to
the site of infection (78, 79).

499

#### 500 Adaptive immune response

In addition, molecules associated with antigen presentation and adaptive 501 immune response were significantly upregulated upon infection. This list includes T 502 and B cell markers (CD4 mentioned before, but also CD2 – both markers of Th cells 503 including Th17, CD9, CD22), genes involved in ubiquitination and antigen processing 504 and presentation (e.g. TRIMs, beta-2-microglobulin, MHC I). Moreover, we found a 505 positive correlation between worm burden and expression of genes involved in 506 antigen processing and presentation (e.g. formation of immunological synapse) and 507 T-cell differentiation, proliferation and apoptosis. These data suggest that 508 509 lymphocytes infiltrate the infected skin. Similarly, T cell marker tetraspanin CD9, B cell receptor CD22, and MHC class I and class II genes were also significantly 510 511 upregulated in skin of orange-spotted grouper infected with C. irritans (see (77)).

Previous studies found associations between the level of infection with 512 513 *Gyrodactylus* and guppy MHC II, both in the field (41) and in controlled experimental infection (1). Here, although MHC class I genes were significantly upregulated in 514 515 differential expression analyses (Supplementary Table S1), we did not find significantly increased expression of MHC II genes in the skin of infected fish. 516 However, constitutive expression of MHC II gene in skin of uninfected fish was 517 518 roughly an order of magnitude higher compared to all other immunity genes, and the lack of differential expression in infected skin is therefore not inconsistent with the 519 role of MHC II in mediating immune response against Gyrodactylus. MHC II 520 expression on the surface of antigen presenting cells (in particular dentritic cells) is 521 regulated by ubigutination (80, 81), and we did find significant changes in expression 522 of several genes involved in ubiguitation and deubiguitation. Finally, we found a gene 523 co-expression module negatively correlated with worm burden and enriched for MHC 524 II biosynthesis, suggesting fish with increased activation of MHC II pathways are 525 more resistant to infection. 526

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

539 Conclusions

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

549

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563

### 564 **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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## 809 Tables

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

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

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**Table 2.** Summary of gene co-expression networks associated with *Gyrodactylus* infection including number of genes per module, significant correlations, module preservation, and gene ontology enrichment. Gene module names denote tissue type in which they were defined (fin 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
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821 **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.* 

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Figure 2. Cytokine-cytokine receptor interactions identified by metabolic pathway 826 analysis (KEGG) to be differentially expressed in fins of infected and uninfected fish. 827 Significantly differentiated genes are in pink; genes which were missed from 828 829 automatic annotation, but which were included into the list of differentially expressed genes in Supplementary Table S3 are framed with red (CXCL1, growth-regulated 830 alpha protein, LOC103476162 in Supplementary Table S2; CCL2, monocyte 831 chemotactic protein 1B-like, LOC103466287 in Supplementary Table S3). Blue boxes 832 indicate automatically annotated but not significantly differentiated genes, while white 833 834 boxes indicate genes which were not annotated in the guppy genome.

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**Figure 3.** IL-17 signalling pathway identified by metabolic pathway analysis (KEGG) 836 to be differentially expressed in fins of infected and uninfected fish. Significantly 837 differentiated genes are in pink; genes which were missed from automatic annotation, 838 but which were included into the list of differentially expressed genes in 839 Supplementary Table S3 are framed with red (CXCL1, growth-regulated alpha 840 protein, LOC103476162 in Supplementary Table S2; CCL2, monocyte chemotactic 841 protein 1B-like, LOC103466287 in Supplementary Table S3). Blue boxes indicate 842 automatically annotated but not significantly differentiated genes, while white boxes 843 indicate genes which were not annotated in the guppy genome. 844

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



#### CYTOKINE-CYTOKINE RECEPTOR INTERACTION













