Proinflammatory cytokine and cytokine receptor gene expression kinetics following challenge with *Flavobacterium psychrophilum* in resistant and susceptible lines of rainbow trout (*Oncorhynchus mykiss*).

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Abbreviations: BCWD, bacterial cold water disease; Fp, *Flavobacterium psychrophilum*; NCCCWA, National Center for Cool and Cold Water Aquaculture; GeXP, GenomeLab Gene Expression Profiler genetic analysis system; TNF, tumor necrosis factor; LOD, limit of detection; CFU, colony forming unit; *i.p.*, intraperitoneal; A.U., arbitrary density units; GE, genome equivalent.

Abstract

Flavobacterium psychrophilum (*Fp*) is the causative agent of bacterial cold water disease (BCWD) which causes appreciable economic losses in rainbow trout aquaculture. We previously reported development of a genetic line, designated ARS-Fp-R that exhibits higher survival relative to a susceptible line, designated ARS-Fp-S, following either laboratory or natural on-farm challenge. The objectives of this study were to determine the temporal kinetics of gene expression between experimentally-challenged ARS-Fp-R and ARS-Fp-S fish and the correlation between gene expression and pathogen load. We developed a GeXP multiplex RT-PCR assay to simultaneously examine expression of immune-relevant genes, concentrating on tumor necrosis factor and interleukin-1 ligand/receptor systems and acute phase response genes. Spleen tissue was sampled at 6h, 24h, 48h and 144h post-challenge and pathogen load quantified by qPCR. Transcript abundance of cytokine genes *tnfa1*, *tnfa2*, *tnfa3*, *il1b1*, *il1b2*, *il11a*; acute phase response genes *saa* and *drtp1*; and putative cytokine receptors *il1r1-like-b*, *il1r2*, *tnfrsf1a*, *tnfrsf9*, *tnfrsf1a-like-b* increased following challenge while the transcript abundance of *il1r-like-1* and *tnfrsf1a-like-a* decreased compared to PBS-injected line-matched control fish. Principal component analysis identified transcript levels of genes *il1r-like-1* and *tnfrsf1a-like-a* as exhibiting differential expression between genetic lines. In summary, *Fp* i.p. injection challenge elicited a proinflammatory cytokine gene expression response in the spleen, with ARS-Fp-R line fish exhibiting modestly higher basal expression levels of several putative cytokine receptors. This study furthers the understanding of the immune response following *Fp* challenge and differences in gene expression associated with selective breeding for disease resistance.

Keywords: *Flavobacterium psychrophilum*, bacterial cold water disease, genetic resistance, selective breeding, proinflammatory cytokines, and cytokine receptors.

Introduction

Genetic improvement programs contribute to increased aquaculture productivity and animal welfare through selection and generation of animals with improved resistance/tolerance against infectious disease-causing microorganisms [1-5]. In 2005, a family-based selective breeding program was initiated at the National Center for Cool and Cold Water Aquaculture (NCCCWA) to improve rainbow trout (*Oncorhynchus mykiss*) survival following exposure to the gram-negative bacterium, *Flavobacterium psychrophilum* [6]. This pathogen is the etiologic agent of bacterial cold water disease (BCWD) and rainbow trout fry syndrome that causes considerable losses to the U.S. rainbow trout aquaculture industry and to trout and salmon populations worldwide [7, 8]. Infection of rainbow trout with *F. psychrophilum* typically results in mortality, ranging from 2 to 30% of the population, with higher losses caused by coinfection with the infectious hematopoietic necrosis virus. Disease prevention is difficult as the pathogen is geographically widespread, fish are often affected at early life-stage, and limited chemotherapeutants are available for treatment. There is currently no commercial vaccine available in the U.S, although killed, subunit, and live-attenuated vaccines are actively being evaluated, and several vaccines have demonstrated protection under laboratory and field conditions [9, 10].

A pedigreed line of rainbow trout, designated ARS-Fp-R, has been subjected to five generations of family-based selection and demonstrates increased survival following experimental injection challenge [6, 11, 12] and natural exposure [13, 14], compared to a disease-susceptible line designated ARS-Fp-S. Current research goals at the NCCCWA include elucidating the intrinsic factors associated with the ARS-Fp-R line to understand the mechanism(s) of how selection has altered the genetic control of survival post-challenge. Measurement of gene expression differences between fish families, or genetic lines, has

contributed to the understanding of the genetic basis of immunological responsiveness [15-25]. Previously, we reported analysis of whole-body gene expression, using RNA-Seq, to quantify changes in gene transcript abundance between genetic lines [26] and identified 1884 genes (4.0% of the protein coding transcripts identified in the rainbow trout genome) that exhibited differential transcript expression between infected and mock-challenged fish (FDR<0.05). Regulated genes included interleukins, tumor necrosis factor (TNF) receptor superfamily members, chemokines, complement components, nod-like receptor family members, and genes putatively involved in metabolism and wound healing. ARS-Fp-S line fish exhibited a greater number of regulated genes with expression levels that correlated to *Fp* load measured at day 5 post-infection. A limitation of this study was that samples were pooled by genetic-line thereby obscuring inter-individual variation and the direct correlation of gene expression with pathogen load. Also, RNA was extracted from the whole-body of small fish (avg. bwt. 1.1 g) and thus low-abundance and organ-specific transcripts were possibly underrepresented or absent within the dataset.

Following gram-negative, bacterial pathogen exposure, mammals and teleost fish produce proinflammatory cytokines and an acute phase response leading to cellular activation and elicitation of effector pathways involved in bacterial killing, clearance, tissue resolution and healing [27-29]. TNF and interleukin-1 (IL-1), as well as several related family members, are important mediators of the mammalian initial proinflammatory response. Inhibition of human TNF or IL1 β bioactivity is able to counteract mortality associated with endotoxin-induced shock [30, 31]. Thus, appropriate regulation of these cytokines and their cognate receptors can be critical for survival. At present, it is unclear whether transcriptional regulation of

proinflammatory cytokines, and associated receptors, contribute to the survival difference between the ARS-Fp-R and ARS-Fp-S genetic lines in response to *Fp* challenge.

Measurement of paralogue specific gene-expression in salmonid fish is challenging due to the putative whole-genome duplications occurring in the basal teleost lineage and the salmonid-specific lineage [32, 33], as well as subsequent, local duplication(s) [34]. Multiple paralogs with complex evolutionary history are often present that are closely related in sequence. The availability of the recently published rainbow trout genome sequence [32] has facilitated initial efforts toward a comprehensive tabulation of immune-related genes [26]. In this manuscript, we investigate proinflammatory cytokine gene expression focusing on rainbow trout paralogs of *tnf* and *il1* and putative cognate receptors. In order to simultaneously investigate expression changes in this suite of genes, we developed a multiplex GeXP assay [35] that measures multiple rainbow trout cytokines and putative receptor transcripts in a single reaction. A similar system has been recently used to dissect cytokine responsiveness in *Takifugu* [36]. The rainbow trout assay also includes genes encoding acute phase proteins, serum amyloid a (*saa*) and differentially regulated protein 1 (*drtp1*), as well as interleukin-11 (*il11a*). These genes were previously identified as upregulated in whole-body lysates following *Fp* challenge [26] and *il11a* is known to be upregulated in the rainbow trout spleen following bacterial challenge [37]. Herein, we analyze the expression kinetics of a suite of immune genes in spleen tissue of juvenile (avg. bwt. 270g) rainbow trout following *i.p.* injection of *Fp*. The correlation between gene expression and pathogen load was determined as well as differential basal and induced responses between the two genetic lines.

2. Material and Methods

2.1. *Experimental animals: Generation of rainbow trout ARS-Fp-R and ARS-Fp-S genetic lines.*

Fish were maintained at the National Center for Cool and Cold Water Aquaculture (NCCCWA) and animal procedures were performed under the guidelines of NCCCWA Institutional Animal Care and Use Committee Protocol #096. Briefly, ARS-Fp-R and ARS-Fp-S genetic lines were derived from the same base population, and thus differed only as a result of artificial selection for BCWD post-challenge survival [38]. All fish utilized in this study were from the 2014 year class. Single-sire $\times$ single-dam matings were made within genetic lines between 3-year-old females and 1-year-old neomales as previously described [39]. Water temperatures in the egg incubation jars were manipulated so that all families hatched within a one-week period [12]. Eggs were pooled within-line at the eyed stage and reared in $\sim 12.5^{\circ}\text{C}$ flow-through spring water. The ARS-Fp-R egg pool consisted of contributions from 90 full-sib families and the ARS-Fp-S egg pool consisted of 7 full-sib families. Eggs were pooled from a larger number of resistant-line families as more families were generated within the breeding program to supply a large-scale farm trial, and thus this sampling design more accurately captured the genetic diversity within the resistant-line. The resistant-line eggs were progeny of dams that had undergone four generations of BCWD selection while the sires had undergone five generations of selection. The susceptible-line eggs were progeny of parents that had undergone one generation of selection for increased susceptibility (2007 year-class) and, thereafter randomly bred to maintain genetic diversity and minimize inbreeding accumulation [38].

2.2. *Sampling*

Prior to challenge, fish ($n=22$ per tank) were allowed 2 weeks to acclimate to challenge tanks (45 L). Replicate tanks were used for each treatment (genetic line, *Fp* challenge; $n=8$ tanks total).

Photoperiod was adjusted weekly in the challenge facility to maintain a natural lighting cycle, and at the time of tissue collection was 13h light and 11h dark. Water quality parameters at the NCCCWA have been described previously [38]. Fish were challenged by intraperitoneal injection with 500 μ L of PBS alone, or PBS containing $2.3 \pm 0.4 \times 10^8$ CFU of the genome-sequenced strain of *F. psychrophilum* CSF259-93 [40]. During the experimental challenge period, one ARS-Fp-S line fish died on day 4 and *Fp* was confirmed by plate culture using TYES media.

At 6h, 24h, 48h and 144h post-injection, fish were sampled at random (n=5 per tank) and euthanized using 200 mg/L tricainemethanesulfonate (tricane-S; Western Chemical, Inc., Ferndale, WA, USA). Fish were then weighed and a blood sample collected in a lithium vacutainer through the caudal vasculature as described [41]. Body weight of ARS-Fp-R line fish averaged 265 ± 70 g, and the ARS-Fp-S line averaged 271 ± 52 g (± 1 SD). Following blood plasma collection, the tail was removed to drain excess circulatory blood. Dulbecco's PBS was added to suspend and capture peritoneal cavity cells, and once completed, the spleen removed and weighed. Spleen weight was normalized to total body weight to calculate spleen-index (SI) as described previously [11]. Two spleen tissue samples (10 and 100 mg) and a kidney tissue sample (100 mg) were dissected, placed in labeled tubes and frozen in liquid nitrogen. Peritoneal cavity cells were pelleted for 10 min at 500 x g at 4°C and flash frozen. Sample storage tubes were transferred from liquid nitrogen to storage at -80 °C. Each 100-mg spleen sample was subjected to RNA extraction, and each 10-mg spleen sample to DNA extraction for quantification of *Fp* genomic DNA.

2.3. qPCR detection of *F. psychrophilum* load in the spleen.

DNA was extracted from 10 mg spleen tissue using the DNeasy Blood & Tissue Kit (Qiagen) with a final elution in 200 μ L 10 mM Tris/ 1 mM EDTA, and the purified DNA was quantified using a Nanodrop ND-1000 spectrophotometer (Thermo Scientific). Polymerase chain reaction was performed using hydrolysis probes (TaqMan®, Applied Biosystems) targeting a conserved single-copy, sequence in the *F. psychrophilum* genome as previously described [42]. Reaction mixtures consisted of 7.5 μ L TaqMan Universal Master Mix II+UNG (Life Technologies), 0.67 μ M forward and reverse primers, 0.17 μ M probe, 1.5 μ L (standard curve) or 4.5 μ L (samples), and nuclease-free water added to a total volume of 15 μ L. Samples were run in duplicate with water used as a no-template control. An ABI 7900HT real-time thermal cycler (Applied Biosystems) was used to amplify the target sequence using absolute quantification with the machine's default program of initial denaturation for 15 min at 95°C and 40 cycles of 15 seconds at 95°C and 1 minute at 60°C. Results were normalized to *F. psychrophilum* genome equivalents (GE) per 100 ng of sample DNA to account for variation in tissue quantity and DNA extraction efficiency [42]. Of the 80 *Fp* challenged fish, a total of 9 ARS-Fp-R line fish (n=4 from 24h, n=2 from 48h, n=3 from 144h sampling time point) and 1 ARS-Fp-S line (144h) fish had qPCR cycle threshold (Ct) values below detection (assay sensitivity of 3 GE per reaction with an average limit of detection (LOD) = 0.19 GE 100 ng⁻¹ for all samples). The ten infected, qPCR-negative fish were conservatively assumed to have not completely cleared the pathogen and thus *Fp* loads were estimated based on the LOD divided by the square root of 2 as described [43] using the formula:

$$\text{Estimated GE } 100 \text{ ng}^{-1} = ((3 \text{ GE sample} * 100) / (\text{ng DNA tested in the reaction})) / \text{sqrt } 2.$$

Since these samples were primarily from the ARS-Fp-R line fish, the estimates may have reduced the actual *Fp* load difference between the two lines. A total of 20 PBS-injected fish

were chosen from the 48h and 144h sampling time points and these were below the level of detection and assigned a value of zero *Fp* GE 100 ng DNA⁻¹ for data analyses (see Table 2 in [44]).

2.4. RNA isolation.

To isolate RNA, 75–100 mg of spleen tissue was homogenized in 1mL TRIzol (Invitrogen; Carlsbad, CA, USA), using a 5-mm steel bead and a multi-tube shaker, followed by phenol extraction per the manufacturer's protocol. The RNA pellet was washed twice with 75% ethanol, dried, and suspended in nuclease-free water. RNA concentration and purity were determined by measuring absorbance, and the A260/A280 ratio varied between 1.99 and 2.08. RNA quality was evaluated by agarose gel electrophoresis.

2.5. Gene expression analysis

We utilized the GenomeLab Gene Expression Profiler (GeXP) genetic analysis system (Beckman Coulter Inc.; Pasadena, CA, USA) to quantify transcript abundance using reverse transcription (RT)-PCR. In this system, capillary electrophoresis was used to separate and quantify the size and fluorescence intensity of the amplicons produced by a single endpoint PCR reaction. Primers were either derived from previously published studies [45-48] or designed de novo using eXpress Designer software (Beckman Counter Inc.; Pasadena, CA, USA) to produce amplicons between 100 – 400 base pairs, each separated by a minimum of three bases (Table 1). Within the multiplex, 21 primer pair's amplified genes associated with the fish immunological response and four served as reference genes (Table S1). Primer sequences were compared against other rainbow trout gene sequences using the BLAST function within the NCBI database

to reduce unintended sequence amplification. The pair of primers designed to amplify the gene(s) annotated as *il1r-like-1* did not discriminate between gene paralogues (GSONMT00035358001, GSONMT00021943001, and GSONMT00035357001) (Table 1). Subsequent analysis of an improved assembly of the rainbow trout genome (Palti et al. unpublished data) uncovered that several primer sets amplify multiple paralogues of *saa*, *drtp1*, *il11a*, *il1r-like-1* and the housekeeping genes (Table S1). For convenience, we refer to each primer pair as amplifying a gene(s) having a common annotation. Optimization of the multiplex, standard curve dilutions (3 – 50 ng per reaction), RT and PCR reactions, and capillary electrophoresis were performed as recommended by the manufacturer (GeXP Chemistry protocol A29143AC; February, 2009) with reagents provided in the GeXP Start Kit (Beckman Coulter Inc.; Pasadena, CA, USA). The size of each amplicon was confirmed with its expected length with the exception of the *il1b4* gene transcript which was not detected. Representative amplification profiles are presented (see Figure 1 in [44]). GeXP gene expression values for *il11a* and *tnfrsf5* were significantly correlated with gene expression values measured by conventional RT-PCR, albeit the GeXP assay for *il11a* was less sensitive in measuring low amounts of gene expression (see Figure 2 in [44]). Gene names, gene symbols, GenBank accession numbers, and database reference numbers are listed (Table 1). Primer sequences that include universal tags, genomic location and standard curve efficiencies are provided in a supplementary data file (Table S1).

Reverse transcription reactions included 100 ng of DNase (Promega, Madison, WI) treated RNA in 1 μ L, 2 μ L 5X RT buffer, 1 μ L gene-specific reverse primer mix, 0.5 μ L RT enzyme, and 1.25 μ L kanamycin RNA (internal control) in a 10 μ L reaction. The RT was incubated according to kit instructions at 48 °C for 1 min, 42 °C for 60 min, then 95 °C for 4 min. An aliquot (4.65 μ L) of the resultant cDNA was used in a PCR reaction with 2 μ L 25 mM MgCl₂, 2

μL 5X PCR buffer, 1 μL forward primer mix, and 0.35 μL DNA Taq polymerase. The PCR was incubated according to kit instructions at 95 °C for 10 min then 34 cycles of 94 °C for 30 sec, 55 °C for 30 sec, and 70 °C for 60 sec. One μL of PCR products was combined with 38.5 μL sample loading solution and 0.25 μL size standard 400. The PCR products were separated by capillary electrophoresis in the GeXP Genetic Analysis System using the Frag-3 protocol with a separation voltage of 6.0 kV for 45 min.

The amount of fluorescence associated with each amplicon was exported to eXpress Profiler software (Beckman Coulter, Inc.; Pasadena, CA, USA) for analysis and normalization to the internal kanamycin control. The amount of fluorescence was quantified using a pooled sample, standard curve (n=3 technical replicates, comprising 8 dilutions of cDNA). For each gene, the transcript concentration was measured in arbitrary density units (A.U.). Standard curve R^2 values were between 0.81-0.97 (Table S1) with the exception of *tnfrsf1b* which was not reliably quantified and thus excluded from further analysis. The three most stable reference genes were identified by GeNorm software as *arp*, *gapdh*, and *eflα*, with M-values of 0.810, 0.697, and 0.620. Data from *actb* were not used. GeNorm software calculated the geometric mean of the reference genes and generated a normalization factor for each sample. Transcript abundance for all genes was normalized to the reference genes by dividing transcript concentrations by the normalization factor. One *Fp*-injected, ARS-*Fp*-R line fish (fish #65, 24h) failed to produce measureable PCR products and this fish was excluded from gene expression analyses.

2.6. Statistical analysis

Gene expression and *Fp* load data were increased by a value of 1 and then $\log_2$ transformed. *Fp* load data were analyzed using a two-way ANOVA to identify significant differences in

bacterial loads between genetic lines and between sample time points using GraphPad Prism version 6.0 (GraphPad Software, Inc.). Separate two-way ANOVA were likewise used to evaluate the effects of 1) genetic line (within treatment), time of sampling, and their interaction, and 2) treatment (within genetic line), time of sampling, and their interaction, on gene expression. The null hypothesis (no difference between groups) was rejected if $P < 0.05$. Principal component analysis (PCA) and heat maps were generated using Qlucore Omics Explorer (v3.1). Initial data inspection identified three PBS-injected fish from tank 4 (24h) that had elevated proinflammatory gene expression profiles and gene expression values from these fish were subsequently eliminated from further analyses (Fish #51, #53 and #55, see Table 2 in [44]). In addition, two fish with unusually large spleen index values were excluded from spleen size analyses (Fish #130 and #133, see Table 2 in [44]). For heat map generation of each variable, all data were normalized to a mean =1 and variance =1 (yellow color high, blue color low relative expression). To identify infection-regulated genes, a two-group comparison of infected vs PBS-injected fish was filtered using a false discovery rate of $q < 0.05$ and a fold-change >2.0 ($P=0.006$). Using these filters, PCA component 1 accounted for 50% of the variation (Figure S2). To identify genes differentially regulated between genetic lines, a two-group comparison of ARS-Fp-R line vs ARS-Fp-S line was filtered using a false discovery rate of $q < 0.05$ and a fold-change >2.0 ($P=0.002$). Using these filters, PCA component 1 accounted for 40% of the variation. Coordinated gene expression was identified by hierarchal clustering of gene-expression values. Pearson correlation (r) was used to evaluate the pair-wise relationships between *log*-transformed gene expression, bacterial load and spleen size. Correlations were determined using GraphPad Prism (v6.0) and considered significant if $P < 0.05$.

3. Results

3.1. Kinetics of *Fp* load change in ARS-Fp-R and ARS-Fp-S line spleen tissue following challenge.

The *Fp* GE in spleen tissue was affected by genetic line ($P=0.015$) and time ($P=0.018$) with no interaction between line and time ($P=0.125$). Pairwise analyses identified significantly higher *Fp* loads in ARS-Fp-S line fish spleens at 48h post-infection (Figure 1). Following challenge, spleen-index significantly increased in both lines at 144h, with a mean increase of 0.508 SI units in the ARS-Fp-R line ($P<0.0001$) and 0.364 units in the ARS-Fp-S line ($P=.003$) relative to the respective PBS-injected controls (Figure S1A). Body weight was not different between PBS-injected or *Fp*-injected fish or between genetic lines ($P=0.573$, Figure S1B). These data indicate that the pathogen load difference between lines and spleen size increase following challenge was consistent with previous observations [11, 13].

3.2. Principal component analysis of changes in gene expression influenced by infection.

Principle component analysis of global gene expression using the dataset of 156 fish identified fifteen immune-relevant genes that were differentially regulated in response to *Fp* challenge. These included the cytokines *tnfa1*, *tnfa2*, *tnfa3*, *il1b1*, *il1b2*, *il11a*, the putative receptors *tnfrsf1a*, *tnfrsf1a-like-a*, *tnfrsf1a-like-b*, *tnfrsf9*, *il1r-like-1*, *il1r1-like-b*, *il1r2*, as well as acute phase response genes *saa* and *drtp* (Qlucore Omics Explorer, two-group comparison of PBS-injected vs infected fish, false discovery rate of $q < 0.05$ and a fold-change >2.0 , $P=0.006$). Hierarchical clustering identified five general patterns of expression (Figure 2). Group I genes were up-regulated at the 6h time point, remained elevated at 24h and 48h and exhibited decreased expression at 144h. This group included *tnfa3*, *il1b1*, *tnfa1*, *tnfrsf9*, *il11a*, *il1r2* and

il1r1-like-b. Group II genes were modestly elevated at 6h and by 24h through 144h exhibited robust upregulation and included acute phase response genes *saa* and *drtp*. Group III genes, *tnfa2* and *il1b2*, were upregulated at 6h but with diminished relative expression at 24 h and 48 h time-points. Group IV genes, *tnfrsf1a-like-b* and *tnfrsf1a* were not changed at 6h but upregulated at 24h in ARS-Fp-S line fish. Group V genes *tnfrsf1a-like-a*, and *il1r-like-1(ex3-5)*, were unchanged at 6h and down-regulated at 48h post-challenge.

3.3. Pairwise-analysis of gene expression in response to *Fp* infection.

To more precisely characterize gene expression kinetics of the thirteen infection up-regulated genes (Groups I-IV), we analyzed each gene using two-way ANOVA followed by pair-wise comparison between PBS and *Fp*-injected fish by genetic line (Figure 3 A-X). Transcript abundance of *saa* was significantly elevated in infected fish at all time points in both lines (Figure 3 A, B), and *drtp1* that was significantly increased in infected fish at 48h and 144h in the ARS-Fp-R line and 24h, 48h and 144h in the ARS-Fp-S line (Figure 3 C, D). *Saa* expression was significantly correlated with *drtp1* expression ($r=0.81$), and the expression of both genes significantly correlated with pathogen load (*saa* $r=0.49$; *drtp1* $r=0.42$; Table S3). Two-way ANOVA identified a significant effect of genetic line for *saa* and *drtp1* with higher transcript abundance in *Fp* challenged ARS-Fp-S line compared to challenged ARS-Fp-R line fish (Table S2, 2-way ANOVA; *saa*, $P=0.0086$; *drtp1*, $P=0.0104$). At the 24h time point, *saa* expression was significantly higher in the *Fp* challenged ARS-Fp-S line than ARS-Fp-R line (Table S2).

Transcript abundance of genes *il1r2*, *il1r1-like-b*, and *tnfrsf9* were significantly elevated by infection in both lines (Table S2, 2-way ANOVA, *il1r2*, $P<0.0001$; *il1r1-like-b*, $P<0.0001$; *tnfrsf9*, $P<0.0001$). The receptor genes were upregulated early (6h) in both lines, *il1r2* (Figure 3

E, F), *il1r1-like-b* (Figure 3 G, H), and *tnfrsf9* (Figure 3 I, J). *Il1r2* transcript abundance was significantly increased at 6h and 48h in the ARS-Fp-R line and at all time-points in the ARS-Fp-S line. *Il1r1-like-b* was significantly increased at 6h and 48h in ARS-Fp-R line and 6h, 24h and 48h in the ARS-Fp-S line. *Tnfrsf9* transcript abundance was significantly increased at 6h and 48h in the ARS-Fp-R line and at 6h, 24h and 48h in the ARS-Fp-S line (Figure 3 I, J). Expression of *il1r2* was significantly correlated with *il1r1-like-b* ($r=0.92$) and *tnfrsf9* ($r=0.80$) (Table S3). Expression of *il1r1-like-b* was significantly correlated with *tnfrsf9* ($r=0.73$). Two-way ANOVA identified a significant effect of genetic line on expression of genes *il1r2*, *il1r1-like-b*, and *tnfrsf9* with higher transcript abundance in challenged ARS-Fp-S line compared to challenged ARS-Fp-R line fish (Table S2, 2-way ANOVA, *il1r2*, $P=0.0158$; *il1r1-like-b*, $P=0.0416$; *tnfrsf9*, $P=0.0101$).

Proinflammatory cytokine genes, *tnfa1*, *tnfa2*, *tnfa3*, *il1b1*, and *il1b2* generally exhibited early (6h) up-regulation. *Tnfa1* was significantly increased at 6h and 48h in ARS-Fp-R and -S lines (Figure 3 K, L). *Tnfa2* was significantly increased at only 6h in the ARS-Fp-S line (Figure 3 M, N); and *tnfa3* was significantly increased at 6h and 48h in the ARS-Fp-R line and at 6h, 24h, 48h in ARS-Fp-S line (Figure 3 O, P). Transcript abundance of *il1b1* was significantly increased at 6h and 48h in the ARS-Fp-R line and 6h, 24h, 48h in the ARS-Fp-S line (Figure 3 Q, R). *Il1b2* was significantly increased at 6h and 48h in the ARS-Fp-R line and 6h and 24h in ARS-Fp-S line (Figure 3 S, T). *Tnfa1*, *tnfa2* and *tnfa3* gene expression were highly correlated ($r=0.63$ to 0.80) and significantly correlated with *il1b1* and *il1b2* ($r=0.55$ to 0.89) (Table S3). These five genes were all significantly correlated with *Fp* load ($r=0.34$ to 0.63). There was no correlation between these five genes and *il1b3* transcript abundance. Two-way ANOVA

identified a significant effect of genetic line only for *il1b1* with higher expression in ARS-Fp-S line fish compared to challenged ARS-Fp-R line fish (Table S2, 2-way ANOVA, line $P=0.0147$).

Up-regulation of *il11a* gene occurred at 6h and 48h in ARS-Fp-R line fish and at 6h, 24h, and 48h in ARS-Fp-S line fish (Table S3). Two-way ANOVA identified a significant effect of genetic line with higher expression in challenged ARS-Fp-S line fish compared to challenged ARS-Fp-R line fish (Table S2, 2-way ANOVA, line $P=0.0033$). *il11a* gene expression was significantly correlated with other Group I genes, with the highest correlations with *il1b1* ($r=0.77$) and *tnfa3* ($r=0.75$).

Up-regulation of receptors *tnfrsf1a* (Figure 3 U, V) and *tnfrsf1a-like-b* (Figure 3 W, X) were observed only in the ARS-Fp-S line at the 24h time-point and the expression of these genes were highly correlated ($r=0.85$) (Table S3).

Tnfrsf5 (*cd40*) was not regulated in response to challenge nor did expression differ between genetic lines (Table S2).

3.4. Comparison of the response to *Fp*-infection between genetic lines

Two genes exhibited a general pattern of down-regulation in response to infection: *tnfrsf1a-like-a*, and *il1r-like-1* (Figure 2). The two primer sets targeting different regions of *il1r-like-1*, either exons 3-5 or 9-11, identified a similar trend of downregulation but not identical values at time points (Figure 2, Table S2). Interestingly, when the dataset was filtered by principle component analysis for genes that significantly differed between genetic lines, both *tnfrsf1a-like-a* and *il1r-like-1* genes were identified (including both *Fp*-infected and PBS-treated fish in the analyses) (Figure 4). Within this global analysis, spleen weight and spleen index also significantly differed between genetic lines (Figure 4) even after accounting for infection and

time. The ARS-Fp-R line fish tended to exhibit larger relative spleen size and higher expression of genes *tnfrsf1a-like-a*, and *il1r-like-1*. Gene expression of *tnfrsf1a-like-a* was significantly correlated with both *il1r-like-1(ex3-5)* ($r=0.49$) and *il1r-like-1(ex9-11)* ($r=0.32$). *Tnfrsf1a-like-a*, and *il1r-like-1* were negatively correlated with *Fp* load ($r= -0.32$, -0.42 (*ex3-5*) and -0.20 (*ex9-11*) respectively). However, neither of these genes were significantly correlated with spleen weight or spleen index values suggesting that these are independent characteristics exhibited by the genetic lines in response to *Fp* challenge (Table S3).

3.5. Comparison of baseline and induced gene expression between genetic lines.

We next explored whether the differences between genetic lines were the result of baseline or induced differences. When the entire data set of PBS-injected animals was analyzed by pair-wise comparison, *tnfrsf1a-like-a*, *il1r-like-1(ex3-5)* and *il1r-like-1(ex9-11)* were all significantly higher in the ARS-Fp-R line compared to the ARS-Fp-S (Figure 5 A, B and C). When analyzed by time-point, significant differences in gene expression between PBS-injected ARS-Fp-R and ARS-Fp-S line fish were identified only at 6h post-injection for *il1r-like-1 (ex3-5 and ex9-11)* (Table S2). Thus, subtle differences in gene expression were detected in PBS-injected fish. Comparison of the *Fp*-injected ARS-Fp-R and ARS-Fp-S line fish identified modest, but significantly higher relative expression (absence of down-regulation) in the ARS-Fp-R line compared with ARS-Fp-S line (Figure 5 D-F). We next examined if expression also differed between lines of infected animals. Gene expression of *tnfrsf1a-like-a* and *il1r-like-1(ex9-11)* were significantly higher at 48h in the ARS-Fp-R line as compared to the ARS-Fp-S line (Figure 5 D, E, Table S2). For *il1r-like-1(ex3-5)*, expression in the ARS-Fp-S line was lower at 6h and 24h (Figure 5 F, Table S2)

4. Discussion

The genetic control of fish disease resistance and the impact of selective breeding on immunological responsiveness remain poorly understood. Herein, we describe a new multiplex assay that measures a suite of immune-relevant genes and we used this assay to examine expression differences between PBS- and *Fp*-injected fish from two genetic lines of rainbow trout exhibiting a well-characterized phenotypic difference in post-challenge survival. This study utilized juvenile rainbow trout in order to examine inter-individual and tissue-specific immunological responsiveness. We report gene expression differences occurring in the spleen, as part of a larger study examining gene expression differences occurring simultaneously within infiltrating peritoneal cavity cells and anterior kidney tissue, as well as, changes in plasma biochemistry and plasma proteins. We chose to initially investigate the spleen as it is a dynamic site of disease manifestation and substantial histological changes occur following *Fp* injection including an increased inflammatory infiltrate, perisplenitis, splenic necrosis, and increase in average and total splenic ellipsoid area [39]. The degree of perisplenitis and necrosis are higher in ARS-Fp-S line fish compared to ARS-Fp-R line fish, consistent with differential pathogen control [39]. The primary finding reported in this manuscript is the identification of fifteen differentially-regulated, immune-relevant genes following *Fp* challenge. These included the cytokines *tnfa1*, *tnfa2*, *tnfa3*, *il1b1*, *il1b2*, *il11a* the putative receptors *tnfrsf1a*, *tnfrsf1a-like-a*, *tnfrsf1a-like-b*, *tnfrsf9*, *il1r-like-1*, *il1r1-like-b*, *il1r2* as well as acute phase response genes *saa* and *drtp1*. Our previous study, measuring gene expression differences across the whole body, identified only a subset of these genes (n=10) as differentially regulated including *tnfa1*, *il1b1*, *il11a*, *il1r1-like-a*, *il1r2*, *tnfrsf1a*, *tnfrsf1a-like-b*, *tnfrsf9*, *drtp1* and *saa* (Table S4), and it is likely that including an earlier sampling time point (6h) as well as sampling a specific tissue as

compared to whole-body RNA-seq allowed detection of low abundance or rare cell-specific transcripts. In this study, we did not identify *tnfrsf5(b)* (GQ169788.1, GSONMT00012579001) as differentially regulated in contrast to our study of whole body gene expression and this difference remains unexplained. One possibility is that regulation occurred in a tissue other than the spleen or that fish size or developmental stage may be important [49, 50]. This study identified two down-regulated genes, *il1r-like-1* and *tnfrsf1a-like-a*, that exhibited differential expression between genetic lines. The measurement of individual fish sampled at the four early time-points allowed a temporal model of responsiveness and the dissection of differences between genetic lines.

4.1. Interleukin and interleukin-receptor gene expression and kinetics

Interleukin-1 β is an early-response, pro-inflammatory cytokine [51, 52], and IL-1 family members also promote the adaptive immune response by enhancing the functions of B cells and the T helper cell subsets [53]. Two types of *il1b* genes are present in teleost fish [48]. Type I genes include *il1b3* and *il1b4*, and type II genes include *il1b1* and *il1b2* [48, 54]. Our data identify early upregulation of *il1b1* and *il1b2* gene transcripts in the spleen following *Fp* challenge. This is consistent with previous reports of *il1b1* and *il1b2* upregulation in response to LPS and gram-negative bacterial pathogen challenge [48, 55]. The expression of *il1b3* has been less well studied. We report abundant *il1b3* basal gene expression but no upregulation in response to *Fp* exposure thus indicating differential control of gene expression compared to *il1b1* and *il1b2* paralogues. Basal *il1b3* expression has been previously reported to be relatively high in the spleen [48], and robust expression was detected in head-kidney macrophages exposed to LPS and in head-kidney tissue following VHSV infection [48]. A fourth paralogue, *il1b4* was

previously identified as a pseudogene in Atlantic salmon [48]. Consistent with this possibility, *il1b4* gene expression in the rainbow trout whole-body RNA-Seq study revealed undetectable, or in a few samples, a very low number of mapped reads [24]. In this study we observe no detectable expression of *il1b4* in spleen tissue in a sample size of 159 fish. In summary, our results are consistent with the proposed gene-expression sub-functionalization of *il1b1*, *il1b2* and *il1b3* in response to different bacterial and viral pathogen challenge, and the loss of *il1b4* expression as a likely pseudogene [48], at least with respect to the fish species, populations and conditions studied.

In mammals, the response to interleukin-1 family members is initiated when a ligand binds to its primary receptor subunit, which for IL-1 is IL-1 receptor type I (IL-1R1). Ligand binding facilitates association of a second receptor subunit; the IL-1R accessory protein (IL-1RAP) and signaling is transmitted via dimerization of the TIR domains (reviewed in [53, 56]). Regulation of the IL-1 receptor family is complex, with four activating receptors IL-1R1, IL-1RL2, ST2, and IL-18R as well as respective accessory proteins IL-1RAP and IL-18RAP, in addition to decoy receptors, receptor antagonists and binding proteins. Within this study, we identified 8 putative interleukin-1 receptor-related genes, and of these, 6 contained TIR domains (Table 1, Figure S3). We were able to design primers specific to 4 of these genes, with two primer-sets that cross-amplified three highly-related genes sequences (GSONMT00035358001, GSONMT00021943001 and GSONMT00035357001). All three of these genes are located adjacent to one another on the same Chr Omy3 scaffold and thus likely represent a possibly recent, tandem duplication within the salmonid lineage (Table S1). The gene encoding interleukin-1 receptor type 1-like (GSONMT00016995001) was not measured, nor was it previously identified as regulated [26]. In this study, due to the number and complexity of the

interleukin-1 receptor accessory genes within the genome, we did not include primers to the 11 identified putative receptor accessory genes (Table S4), although we note that several are of interest as four (GSONMT00051039001, GSONMT00007860001, GSONMT00067362001, and GSONMT00029719001) were identified as regulated following challenge [26].

Mammalian IL-1 family member signaling is modulated by both receptor antagonists (IL-1Ra) as well as membrane and soluble decoy receptors (IL-1R2, SIGIRR). In this manuscript, we identify *il1r2* (GSONMT00066304001) and *il1r1-like-b* (GSONMT00032852001) genes as upregulated following *Fp* challenge. The trout *ilr2* gene lacks a TIR domain [57] and thus may function as a decoy receptor consistent with the lack of a TIR domain in other fish species [58-60] and upregulation in rainbow trout in response to bacterial infection [61]. Several putative receptor genes exhibited down-regulation following challenge. We identified *Il1r1-like-a* (GSONMT00066303001) gene expression as down-regulated in our previous study using whole fish and also in this study, albeit to a limited extent, at the 48h time point and in only the ARS-Fp-S line.

The most interesting down-regulated gene was *il1r-like-1* representing three sequence related, adjacent genes (GSONMT00035358001, GSONMT00021943001, GSONMT00035357001). The two primer sets used in this paper amplify across different regions of the three genes: either exons 3-5 or exons 9-11. The primers that amplify across exons 3-5 amplify 2 of the 3 sequence-related genes and have a 2 bp mismatch with gene GSONMT00021943001, while primers that amplify across exons 9-11 amplify all three genes. The amplification profiles using primer sets to exons 3-5 and 9-11 differed and it is likely that differential gene-set amplification and/or differences in expression between the three copies may explain these results. It is also possible that alternative splicing may contribute to these

differences and the development of tools (primers or sequencing strategies) to distinguish between these gene copies is needed to resolve these possibilities. The predicted proteins share high (95-99%) sequence identity to trout ST2L protein (CDG03205.1). Interestingly, the interleukin 1 receptor-like 1 gene (ST2L protein, 599aa) was previously identified as having 2.2-fold lower expression in the rainbow trout strain BORN, a 37 year selection line bred in semi-closed aquaculture as compared to commercially-available Troutlodge strain (TCO) [62]. The BORN line is reported to exhibit higher survival following bacterial or viral challenge [62, 63]. The contribution of ST2L to the BORN line resistance phenotype is unknown as there were over 800 differentially-regulated genes between the two lines identified in spleen tissue from healthy animals. A ST2L homologue from Atlantic salmon (*Salmo salar*) is constitutively expressed in spleen, brain, blood cells, head kidney, liver, gills and muscle [64]. In vivo stimulation of salmon with bacterial lipopolysaccharide did not affect the expression of the salmon ST2L gene [64]. In this study, the expression of *il1r-like-1(ex9-11)* was negatively correlated with expression of *il1b1* (-0.23), while expression levels of *il1r1-like-b* and *il1r2* were positively correlated with both *il1b1* (0.83 and 0.85 respectively) and *il1b2* (0.62 and 0.59 respectively) (Table S3). In uninfected rainbow trout, we previously observed, using RNA-seq, that expression of the *il1r-like-1* gene (GSONMT00035358001) was highest in the ARS-Fp-R line, intermediated in the ARS-Fp-C line, and lowest in the ARS-Fp-S line fish consistent with the possibility that artificial selection for BCWD resistance may have led to increased basal gene expression [26]. It is notable that a previously identified BCWD QTL on Omy3 [65] has been recently localized within close proximity to these genes (Vallejo et al. unpublished data). The further characterization of the genomic location, ligand interaction(s), and protein abundance following *Fp* challenge is of future interest.

4.2. *Tnf* and *tnfrsf* gene expression.

The TNF superfamily of ligands and receptors are critical immune response molecules involved in a number of biological activities including the canonical proinflammatory response in teleost fish [52, 66, 67]. Teleost fish can have multiple tumor necrosis factor encoding genes [67], and three *tnfa* genes have been reported in rainbow trout: *tnfa1*, *tnfa2*, and *tnfa3* [47]. In this study, expression of all three trout tumor necrosis factor genes were upregulated following *Fp* challenge, albeit the *tnfa2* gene exhibited weaker up-regulation and a different pattern compared to *tnfa1* and *tnfa3* (Figure 2). The *tnfa3* protein sequence is more distantly related to *tnfa1* and *tnfa2* proteins suggesting the potential for subfunctionalization, although all three genes are upregulated in response to LPS or following bacterial challenge with the gram-negative pathogen *Y. ruckeri* [47]. *Tnfa3* gene expression was found to be earlier and transiently expressed following either stimulation in the RTS-11 cells, in a mixed lymphocyte response, or following VHS virus challenge [47]. In this study, all three *tnfa* genes exhibited increased abundance by the earliest time-point measured, 6h post-injection.

In humans, TNF exhibits high-affinity binding to two receptors, TNF-R1 (TNFRSF1A) and TNF-R2 (TNFRSF1B), whose major difference is the presence of a cytosolic death domain (present in TNF-R1) [68, 69]. The identification of the receptor(s) bound by rainbow trout *tnfa* gene products have not been unambiguously identified and analysis is complicated by a large family of at least 59 *tnfrsf* encoding genes (including partial genes) in the rainbow trout genome [26] and thus is more complicated than previously identified in zebrafish n=33 [67] and Fugu n=9 [70]. We identified a select number of *tnfrsf* for further expression analysis based on sequence-based likelihood as candidate *tnfa* receptor(s) (Figure S4). One *tnfrsf1a* and two

tnfrsf1a-like genes are present in the genome with associated death domains, albeit GSONMT00061855001 is likely either an incompletely assembled gene, a pseudogene, or an inhibitory receptor due to the partial sequence and presence of a death domain (Figure S4). The gene *tnfrsf1a* is expressed at high levels in the spleen (see Table 2 in [44]) and whole body (Table S4) consistent with the expression of the *TNFR1* gene product which is present on most mammalian cell types. The three genes exhibit differential patterns of expression following challenge: *tnfrsf1a* and *tnfrsf1a-like-b* are up-regulated while *tnfrsf1a-like-a* is down-regulated in the spleen (Figure 2). Two putative *tnfrsf1b* receptor orthologues were identified, both of which lack death domains (Figure S4), but expression was not quantified in this study. In our prior analyses of these genes, both were expressed at low level (see Table 2 in [44]) and were not regulated following *Fp* challenge [26].

In this paper, we confirm that *tnfrsf9* exhibits robust up-regulation following challenge (Figure 2). This gene is a putative orthologue of the mammalian receptor gene encoding 4-1BB. In mammals, this protein functions as a co-stimulatory molecule expressed on activated T cells, dendritic cells, NK cells, and granulocytes. A putative ligand, identified in several fish species, clusters phylogenetically with mammalian 4-1BBL but a clear orthologue, to our knowledge, has not been identified in rainbow trout [71]. The function of these proteins in salmonid fish is unknown.

4.3. Expression of acute phase response genes *saa* and *drtp1*

The acute phase response (APR) and the inflammatory response are overlapping components of the reaction to infection and injury [27, 72]. In humans, the pro-inflammatory SAA is a major acute phase protein (APP), whose expression is induced by inflammatory cytokines IL1B, IL6

and TNF [73, 74]. SAA is involved in diverse defensive functions, such as induction of cytokine synthesis, leukocyte recruitment, activation of epithelial cell immunity, neutrophil priming, opsonization of Gram-negative bacteria, activation of antimicrobial functions in polymorphonuclear cells and antiviral activity [75-82]. In rainbow trout it is upregulated after stimulation with LPS as well as during infection with gram-negative pathogens including *Fp* [55, 83, 84]. In the present study, expression level of *saa* was upregulated early (6h) in both trout lines and remained high at all time-points (Figure 3). Increased expression of *saa* in the peritoneal cavity of rainbow trout during infection with *Aeromonas salmonicida* correlated with the increased level of *il1b* and *tnfa* and other acute phase proteins [85]. We found a similar significant and positive gene expression correlation between *saa* and the three *tnfa* genes as well as *il1b1* and *il1b2* gene expression (Table S3).

Drtp1 belongs to APR and may participate in phospholipase inhibition, pathogen response, and the stress reaction [86]. Previously, it was shown that *drtp1* gene expression level increased in the liver of ayu (*Plecoglossus altivelis*) during *Listonella anguillarum* infection [87]. In Atlantic salmon, *drtp1* is upregulated in the gill, liver, spleen and head kidney after challenge with the bacterial pathogen *A. salmonicida* [88, 89]. In this study, the level of *drtp1* increased following *Fp* injection. However, *drtp1* expression level in ARS-Fp-S line increased earlier (24h) as compared to the ARS-Fp-R line (48h).

5. Conclusion

The GeXP assay described here is a cost-effective method for simultaneous measurement of transcript abundance of multiple immune-relevant genes and useful for confirming gene expression signatures identified by RNA-seq as well as for examining inter-individual variation

in responsiveness to pathogen exposure. Herein, we described 15 differentially-regulated genes of which 13 were upregulated in response to *Fp* challenge, and these exhibited four general patterns of gene expression over the six day time period (Figure 2). Principle component analysis of variation, discriminating between the ARS-Fp-R and ARS-Fp-S lines, identified higher basal gene expression levels of three putative cytokine receptors *tnfrsf1a-like-a*, and *il1r-like-1* in the ARS-Fp-R line, as well as less down-regulation following challenge. Our working model is that the ARS-Fp-R line has more beneficial response, not due to greater proinflammatory cytokine gene expression, but possibly due to higher transcript abundance of putative cytokine receptors, albeit these may be decoy receptors. While variable between individual fish, pathogen load increases to higher mean levels in ARS-Fp-S line fish following challenge and it is not unexpected that we observed differences between genetic lines in the APR response (*saa* and *drtp1*), cytokine receptor (*il1r2*, *il1r1-like-b*, and *tnfrsf9*) and cytokine (*il1b1*) gene transcript abundance that correlated with elevated pathogen load. Consistent with other reports, the induction of proinflammatory genes *tnfa1-3* and *il1b1-2* and putative anti-inflammatory *il11a* temporally precedes the elevated transcript abundance of *saa* and *drtp1*.

An important caveat of this study is that we selected gene candidates based on primary sequence comparison, automated annotation and previous expression analyses using whole fish. Detailed evolutionary and functional analyses remain to confirm these are functional receptors (or decoys) as well as receptor accessory proteins implied by the automated annotation nomenclature. Furthermore, several of the genes we measured in this study (i.e. *il1r-like-1*) were incompletely assembled in the current version of the rainbow trout genome (32) and it is likely that genes models will continue to be modified as improvements are made to the genome assembly. Finally, our analyses do not rule out changes in infiltrating cells (or exit of cells)

accounting for differences in gene expression. Our current approach to confirm the relationship of the identified differentially expressed genes with the disease resistance phenotype is through continued fine-mapping of BCWD quantitative trait loci [65, 90-93].

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Figure Captions

Figure 1. Comparison of *Fp* load in spleen tissue between ARS-Fp-R and -S line fish at 6, 24, 48h and 144h post-injection challenge. Values represent mean *Fp* GE 100 ng DNA⁻¹ ($\pm$ 1 SEM, n=10 fish). Two-way ANOVA identified a significant effect of time and genetic line. Pair-wise comparison at each of the 4 time points identified a significant difference in *Fp* load at 48h between genetic lines ($P<0.05$). n.s. = not significant.

Figure 2. Heat map showing normalized gene expression of significantly regulated genes in spleen tissue of *Fp* injection-challenged and PBS-injected fish. Yellow heat-map bars represents higher gene expression and blue bars lower gene expression in individual fish (n=156). Differentially regulated genes (listed to right) were identified by principal component analysis as exhibiting >2-fold expression difference between infected and PBS injected animals with a false discovery rate of ($q=0.05$) and are ordered by hierarchical clustering (left). The top track represents pathogen load measured by qPCR and expressed as *Fp* GE 100 ng DNA⁻¹. Legend above the heat-map shows time post-injection (h), genetic line (ARS-Fp-R line, blue; ARS-Fp-S line, red), fish infection status (PBS-injected, yellow; *Fp*-injected, orange). Genes are divided into five groups based on their overall expression pattern.

Figure 3. Comparison of gene expression (GE) kinetics between *Fp* injected with PBS injected fish by genetic line. Genes *saa* (panels A, B), *drtp1* (panels C, D), *il1r2* (panels E, F), *il1r1-like-b* (panels G, H), *tnfrsf9* (panels I, J), *tnfa1* (panels K, L), *tnfa2* (panels M, N), *tnfa3* (panels O, P), *il1b1* (panels Q, R), *il1b2* (panels S, T), *tnfrsf1a* (panels U, V), *tnfrsf1a-like-b* (panels W, X). Data are from the ARS-Fp-R line (panels A, C, E, G, I, K, M, O, Q, S, U, W) and ARS-Fp-S line

(B, D, F, H, J, L, N, P, R, T, V, X). In each graph, *Fp*-challenged fish are denoted by a solid line, PBS-injected fish are indicated by a dashed line. The results represent the mean ($\log_2$) $\pm$ 1 SEM. Asterisk annotation: * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; and **** $P < 0.0001$.

Figure 4. Identification of significantly regulated genes between genetic lines using principal component analysis (>2-foldcut-off and false discovery rate $q = 0.05$). Legend above the figure denotes individual fish (ARS-Fp-R line, blue color; ARS-Fp-S line, red color). Variables, genes and spleen size measurements, are grouped by hierarchal clustering. Individual fish are ordered along the horizontal, based on PCA1 values which account for 40% of the variation.

Figure 5. Differences in gene expression of *tnfrsf1a-like-a* (A, D), *il1r-like-1(exons3-5)* (B, E), *il1r-like-1(exons9-11)* (C, F) between ARS-Fp-R and ARS-Fp-S lines. The results represent the mean ($\log_2$) $\pm$ 1 SEM. A-C) comparison between PBS injected fish from both lines. D-F) comparison between lines of *Fp* challenged fish (solid line, ARS-Fp-R line; dashed line, ARS-Fp-S line). Asterisk annotation: * $P < 0.05$; and ** $P < 0.01$.

Table 1. Immune relevant and house-keeping genes targeted by the PCR multiplex assay.

Category	Gene/Protein Description	Gene Abbreviation	Accession # or Genome locus transcript	PCR Product(bp)
Acute Phase Protein	serum amyloid A	<i>saa</i>	GSONMT00016296001	247
	differentially regulated protein 1 precursor	<i>drtp1</i>	GSONMT00048193001	151
Interleukins (IL)	interleukin-1 beta-1	<i>il1b1</i>	NM_001124347	223
	interleukin-1 beta-2	<i>il1b2</i>	AJ245925	279
	interleukin-1 beta-3	<i>il1b3</i>	AJ557021	232
	interleukin-1 beta 4 (pseudogene)	<i>il1b4</i>	GSONMT00003908001	179
	interleukin-11a	<i>il11a</i>	GSONMT00009406001	251
IL-receptors	interleukin-1 receptor-like 1 ¹	<i>il1r-like-1</i>	GSONMT00035358001	168
	interleukin-1 receptor-like 1	(Exons 9-11)	GSONMT00021943001	
	interleukin-1 receptor-like 1		GSONMT00035357001	
	interleukin-1 receptor-like 1 ²	<i>il1r-like-1</i>	GSONMT00022297001	307
	interleukin-1 receptor type 1-like	(Exons 3-5) <i>il1r1-like-a</i> ³	GSONMT00066303001	238
	interleukin-1 receptor type 1-like	<i>il1r1-like-b</i> ³	GSONMT00032852001	241
	interleukin-1 receptor type 2 ⁴	<i>il1r2</i>	GSONMT00066304001	270
TNF superfamily	tumor necrosis factor alpha 1	<i>tnfa1</i>	NM_001124357	136
	tumor necrosis factor alpha 2	<i>tnfa2</i>	NM_001124374	143
	tumor necrosis factor alpha 3	<i>tnfa3</i>	HE798544	184
TNF receptor superfamily	tnf receptor superfamily 1a	<i>tnfrsf1a</i>	GSONMT00019008001	349
	tnf receptor superfamily 1a like	<i>tnfrsf1a-like-a</i> ³	GSONMT00061855001	175
	tnf receptor superfamily 1a like	<i>tnfrsf1a-like-b</i> ³	GSONMT00061996001	337
	tnf receptor superfamily 1b	<i>tnfrsf1b</i>	GSONMT00074093001	147
	tnf receptor superfamily 5	<i>tnfrsf5</i>	GSONMT00012579001	155
	tnf receptor superfamily 9	<i>tnfrsf9</i>	GSONMT00057532001	275
Housekeeping Genes	60S acid ribosomal protein p2	<i>arp</i>	BT074359	192
	actin-beta (aortic smooth muscle)	<i>actb</i>	NM_001124235	218
	glyceraldehyde-3-phosphate dehydrogenase	<i>gapdh</i>	NM_001124246	287

¹ Automated annotation of transcript GSONMT00035358001, Marancik et al. (26), was changed from “interleukin-1 receptor-like 1-like” to “interleukin-1 receptor 1-like” based on analysis of the omy03 genomic locus that contains three sequence-related, tandem genes (GSONMT00035358001, GSONMT00021943001 and GSONMT00035357001). The primers anneal to exon 9 and exon junction 10/11 in all three genes.

² Automated annotation of transcript GSONMT00022297001, Marancik et al. (26), was changed from “interleukin-1 receptor type 2” to “interleukin-1 receptor 1-like” based on analysis of genomic locus from omy03. This gene is actually the 5’ region of gene GSONMT00035357001. The primers designed against GSONMT00022297001 also amplify the 5’ region belonging to GSONMT00035358001 but have 2 bp mismatch with the 5’ region belonging to GSONMT00021943001.

³ The designations “-a” or “-b” were added to the automated annotation to distinguish between putative paralogues.

⁴ Automated annotation of transcript GSONMT00066304001, Marancik et al. (26), was changed from “interleukin-1 receptor type 2-like” to “interleukin-1 receptor type 2”.

Oncorhynchus mykiss

Flavobacterium psychrophilum

GeXP multiplex, RT-PCR assay