



Comparative transcriptomics reveal conserved impacts of rearing density on immune response of two important aquaculture species

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1 *Fish & Shellfish Immunology: Research article*

2 **Comparative transcriptomics reveal conserved impacts of rearing density on immune**
3 **response of two important aquaculture species**

4

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16 **Keywords:** rearing density, stress, immunity, transcriptome, comparative transcriptomics,
17 Atlantic salmon, Nile tilapia, *Saprolegnia parasitica*, Th17 responses

18

19 Abstract

20 Infectious diseases represent an important barrier to sustainable aquaculture development.
21 Rearing density can substantially impact fish productivity, health and welfare in aquaculture,
22 including growth rates, behaviour and, crucially, immune activity. Given the current emphasis
23 on aquaculture diversification, stress-related indicators broadly applicable across species are
24 needed. Utilising an interspecific comparative transcriptomic (RNAseq) approach, we
25 compared gill gene expression responses of Atlantic salmon (*Salmo salar*) and Nile tilapia
26 (*Oreochromis niloticus*) to rearing density and *Saprolegnia parasitica* infection. Salmon reared
27 at high-density showed increased expression of stress-related markers (e.g. *c-fos* and *hsp70*),
28 and downregulation of innate immune genes. Upon pathogen challenge, only salmon reared
29 at low density exhibited increased expression of inflammatory interleukins and lymphocyte-
30 related genes. Tilapia immunity, in contrast, was impaired at low-density. Using overlapping
31 gene ontology enrichment and gene ortholog analyses, we found that density-related stress
32 similarly impacted salmon and tilapia in key immune pathways, altering the expression of
33 genes vital to inflammatory and Th₁₇ responses to pathogen challenge. Given the challenges
34 posed by ectoparasites and gill diseases in fish farms, this study underscores the importance
35 of optimal rearing densities for immunocompetence, particularly for mucosal immunity. Our
36 comparative transcriptomics analyses identified density stress impacted immune markers
37 common across different fish taxa, providing key molecular targets with potential for monitoring
38 and enhancing aquaculture resilience in a wide range of farmed species.

39 **1. Introduction**

40 Sustainable aquaculture development continues to be at the forefront of priorities for meeting
41 protein demands of a growing human population (1, 2) and therefore remains the fastest
42 growing food sector (3). A staggering 598 aquatic species are commercially cultured
43 worldwide today, up by 26.7% in the last 10 years alone (3), vastly outweighing the diversity
44 of terrestrial animal production (4). Arguably one of the greatest challenges to the current level
45 of farmed aquatic species diversity and future diversification of aquaculture is to identify and
46 reliably assess the optimum conditions for each species' health, welfare, and productivity.

47 Rearing density is considered one of the pivotal factors determining aquaculture productivity
48 and profitability (5-7). While overcrowding and/or under-stocking can significantly impact overt
49 measures of fish performance such as growth rate (8, 9), size uniformity (10), and
50 aggressive/unwanted behaviours (11-13), it can also adversely affect less obvious
51 physiological parameters such as stress levels (9, 14), circulating hormones (14, 15), and flesh
52 quality/composition (16). It is increasingly apparent that suboptimal rearing densities have
53 negative consequences for fish immunity (17-19) and thus increase susceptibility to pathogens
54 (10, 20). Infectious disease is currently one of the greatest barriers to sustainable aquaculture
55 intensification (21), and a substantial economic burden on the industry (22). Therefore, it is
56 important to know if the underlying effects of rearing density on fish health are conserved
57 across fish species, and whether broadly applicable key stress indicators can be applied for
58 management of density-related stress in aquaculture.

59 RNAseq methods have proved valuable tools for assessing the wider impacts of
60 environmental stressors and pathogens on animal health at the functional genomic level (23-
61 25). Interspecific comparative transcriptomics (comparison of gene expression responses
62 across multiple species) has, as yet, been little used in the context of aquaculture. However,
63 the utility of interspecific comparative transcriptomics to address fundamental questions in fish
64 biology and evolution (26, 27), and reveal key species differences in response to shared

65 pathogens in vertebrates (28, 29), indicates its potential value as a tool for refining aquaculture
66 practices.

67 Nile tilapia (*Oreochromis niloticus*) and Atlantic salmon (*Salmo salar*) are two of the most
68 important farmed finfish species worldwide, accounting for 8% and 4% of global annual
69 production, respectively (3). Suboptimal rearing densities have been shown to negatively
70 affect salmon and tilapia welfare (30-32), health (33, 34) and productivity (35). Salmon tend to
71 experience greater stress at high rearing densities (31, 36). Conversely, tilapia show increased
72 aggression, and are therefore considered more stressed, at low densities (20, 32, 37). For
73 both tilapia and salmon production, outbreaks of *Saprolegnia parasitica* – a fungal-like
74 pathogen that parasitizes the skin, fins and gills of fish (38) – are a substantial economic
75 burden (38-40), with limited effective treatment options approved for aquaculture (38).

76 This study compares the impact of rearing density on the functional genomic responses of
77 salmon and tilapia to *S. parasitica* challenge, utilising an interspecific comparative
78 transcriptomic (RNAseq) approach. We assess the commonalities of density-specific
79 pathogen responses of these two key – yet highly divergent (ca. 225 MYA; (41)) – aquaculture
80 species, with the aim of identifying stress-related transcriptomic-level markers broadly
81 applicable across the diversity of cultured fish.

82 **2. Results**

83 This study first examines Atlantic salmon (*S. salar*) transcriptome-wide expression responses
84 to rearing density and infection challenge. These data were then analysed alongside
85 comparable, previously reported data (20) on Nile Tilapia (*O. niloticus*) to assess
86 commonalities of density-specific immune responses across divergent fish species, which
87 respond differently to stocking-density.

88 **2.1 Salmon transcriptomic responses to rearing density and *Saprolegnia* challenge**

89 No salmon developed visible signs of *Saprolegnia* infection (i.e., mycelial growths or lesions)
90 during the current experiment. *Saprolegnia*-challenged salmon are known to show immune

91 gene expression changes despite an absence of visible signs of infection (54). However, we
92 are careful to refer to our *Saprolegnia* treatment groups as “*Saprolegnia*-challenged”. As we
93 are examining early responses (24 hr post-exposure) with no external signs of pathology, we
94 do not assume established/successful infection. Nonetheless, by directly comparing
95 *Saprolegnia*-challenged groups with a control group (those sham-challenged), these data
96 allow us to determine how salmon respond to pathogen exposure, irrespective as to whether
97 the pathogen successfully establishes long-term in the host.

98 Illumina RNAseq achieved an average 31.6 million reads pairs per sample (range 29.3 to 32.1
99 million). In the gills, comparison of healthy salmon at high and low density (i.e., unchallenged
100 controls) gene expression found 1,163 genes significantly differentially expressed. We found
101 significant enrichment of genes with higher expression in low-density salmon (568 genes) for
102 124 biological process GO terms including several immune-related functions such as “innate
103 immune response” (GO:0045087, e.g. *hck*), “neutrophil mediated immunity” (GO:0002446,
104 e.g. *rac2*), and “dendritic cell differentiation” (GO:0097028, e.g. *lyn*). These genes were also
105 enriched for “cortisol metabolic process” (GO:0034650, e.g. *hsd11b2*). Genes with higher
106 expression in healthy high-density salmon (compared to healthy low-density fish, 595 genes)
107 were enriched for 93 biological process GO terms including a number related to physiological
108 stress such as osmotic (e.g. GO:0042538, GO:0042539) and starvation responses (e.g.
109 GO:0042594). This gene set also included hormonal responses, including “response to growth
110 hormone” (GO:0060416), “thyroid hormone transport” (GO:0070327), and “response to
111 estradiol” (GO:0032355). Full lists of differentially expressed genes and GO terms are
112 provided in Supplementary File S1.

113 Comparison of gill tissues in healthy (unchallenged control) and *Saprolegnia*-challenged
114 salmon at high and low density revealed 1,859 and 1,649 differentially expressed genes
115 respectively. Genes exhibiting increased expression in *Saprolegnia*-challenged salmon at
116 both densities were highly enriched for GO terms related to immune functions including
117 “inflammatory response” (GO:0006954), “lymphocyte chemotaxis” (GO:0048247), “T cell

118 proliferation” (GO:0042098), and “cellular response to interleukin-1” (GO:0071347).
119 Enrichment of downregulated genes in *Saprolegnia*-challenged salmon (59 GO terms) was
120 predominantly developmental processes (e.g. GO:0048706 embryonic skeletal system
121 development, GO:0050793 regulation of developmental process, GO:0048538 thymus
122 development). However, this gene set also included immune (GO:0045624 positive regulation
123 of T-helper cell differentiation, GO:0072679 thymocyte migration) and circadian rhythm
124 functions (GO:0032922 circadian regulation of gene expression, GO:0045475 locomotor
125 rhythm). Upregulated expression specific to high-density challenged salmon was rich in GO
126 terms related to mast cell responses, including mast cell mediator production (e.g. leukotriene:
127 GO:0006691, prostaglandin: GO:0001516), chemotaxis (GO:0071624) and activation
128 (GO:0033004, GO:0043303). In contrast, genes found only to be significantly increased in
129 low-density challenged salmon were enriched for adaptive immune functions including
130 lymphocyte differentiation and aggregation (GO:0030098, GO:0071593), T helper cell
131 development (GO:0045064, GO:0072540), and B cell chemotaxis (GO:0035754). Full lists of
132 differentially expressed genes and GO terms are provided in Supplementary File S1.

133 **2.2 Interspecific comparison of density and *Saprolegnia* transcriptomic responses**

134 **2.2.1 Impact of density on uninfected salmon and tilapia**

135 Comparison of functional enrichment of differential expression between high- and low-density
136 treatments in unchallenged (healthy) salmon and tilapia reveal a small number of biological
137 processes shared between these species (Supplementary File S2), including “innate immune
138 response” (GO:0045087) in genes showing higher expression in high-density tilapia and low-
139 density salmon. Using a reciprocal best hit approach, we found 21,745 1:1 orthologs between
140 published salmon and tilapia transcriptomes, of which 13,364 were expressed in gill tissues of
141 both species. Twenty-three gene orthologs were found to be differentially expressed between
142 healthy high- and low-density fish in both species (Table 1), including several genes involved
143 in regulation of transcription (*dlx3*, *mef2d*, *npas2*, *med12*).

144 2.2.2 *Saprolegnia* responses in salmon and tilapia

145 To assess overlap of functional responses to challenge with *Saprolegnia*, we compared
146 differential expression GO term enrichment between salmon (unchallenged control vs
147 *Saprolegnia* challenged) and tilapia (20). All GO terms found to be shared among salmon and
148 tilapia treatment groups are summarized in Table 2. For the purposes of this study, we
149 focussed our attention to those related to immunity. Salmon challenged with *Saprolegnia*
150 (though no visible signs of saprolegniasis) showed increased expression of genes associated
151 with “immune response” (GO:0006955) at both densities. In contrast, this GO term was
152 enriched in genes with decreased expression in tilapia at both densities challenged with
153 *Saprolegnia* (and exhibiting signs of saprolegniasis). Increased expression of genes involved
154 in Fcε receptor signalling (GO:0038095) was found at both densities in both species (Table
155 2). Mast cell degranulation genes (GO:0043303) were upregulated in *Saprolegnia*-challenged
156 tilapia in both density treatments, but only observed in high-density salmon (Table 2). Genes
157 with increased expression in low-density salmon and high-density tilapia shared GO
158 enrichment for “T-helper 2 cell differentiation” (GO:0045064), “T-helper 17 cell lineage
159 commitment” (GO:0072540), “myeloid dendritic cell differentiation” (GO:0043011), “defence
160 response to protozoan” (GO:0042832), and “isotype switching” (GO:0045190) (Table 2).

161 Examining expression patterns of 1:1 gene orthologs, we found 41 genes with significantly
162 higher expression in *Saprolegnia*-challenged salmon and tilapia. These included genes
163 involved in antigen presentation (*ap1m1*, *ap1s2*, *ap1s3*), neutrophil activity (*lect2*, *serpinb1*)
164 and inflammation (*il1rap*) (Table 3). Twenty-two genes were found to have increased
165 expression in challenged fish in both species at only low densities, including two tumor
166 necrosis factor superfamily members involved in immune responses (*tnfsf9*, *tnfsf15*). Mucin
167 genes also exhibited similar responses with density in both species; *muc5ac* had higher
168 expression at low density in both salmon and tilapia. A “integumentary mucin” transcript
169 (XP_014011243.1) had lower expression in high density challenged fish of both species.
170 Several genes exhibited contrasting density-specific *Saprolegnia* challenge responses (22

171 increased in high-density salmon/low-density tilapia, 21 increased in low-density salmon/high-
172 density tilapia) were related to T helper cell activity/maintenance (e.g. *il17c*, *lag3*, *tnfaip8l2*,
173 *batf*; Figure 1).

174 An alternative to looking at the overlap of individual gene differential expression and gene
175 ontology enrichment, is to examine cross-species preservation of weighted gene co-
176 expression modules (28, 42). WGCNA of expressed tilapia genes with salmon 1:1 orthologs
177 ($n = 13,364$) found 18 genes modules, of which 4 were associated with *Saprolegnia*-challenge
178 status and significantly preserved in salmon (i.e., also responsive to *Saprolegnia* status in
179 salmon, Supplementary File S2). By defining gene co-expression networks (13 modules) in
180 salmon, six modules were significantly associated with *Saprolegnia* status and were preserved
181 in tilapia (Supplementary File S2). Although in both species single gene modules were found
182 to be associated with density and *Saprolegnia* (Supplementary File S2), neither were
183 significantly preserved in the other species.

184 **3. Discussion**

185 Rearing density is a critical factor for intensive aquaculture productivity, with suboptimal
186 conditions known to impact fish, from growth (8, 9) and quality (16) to health (17, 19) and
187 welfare (9, 30, 31, 43). Here, we used a transcriptome-wide approach to assess the effect of
188 rearing density on pathogen responses in Atlantic salmon, revealing suppression of
189 immunologically-important gill gene expression responses at high density. Strikingly, we
190 identified conserved disruption of Th₁₇ responses in salmon and Nile tilapia (44) when subject
191 to density stress. This study highlights the potential of interspecific comparative
192 transcriptomics to identify broadly-applicable indicators of fish health.

193 In our study, comparison of expression profiles of unchallenged (i.e., “healthy”) salmon reared
194 at high and low densities revealed a substantial number of differentially expressed genes in
195 the gills ($n = 1,163$). High density salmon (four-fold higher than “low-density” treatment, though
196 still within recommended welfare limits (45)) had increased expression of key markers for

197 stress in vertebrates including *c-fos* (46-48) and *hsp70* (49, 50). Previous studies on the
198 effects of rearing density on immunity in salmonids have primarily focussed on levels of serum
199 antibodies (typically IgM) or antibody-producing cells, with suppression generally found at
200 higher densities (8, 36). In addition, non-specific innate immune markers such as serum
201 lysozyme activity have been shown to be influenced by rearing density (43). Here, we found
202 that salmon raised at high density had lower expression of genes related to immune responses
203 including neutrophil (e.g. *rac2*), dendritic cell (e.g. *cd209*, *ctss*), and B cell immunity (e.g. *lyn*,
204 *cd22*, *blnk*) plus inflammatory interleukins (*il-12*, *il-17*) (Supplementary File S1). A previous
205 study of rainbow trout head-kidney gene expression during crowding stress also showed
206 increased *hsp70* stress marker expression, but found different immune gene suppression
207 (*lyzll*, *tnf-1 α* , *il-1 β* , *il-8* and *ifn- γ 1*) (18). This suggests that crowding stress in salmonids may
208 result in tissue-specific suppression of immune factors, which must be considered for their
209 potential impacts on disease susceptibility. Clearly overcrowding in salmonids has a wider
210 impact on their immune system expression than previously reported. The effects of crowding
211 stress on immune health caused by high rearing densities may explain in part the failure of
212 supportive breeding in salmon conservation (51-53).

213 We found over 1,500 genes differentially expressed in the gills of *Saprolegnia*-challenged and
214 control (sham-challenged) fish at both densities, although no salmon at either density showed
215 visual signs of saprolegniasis (e.g. mycelial growth). Fish at both densities exhibited
216 expression profiles indicating initiation of inflammatory (particularly interleukin-1 β mediated)
217 responses and lymphocyte migration (Supplementary File S1), in line with previous studies of
218 salmonids (54-56). A previous targeted immune gene study of *Saprolegnia*-challenged Atlantic
219 salmon also showed differential expression profiles in gills despite an absence of visible signs
220 of infection (54). Our results, however, indicate a far wider impact of sublethal *Saprolegnia*
221 challenge; in addition to altered expression of immune genes, we found disruption of
222 expression related to a wide range of physiological processes including development and
223 circadian functioning (Supplementary File S1). Disruption of circadian rhythms is increasingly

224 recognised as detrimental to vertebrate health (57), yet we are only beginning to consider this
225 in the context of teleost immunity in aquaculture (20, 58, 59). Furthermore, *Saprolegnia*
226 species are considered ubiquitous in freshwaters (38) and our results suggest that in
227 aquaculture facilities even sublethal levels of *Saprolegnia parasitica* (or other related
228 pathogenic species) may substantially impact fish health and productivity.

229 A key factor to *Saprolegnia* virulence is the pathogens' ability to suppress fish adaptive
230 immunity such as T helper cell responses and immunoglobulin production (54). While we
231 found transcriptomic evidence of adaptive immunosuppression at both densities (e.g.
232 downregulation of T helper cell differentiation, Supplementary File S1), the extent of
233 suppression appeared to be density-specific. Genes upregulated in response to *Saprolegnia*
234 found only in salmon reared at the lower density included those important to lymphocyte
235 development and migration (including both B and T cells). In contrast, infection responses of
236 fish raised at high density suggest a greater reliance on mast cells (Supplementary File S1),
237 mediators of acute inflammation and non-specific antimicrobial production in teleosts (60, 61).
238 Given the dramatic impact Saprolegniasis has on salmon aquaculture, accounting for at least
239 1 in 10 reported mortalities (62), our findings highlight the potential importance for optimal
240 rearing densities to mitigate against this devastating fish pathogen.

241 The great diversity of fish species now cultured (3) poses a challenge to identify biomarkers
242 broadly applicable to monitor optimal husbandry conditions and/or fish health and welfare
243 status in aquaculture. Cortisol is most commonly used as an indicator of stress in fish and is
244 widely implicated in suppression of immunity (63). Cortisol levels do not, however, necessarily
245 correlate with immune function and parasite susceptibility (64), and cortisol effects on immune
246 levels can be inconsistent (63). Our approach to uncover common biomarkers for the impacts
247 of suboptimal husbandry conditions and their effects on fish immunity, was to compare the full
248 transcriptional responses of two highly divergent fish species to density and pathogen
249 challenge. Examining gene ontology (GO) enrichment in density responses from our salmon
250 data and a previous, comparable study of Nile tilapia (20) revealed a number of GO terms

251 shared between healthy salmon and tilapia (Supplementary File S2). Importantly, we found
252 low-density tilapia and high-density salmon both have lower expression of genes classed as
253 GO term “innate immune response”, indicating a broad-scale signal of innate immune
254 suppression due to density-dependent stress. Although we found only a small number of
255 salmon-tilapia gene orthologs sharing differential expression due to density alone (Table 1),
256 these included a homolog of *dok1*, a known negative regulator of inflammatory pathways and
257 innate lymphocytes in vertebrates (65, 66). This gene had higher expression in high-density
258 salmon and low-density tilapia, further indicating that suboptimal rearing density suppresses
259 innate immune levels.

260 Comparing expression responses to *Saprolegnia* between salmon and tilapia revealed an
261 upregulation of genes related to “Fcε receptor signalling” in both species at both densities
262 (Table 2). While teleosts lack IgE antibodies, and so do not appear to possess true Fcε
263 receptors, fish mast cells do express their homologs (61). Indeed, “mast cell degranulation”
264 was found in upregulated genes of both densities of *Saprolegnia*-challenged tilapia, and high-
265 density salmon (Table 2). Mast cells release mediators thought to be critical in responses
266 against the fungal-like pathogen *Saprolegnia* (54). There is increasing recognition for the
267 involvement of mast cells in fungal infections in other vertebrates (67), and more generally
268 their importance in fish immune systems (61). We propose this cell set should be considered
269 more closely for understanding innate resistance/susceptibility to saprolegniasis, particularly
270 in salmonids where their functioning appears to be impacted by rearing density.

271 We found intriguing conservation in the impacts of rearing density on immune responses of
272 fish. At the rearing density least stressful for each species (salmon; low-density, tilapia; high-
273 density), we found expression patterns consistent with enhanced T helper cell activity. GO
274 term enrichment for Th₂ cell differentiation and Th₁₇ cell lineage commitment were found in
275 genes with increased expression in both these groups. In addition, *batf* – a transcription factor
276 crucial to Th₁₇ cell differentiation (68) - was significantly upregulated in response to
277 *Saprolegnia* challenge only at these “non-stressful” densities (Figure 1). In contrast, the

278 “stressed” fish density groups (high-density salmon, low-density tilapia) both exhibited
279 increased expression of *lag3*, a negative regulator of T cell expansion (69), whose activity has
280 been implicated in reduced parasite clearance in other vertebrates (70). Moreover, these fish
281 also had increased expression of *tnfaip8l2* (Table 3), a suppressor of inflammation that is
282 typically downregulated (i.e., to induce inflammation) during pathogen challenge in vertebrates
283 including fish (71, 72). Taken together, these results indicate suboptimal rearing densities can
284 disrupt beneficial Th₁₇/inflammatory transcriptional responses to pathogens, and these genes
285 provide potential new markers for measures of health under different rearing densities, across
286 a wide range of teleost species. Interestingly, in mammals, Th₁₇ responses are increasingly
287 recognised for their importance in mucosal (73) and vaccine-induced immunity (74). This
288 appears to hold true for fish (75), which raises the question as to whether optimising rearing
289 densities in aquaculture may in turn increase vaccine efficacy.

290 **4. Conclusions**

291 Rearing density of Atlantic salmon can significantly impact their immune status with suboptimal
292 rearing density broadly suppressing gill innate immune gene expression, but also key adaptive
293 immune responses to pathogen challenge. In addition, we found density-driven disruption of
294 Th₁₇ responses – key to mucosal immunity – to be similar between Atlantic salmon and Nile
295 tilapia, suggesting these genes may be useful transcriptional indicators of rearing density
296 impacted immunity across a broad range of fish species. We propose maintaining fish at
297 suitable densities may not only improve natural immunocompetence, but could improve
298 vaccination efficacy, and recommend this as a valuable line of future research for mitigating
299 disease in aquaculture. As the species diversity of aquaculture increases, whilst disease
300 remains a barrier to sustainable intensification of the industry, the key molecular targets
301 identified here have the potential for monitoring and enhancing aquaculture resilience across
302 the range of farmed species.

303 **5. Methods**

304 **5.1 Salmon rearing conditions**

305 Salmon fry (average weight = 2.55 g, average standard length = 6.52 cm), obtained from
306 Landcatch Natural Selection (10 families; 1:1 crosses), were maintained in a re-circulating
307 aquaculture system in CSAR, Swansea University (water temperature 10.5 ± 0.5 °C, pH $7.5 \pm$
308 0.2). On arrival, fish were subject to routine visual health screening to ensure no abnormalities
309 or existing health issues. Fry were fed with a commercial salmon feed (Nutraparr, Skretting,
310 UK) and kept under a 12:12h photoperiod. Water oxygen saturation (>90%), ammonia (<0.02
311 mg/L), nitrite (<0.01 mg L⁻¹) and nitrate (<15 mg L⁻¹) were maintained within an appropriate
312 range. The density experiment was conducted for 16 weeks. Fry were randomly assigned to
313 low- and high-density groups, within two replicate 260 L tanks per treatment. Each low-density
314 tank contained 130 fish (initial density 1.3 g L⁻¹, final density 3.6 g L⁻¹), and each high-density
315 tank contained 520 fish (initial density 5.1 g L⁻¹, final density 14.6 g L⁻¹). These densities fall
316 within current farming practices and UK welfare recommendations (up to 30 g L⁻¹ for 5 to 30 g
317 juvenile fish (44)). All experiments were performed with the approval of the Swansea Animal
318 Welfare and Ethical Review Body (Approval Number IP-1415-2), and infection challenges
319 were approved by Cardiff University Animal Ethics Committee and conducted under UK Home
320 Office License PPL 302876.

321 **5.2 Saprolegnia challenges**

322 *Saprolegnia parasitica* maintenance and zoospore production followed Ellison et al (44) and
323 zoospore suspensions were equilibrated to 10.5 °C before use (76). Fish were simultaneously
324 challenged with *S. parasitica* within their treatment groups to avoid the masking effects of
325 acute stress due to confinement and individual isolation (77). The exposure trials were
326 conducted in 22L tanks, with 2 replicate tanks per group containing 96 fish/tank (4 fish L⁻¹,
327 29.2 g L⁻¹) for high density groups (2 control tanks, 2 Saprolegnia-challenge tanks), and 24
328 fish/tank (1 fish L⁻¹, 7.3 g L⁻¹) for low density groups (2 control tanks, 2 Saprolegnia-challenge
329 tanks).

330 Following Ellison et al (44), all fish were net shaken to facilitate infection (78) and live
331 zoospores were added directly to high-density aquaria to achieve a concentration of 5×10^6
332 zoospores L^{-1} . A mixture of 1:3 live:heat-killed zoospores was added directly to each low-
333 density aquarium to achieve a concentration of 5×10^6 zoospores L^{-1} , controlling for
334 equivalent 1) number of infective zoospores per individual and 2) concentration of organic
335 matter between density treatment groups. Water and zoospore solutions were completely
336 changed every 6 h during 24 h exposure period and fish in unchallenged control groups
337 received the same handling (e.g. net shaking) and maintenance regime.

338 Fish were visually inspected hourly (under red light during dark periods) throughout the
339 experiment and those challenged with *S. parasitica* from both density groups displayed signs
340 of lethargy (reduced swimming activity, increased resting on the bottom of the tank) ~12 h
341 after infection, but there were no signs of mycelial growth. At 24 h post-exposure, six fish per
342 treatment tank (high/low density, challenged/sham-challenged, 2 tank replicates) were
343 euthanised with an overdose of Phenoxyethanol ($0.5 \text{ ml } L^{-1}$) and samples of gill tissues (all
344 arches) were immediately preserved in RNAlater and stored at $-80^\circ C$ until RNA extraction.
345 Gill tissues were chosen as they are one of the primary sites of infection of *S. parasitica* (55),
346 and critical to fish mucosal immunity and antibody-producing cell production (79).

347 **5.3 Salmon transcriptome sequencing and gene expression analyses**

348 Sample preparation and sequencing followed Ellison et al. (44). Briefly, total RNA was
349 extracted from each tissue sample separately using AllPrep DNA/RNA Micro kit (Qiagen).
350 RNA was quantified using Qubit High-Sensitivity RNA assays (ThermoFisher Scientific) and
351 integrity was determined using Agilent 4200 TapeStation RNA assays (Agilent Technologies).
352 All samples had RNA integrity values greater than 8.0. Libraries were generated using the
353 Illumina Stranded TruSeq mRNA sample preparation kits (high-throughput protocol), as per
354 the manufacturer's instructions (Illumina, San Diego, CA). Libraries' quality was quantified and
355 assessed using Agilent 4200 TapeStation prior equimolar pooling. The library pool was run
356 four times on Illumina NextSeq500 ($2 \times 75 \text{ PE}$) to achieve a minimum of 25 million read pairs

357 per sample. Raw reads are available at the NCBI Short Read Archive under Accession
358 Number PRJNA552428. Trimmed reads were mapped to the *Salmo salar* genome
359 (International Cooperation to Sequence the Atlantic Salmon Genome, version 2) using
360 HISAT2 version 2.0.5 (81) and quantified using RSEM version 1.2.30 (82). Transcripts were
361 filtered to include only those with at least two counts per million mapped reads (TPM) in at
362 least two individuals. Differential expression tests were performed using the R package limma
363 (83), comparing 1) high- and low-density control (uninfected) fish, and 2) infected and
364 uninfected (control) fish. Potential tank effects were explicitly accounted using the
365 duplicateCorrelation function (83). Only tests resulting in FDR-corrected P-values of less than
366 0.05 was considered differentially expressed. Overlap of differentially expressed genes
367 between salmon treatment groups were determined using Venny version 2.1 (84). Gene
368 ontology (GO) functional enrichment tests (with FDR corrected P-values <0.05 considered
369 significant) were carried out via the R package TopGO (85) to detect significantly
370 overrepresented biological processes of groups of differentially expressed genes
371 shared/unique to particular treatment groups.

372 **5.4 Interspecific comparisons of gill transcriptomic responses to *Saprolegnia* challenge**

373 To examine the similarity of rearing density impacts on transcriptomic responses to pathogen
374 challenge across divergent fish species, we compared salmon gene expression profiles to
375 those previously characterised in Nile tilapia (*Oreochromis niloticus*) by Ellison et al. (2018,
376 24 h sample data only). For this, we used three methods: 1) overlap of GO term enrichment
377 of differentially expressed genes using the full transcriptome of both species, 2) overlap of
378 differentially expressed 1:1 gene orthologs, and 3) preservation of weighted gene co-
379 expression networks defined using 1:1 gene orthologs. These two datasets were broadly
380 comparable as in both 1) “high-density” rearing treatments were 4 times that of the “low-
381 density” treatments (4 fish L⁻¹ and 1 fish L⁻¹ respectively), 2) the same tissue (gill) was studied,
382 3) tissue samples were taken 24 h post-*Saprolegnia* exposure, and 4) the same *S. parasitica*
383 isolate, inoculation dose and challenge procedures were used.

384 Biological processes GO term lists from functional enrichment tests comparing healthy and
385 *Saprolegnia*-infected tilapia at 24 h post-exposure were compared to those in salmon using
386 Venny version 2.1 (84). We performed a reciprocal best-hit analysis to identify 1:1 gene
387 orthologs between the two species. We used BLASTP with an E-value threshold of 1×10^{-6}
388 to search all protein sequences from one species against the other (86). Only sequences that
389 were the reciprocal best hit between both species were retained for further analyses.

390 Weighted gene co-expression networks (gene modules) were defined and correlated with
391 treatments following methods of Ellison et al. (44) in tilapia and salmon using only genes with
392 a 1:1 ortholog in the other species. To assess the degree to which gene modules were
393 conserved in the other species, module preservation statistics were computed using the
394 modulePreservation function (500 permutations) (42, 87). Network module preservation
395 statistics quantify how density and connectivity patterns of modules defined in a reference
396 data set are preserved in a test data. A Z_{summary} score of 2.0 to 10.0 was considered weak to
397 moderately preserved, and Z_{summary} above 10.0 was considered highly preserved among
398 species (42, 87).

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407 **Declarations**

408 *Ethics approval:* All experiments were performed with the approval of the Swansea Animal
409 Welfare and Ethical Review Body (Approval Number IP-1415-2), and infection challenges

410 were approved by Cardiff University Animal Ethics Committee and conducted under UK Home
411 Office License PPL 302876 in accordance with ARRIVE guidelines.

412 *Availability of data and materials:* All sequence data have been submitted to the NCBI
413 Sequence Read Archive (Accession: PRJNA552428) and will be made publicly available upon
414 acceptance of this manuscript for publishing. All other data are available as additional files
415 with this article.

416 *Competing interests:* The authors declare that they have no competing interests.

417 *Author contributions:* All authors designed the study. SC, CGL, JC and POW organized
418 funding. AE, TUW and DRB collected data. AE performed analyses. AE wrote the manuscript
419 with contributions and edits from all authors.

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657

658 **Table 1.** Summary of Atlantic salmon and Nile tilapia gene orthologs that exhibit density-
 659 specific expression in response to rearing density in both species. Shaded boxes indicate the
 660 treatment group (H = high-density 4 fish/L, L = low-density 1 fish/L) with higher expression.

Gene	Function	Salmon		Tilapia	
		H	L	H	L
dihydropyrimidinase-related	axon repair/development, cell migration, filipodia				
large neutral amino acids transporter small subunit 4-like	amino acid transport				
aquaporin FA-CHIP-like	ammonium, CO ₂ , water transport				
complement decay-accelerating factor-like	cell adhesion, motility, angiogenesis				
GTP-binding 2	GTPase activity				
ras-related and estrogen-regulated growth inhibitor	GTPase activity				
inositol-trisphosphate 3-kinase C	inositol phosphate biosynthetic process				
egl nine homolog 2-like	oxidation-reduction				
death-associated kinase 3	protein kinase				
transmembrane protease serine 4-like	proteolysis				
neuronal PAS domain-containing 2-like	regulation of transcription				
homeobox DLX-3	regulation of transcription				
myocyte-specific enhancer factor 2D	regulation of transcription				
mediator of RNA polymerase II transcription subunit 12	regulation of transcription				
endothelin B receptor-like	vasoconstriction, cartilage development				
PDZ and LIM domain 4	zinc ion binding, protein binding				
docking 1-like	insulin receptor binding				
transmembrane 268-like	unknown				

poly [ADP-ribose] polymerase 9	NAD+ ADP-ribosyl transferase activity				
claudin-4-like	structural molecule activity				
aldehyde dehydrogenase family 3 member B1-like	aldehyde metabolic process				
non-lysosomal glucosylceramidase	axonogenesis				
nucleotide exchange factor SIL1	binding				

661

662 **Table 2.** Summary of Gene Ontology (GO) term enrichment overlap of differentially expressed
663 genes compared between control (sham-challenged) and *Saprolegnia*-challenged Atlantic
664 salmon and Nile tilapia. Arrows indicate treatment groups (H = high-density 4 fish/L, L = low-
665 density 1 fish/L, HL = both densities) in which significant enrichment was found. Arrow
666 direction indicates direction of expression (\downarrow = decreased expression, \uparrow = increased
667 expression).

Salmon			Tilapia			GO biological process
HL	H	L	HL	H	L	
\downarrow	\downarrow	\downarrow	\downarrow	\downarrow	\downarrow	regulation of Rho protein signal transduction
\uparrow	\uparrow		\uparrow	\uparrow		spliceosomal snRNP assembly
	\uparrow		\downarrow	\downarrow	\uparrow	regulation of apoptotic process
\uparrow			\uparrow	\uparrow	\uparrow	fructose metabolic process
\downarrow	\downarrow		\downarrow		\downarrow	peptidyl-tyrosine phosphorylation
\downarrow		\downarrow	\downarrow	\uparrow		DNA replication
\downarrow		\downarrow	\downarrow		\downarrow	protein phosphorylation
	\downarrow		\downarrow	\downarrow	\uparrow	regulation of cell growth
\uparrow	\uparrow		\uparrow			isoprenoid biosynthetic process
	\uparrow	\uparrow			\downarrow	spermine biosynthetic process, embryonic neurocranium morphogenesis

				protein peptidyl-prolyl isomerization, protein folding, glycolytic process, pentose-phosphate shunt, oxidation-reduction process
↑	↑	↑		
↑	↑↓			protein methylation
↑	↑	↑		Arp2/3 complex-mediated actin nucleation
↑	↓	↑		cellular response to xenobiotic stimulus
↑↓			↓	cell cycle
↑	↓		↓	determination of ventral identity
↑	↑		↑	rRNA modification
↑	↑	↑		defence response to Gram-negative bacterium
↑	↓	↓		convergent extension involved in gastrulation
↑		↑	↑	pseudouridine synthesis
↑		↑	↑	fructose 2,6-bisphosphate metabolic process
↑		↓	↓	immune response
↓	↓		↓	locomotor rhythm
↓	↓		↓	embryonic skeletal system development, transmembrane receptor protein tyrosine kinase signalling pathway
↓		↓	↓	regulation of ARF protein signal transduction
↓		↓	↓	protein kinase C-activating G protein-coupled receptor signalling pathway, retinal ganglion cell axon guidance
	↓	↑	↓	regulation of transcription by RNA polymerase II
	↓	↓	↓	positive regulation of GTPase activity, negative regulation of transcription (DNA-templated)
	↓	↓	↓	negative regulation of angiogenesis
	↓	↓	↓	cell adhesion
	↓	↓	↓	axon guidance
	↑	↑	↑	ribosome biogenesis, nucleoside metabolic process

↑	↑	↑	protein O-linked mannosylation
↓	↑	↑	DNA recombination
↓	↓	↓	signal transduction
↑		↑	proton transport
↑	↑		mast cell degranulation, response to lipopolysaccharide, peptidyl-lysine methylation, galactose metabolic process, proton-transporting ATP synthase complex assembly
↑	↓		embryonic digestive tract morphogenesis, proepicardium development, regulation of vascular endothelial growth factor receptor signalling pathway
↑		↓	regulation of alternative mRNA splicing via spliceosome
↑		↑	fucosylation, RNA phosphodiester bond hydrolysis, intestinal cholesterol absorption, peptide cross-linking
↑		↓	stabilization of membrane potential
↑		↑	spliceosomal complex assembly, mRNA transport, exonucleolytic trimming, positive regulation of cell division
↑	↑		Fc-epsilon receptor signalling pathway, viral entry into host cell, maturation of LSU-rRNA, asparagine biosynthetic process, nuclear import, isocitrate metabolic process, regulation of translational initiation, histone mRNA metabolic process, cyclooxygenase pathway, mitotic sister chromatid cohesion, ribosomal subunit export from nucleus
↑	↓		cell migration involved in gastrulation, spermatid development, cell chemotaxis
↑		↓	positive regulation of gene expression, positive regulation of ERK1 and ERK2 cascade, gastric inhibitory peptide signalling pathway
↑		↑	carbohydrate phosphorylation, melanosome transport, phospholipid transport

↑		↓	NADP biosynthetic process
↓		↑	regulation of skeletal muscle cell differentiation, adherens junction assembly
↓		↓	positive regulation of cell proliferation, negative regulation of canonical Wnt signalling, fibroblast growth factor receptor signalling, smoothened signalling pathway, dorsal root ganglion development, positive regulation of transcription
↓		↓	phosphate ion transmembrane transport, endothelial cell chemotaxis
↓		↓	de novo' actin filament nucleation, lipoprotein metabolic process
↓		↑	regulation of stress fiber assembly
↓		↓	proteolysis, heart development, retinol metabolic process, response to axon injury, homophilic cell adhesion via plasma membrane
↓		↓	regulation of cell proliferation, mesenchyme migration, somatic muscle development, inactivation of MAPK activity, positive regulation of protein kinase A signalling, angiogenesis, actin filament organization
↓		↓	phagocytosis, protein autophosphorylation, reverse
↓		↑	cholesterol transport, negative regulation of ERK1 and ERK2 cascade, Rho protein signal transduction
↓		↓	Roundabout signalling pathway, notochord morphogenesis, regulation of calcineurin-NFAT signalling, calcium ion import
	↑	↑	T-helper 2 cell differentiation, T-helper 17 cell lineage commitment, myeloid dendritic cell differentiation, defence response to protozoan, isotype switching, rRNA methylation, hematopoietic stem cell differentiation, ribonucleoside

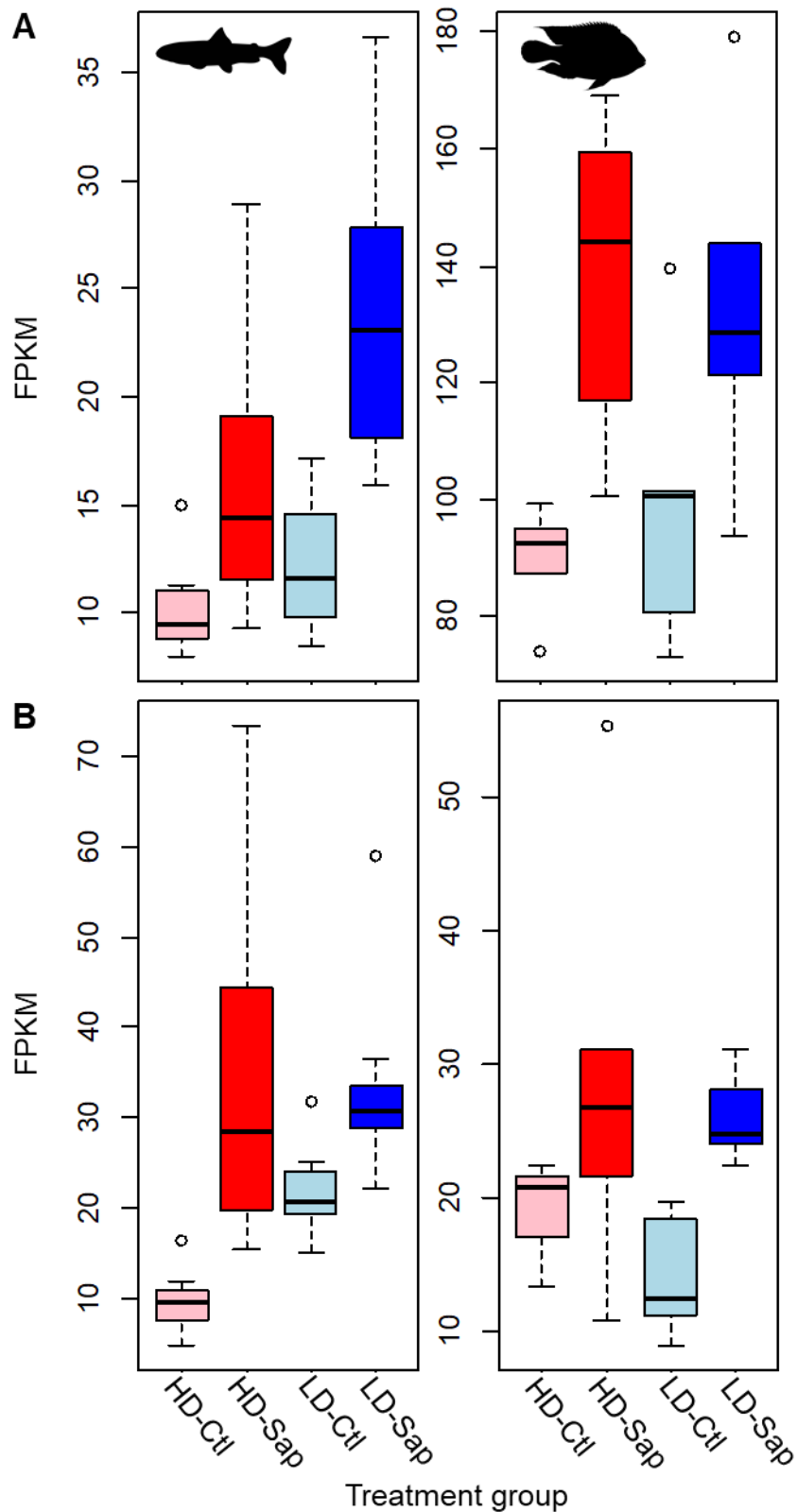
			monophosphate biosynthetic process, fatty acid biosynthetic process, threonine catabolic process
	↑	↑	regulation of defence response to virus by virus
	↑	↓	NLS-bearing protein import into nucleus, regulation of RNA splicing
	↓	↑	peptidyl-diphthamide biosynthetic process, 'de novo' CTP biosynthetic process
	↓	↓	regulation of protein localization, retinoic acid catabolic process
	↓	↑	regulation of Rho guanyl-nucleotide exchange factor activity, regulation of ephrin receptor signalling, cytoplasmic microtubule organization, peripheral nervous system myelin maintenance, Kit signalling pathway, drug transmembrane transport
	↓	↓	positive regulation of non-canonical Wnt signalling pathway, negative regulation of vascular endothelial growth factor receptor signalling pathway, reelin-mediated signalling pathway, histone H4-K16 acetylation, positive regulation of Wnt signalling pathway

668

669 **Table 3.** Summary of gene orthologs sharing differential expression between control (sham-
670 challenged) and *Saprolegnia*-challenged Atlantic salmon and Nile tilapia. Arrows indicate
671 treatment groups (H = high-density 4 fish/L, L = low-density 1 fish/L) in which significant
672 enrichment was found. Arrow direction indicates direction of expression (↓ = decreased
673 expression, ↑ = increased expression).

Salmon		Tilapia		ID	Gene
H	L	H	L		

↑	↑	XP_013995411.1	growth differentiation factor 15		
↑	↑	XP_014004629.1	interleukin-17C		
↑	↑	XP_014009356.1	lymphocyte activation gene 3		
↑	↑	XP_014056305.1	tumor necrosis factor alpha-induced protein 8-like 2		
↑	↑	XP_003455658.1	basic leucine zipper transcriptional factor ATF-like		
↓	↓	XP_014027980.1	perforin-1		
↑	↑	XP_014068485.1	stimulator of interferon genes		
↓	↓	XP_014011243.1	integumentary mucin		
↑	↑	XP_014015941.1	tumor necrosis factor ligand superfamily member 15-like		
↑	↑	XP_014001973.1	tumor necrosis factor receptor superfamily member 9-like		
↑	↑	XP_014064920.1	mucin-5AC-like		
↑	↑	↑	↑	NP_001117024.1	interleukin 1 receptor accessory protein
↑	↑	↑	↑	XP_014036616.1	leukocyte elastase inhibitor
↑	↑	↑	↑	XP_014067437.1	leukocyte cell-derived chemotaxin 2 precursor
↑	↑	↑	↑	XP_014071691.1	AP-1 complex subunit mu-1
↑	↑	↑	↑	XP_014047427.1	AP-1 complex subunit sigma-2
↑	↑	↑	↑	NP_001134642.1	AP-1 complex subunit sigma-3



675

676 **Figure 1.** Boxplots of A) *batf*, and B) *il17c* gene expression (FPKM; Fragments Per Kilobase
 677 of transcript per Million mapped reads) in Atlantic salmon (left, n = 8; 4 per tank) and Nile
 678 tilapia (right, n = 5 fish per group). Colours indicate density treatment (red = high-density; “HD”,

679 blue = low-density; "LD") and colour intensity *Saprolegnia* status (light = sham-challenged;
680 "Ctl", dark = *Saprolegnia*-challenged; "Sap"). Shown in the boxplots are minimum, first quartile,
681 median, third quartile, and maximum values. Extreme values are shown by closed circles.