Fish & Shellfish Immunology: Research article

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

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Abstract

Infectious diseases represent an important barrier to sustainable aquaculture development. Rearing density can substantially impact fish productivity, health and welfare in aquaculture, including growth rates, behaviour and, crucially, immune activity. Given the current emphasis on aquaculture diversification, stress-related indicators broadly applicable across species are needed. Utilising an interspecific comparative transcriptomic (RNAseq) approach, we compared gill gene expression responses of Atlantic salmon (*Salmo salar*) and Nile tilapia (*Oreochromis niloticus*) to rearing density and *Saprolegnia parasitica* infection. Salmon reared at high-density showed increased expression of stress-related markers (e.g. *c-fos* and *hsp70*), and downregulation of innate immune genes. Upon pathogen challenge, only salmon reared at low density exhibited increased expression of inflammatory interleukins and lymphocyte-related genes. Tilapia immunity, in contrast, was impaired at low-density. Using overlapping gene ontology enrichment and gene ortholog analyses, we found that density-related stress similarly impacted salmon and tilapia in key immune pathways, altering the expression of genes vital to inflammatory and Th_{17} responses to pathogen challenge. Given the challenges posed by ectoparasites and gill diseases in fish farms, this study underscores the importance of optimal rearing densities for immunocompetence, particularly for mucosal immunity. Our comparative transcriptomics analyses identified density stress impacted immune markers common across different fish taxa, providing key molecular targets with potential for monitoring and enhancing aquaculture resilience in a wide range of farmed species.

1. Introduction

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

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

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

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

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

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

2. Results

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

2.1 Salmon transcriptomic responses to rearing density and Saprolegnia challenge

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

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

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

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

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

2.2 Interspecific comparison of density and Saprolegnia transcriptomic responses

2.2.1 Impact of density on uninfected salmon and tilapia

Comparison of functional enrichment of differential expression between high- and low-density treatments in unchallenged (healthy) salmon and tilapia reveal a small number of biological processes shared between these species (Supplementary File S2), including "innate immune response" (GO:0045087) in genes showing higher expression in high-density tilapia and low-density salmon. Using a reciprocal best hit approach, we found 21,745 1:1 orthologs between published salmon and tilapia transcriptomes, of which 13,364 were expressed in gill tissues of both species. Twenty-three gene orthologs were found to be differentially expressed between healthy high- and low-density fish in both species (Table 1), including several genes involved 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 Th_{17} 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

stress in vertebrates including *c-fos* (46-48) and *hsp70* (49, 50). Previous studies on the effects of rearing density on immunity in salmonids have primarily focussed on levels of serum antibodies (typically IgM) or antibody-producing cells, with suppression generally found at higher densities (8, 36). In addition, non-specific innate immune markers such as serum lysozyme activity have been shown to be influenced by rearing density (43). Here, we found that salmon raised at high density had lower expression of genes related to immune responses including neutrophil (e.g. *rac2*), dendritic cell (e.g. *cd209, ctss*), and B cell immunity (e.g. *lyn, cd22, blnk*) plus inflammatory interleukins (*il-12, il-17*) (Supplementary File S1). A previous study of rainbow trout head-kidney gene expression during crowding stress also showed increased *hsp70* stress marker expression, but found different immune gene suppression (*lyzII, tnf-1α, il-1β, il-8 and ifn-γ1*) (18). This suggests that crowding stress in salmonids may result in tissue-specific suppression of immune factors, which must be considered for their potential impacts on disease susceptibility. Clearly overcrowding in salmonids has a wider impact on their immune system expression than previously reported. The effects of crowding stress on immune health caused by high rearing densities may explain in part the failure of supportive breeding in salmon conservation (51-53).

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

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

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

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

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

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

We found intriguing conservation in the impacts of rearing density on immune responses of fish. At the rearing density least stressful for each species (salmon; low-density, tilapia; high-density), we found expression patterns consistent with enhanced T helper cell activity. GO term enrichment for Th_2 cell differentiation and Th_{17} cell lineage commitment were found in genes with increased expression in both these groups. In addition, *batf* – a transcription factor crucial to Th_{17} cell differentiation (68) - was significantly upregulated in response to *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 Th_{17}/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, Th_{17} 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 Th_{17} 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**

5.1 Salmon rearing conditions

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

5.2 Saprolegnia challenges

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

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

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

5.3 Salmon transcriptome sequencing and gene expression analyses

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

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

5.4 Interspecific comparisons of gill transcriptomic responses to Saprolegnia challenge

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

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

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

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Declarations

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

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

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

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

Author contributions: All authors designed the study. SC, CGL, JC and POW organized funding. AE, TUW and DRB collected data. AE performed analyses. AE wrote the manuscript 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				

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

Salmon			Tilapia			GO biological process
HL	H	L	HL	H	L	
↓	↓	↓	↓	↓	↓	regulation of Rho protein signal transduction
↑	↑		↑	↑		spliceosomal snRNP assembly
	↑		↓	↓	↑	regulation of apoptotic process
↑			↑	↑	↑	fructose metabolic process
↓	↓		↓		↓	peptidyl-tyrosine phosphorylation
↓		↓	↓	↑		DNA replication
↓		↓	↓		↓	protein phosphorylation
	↓		↓	↓	↑	regulation of cell growth
↑	↑		↑			isoprenoid biosynthetic process
	↑	↑			↓	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

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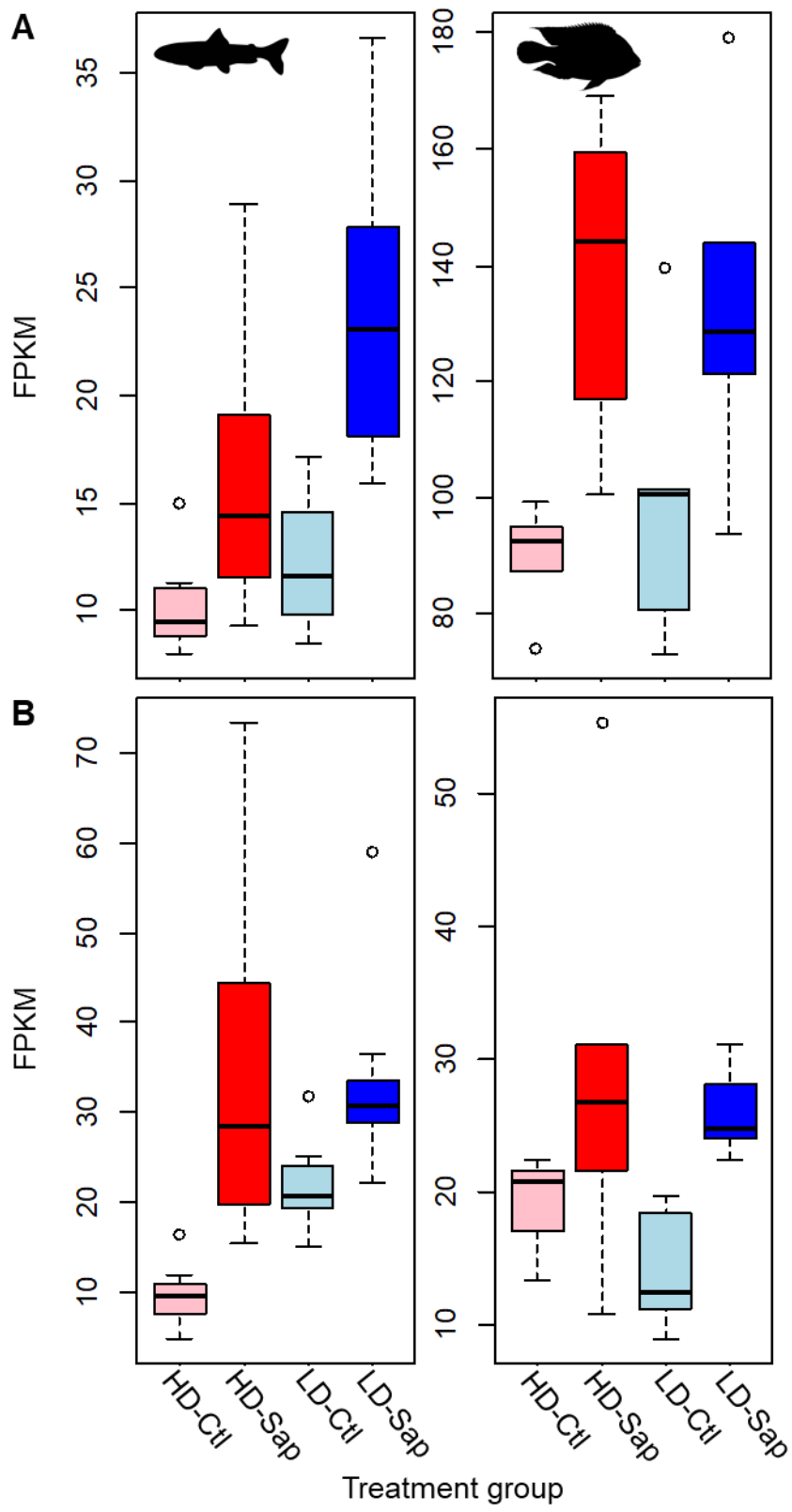

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676 **Figure 1.** Boxplots of A) *batf*, and B) *il17c* gene expression (FPKM; Fragments Per Kilobase
677 of transcript per Million mapped reads) in Atlantic salmon (left, n = 8; 4 per tank) and Nile
678 tilapia (right, n = 5 fish per group). Colours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