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Impact of freshwater rearing history on Atlantic salmon gill response to viral stimulation post seawater transfer☆

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ABSTRACT

Land-based recirculating aquaculture systems (RAS) have risen in prevalence in recent years for Atlantic salmon production, enabling intensive production which allows increased growth and environmental control, but also having the potential for reducing water use and eutrophication. The Atlantic salmon has an anadromous life history with juvenile stages in freshwater (FW) and on-growing in seawater (SW), enabled by a transformational process known as smoltification. The timing of smoltification and transfer of smolts from FW to SW is critical under commercial production with high mortalities during this period. The impact of FW rearing system on immune function following seawater transfer (SWT) is not well understood. In this study parr were raised in either RAS or a traditional open-LOCH system until smolting and then transferred to a common marine environment. Two-weeks post-SWT fish were immune stimulated with a viral mimic (poly I:C) for 24 h to assess the ability to mount an antiviral immune response, assessed by whole transcriptome analysis of gill tissue, an important immune organ in fish. We show that unstimulated smolts reared in the LOCH had higher immune gene expression than those reared in RAS as determined by functional analysis. However, following stimulation, smolts reared in the RAS mounted a greater magnitude of response with a suite of immune genes displaying higher fold induction of transcription compared to LOCH reared smolts. We suggest RAS smolts have a lower steady state immune-associated transcriptome likely due to an unvarying environment, in terms of environmental factors and lack of exposure to pathogens, which shows a compensatory mechanism following stimulation allowing immune 'catch-up' with those reared in the LOCH. Alternatively, the RAS fish are experiencing an excessive response to the immune stimulation.

1. Introduction

As the global population continues to rise, so too does per capita fish consumption. Fish produced in aquaculture now constitute more than half of all consumption fish produced worldwide, out-producing wild-capture fisheries. From an economic standpoint, Atlantic salmon (*Salmo salar*) is the most valuable species cultured worldwide [1]. The anadromous life cycle of this fish dictates a need for periods of culture in both freshwater (FW) and seawater (SW). An important transition known as smoltification, regulated by water temperature and daylength, occurs in FW juvenile parr prior to SW migration and encompasses a suite of physiological, morphological and behavioural changes to become SW adapted juveniles called smolts [2]. In aquaculture, light regimes need to be carefully considered to balance growth with optimum development of SW tolerance, as photoperiod regime has been shown to impact gene expression in important osmoregulatory tissues such as gill [3–5]. Production of robust, well-adapted smolts is vital to success post-transfer to SW for on-growing and mistiming of smoltification or seawater transfer (SWT) results in high mortalities [6].

The on-going expansion of aquaculture production has meant investment in systems technologies with a view to reducing environmental impacts, increasing fish welfare, and promoting sustainability in the

☆ SAMM, JT, and HM designed the experiment. SAMM, JT, SM and HM were responsible for project management. LC, and MC conducted the sample collection. MLR performed the RNA extraction and preparation of samples for mRNA sequencing and downstream bioinformatic data analyses, and wrote the initial manuscript draft which was reviewed and edited by SM, HM and SAMM. All authors contributed to the article and approved the submitted version.

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industry. In the Scottish aquaculture sector, cage systems open to the environment situated in large inland water bodies called lochs have traditionally been used to rear freshwater juveniles to the smolt stage and subsequent transfer to sea, exposing fish to natural freshwater as well as ambient photoperiod, temperature and seasonality. However, a shift from production of smolts in open-water (LOCH) or flow-through systems (FTS) to extended production to post-smolt stage in land-based recirculating aquaculture systems (RAS) has occurred in recent years [7]. RAS ensure year-round smolt production in a carefully controlled environment alongside increased biosecurity and prevention of eutrophication of the surrounding environment but are energetically costly to operate [8,9]. Despite the wide use of RAS, their impacts on physiology of smolts reared in these tightly controlled non-natural systems, one important aspect of which is the immune system, is still poorly understood.

Atlantic salmon have highly functioning innate and adaptive immune systems acting at local and systemic levels [10]. Mucosal surfaces are the first line of host defence against potential environmental pathogens and distinct mucosa-associated lymphoid tissues (MALT) persist in distinct tissues including the gill (GiALT) [11]. The GiALT can be sub-divided into interbranchial lymphoid tissue (ILT) and amphibranchial lymphoid tissue (ALT) in salmonids [12]. Within GiALT, local innate immune mediators include mucins, antimicrobial peptides, immune-related enzymes, lectins, complement proteins, phagocytic cells, pathogen recognition receptors (PRRs) and local cytokine signalling that regulates local inflammation [13,14]. The adaptive immune system in the gill comprises mucosal immunoglobulins (IgT/IgM) in addition to CD_4+ (helper cells), CD_8+ (cytotoxic) and $FOXP_3$+ (regulatory) T-cells [14,15]. The gill is a complex organ with multiple functions including gas exchange, pH balance and osmoregulation [16]. Due to its large surface area and its constant and direct contact with the environment, it often is subject to inflammation driven by abiotic and biotic factors during the SW on-growing phase in aquaculture [17].

The post-SWT period in aquaculture production is a time when fish can experience poor health and increased disease burden and mortality [18,19]. Increased susceptibility to disease has been attributed to stress, exposure to novel pathogens, adaptation to the SW environment, and immune suppression. Suppression of the immune system during smoltification is a phenomenon which has been independently identified in different cohorts of fish and in tissues including head kidney, gill, skin and intestine [20–23]. Downregulation of immune genes has been suggested as a side effect of large changes in endocrine regulation and a balance between immunity and transformation of osmoregulatory systems during smoltification [24].

Exposure to pathogens and other antigens in the environment during immune system development may 'prime' the system for encounters later in life and thus the rearing environment is likely to impact the development of the immune system. In this study we utilised RNA sequencing to identify a panel of genes with differing expression profiles in the gills of fish reared in RAS or LOCH environments two-weeks post-transfer to SW. Fish were additionally stimulated with a viral mimic in the form of the pathogen associated molecular pattern (PAMP) poly I:C to illicit an immune response and identify any differences in the response of genes involved in immune protection post-SWT in fish from different FW backgrounds.

2. Materials and methods

2.1. Fish

The Atlantic salmon utilised in this study were obtained from a commercial salmon production company (MOWI, Scotland) and supplied as sea-ready smolts from either a RAS or LOCH site to the Machrihanish Marine Environmental Research Laboratory (MERL). Smolts were produced out of season by using a standard smoltification regime in both RAS (9.8 °C–11.5 °C) and LOCH populations (natural temperature ranging from 5.6 °C to 7.5 °C) consisting of 400° days 12L:12D for the RAS population and at ambient photoperiod for the LOCH population followed by continuous light (LL, starting on January 10, 2020 and December 31, 2020, respectively). Smoltification was monitored through gill Na^+, K^+-ATPase activity and blood chloride analyses performed every 100° days from the onset of spring photoperiod (LL). No smoltification diets were used with both parr populations fed on Skretting diet. Fish in both systems were vaccinated with AlphaJect Micro 6 (Pharmaq) in early December 2019. There were no additional disease control measures taken for these fish. Smolts from the RAS facility were transferred to MERL on February 14, 2020 (375° days (dd); mean weight = 92.2 g; mean length = 200.2 mm) while LOCH-reared smolts were transferred on March 5, 2020 (430 dd; mean weight = 101.2 g; mean length = 210.1 mm). Smolts, regardless of freshwater origin, were stocked into 2 m diameter stock tanks (1 freshwater origin and ~120–150 fish $tank^{-1}$). Fish were maintained under ambient temperature (min 7 °C; max 12 °C) with aeration provided by air stones. The difference in time of seawater entry was related to the predicted peak time for transfer. All fish were from the same genetic background.

2.2. Poly I:C stimulation

The experimental design is outlined in Fig. 1. Fish from both RAS and LOCH origins (average weight: RAS 86.4 g; LOCH 102.4 g) were subject to experimental viral stimulation at two weeks post-seawater transfer. Prior to the stimulation, fish were randomly allocated into 1.4 m diameter (ca. 750 L) tanks (three tanks freshwater $origin^{-1}$; 18 fish $tank^{-1}$) for seven days and allowed to acclimate. Fish were anaesthetised prior to injection (MS-222, 50 ppm, PHARMAQ, Norway). Six fish per tank received an intraperitoneal injection of poly I:C (P1530, Sigma-Aldrich, UK) while another six received phosphate buffered saline (PBS). Poly I:C was prepared in 0.02 M PBS to a working solution of 5 mg mL^{-1} and administered at a final concentration of 5 mg/Kg body weight. Poly I:C and PBS fish were marked with panjet (0.0652 g alcian blue ml^{-1}, Sigma-Aldrich, UK) to differentiate between treatments. Following injection, fish were returned to tanks to recover and were sampled after 24 h. Prior to sampling, fish were humanely culled by an overdose of anaesthetic (MS-222, 1000 ppm). Weight and length of all fish were recorded. Sections of gill (100 mg) from second gill arch were excised into RNAlater™ (Ambion Inc., United States), stored at 4 °C for 24 h before further storage at −20 °C until gene expression analyses.

2.3. RNA extraction

RNA was extracted from gill filaments using a standard TRIzol® reagent (Ambion by Life Technologies, Carlsbad, CA, United States) extraction protocol as previously described (Król et al., 2020). RNA quantity was determined by spectrophotometry (NanoDrop Technologies, Santa Clara, CA, United States) and RNA integrity determined by electrophoresis (Agilent Technologies, Santa Clara, CA, United States). All gill RNA samples met the criteria for RNA sequencing with all 260/280 and 260/230 ratios >1.8 and RIN >9.

2.4. RNA-seq library preparation and sequencing

For mRNA sequencing, n = 6 fish (n = 2 per triplicate tank) were randomly selected from the stimulated (poly I:C) and unstimulated (PBS) groups from RAS and LOCH reared fish (n = 24 total). Gill total RNA samples were normalized to a concentration of 50 ng/μL in 20 μL RNase-free water and (1000 ng total). mRNA selection (poly A enrichment), library construction and sequencing were performed by commercial company Novogene. Sequencing was performed on a NovaSeq 6000 platform (PE150, 6G raw data per sample).

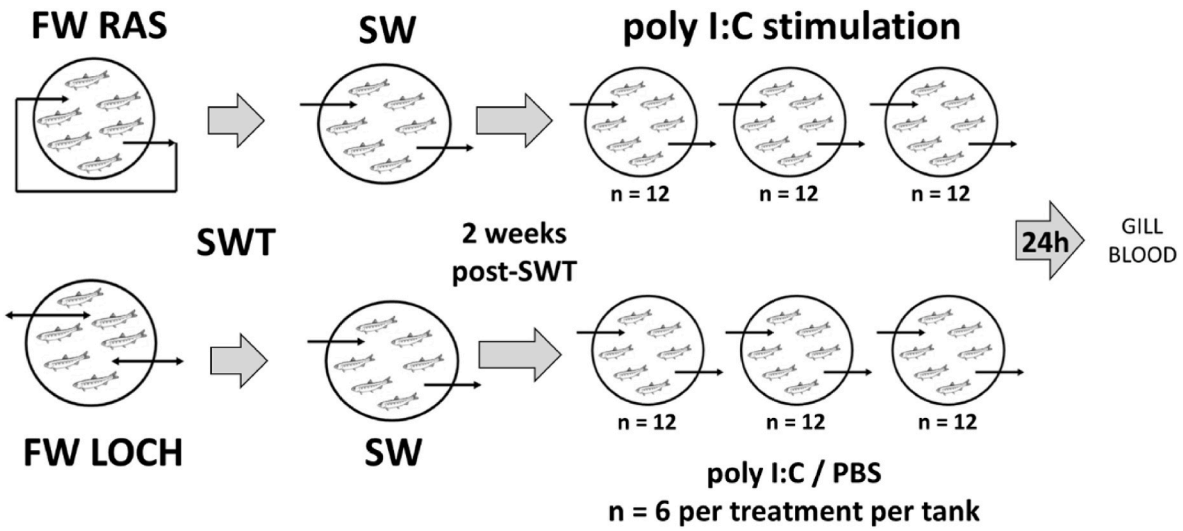

Fig. 1. Experimental design outline. Fish were supplied as smolts from either a RAS or LOCH site to seawater (SW) tanks at the Machrihanish Marine Environmental Research Laboratory (MERL). At MERL Smolts were stocked into 2 m diameter stock tanks (1 freshwater origin and ~120–150 fish tank^{-1}). Prior to stimulation at 2 weeks post-seawater transfer (SWT), fish were randomly allocated into 1.4 m diameter (ca. 750 L) tanks (three tanks freshwater origin^{-1}; 12 fish tank^{-1}) for seven days and allowed to acclimate. Under light anaesthetic, six fish per tank received an intraperitoneal injection of poly I:C at a final concentration of 5 mg/Kg body weight and six of PBS. Fish were sampled after 24 h for plasma and gill.

2.5. Sequencing data quality control and read mapping

Pre-processing of sequencing data included quality assessment with FASTQC v0.11.9 and adapter removal and quality trimming with TrimGalore! v0.6.6. Genome indexes were generated with STAR and RSEM using the Atlantic salmon reference genome ICASG_v3 (GCA_905237065.2) and reads were mapped using the parameter –aligner star_rsem. Trimmed sequencing data was analysed using the nfcore RNA-seq pipeline v3.3 (github.com/nf-core/rnaseq v3.3).

2.6. Identification of differentially expressed genes (DEGs)

Differential expression analysis was performed using the package DESeq2 v1.6 [25] in R v3.6.1 (R Core Team, 2018). An estimated gene count matrix produced using featureCounts from the Rsubread package [26] was used as input to DESeq2 which performs internal normalization to library size. Based on exploratory data analysis, one RAS poly I:C library (sample A25) was identified as an outlier and removed from the subsequent analysis. Pre-filtering was carried out to remove genes with no transcript per million (TPM) counts in n = 3 or more individuals per treatment group. Both rearing history and treatment were included in the DESeq2 general linear model as fixed effects. In total, four contrasts were generated from the model: (1) RAS control vs LOCH control; (2) RAS poly I:C vs LOCH poly IC; (3) RAS control vs RAS poly I:C; (4) LOCH control vs LOCH poly I:C. Differentially expressed genes (DEGs) were identified at false discovery rate (FDR) < 0.05 and $\log_2$ fold change ($\log_2$FC) > 1 or < -1.

2.7. Functional enrichment analysis genes for biological processes

To facilitate functional analysis using gene ontology tools, Atlantic salmon genes were mapped to human orthologues using BLAST and annotated with HUGO Gene Nomenclature Committee (HGNC) identifiers where possible, as previously described [27]. Gene ontology analysis was conducted using the Database for Annotation, Visualization and Integrated Discovery (DAVID 2021). We also used Ingenuity Pathway Analysis (IPA; Qiagen) to identify enriched canonical pathways. DAVID accepts a list of HGNCs as an input but does not consider fold change magnitude or direction. As such when using DAVID analyses of up- and down-regulated mRNA transcripts were conducted separately. In IPA analysis, HGNCs and corresponding fold changes are entered as input. The fold changes associated with duplicate HGNCs were averaged prior to IPA analysis. In cases where fold changes were in opposite directions, the HGNC was excluded from analysis.

3. Results

3.1. Fish growth and condition factor

Fish weight, length and condition factor at the time of sampling did not differ significantly between FW history or treatment groups (Table 1).

3.2. Sequencing outputs

An average of 53,471,120 ± 6,158,223 raw reads were obtained from the 24 gill RNA samples sequenced. On average 92.8 ± 0.38 % of reads were retained following filtering at Q30, resulting in an average of 49,638.580 ± 1172.446 reads per sample for analysis. Reads mapped to 35,894 gene IDs in the Atlantic salmon genome, of which 32,986 had corresponding protein IDs. Of these protein IDs, 29,392 had corresponding annotation against the Atlantic salmon genome and 27,987 of these were mapped to a human gene identifier (HGNC).

3.3. Higher mRNA expression from immune genes in fish reared in FW LOCH compared to RAS

We first considered the effect of FW rearing history on mRNA expression in unstimulated (control) fish 2-weeks post-SWT. LOCH was

Table 1
Fish length, weight and condition factor (k) at the time of sampling (n = 6 fish, means ± SD). Fork length was measured in millimetres and weight in grams. Lack of significant differences in metrics between groups were confirmed by ANOVA.

System	Treatment	Weight (g)	Length (mm)	k	n
RAS	**Control**	96.1 ± 19.8	200.7 ± 12.2	1.10 ± 0.07	6
RAS	**Poly I:C**	88.7 ± 14.1	200.0 ± 10.1	1.10 ± 0.03	6
LOCH	**Control**	116.8 ± 28.2	208.5 ± 21.7	1.09 ± 0.07	6
LOCH	**Poly I:C**	96.3 ± 14.0	203.2 ± 12.0	1.07 ± 0.06	6

the baseline in the model, thus genes showing higher mRNA expression in RAS than LOCH have $\log_2FC > 1$ while those with lower expression in RAS (i.e. higher expression in LOCH) have $\log_2FC < -1$. In control fish, 596 differentially expressed genes (DEGs) were identified between RAS and LOCH reared fish. The majority of DEGs had higher mRNA expression in LOCH reared fish (n = 466, 78.2 %) than in RAS reared fish, with the remainder (n = 130, 21.8 %) having higher expression in RAS fish (Fig. 2). The full list of DEGs is presented in Table S1.

To understand the potential functional implications of rearing history on performance post-transfer to SW, gene ontology analysis (DAVID) was conducted using DEGs between unstimulated LOCH and RAS fish 2-weeks after transfer to the same SW facility. Considering only genes showing higher mRNA expression in LOCH reared fish compared to those from RAS (n = 466, 78.2 % of all DEGs), 6 enriched biological processes were identified at FDR <0.05 (Fig. 3, Table S2). The identified pathways were related to the immune response, both innate and adaptive immunity, including T cell activation and inflammatory response.

The annotated *S. salar* gene IDs which mapped to HGNCs associated with the immune-related GO terms 'T cell activation', 'innate immune response', 'inflammatory response', and 'adaptive immune response' are presented in Tables 2–5, respectively. In all tables genes are ordered by fold change and In the case of 'innate immune response' and 'inflammatory response' only the top 20 genes are tabulated.

For further investigation of enriched canonical pathways we utilised Ingenuity Pathway Analysis (IPA) that includes the magnitude of expression. IPA revealed 19 canonical pathways which were enriched in fish from a LOCH background compard to those reared in RAS. The full list of pathways is in Table S3. IPA also identified 84 downstream diseases or functions that differed significantly based on the differentially expressed genes between RAS or LOCH backgrounds. Of these 80 (95.2 %) diseases or functions were deemed decreased while the remaining 4 (4.8 %) were increased in RAS compared to LOCH fish and were defined as 'Parasitic Infection', 'Infection of mammalia' 'Organismal death' and 'Quantity of cytokine'. The full list of pathways is presented in Table S4. For decreased function we find 'Immune Cell Trafficking', 'Cell-To-Cell Signaling and Interaction' and 'Inflammatory Response' were all

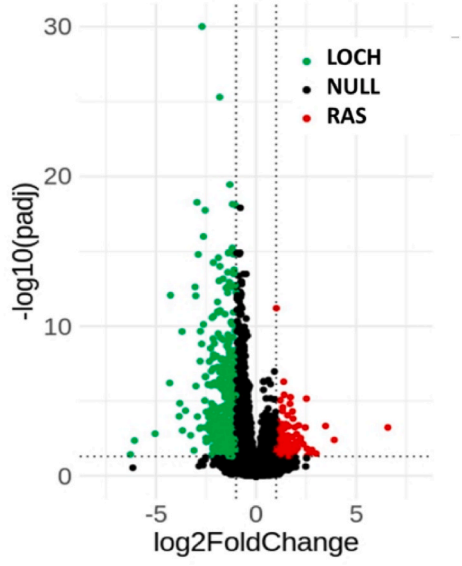

Fig. 2. Differential gene expression in relation to FW history in control fish 2-weeks post-SWT. Volcano plot showing DEGs with higher mRNA expression in LOCH (green) or RAS (red) reared fish 2-weeks post-transfer to seawater. Genes were considered differentially expressed when $\log_2FC > 1$ or < -1 and adjp <0.05. LOCH was the baseline in the model, thus genes more highly expressed in RAS than LOCH have $\log_2FC > 1$ while those with lower expression in RAS (i.e. higher expression in LOCH) have $\log_2FC < -1$. Genes in black were not considered to have differential mRNA expression levels.

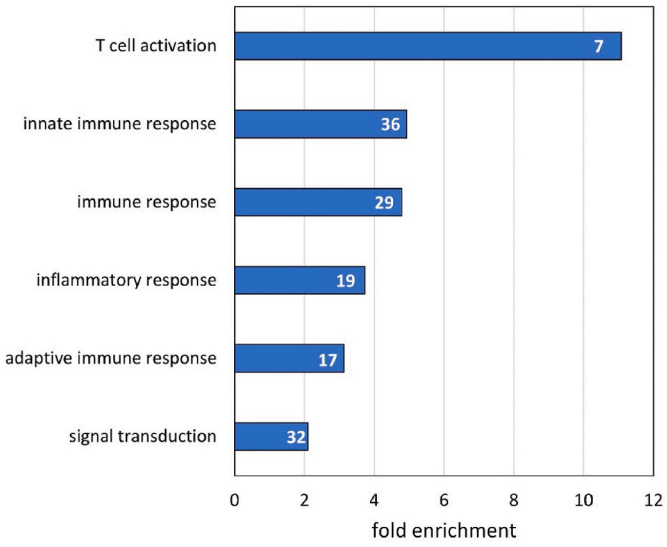

Fig. 3. Gene ontology analysis of gnes with higher mRNA expression in LOCH reared fish. DAVID bioloical processes differing between RAS and LOCH unstimulated groups post-SWT. Considering only differentially expressed genes (DEGs) which had higher expression in LOCH than in RAS, 253 HGNCs were accepted as a gene list against a *Homo sapiens* background. The number of input DEGs involved in each pathway is displayed at the end of each column. BP_DIRECT and FDR <0.05. For genes related to each term see Table S2.

potential outcomes driven by the gene sets. IPA also identified 183 upstream regulators with z-scores indicative of activation or inhibition of which 55 (30.1 %) were deemed to be in a state of activation while the remaining 128 (69.9 %) were inhibitive in RAS compared to LOCH fish. The full list of regulators is in Table S5. Cytokine mediators were prevalent in terms of predicted upstream regulators and constituted 20.3 % (n = 26) of inhibited regulators compared to just 1.8 % (n = 1) of activated.

3.4. Higher induction of immune gene transcription in RAS compared to LOCH reared fish following poly I:C stimulation

Following poly I:C stimulation a strong antiviral response was obtained in both RAS and LOCH reared fish in comparison to their respective unstimulated time-matched controls. We find 2035 and 1870 genes with modulated mRNA expression in RAS and LOCH reared fish respectively (full lists are available in Tables S6 and S7). The majority of DEGs were up-regulated in both groups (Fig. 4A) with 1552 up-regulated and 483 down-regulated in RAS-reared fish, and 1425 up-regulated and 445 down-regulated in LOCH-reared fish and more than 70 % of up-regulated DEGs in each system shared between RAS (72.4 %) and LOCH (78.8 %) reared fish (Fig. 4B). In the case of genes with down-regulated mRNA expression, a higher proportion of DEGs were specific to RAS or LOCH systems with only 41.2 % of RAS and 44.7 % of LOCH down-regulated DEGs shared between fish from the two FW backgrounds. The greatest difference between the four groups relates to immune stimulation by poly I:C with a lesser difference due to FW rearing environment (Fig. 4C). Principal component 1 (PC1) explained 73 % of the variance and separated samples by treatment (control vs poly I:C stimulated) while PC2 explained 6 % of the variance and separated samples by rearing history (RAS vs LOCH).

We next compared the magnitude of response of overlapping genes with significantly up- (n = 1123) or down-regulated (n = 199) mRNA expression between RAS and LOCH fish following poly I:C stimulation. The complete list of genes with a $\log_2FC > 1$ or < -1 difference between the systems is provided in Table S8. This is equivalent to a response to poly I:C stimulation of either double or half in fish from the two FW systems. Of the DEGs identified, mRNA expression was significantly up-

Table 2
Annotated DEGs associated with DAVID GO term 'T cell activation'. DEGs are ordered by log_2 fold change (log_2FC) with LOCH fish as the reference level and an adjusted p-value (padj) cutoff of $q < 0.05$ was applied. Gene IDs refer to v3 of the Atlantic salmon genome.

Gene ID	*Salmo salar* description	HGNC	log_2 FC	padj
ENSSSAG00000093109	cytotoxic T-lymphocyte protein 4	CD28	−1.77	2.30E-04
ENSSSAG00000114820	protein NLRC3	NLRC3	−1.71	8.69E-03
ENSSSAG00000099198	protein NLRC3	NLRC3	−1.61	4.86E-02
ENSSSAG00000009083	interferon regulatory factor 4	IRF4	−1.40	2.30E-05
ENSSSAG00000082171	tumor necrosis factor ligand superfamily member 14	TNFSF14	−1.33	1.11E-07
ENSSSAG00000045680	CD8 beta	CD8B	−1.26	8.26E-04
ENSSSAG00000065860	CD8 alpha	CD8A	−1.14	8.42E-04
ENSSSAG00000005984	phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit delta	PIK3CD	−1.12	1.58E-15
ENSSSAG00000039730	Interferon regulatory factor 4	IRF4	−1.05	1.47E-02
ENSSSAG00000100127	tumor necrosis factor ligand superfamily member 14	TNFSF14	−1.03	5.55E-04
ENSSSAG00000001991	interferon regulatory factor 4	IRF4	−1.01	3.39E-07

regulated in fish from both systems in 115 cases (90 higher in RAS / 25 higher in LOCH), down-regulated in both systems in 3 cases (2 higher in RAS / 1 higher in LOCH) and a single mRNA increased in expression in RAS reared fish but decreased in those reared in the LOCH (ENSSSAG00000006498; interleukin 8). The top 20 annotated genes with the largest mRNA expression differences in response to poly I:C (larger magnitude of response in RAS fish) are presented in Table 6 along with all of those with a larger magnitude of response in LOCH than RAS (n = 14), all genes with down-regulated mRNA expression (n = 3) and the single gene in which mRNA expression was modulated in opposite

Table 3
Annotated DEGs associated with DAVID GO term 'Innate immune response'. DEGs are ordered by log_2 fold change (log_2FC) with LOCH fish as the reference level and an adjusted p-value (padj) cutoff of $q < 0.05$ was applied. Gene IDs refer to v3 of the Atlantic salmon genome.

Gene ID	*Salmo salar* description	HGNC	log_2 FC	padj
ENSSSAG00000028365	pentraxin	APCS	−3.10	2.00E-02
ENSSSAG00000096170	interferon-induced GTP-binding protein Mx	MX1	−2.95	5.37E-19
ENSSSAG00000043705	complement C8 beta chain	C8B	−2.38	1.17E-04
ENSSSAG00000116533	tripartite motif-containing protein 16	TRIM25	−2.34	3.28E-04
ENSSSAG00000119033	tripartite motif-containing protein 47	TRIM25	−2.22	3.92E-02
ENSSSAG00000051905	interferon-induced GTP-binding protein Mx	MX1	−2.14	1.30E-08
ENSSSAG00000108840	radical S-adenosyl methionine domain containing 2	RSAD2	−1.95	1.08E-02
ENSSSAG00000109260	tripartite motif-containing protein 47	TRIM25	−1.91	1.70E-03
ENSSSAG00000110590	nuclear pore complex protein Nup214	TRIM25	−1.86	4.00E-05
ENSSSAG00000076982	nuclear factor 7, brain	TRIM35	−1.82	1.24E-03
ENSSSAG00000071290	src-like-adapter	SLA	−1.78	8.22E-04
ENSSSAG00000064417	nuclear factor 7, ovary	TRIM29	−1.78	3.06E-04
ENSSSAG00000042324	toll-like receptor 7	TLR7	−1.69	1.52E-08
ENSSSAG00000077530	myxovirus resistance 2	MX1	−1.54	9.87E-05
ENSSSAG00000086690	Ig heavy chain Mem5	IGLL1	−1.49	4.56E-05
ENSSSAG00000085879	tripartite motif-containing protein 16	TRIM25	−1.44	3.51E-05
ENSSSAG00000048067	Ribonuclease T2	RNASET2	−1.43	2.40E-13
ENSSSAG00000081800	tripartite motif-containing protein 16	TRIM25	−1.42	2.38E-13
ENSSSAG00000063817	E3 ubiquitin-protein ligase TRIM39	TRIM25	−1.42	6.49E-07
ENSSSAG00000079026	interferon-induced protein with tetratricopeptide repeats 5	IFIT5	−1.37	3.39E-04

Table 4
Annotated DEGs associated with DAVID GO term 'Inflammatory response'. DEGs are ordered by log_2 fold change (log_2FC) with LOCH fish as the reference level and an adjusted p-value (padj) cutoff of $q < 0.05$ was applied. Gene IDs refer to v3 of the Atlantic salmon genome.

Gene ID	*Salmo salar* description	HGNC	log_2 FC	padj
ENSSSAG00000078145	eotaxin	CCL11	−2.24	1.00E-07
ENSSSAG00000066280	C–C motif chemokine 20	CCL13	−2.14	6.55E-08
ENSSSAG00000006380	probable polyketide synthase 1	FASN	−2.13	7.73E-09
ENSSSAG00000071823	C–C motif chemokine 19	XCL2	−2.12	2.16E-11
ENSSSAG00000042324	toll-like receptor 7	TLR7	−1.69	1.52E-08
ENSSSAG00000118511	C–C motif chemokine 13	CCL13	−1.64	3.39E-04
ENSSSAG00000065312	tumor necrosis factor alpha-1 precursor	TNF	−1.47	3.71E-02
ENSSSAG00000075631	galectin-9	LGALS9	−1.42	2.52E-14
ENSSSAG00000003774	C–C motif chemokine 4	XCL2	−1.35	1.31E-09
ENSSSAG00000075658	chemokine (C motif) receptor 1b, duplicate 1	XCR1	−1.33	7.04E-08
ENSSSAG00000066649	toll-like receptor 22	TLR1	−1.29	7.65E-03
ENSSSAG00000108488	toll-like receptor 21	TLR1	−1.26	9.86E-09
ENSSSAG00000115800	C-X-C chemokine receptor type 3	CXCR3	−1.26	8.42E-04
ENSSSAG00000072987	nitric oxide synthase 2	NOS2	−1.25	1.71E-02
ENSSSAG00000042309	TLR8	TLR7	−1.23	4.95E-11
ENSSSAG00000003152	meprin A subunit beta	MEP1B	−1.17	2.02E-06
ENSSSAG00000023874	B-cell linker	BLNK	−1.15	2.36E-07
ENSSSAG00000007182	phospholipase D4	PLD4	−1.13	1.20E-11
ENSSSAG00000005984	phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit delta	PIK3CD	−1.12	1.58E-15
ENSSSAG00000103354	chemokine XC receptor 1	XCR1	−1.11	2.63E-02

Table 5
Annotated DEGs associated with DAVID GO term 'Adaptive immune response'. DEGs are ordered by log_2 fold change (log_2FC) with LOCH fish as the reference level and an adjusted p-value (padj) cutoff of $q < 0.05$ was applied. Gene IDs refer to v3 of the Atlantic salmon genome.

Gene ID	*Salmo salar* description	HGNC	log_2 FC	padj
ENSSSAG00000114455	eomesodermin	EOMES	−1.83	3.04E-02
ENSSSAG00000049121	cytotoxic and regulatory T-cell molecule	CRTAM	−1.80	8.76E-12
ENSSSAG00000039883	B- and T-lymphocyte attenuator	BTLA	−1.50	1.37E-02
ENSSSAG00000068877	cytotoxic and regulatory T cell molecule	CRTAM	−1.36	2.43E-05
ENSSSAG00000045680	CD8 beta	CD8B	−1.26	8.26E-04
ENSSSAG00000048537	Lck interacting transmembrane adaptor 1	LIME1	−1.14	6.18E-08
ENSSSAG00000005984	phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit delta	PIK3CD	−1.12	1.58E-15
ENSSSAG00000099702	major histocompatibility complex class I-related gene protein	HLA-C	−1.11	2.44E-02
ENSSSAG00000009995	*Salmo salar* CD3 gammadelta-B (LOC100137057), mRNA	CD3D	−1.08	1.11E-07
ENSSSAG00000084854	T-cell receptor alpha/delta variable 31.0	TRAV18	−1.06	3.68E-04
ENSSSAG00000007543	rap1 GTPase-activating protein 2	RAP1GAP	−1.05	5.94E-04
ENSSSAG00000085531	H-2 class II histocompatibility antigen, A-Q alpha chain	HLA-DQA1	−1.02	5.19E-06

directions in fish from the two rearing histories.

We also compared the magnitude of response for genes which were only defined as having differential mRNA expression in fish reared in one system or the other (Tables S9 and S10). Of the 429 genes with mRNA expression uniquely up-regulated in fish from the RAS system, 162 were also significantly up-regulated in LOCH, but did not reach the log_2FC of >1 cut-off to be defined as a DEG. Of the 302 genes with mRNA expression uniquely up-regulated in the LOCH system, 109 were significantly up-regulated in RAS, but did not reach the log_2FC of >1 to be defined as a DEG. Of the 284 genes with mRNA expression uniquely down-regulated in fish from the RAS system, 94 were also significantly down-regulated in LOCH, but did not reach the log_2FC of < −1 cut-off to be defined as a DEG. Of the 246 genes with mRNA expression uniquely down-regulated in the LOCH system, 90 were significantly down-

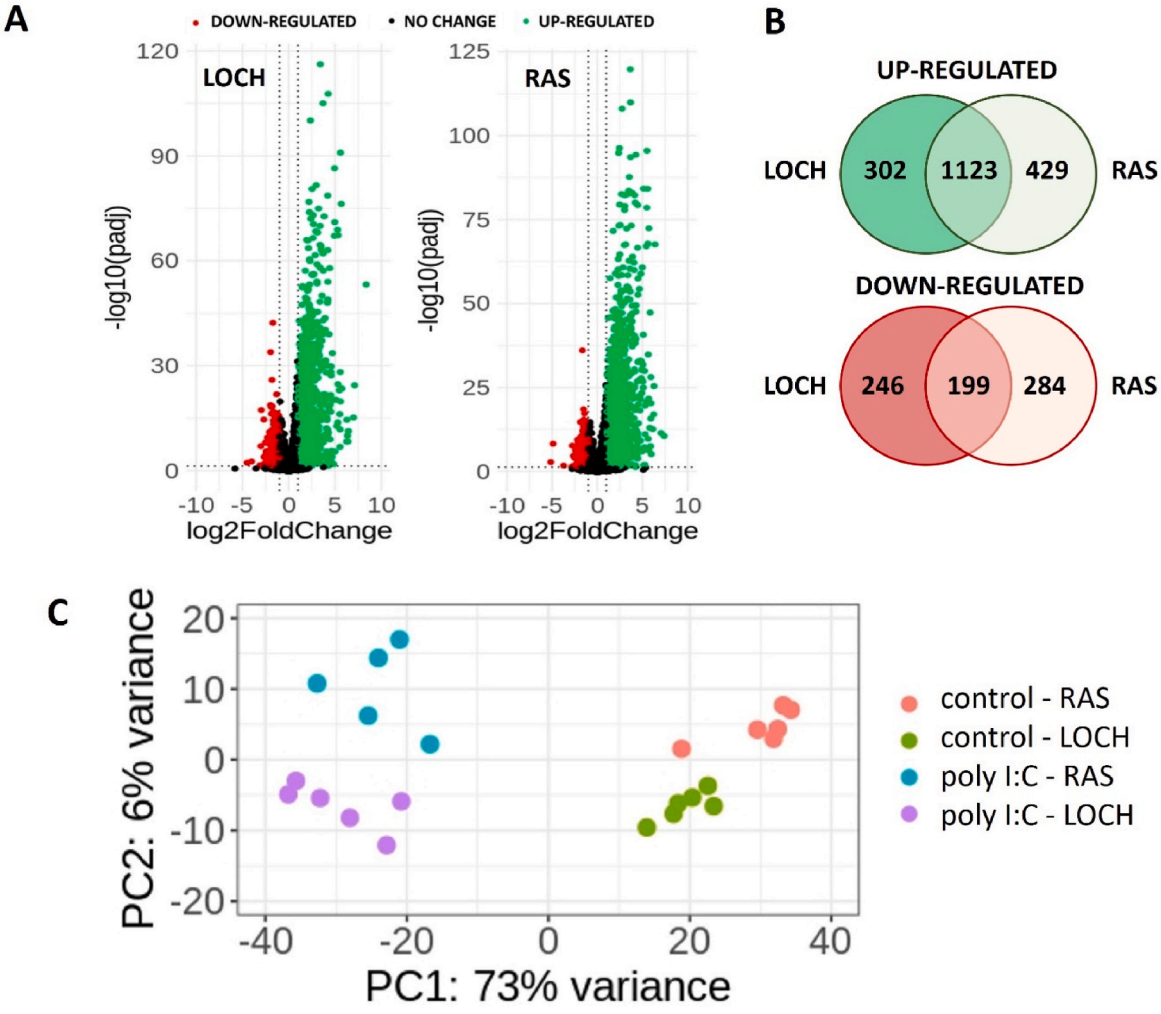

Fig. 4. Differential gene expression in resposne to poly I:C stimulation. (A) Volcano plots showing genes with down (red) and up (green) regulated mRNA expression in response to poly I:C stimulation in fish reared in FW LOCH or RAS. mRNAs were considered differentially expressed if $log_2FC > 1$ or < -1 and adjp <0.05. **(B)** Venn diagram showing unique and shared differentially expressed genes (DEGs) in response to stimulation with poly I:C in fish reared in FW LOCH or RAS systems **(C)** PCA depicting separation between the four treatment groups.

Table 6
Differentially expressed genes which differ in size of mRNA expression response to poly I:C stimulation between fish reared in FW RAS and LOCH systems. Genes which were significantly differentially expressed in both RAS and LOCH reared fish were considered. mRNA expression was categorised as up-regulated, down-regulated or regulated in opposite directions. Genes with a difference in magnitude of response in excess of $\log_2$ fold change (FC) > 1 or < −1 are presented in Table S8 and the annotated genes are presented here.

Gene ID	*Salmo salar* description	HGNC	RAS poly I:C $\log_2$ FC	LOCH poly I:C $\log_2$ FC	FC difference
UP-REGULATED					
ENSSSAG00000053028	Hepcidin-1	hamp1	5.63	3.15	5.58
ENSSSAG00000045029	MARCKS-related protein	MARCKSL1	5.25	3.08	4.48
ENSSSAG00000046729	basic leucine zipper transcriptional factor ATF	BATF	3.81	1.74	4.19
ENSSSAG00000045448	interleukin-1 beta	IL1B	5.30	3.34	3.90
ENSSSAG00000006380	probable polyketide synthase 1	FASN	3.40	1.44	3.89
ENSSSAG00000083879	E3 ubiquitin/ISG15 ligase TRIM25	TRIM25	4.02	2.15	3.67
ENSSSAG00000086219	tripartite motif-containing protein 47	TRIM25	4.39	2.52	3.67
ENSSSAG00000042607	septin-7	SEPTIN7	2.83	1.19	3.12
ENSSSAG00000121058	tripartite motif-containing protein 16	TRIM39	3.86	2.23	3.08
ENSSSAG00000104176	G protein-coupled receptor 84	GPR84	3.21	1.60	3.06
ENSSSAG00000009083	interferon regulatory factor 4	IRF4	2.95	1.36	3.03
ENSSSAG00000041661	E3 ubiquitin-protein ligase RNF138	RNF138	2.79	1.20	3.01
ENSSSAG00000089459	CUB and zona pellucida-like domain-containing protein 1	0	3.64	2.07	2.97
ENSSSAG00000108840	radical S-adenosyl methionine domain containing 2	RSAD2	5.68	4.11	2.97
ENSSSAG00000090282	putative inactive phenolphthiocerol synthesis polyketide synthase type I Pks15	FASN	3.20	1.66	2.91
ENSSSAG00000120145	tripartite motif-containing protein 16	TRIM47	2.63	1.09	2.90
ENSSSAG00000015898	tripartite motif-containing protein 16	TRIM27	3.26	1.73	2.88
ENSSSAG00000066307	regulator of G-protein signalling 5	RGS5	2.92	1.48	2.73
ENSSSAG00000007225	tripartite motif-containing protein 16	TRIM25	3.52	2.07	2.72
ENSSSAG00000056094	interleukin-17F	0	2.60	1.21	2.62
ENSSSAG00000062279	tumor necrosis factor alpha-induced protein 2	TNFAIP2	4.23	2.86	2.59
ENSSSAG00000091114	retinol dehydrogenase 10	RDH10	2.24	3.24	−2.01
ENSSSAG00000000454	stromal cell-derived factor 1-like	CXCL5	1.33	2.45	−2.18
ENSSSAG00000066359	sal-like protein 4	SALL2	2.24	3.39	−2.23
ENSSSAG00000106205	G protein-coupled receptor 101	GPR101	1.66	2.85	−2.27
ENSSSAG00000088069	*Salmo salar* interferon alpha 2 (ifna2), mRNA	IFNA2	4.31	5.51	−2.30
ENSSSAG00000048752	RUN domain-containing protein 3B	RUNDC3B	1.49	2.73	−2.36
ENSSSAG00000069384	T-cell immunoglobulin and mucin domain-containing protein 4	TIMD4	1.76	3.08	−2.49
ENSSSAG00000121489	E3 ISG15—protein ligase HERC5	HECT	4.98	6.45	−2.77
ENSSSAG00000032651	leucine-rich repeat-containing protein 4	LRRC4	2.33	3.87	−2.91
ENSSSAG00000000935	leucine-rich repeat transmembrane protein FLRT3	FLRT3	1.48	3.23	−3.36
ENSSSAG00000109065	leucine-rich repeat-containing protein 4	LRRC4	2.48	4.36	−3.66
ENSSSAG00000095442	neuritin	NRN1	6.39	8.37	−3.95
ENSSSAG00000063668	E3 ISG15—protein ligase HERC5	HERC3	4.86	7.00	−4.39
ENSSSAG00000085838	*Salmo salar* Interferon-induced transmembrane protein 5 (ifm5), mRNA	IFITM3	1.77	4.09	−4.99
DOWN-REGULATED					
ENSSSAG00000112326	short stature homeobox 2	SHOX2	−1.41	−3.00	3.00
ENSSSAG00000075896	iroquois-class homeodomain protein irx-3	IRX3	−1.29	−2.49	2.31
ENSSSAG00000079738	protein phosphatase Slingshot homolog 1	SSH1	−2.11	−1.08	−2.04
OPPOSITE DIRECTIONS					
ENSSSAG00000006498	interleukin 8	CXCL6	1.52	−1.65	9.00

regulated in RAS, but did not reach the $\log_2$FC of < −1 to be defined as a DEG. The genes with up-regulation of mRNA expression unique to RAS fish were widely immune related.

To examine the transcriptome response in terms of gene set enrichment and functional processes (gene ontology using DAVID) following poly I:C stimulation in the gill, DEGs revealed 94 and 66 GO terms in RAS and LOCH fish, respectively. Of these, 58 GO terms were shared between fish from the two rearing histories while 36 were unique to RAS and 8 unique to LOCH fish. In fish from both FW rearing backgrounds, there was a clear association with immune response pathways (Fig. 5). RAS and LOCH fish shared eight of their top 10 pathways and the remaining two in each case were also significant in the other rearing group. Fold enrichment of top GO terms were similar between fish from different rearing histories with the exception of 'response to cytokine' (GO:0034097; 8.9 fold enriched (FE) in RAS/7.2 FE in LOCH). Of the 36 significant GO terms unique to RAS reared fish, one third were related to immunity or defence including 'positive regulation of interleukin-2 production' and 'tumor necrosis factor-mediated signalling pathway', while in the 8 terms unique to LOCH reared fish, only two were related to immunity – 'cellular response to interleukin-6' and 'positive regulation of chemokine production' (Tables S11 and S12).

Using the HGNC identifiers associated with the DAVID GO term 'defence response to virus' (RAS n = 46/LOCH n = 49; 45 overlapping/1 RAS unique/4 LOCH unique) and using these to extract all genes with differential mRNA expression in RAS and LOCH systems in response to poly I:C stimulation, a total of 170 gene IDs were extracted. Of these 156 had differential mRNA expression in response to poly I:C stimulation in RAS and 152 in LOCH (n = 138 overlapping/18 RAS unique/14 LOCH unique). The difference in $\log_2$FCs were calculated; 44 DEGs showed a greater magnitude of response to poly I:C in LOCH reared fish than in RAS reared fish while the opposite was true for the remaining 126 DEGs. Genes with a $\log_2$ fold change difference in magnitude of transcriptomic response to poly I:C between systems of at least 1 are shown in Table 7.

The same process was carried out for the term 'response to cytokine'. 14 HGNCs were common to RAS and LOCH while 4 were unique to RAS (none unique to LOCH). HGNCs mapped to 36 RAS genes and 26 LOCH

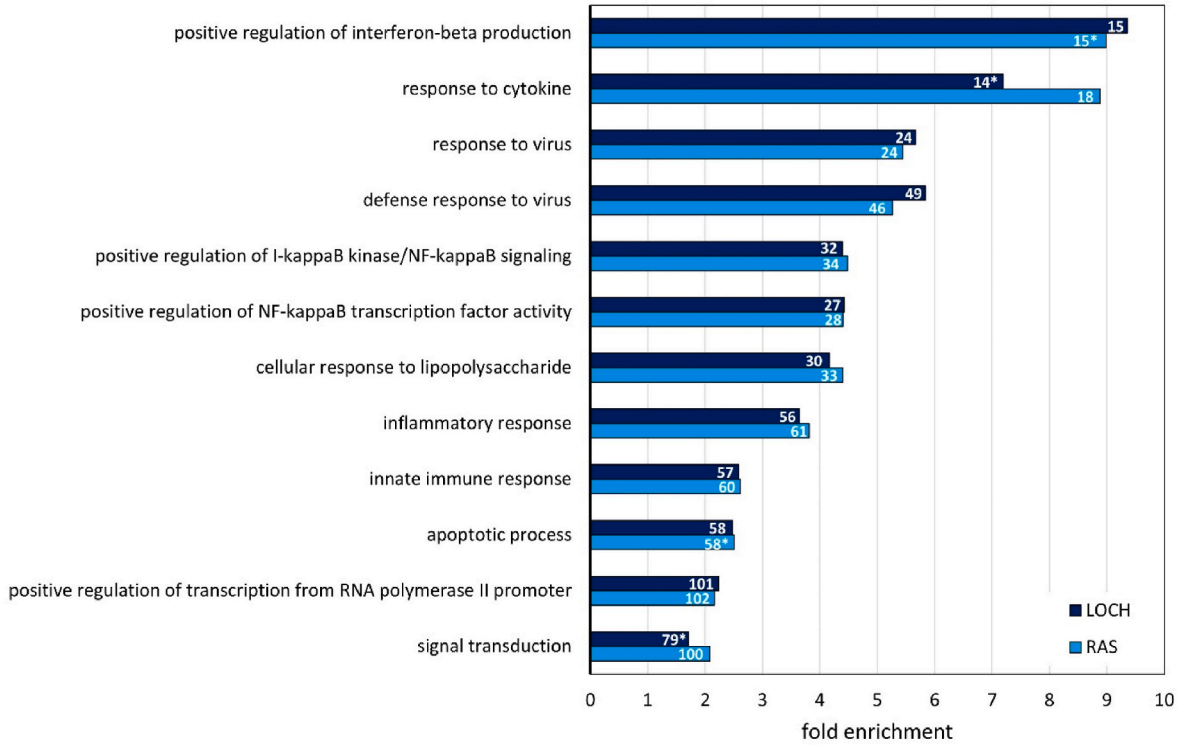

Fig. 5. Gene ontology analysis of genes with up-regulated mRNA expression in response to poly I:C stimulation. Top 15 GO BP terms differing between stimulated and control groups in RAS and LOCH reared fish. The number of input DEGs involved in each pathway is displayed at the end of each column. Asterix indicates not in the top 10 of both systems, but significantly enriched in both. For lists of genes involved in each term see Tables S11 and S12.

Table 7
Genes associated with the GO term 'defence response to virus' with mRNA response to poly I:C stimulation differing by more than $log_2FC = \pm 1$ between RAS and LOCH reared fish. Only genes with a log_2 fold change (FC) difference >1 or < −1 between fish reared in FW RAS and LOCH systems are presented. Cells in grey were not significantly differentially expressed in response to poly I:C stimulation in that system.

Gene ID	*Salmo salar* description	HGNC	RAS poly I:C log_2 FC	LOCH poly I:C log_2 FC	FC difference
ENSSSAG00000031095	aconitate decarboxylase 1	ACOD1	4.12	1.43	6.46
ENSSSAG00000089765	0	TRIM25	2.77	0.78	3.97
ENSSSAG00000083879	E3 ubiquitin/ISG15 ligase TRIM25	TRIM25	4.02	2.15	3.67
ENSSSAG00000086219	tripartite motif-containing protein 47	TRIM25	4.39	2.52	3.67
ENSSSAG00000118289	tripartite motif-containing protein 16	TRIM22	2.03	0.42	3.06
ENSSSAG00000108840	radical S-adenosyl methionine domain containing 2	RSAD2	5.68	4.11	2.97
ENSSSAG00000015795	DNA damage-inducible transcript 4 protein	DDIT4	1.31	−0.18	2.81
ENSSSAG00000007225	tripartite motif-containing protein 16	TRIM25	3.52	2.07	2.72
ENSSSAG00000005439	perforin-1	PRF1	1.34	−0.05	2.62
ENSSSAG00000115299	tripartite motif-containing protein 47	TRIM25	3.30	2.01	2.44
ENSSSAG00000119033	tripartite motif-containing protein 47	TRIM25	2.53	1.27	2.38
ENSSSAG00000122227	tripartite motif-containing protein 47	TRIM25	3.42	2.24	2.27
ENSSSAG00000037858	probable ATP-dependent RNA helicase DHX58	DHX58	5.86	4.71	2.21
ENSSSAG00000099596	0	TRIM25	2.73	1.58	2.21
ENSSSAG00000113770	tripartite motif-containing protein 16	TRIM25	3.46	2.35	2.16
ENSSSAG00000107978	0	TRIM25	2.32	1.28	2.06
ENSSSAG00000038498	interferon-induced protein 44	IFI44L	3.18	2.17	2.02
ENSSSAG00000095160	0	TRIM25	0.55	1.55	−2.00
ENSSSAG00000087907	tripartite motif-containing protein 16	TRIM25	1.02	2.16	−2.20
ENSSSAG00000088069	*Salmo salar* interferon alpha 2 (ifna2), mRNA	IFNA2	4.31	5.51	−2.30
ENSSSAG00000108853	0	TRIM25	1.52	3.34	−3.52
ENSSSAG00000085838	*Salmo salar* Interferon-induced transmembrane protein 5 (ifm5), mRNA	IFITM3	1.77	4.09	−4.99

genes (n = 26 overlapping/n = 10 unique to RAS). Genes with a log_2 fold change difference in magnitude of mRNA response to poly I:C between systems of at least 1 are shown in Table 8. In this case, all DEGs showed a greater magnitude of response to poly I:C in fish reared in RAS compared to LOCH.

For the term 'inflammatory response', 47 HGNCs overlapped between RAS and LOCH while 14 were RAS specific and 9 LOCH specific. HGNCs mapped to a total of 112 genes (96 RAS/86 LOCH). Of these, 70 genes had differential mRNA expression in both RAS and LOCH reared fish while 26 were unique to RAS and 16 unique to LOCH. Genes with a log_2 fold change difference in magnitude of mRNA response to poly I:C between systems of at least 1 are shown in Table 9.

Table 8
Genes associated with the GO term 'response to cytokine' with mRNA response to poly I:C stimulation differing by more than $log_2FC = \pm 1$ between RAS and LOCH reared fish. Only genes with a log_2 fold change (FC) difference >1 or < −1 between fish reared in FW RAS and LOCH systems are presented. Cells in grey were not significantly differentially expressed in response to poly I:C stimulation in that system.

Gene ID	*Salmo salar* description	HGNC	RAS poly I:C log_2 FC	LOCH poly I:C log_2 FC	FC difference
ENSSSAG00000067000	P-selectin	SELE	1.51	−0.27	3.44
ENSSSAG00000046644	proto-oncogene c-Fos	FOS	1.38	−0.08	2.75
ENSSSAG00000045082	Programmed cell death 1 ligand 1	CD274	3.47	2.25	2.33
ENSSSAG00000004552	retinal dehydrogenase 2	ALDH1A2	2.23	1.02	2.32
ENSSSAG00000085238	tumor necrosis factor receptor superfamily member 9-like	TNFRSF11A	4.13	2.96	2.25
ENSSSAG00000068892	nuclear factor of kappa light polypeptide gene enhancer in B-cells 2, p49/p100	NFKB2	2.27	1.13	2.21
ENSSSAG00000054260	transcription factor AP-1	JUN	1.77	0.70	2.11
ENSSSAG00000102030	proto-oncogene c-Rel	REL	1.81	0.80	2.01

Table 9
Genes associated with the GO term 'inflammatory response' with mRNA response to poly I:C stimulation differing by more than $log_2FC = \pm 1$ between RAS and LOCH reared fish. Only genes with a log_2 fold change (FC) difference >1 or < −1 between fish reared in FW RAS and LOCH systems are presented. Cells in grey were not significantly differentially expressed in response to poly I:C stimulation in that system.

Gene ID	*Salmo salar* description	HGNC	RAS poly I:C log_2 FC	LOCH poly I:C log_2 FC	FC difference
ENSSSAG00000006498	interleukin 8	CXCL6	1.52	−1.65	9.00
ENSSSAG00000065312	NA	TNF	2.22	−0.57	6.87
ENSSSAG00000031095	aconitate decarboxylase 1	ACOD1	4.12	1.43	6.46
ENSSSAG00000039686	0	C3	3.69	1.30	5.25
ENSSSAG00000045448	interleukin-1 beta	IL1B	5.30	3.34	3.90
ENSSSAG00000006380	probable polyketide synthase 1	FASN	3.40	1.44	3.89
ENSSSAG00000067000	P-selectin	SELE	1.51	−0.27	3.44
ENSSSAG00000108438	C-X-C chemokine receptor type 3	CXCR3	2.29	0.75	2.92
ENSSSAG00000090282	putative inactive phenolphthiocerol synthesis polyketide synthase type I Pks15	FASN	3.20	1.66	2.91
ENSSSAG00000046644	proto-oncogene c-Fos	FOS	1.38	−0.08	2.75
ENSSSAG00000051405	C–C motif chemokine 4	CCL4	3.55	2.24	2.48
ENSSSAG00000007227	prostaglandin E2 receptor EP1 subtype	PTGER1	1.14	−0.10	2.36
ENSSSAG00000072775	TNF alpha induced protein 3	TNFAIP3	2.45	1.34	2.16
ENSSSAG00000071823	C–C motif chemokine 19	XCL2	2.98	1.93	2.06
ENSSSAG00000102030	proto-oncogene c-Rel	REL	1.81	0.80	2.01
ENSSSAG00000000454	stromal cell-derived factor 1	CXCL5	1.33	2.45	−2.18
ENSSSAG00000088069	*Salmo salar* interferon alpha 2 (ifna2), mRNA	IFNA2	4.31	5.51	−2.30
ENSSSAG00000040467	calcium/calmodulin-dependent protein kinase type IV	CAMK4	−0.91	1.97	−7.38

3.5. RAS reared fish mount a relatively stronger transcriptomic immune response to poly I:C stimulation than LOCH reared fish

A total of 119 genes displayed differential mRNA expression in response to poly I:C stimulation in the gills of both RAS and LOCH fish, but differed in magnitude of mRNA induction by log_2 FC > 1 or < −1 (Table S8). Of these, 34 genes also showed significant differential mRNA expression between RAS and LOCH fish prior to stimulation (Table S13). Only 19 of these genes were annotated against the *S. salar* genome and are presented in Table 10. With the exception of neuritin (ENSSSAG00000095442), all mRNAs were expressed at a lower level in RAS reared fish compared to LOCH prior to stimulation but had a higher magnitude of response to poly I:C stimulation in RAS fish compared to LOCH post-stimulation. The opposite was true for neuritin. There was no correlation between the differential expression in the two comparisons.

4. Discussion

In this study we assessed the impact of freshwater rearing history on the transcriptomic response in the Atlantic salmon gill to stimulation with the viral mimic poly I:C, two weeks post-transfer to seawater. In unstimulated fish several immune-related genes displayed differential mRNA expression between fish reared in RAS or LOCH environments and functional prediction indicated immune suppression in those fish reared in RAS compared to those reared in the open LOCH environment. Fish from both systems mounted a strong transcriptomic immune response following stimulation with poly I:C as expected, highlighting no serious immune disfunction due to the rearing environment. However, the intensity of the response to stimulation at the measured timepoint was stronger in RAS reared fish compared to those from the LOCH. A subset of genes which had a greater magnitude of induction of mRNA expression by poly I:C in RAS fish were inversely transcribed at a lower level prior to stimulation in RAS fish compared to those reared in the LOCH. Most of these genes are involved in aspects of immune function. It may be that fish reared in RAS have a reduced (or suppressed) immune system in terms of baseline mRNA expression, but that they are able to compensate by essentially 'catching up' to their LOCH reared counterparts by mounting a larger response when faced with a pathogen. Alternatively, those in the LOCH environment have higher steady state immune function and are more prepared for immunological insult. However, such amplification of the immune response as seen in the RAS reared fish could also be an over-reaction to viral stimulation and would be energetically costly, likely to divert resources away from other important physiological processes at this vital life history stage such as osmoregulation and growth. Due to the single sampling point nature of this study, further work is required to monitor any temporal patterns of suppression or induction.

4.1. Rearing history impacts immune gene transcription post-SWT

At two-weeks post-transfer to SW, we identified a large differentially expressed gene set in smolts reared in RAS or LOCH systems in FW. Gene set enrichment by gene ontology revealed these DEGs to be related to both innate and adaptive immune response pathways including the

Table 10
Genes with significant differential mRNA expression between RAS and LOCH reared fish and also with a significant response to poly I:C stimulation differing by more than log_2 fold change (FC) > ± 1 between RAS and LOCH reared fish. Control system log_2 FC < −1 indicates lower mRNA expression in RAS than in LOCH reared fish. Only genes with annotation against the *S. salar* genome are presented. For the full list see Table S13.

Gene ID	*Salmo salar* description	HGNC	control system log_2 FC	RAS poly I:C log_2 FC	LOCH poly I:C log_2 FC	FC difference
ENSSSAG00000045029	MARCKS-related protein	MARCKSL1	−1.40	5.25	3.08	4.48
ENSSSAG00000046729	basic leucine zipper transcriptional factor ATF	BATF	−1.76	3.81	1.74	4.19
ENSSSAG00000006380	probable polyketide synthase 1	FASN	−2.13	3.40	1.44	3.89
ENSSSAG00000009083	interferon regulatory factor 4	IRF4	−1.40	2.95	1.36	3.03
ENSSSAG00000108840	radical S-adenosyl methionine domain containing 2	RSAD2	−1.95	5.68	4.11	2.97
ENSSSAG00000120145	tripartite motif-containing protein 16	TRIM47	−1.27	2.63	1.09	2.90
ENSSSAG00000056094	interleukin-17F	0	−2.56	2.60	1.21	2.62
ENSSSAG00000082955	calcium binding and coiled-coil domain 2	calcoco2	−2.49	4.16	2.92	2.36
ENSSSAG00000005181	sterile alpha motif domain-containing protein 9	SAMD9L	−1.85	3.62	2.45	2.25
ENSSSAG00000105945	TYRO protein tyrosine kinase-binding protein	TYROBP	−1.56	3.07	1.97	2.15
ENSSSAG00000073138	uncharacterized LOC106578729	EIF4G1	−1.64	3.48	2.40	2.12
ENSSSAG00000097252	poly [ADP-ribose] polymerase 11	PARP11	−1.55	4.98	3.92	2.09
ENSSSAG00000007886	cytidine/uridine monophosphate kinase 2	CMPK2	−1.15	5.10	4.04	2.08
ENSSSAG00000121778	polyubiquitin	UBB	−2.02	4.56	3.51	2.08
ENSSSAG00000062001	sterile alpha motif domain-containing protein 9	SAMD9	−1.75	3.96	2.91	2.08
ENSSSAG00000071823	C–C motif chemokine 19	XCL2	−2.12	2.98	1.93	2.06
ENSSSAG00000075036	galectin-3-binding protein A	LGALS3BP	−1.93	3.29	2.24	2.06
ENSSSAG00000073361	stathmin-3	STMN3	−1.23	2.85	1.80	2.06
ENSSSAG00000038498	interferon-induced protein 44	IFI44L	−2.21	3.18	2.17	2.02
ENSSSAG00000095442	neuritin	NRN1	1.84	6.39	8.37	−3.95

inflammatory response and T-cell activation. The unstimulated status of transcriptional activity of the fish two weeks following SWT indicate inherent differences in baseline levels of immunity in fish from the two rearing backgrounds following transfer to sea. This is in agreement with other studies on basal immune transcriptional activity following seawater transfer in immune tissues [20] and single cell sequencing of gill where a clear decrease in immune cell types was observed a short time post transfer [23].

Immune suppression occurs during the smoltification process in FW, but becomes further pronounced post-transfer to seawater [20,23,28]. The phenomenon has been shown independently, but only in cultured populations and not in the wild, so could potentially be a result of culture methods. It may seem counter-intuitive that immune function is suppressed post-transfer to the marine environment which is rich in potential pathogens. It has been suggested that the dampening of the immune response could function to avoid immune shock when transferring between environments with distinct pathogen profiles [23]. For the fish to prevent excessive undesired inflammation it may be that tolerance mechanisms are at play during this life history event, although this experimental set up is unable to address this hypothesis. One of the key hormones known to increase during smolting is cortisol which can act as an immune suppressor and is associated with stress. The hormone is also believed to play roles in controlling genes such as Na^+K^+-ATPase involved in osmoregulation [20]. Chronic stress is associated with elevated cortisol and can impact susceptibility to disease by suppression of the innate immune system [29]. Additionally, acute stress during embryogenesis enhanced the transcriptomic immune response to bacterial stimulation in Atlantic salmon fry while chronic stress suppressed it [30].

Two pro-inflammatory genes which differed in mRNA expression levels between unstimulated smolts from RAS and LOCH systems two weeks post-SWT, C–C motif chemokine 19-like (CCL19) and CCL4, were also suppressed in the gill of Atlantic salmon post-smolts 3-weeks after SWT in an independent study [20]. Ingenuity pathway analysis also identified immune genes as potential upstream regulators of the differentially expressed gene sets in unstimulated LOCH and RAS fish, for example, interferons and interleukins, as well as MAMPs such as lipopolysaccharide (a strong immunostimulant) and dexamethasone, an anti-inflammatory agent with the same function as cortisol. Breeding for rapid growth has been suggested to suppress immunity in Atlantic salmon [28] where energy allocation has been selected for somatic growth and not immune system. Accordingly, the artificial constant light regimes often used in RAS to stimulate smoltification could drive abnormal immunosuppression [23]. Constant light is known to have a distinct impact on mammalian immune defences [31], however, in smolts produced in RAS under either constant light or given a traditional signal and transferred to sea at different weights, no significant differences were identified in a panel of immune genes [32].

Despite the apparent gap in steady-state gill transcriptome-level immune status following SWT, no visible differences in smolt health were determined in this study. A study comparing performance and welfare indicators of smolts produced in RAS or FTS also found comparable survival rates post-SWT in fish reared in the two different systems [33]. The critical level of suppression of immune genes is not well understood in terms of influencing disease resistance or susceptibility. In a study in which expression from an immune multigene expression assay (MGE) was compared to a gill reference data set in an attempt to detect deviation from a 'normal' immune status, good immune status was determined in fish reared in both flow-through and RAS [24].

4.2. Stimulation with a viral PAMP induced transcriptomic immune responses in RAS and LOCH reared fish

To assess immune capacity in smolts reared in FW RAS or LOCH, fish were stimulated for 24 h with a viral mimic two-weeks after transfer to SW. In the gills of fish from both FW backgrounds, functional analysis revealed robust antiviral responses with similar gene set enrichment of functional pathways including defence response to virus, inflammatory response and innate immune response. The interferon response is considered as the primary antiviral defence system in fish and in other vertebrates [34]. mRNA expression levels of key interferon and interferon-stimulated genes including interferon inducible Mx protein (Mx), ISG15 ubiquitin-like modifier (ISG15), viperin (vip-2 or RSAD2), interferon alpha 2 (IFNa2), interferon regulatory factor 4-like (IRF4), interferon-inducible protein gig2 (gig2) and CCL19 were up-regulated in response to poly I:C smolts reared in both FW systems. A similar suite of antiviral genes (also termed Interferon Stimulated Genes (ISGs) [27]) varied between susceptible and resistant fish challenged with infectious pancreatic necrosis virus (IPNV), with the up-regulation of interferon-response genes more pronounced in susceptible fish [35]. Proinflammatory cytokines also play a role in antiviral defences [36] and interleukin-1 beta-like (IL-1β) was strongly induced in the gills of

fish reared in RAS and LOCH in response to poly I:C stimulation.

4.3. Immune system 'catch-up' in RAS-reared fish?

Despite strong immune responses being mounted in fish from both rearing backgrounds, many of the genes with up-regulated mRNA expression were more strongly induced in the gills of fish from one background or another and the functional pathway 'response to cytokine' showed a greater fold enrichment in smolts reared in RAS. A total of 115 genes had increased mRNA expression in response to viral stimulation in smolts from both systems showed a difference in induction of at least 2-fold between RAS and LOCH smolts with the majority (78.3 %) showing a greater magnitude of response in RAS fish compared to LOCH. This included antiviral and pro-inflammatory makers (for example, ISG15, RSAD2, IRF4, CCL4, CCL19, IL1β and IL17F). A subset of 34 mRNAs that differed in extent of viral induction were also differentially expressed between unstimulated fish reared in RAS and LOCH post-SWT. In all but one case, these genes had lower mRNA expression in fish reared in RAS compared to LOCH prior to viral stimulation, but following stimulation, a larger magnitude of response was identified in RAS-reared smolts compared to their LOCH counterparts.

The stronger response may be a result of excessive up-regulation of the immune response in RAS-reared smolts in response to a viral challenge. Induction of antiviral genes does not always result in successful eradication of a viral infection. In an infection with pilchard orthomyxovirus (POMV), strong up-regulation of IFNa, IFN-induced genes (Mx1-3, ISG15) and multiple pro-inflammatory cytokines and chemokines was not sufficient to suppress viral replication and mortality [37] and similar outcomes were found in ISAV-infected fish [38]. A comparison of smolts with low and high mortality determined a panel of inflammatory genes associated with high mortality [39]. An excessive inflammatory response, in combination with lack of control of the anti-inflammatory response, can result in so-called 'cytokine storm' which can amplify pathology during viral infections [40,41]. High cortisol levels are known to suppress innate immunity in salmonids [29]. In the gills of salmon injected with hydrocortisone to induced the stress- and smoltification-related hormone cortisol, antiviral Mx and ISG15 induction was delayed in comparison to non-hydrocortisone injected fish during infection with salmon gill poxvirus (SGPV), but then surged along with the viral peak, reaching far higher levels than in control fish [42].

Alternatively, it may be the case that the greater magnitude of transcriptional response during viral stimulation acts to compensate for the lowered baseline expression, effectively allowing RAS fish to 'catch-up' to their 'more natural environment' LOCH counterparts in terms of immune competence. This may be costly at a sensitive life history stage, potentially diverting resources away from other physiological processes including growth, metabolism or osmoregulation. The number of genes with down-regulated mRNA expression in the two systems mapped to HGNCs did not allow for a coherent functional analysis to be conducted, but a gene coding for fatty acid amide hydrolase-1-like (FAAH) had down-regulated mRNA expression in viral stimulation smolts reared in RAS. The immune system of fish reared in RAS compared to a cohort in FTS was described as having higher reactivity of the immune system three-weeks post-SWT, but this became comparable between cohort by three months in SW [24]. Fish that appear to perform equally and do not have visible health problems can have varying degrees of immune system competence [39]. Understanding the molecular pathways behind immune system competence could help to evaluate and mitigate risks, for example, higher susceptibility to opportunistic infection. The single timepoint nature of this study did not allow examination of any temporal patterns of gill transcriptome or immune status and we cannot be certain that we have captured the peak magnitude and/or intensity of response to viral stimulation in fish from differing rearing backgrounds. Temporal analysis would shed more light on the potential for rearing systems to shape immune competence both prior to and following transfer to sea.

5. Conclusions

Fish reared in RAS had lower steady state transcription of immune-related genes in the gill at two weeks post-SWT compared to fish reared in a LOCH system. When stimulated with a viral PAMP, RAS-reared fish mounted a stronger immune response at 24 h post-challenge relative to those reared in the LOCH at a transcriptional level. We hypothesise that in the first weeks following transfer to SW an early immune response develops in the gills of LOCH-reared fish, stimulated by the transition to a new environment, which is absent or suppressed in RAS reared fish. RAS fish mounted a stronger gill-based immune response, in terms of viral PAMP-induced changes related to increased magnitude and intensity of transcriptomic responses, than LOCH fish and thus appear to be able to 'catch up' with LOCH counterparts. Further work is needed to ascertain if mounting a larger immune response negatively impacts other aspects of RAS fish physiology, and studies with multiple sampling points are required to elucidate the temporal succession of such responses.

Availability of data and materials

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://www.ncbi.nlm.nih.gov/bioproject/PRJNA852873.

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Declaration of competing interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Data availability

The datasets presented in this study can be found in online repositories. The names of the repository and accession number(s) can be found below: https://www.ncbi.nlm.nih.gov/bioproject/PRJNA852873.

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Appendix A. Supplementary data

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