Gene expression response to sea lice in Atlantic salmon skin: an RNA-Seq comparison between resistant and susceptible animals

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ABSTRACT

Background: Sea lice are parasitic copepods that cause large economic losses to salmon aquaculture worldwide. Frequent chemotherapeutic treatments are typically required to control this parasite, and alternative measures such as breeding for improved host resistance are desirable. Insight into the host-parasite interaction and mechanisms

of host resistance can lead to improvements in selective breeding, and potentially novel treatment targets. In this study, RNA sequencing was used to study the skin transcriptome of Atlantic salmon parasitized with sea lice (*C. rogercresseyi).* The overall aims were to compare the transcriptomic profile of skin at louse attachment sites and 'healthy' skin, and to assess differences between animals with varying levels of resistance to the parasite.

Results: Atlantic salmon were challenged with *C. rogercresseyi*, growth and lice count measurements were taken for each fish. 21 animals were selected and RNA-Seq was performed on skin from a louse attachment site, and skin distal to attachment sites for each animal. These animals were classified into family-balanced groups according to the traits of resistance (high vs low lice count), and growth during infestation (an indication of tolerance). Overall comparison of skin from louse attachment sites versus healthy skin showed that 4,355 genes were differentially expressed, indicating local up-regulation of several immune pathways and activation of tissue repair mechanisms. Comparison between resistant and susceptible animals highlighted expression differences in several immune response and pattern recognition genes, and also myogenic and iron availability factors. Genomic regions showing signs of differentiation between resistant and susceptible fish were identified using an Fst analysis.

Conclusions: Comparison of the skin transcriptome between louse attachment sites and healthy skin has yielded a detailed profile of genes and pathways with putative roles in the local host immune response to *C. rogercresseyi*. The difference in skin gene expression profile between resistant and susceptible animals led to the identification of several immune and myogenic pathways potentially involved in host resistance. Components of these pathways may be targets for studies aimed at improved or novel

treatment strategies, or to prioritise candidate functional polymorphisms to enhance

genomic selection for host resistance in commercial salmon farming.

Keywords: *Caligus rogercresseyi*, *Salmo salar*, aquaculture, disease, parasite, RNA-

Seq, transcriptome, differential expression

BACKGROUND

Aquaculture is currently the fastest growing food industry [1] and is essential to meet

increasing global demands for fish. However, the sustainability and prolonged success

of any farming industry depends on effective disease prevention and control, and this

tends to be particularly challenging for aquaculture. The aquatic environment and high

stock density can expedite pathogen spread, which has historically resulted in periodic

mass mortality events and ongoing challenges in disease prevention and control. While

biosecurity measures, vaccination, nutrition and medicines all play vital roles for several

diseases, selective breeding to produce more resistant and tolerant aquaculture stocks is

rapidly becoming a key component of the battle to prevent these outbreaks [2, 3].

Sea lice, ectoparasites of the family Caligidae, are one of the major disease problems

facing the aquaculture industry, and specifically for salmon farming. Atlantic salmon is

the most important species in aquaculture with a production value of 14.7 billion US

dollars in 2014 [1], therefore control of sea lice is a primary goal for the industry. Sea

lice-related economic losses to worldwide salmonid aquaculture were estimated at ~430

million USD per annum [4]. Two lice species present the primary concerns for salmon

farming: *Lepeophtheirus salmonis* in the Northern Hemisphere and *Caligus*

rogercresseyi in the Southern [5]. These copepods parasitize salmon during the marine

phase of the lifecycle by attaching to their skin or fins, and feeding on the blood and

tissue. This leads to open wounds which can facilitate the entry of other pathogens. The

impaired growth and secondary infections cause significant negative animal welfare and

economic impact [6]. Interestingly, the outcome of sea lice infestation varies for

different salmonid species, with coho salmon (*Oncorhynchus kisutch*) showing rapid

inflammatory response and epithelial hyperplasia, leading to parasite encapsulation and

more than 90% reduction in lice loads [7]. In comparison, Atlantic salmon (*Salmo*

salar) is highly susceptible to sea lice infestation and seemingly cannot mount an

effective immune response [7].

Despite extensive use of both chemical and non-chemical treatments to control sea lice,

their negative impact on salmon aquaculture has increased in the past years [8]. Various

sea lice populations have been reported to be resistant to the most common chemicals

available for therapeutic control [9]. Therefore, alternative methods to control sea lice

are currently being studied, including the use of probiotics to reduce salmon

attractiveness for sea lice [10] or cohabitation with lice-eating species [11, 12]. A

promising and potentially complementary approach to existing control measures is to

exploit natural genetic variation in farmed salmon populations to breed stocks with

enhanced resistance to the parasite. Current evidence indicates that host resistance to sea

lice in Atlantic salmon has a highly polygenic genetic component [13, 14, 15, 16, 17].

The presence of significant genetic variation for resistance against *Caligus*

royercresseyi, with heritability values ranging between 0.1 up to 0.34, demonstrates the

feasibility of improving this trait by selective breeding in Atlantic salmon [18, 19].

While selective breeding for resistance can be effective without any prior knowledge of

the underlying genes, understanding the functional basis underpinning genetic

resistance is a major research goal. The benefits of this include the prioritisation and

testing of putative functional (causative) variants which may be directly affecting host

resistance, for which the new Functional Annotation of All Salmonid Genomes

(FAASG) project will be integral [20]. Such variants can enhance selective breeding by

improving accuracy of prediction of the genetic merit, particularly across distant

relatives or populations. Finally, these causative variants could be introduced into

populations or species where it has never been present through the use of genome

editing, for example using CRISPR-Cas9 technology which has been successfully

applied in salmon [21].

In addition to the value to selective breeding programs, knowledge of the interaction

between salmon and sea lice can help devise more effective prevention and treatment

strategies. RNA sequencing can provide a relatively holistic view of the host response

to parasite infection, which in turn can highlight specific genes, pathways and networks

involved in the host-parasite interaction. RNA-Sequencing can also be used to identify

genetic markers in transcribed regions, and to assess the putative impact of those

markers on transcript and protein function. The effect of these markers on gene

expression (and ultimately host resistance) can be assessed, leading to a shortlist of

candidate functional variants. The overall aims of the current study were to compare the

transcriptome profile of salmon skin at louse attachment sites and 'healthy' skin, and to

evaluate differences in these profiles between animals with varying levels of resistance

to the parasite. To achieve this, challenged animals were classified into family-balanced

groups according to the traits of resistance (based on high vs low lice count), and

growth during infestation (an indication of tolerance) and RNA-Seq was performed on

individual samples. While individual growth rate during challenge is not strictly

'tolerance' (because it is not scaled according to parasite burden [22]), this is the term

used herein to refer to this trait. By comparing these groups, genes, pathways and

genetic variation related to local immune response and host resistance were identified

and discussed.

RESULTS AND DISCUSSION

Disease challenge

A total of 2,632 fish belonging to 105 families from a commercial breeding program

were challenged with *Caligus rogercresseyi*, and killed for sampling eight days post

challenge. Average lice burden per fish was 38 ± 16. Fish were selected for RNA

sequencing based on the traits of resistance, measured as number and concentration of

lice per fish, and tolerance, defined in this study as weight and length gain since the

start of the challenge. The selected fish allowed for 8 vs 8 comparisons between family-

matched fish showing differential resistance (26.2 ± 5.5 vs 54.9 ± 13.5 sea lice per fish)

and differential tolerance (7.0 ± 4.3 vs 28.8 ± 12.3 weight gain percentage). A total of

42 samples (21 fish, skin from sites of louse attachment and healthy skin) were

sequenced, resulting in an average of $\sim 27.9 \pm 2.7$ million reads per sample. After

trimming, these were aligned against the salmon reference genome (ICSASG_v2;

Genbank Accession GCF_000233375.1 [23]) and levels of gene expression were

estimated according to the official salmon genome annotation (NCBI *Salmo salar*

Annotation Release 100). 25% of the aligned reads were discarded due to

multimapping, and the position of an additional 11% did not overlap with any known

gene. Following these filters, an average of 19 M reads per sample were assigned to

genes and used for downstream analyses of gene expression. All raw sequence data is

available in NCBI's Sequence Read Archive (SRA) under BioProject accession number

SRP100978, and may be a useful contribution to the functional annotation of all

salmonid genomes initiative (FAASG [20]).

Louse attachment sites versus healthy skin

Hierarchical clustering of samples according to their normalized gene expression did

not initially reveal an obvious clustering according to any of the measured phenotypes,

nor between samples from louse attachment sites and those from healthy skin

(Additional file 1). Despite this, after a single outlier sample was removed, a total of

1,711 differentially expressed (DE) genes were identified between healthy and injured

skin (Figure 1A). These DE genes showed relatively small log2 fold change (FC)

differences. The expression levels for the most significant DE genes from this list ($p <$

0.001, 156 genes) are shown in a heatmap (Figure 1B), which highlights a gene

expression signature of lice attachment sites and healthy skin. However, a small number

of lice attachment site samples did not cluster as expected and were removed. The

differential expression was repeated using 13 vs 13 samples (Figure 1C). Despite the

lower statistical power due to the reduced sample size, the number of significant DE

genes was substantially larger (n = 4,355, Additional file 2), with a higher number of

up-regulated than down-regulated genes when comparing attachment sites to healthy

skin (n = 3,114 vs n = 1,241).

Among these DE genes were well-known components of the innate immune response

like interleukins, interferon response factors and complement components (Figure 2A).

GO term and KEGG pathway analyses (Additional file 2) revealed a clear enrichment of

immune pathways and functions among the up-regulated genes (Figure 2B),

highlighting a localised immune response strongly related to cytokines. A similar

scenario has been observed in other salmonids such as coho salmon where resistance to

sea lice has been associated with early inflammation in skin and head kidney, which

results in epithelial hyperplasia and often parasite encapsulation and removal of the sea

lice within two weeks [24, 25]. In pink salmon (*Oncorhynchus gorbuscha*), an early and

high expression of pro-inflammatory genes (IL-8, TNFα-1, IL-1β) has been suggested

as a mechanism of rapid louse rejection [26]. The classical complement pathway has

also been linked to resistance of host fish to parasitic copepod infection [7]. The results

presented here indicate that despite a marked up-regulation of the local inflammatory

response and complement pathway in Atlantic salmon, resembling those of coho salmon

or pink salmon, the response does not seem to be sufficient to successfully respond to

the louse attachment and feeding.

In addition to the expected innate immune response observed above, cell division

related processes were also clearly up-regulated at louse attachment sites, and well-

characterised genes involved in tissue repair such as fibroblast growth factor-binding

protein 1 and Epigen showed significant differences between lice attachment sites and

healthy skin (FC > 3). Several genes related to the cell matrix and cell adhesion also had

higher expression at attachment sites (i.e. cadherin-13, integrin alpha-2, desmoplakin, or

various keratin and collagen genes). Cell proliferation is the main response to skin

wounds in fish [27], and these results are consistent with those previously found in the

early response to *Lepeophtheirus salmonis* [28]. Several mucins were also found to have

higher expression at attachment sites, pointing towards increased mucus production and

secretion which can also be a typical response to wounding in fish [7].

Resistance

The Atlantic salmon samples used for RNA-Seq were taken from a broader experiment

relating to genetic resistance to sea lice, and were measured for several traits of

relevance to resistance and host response to infection. These traits were defined as

resistance, measured as number and concentration of lice per fish, and tolerance,

defined in this study as weight gain since the start of the challenge. The samples for

RNA sequencing were chosen to enable 8 vs 8 comparisons between family-matched

fish with high and low values for both resistance (26.2 ± 5.5 vs 54.9 ± 13.5 sea lice per

fish) and tolerance (7.0 ± 4.3 vs 28.8 ± 12.3 weight gain percentage).

Differential expression

There were 43 genes significantly differentially expressed between resistant and

susceptible fish, although all but one were from comparison of healthy skin samples

between the two groups (Additional file 3). The susceptible group had higher expression

levels for genes involved in muscle contraction like troponins and myosins, which was

also highlighted by GO enrichment analyses (Figure 3). Myosins and troponins have

previously been identified as genes that respond to sea lice attachment in salmon skin

[29]. Further, *Caligus* infection is known to induce increased enzyme activity in muscle

tissue [30], and behavioural changes in the fish such as flashing and jumping are

associated with ectoparasite removal [31, 32]. It has been recently reported that

inactivity or reduced swimming activity contribute to resistance to sea lice [33], so it is

possible that the high lice counts of susceptible fish in this study are due to higher

activity levels with associated expression of muscle contraction related genes. In turn,

high lice burden can provoke behavioural responses increasing fish activity, which

results in the up-regulation of muscle genes, increasing the expression differences

between resistant-passive-low lice fish and susceptible-active-high lice fish.

Two heme oxygenase genes (HO), encoding enzymes which catalyze the degradation of

heme, also had higher expression levels in susceptible samples (Figure 3). These genes

have been previously shown to be up-regulated in response to *Caligus* infection [34].

Reduction of iron availability within the host has been observed in Atlantic salmon

infected with *L. Salmonis* [35], as has reduced hematocrit and anemia in Pacific salmon

[36]. Finally, three immune receptors showed higher expression in susceptible samples

(Figure 3); C-X-C chemokine receptor type 2 (CXCR2) is a receptor for IL-8, its

binding causes activation of neutrophils; while C type lectin receptors A and B

(CLECA, CLECB) are involved in pathogen recognition and immunity [37]. While it is

clear that resistance and host response to sea lice is multifactorial in nature, these genes

related to muscle contraction, iron availability and immunity may be targets for

functional validation in future studies, and for cross-referencing with genome-wide

association analyses to identify candidate causative genes and variants.

Network analysis

As an alternative approach to investigate the gene expression profiles associated with

resistance and tolerance to sea lice in these samples, a network correlation analysis was

performed. For this analysis, instead of categorizing the animals into two groups and

testing differential expression, gene expression values were used to group genes with

similar expression profiles into different networks. The average expression profile of

each network was then correlated with the measured traits to identify significant

associations between groups of genes and the resistance phenotypes (Figure 4). One

gene network with a total of 76 genes was associated with resistance (as measured by

lice counts) in healthy skin (Additional file 3). A single gene from this network was also

differentially expressed in the previous analysis between high and low resistant fish, C-

type lectin receptor A (FC = - 1.34). As previously mentioned, this gene is a receptor

involved in antigen recognition and immune response [37]. Four other immune

receptors involved in innate immunity and inflammation were assigned to this network

and found to be positively correlated with sea lice counts (Table 1). Macrophage

mannose receptor 1 (MRC1) shows the highest expression difference between louse

attachment vs healthy skin DE genes (FC = 4.79) and also the highest positive

correlation with number of sea lice (r = 0.87). MRC1 is also a c-type lectin receptor,

expressed in macrophages, dendritic cells and skin in humans. MRC1 plays a role both

in innate and adaptive immunity and also acts as a recognition receptor for different

pathogens such as bacteria, virus or fungi [38]. Lectins such as MRC1 and CLEC4E

have been found to be induced by glucosinolate-enriched feeds in Atlantic salmon,

which also reduced lice counts between 17-25% [39]. Lectins have been reported to

activate the immune system in response to different parasites in different species [40,

41], therefore modulation of these genes represents a possible route to enhance Atlantic

immune responses to sea lice. Two immune receptors were negatively correlated with

number of sea lice, CD97 (r = -0.84) and suppressor of cytokine signalling 5 (SOCS5; r

= -0.76). CD97 regulates cytokine production and T-cell activation and proliferation

[42, 43]; while SOCS5 is part of the cytokine-mediated signalling pathway, and acts as

a negative regulator of inflammatory response and other immune-related pathways [44].

The correlation of these genes with number of sea lice is probably indicating that the

immune system of the host responds proportionally to the degree of infestation, but

nonetheless may be important in the louse-salmon interaction.

Table 1. Immune receptors showing correlation with sea lice counts

Gene	Full name	Corr. (r)	GO terms

MRC1	Macrophage mannose receptor 1	0.87	- Cellular response to interferon gamma - Cellular response to interleukin-4
CLEC4E	C-type lectin receptor A	0.81	- Innate immune response - Positive regulation of cytokine secretion
CR2	Complement receptor type 2	0.78	- Innate immune response - Complement activation, classical pathway
LIFR	Leukemia inhibitory factor receptor	0.78	- Cytokine-mediated signalling pathway
CD28	T-cell-specific surface glycoprotein CD28	0.77	- Positive regulation of inflammatory response to antigenic stimulus - Cytokine biosynthethic process
SOCS5	Suppressor of cytokine signaling 5	-0.76	- Cytoine-mediated signalling pathway -Negative regulation of inflammatory response
CD97	CD97 antigen	-0.84	- Inflammatory response

Amongst genes without a (well-known) immune function, there was an association between SUMO1 (r = 0.76) and SUMO3 (r = -0.91) expression and resistance. Small Ubiquitin-like Modifier (SUMO) proteins are small proteins similar to ubiquitins that are covalently attached to other proteins to modify their function. According to the gene expression data, SUMO1 seems to be preferred over SUMO3 in salmon upon sea lice infestation. Although post-translational modifications have been barely explored in fish, in mice SUMOylation has been shown to be involved in modulation of host innate immune response to pathogens [45]. SUMOylation is also a very active field of research in plants, where SUMO is known to be involved in many important processes such as plant response to environmental stresses, including pathogens [46]. It would be

interesting to further study the role of SUMO in modulating Atlantic salmon responses

to sea lice.

Tolerance

Differences in weight gain percentage from the start to the end of the trial were also

investigated; a trait which was defined as tolerance. Tolerance did not show any

significant correlation with initial weight (r = -0.27, p = 0.10), sea lice counts (r = 0.12,

p = 0.45) or sea lice density (r = 0.19, p = 0.24) in our dataset, and the means for these

three traits are not significantly different between our tolerant and not-tolerant groups (t-

test p-values > 0.35).

Differential expression

A total of 24 and 1 genes were found differentially expressed between fish showing

high and low tolerance in healthy and sea louse attachment site samples respectively

(Additional file 4). The gene differentially expressed in injured skin, solute carrier

family 15 member 1 (SLC15A1), also showed the lowest p-value and highest fold

change in healthy skin (FC = 3.38, p = 0.003). SLC15A1 protein is a membrane

transporter that mediates the uptake of dipeptides and tripeptides, in humans this gene is

expressed in the intestinal epithelium and plays a major role in protein absorption [47].

Another interesting DE gene is myogenic regulatory factor 6 (MYF6; FC = 0.72, p =

0.04). Myogenic regulatory factors are transcription factors that regulate muscle

development [48]; in *Senegale sole* decreased expression of these factors was observed

in fast muscle when fed with a high-lipid content diet which caused reduced growth

[49]. While skin is unlikely to be a highly suitable tissue to study genes underlying fish

growth during sea lice infestation, both myogenic factors and increased nutrient

absorption, and specifically MYF6 and SLC15A1, are good candidates to better

understand growth impairment differences under sea lice infestation.

Network analysis

A gene network correlation analysis was also performed for tolerance, finding five

associated networks, one of them common between healthy and injured datasets (Figure

4). A total of 373 genes showed correlation values > 0.75 with weight gain percentage

(Additional file 4). These genes are fairly heterogeneous, showing no GO or KEGG

enrichment. This is perhaps not that surprising; here we have defined tolerance as

impact of sea lice on growth performance, but most likely growth performance is only

one of the aspects of tolerance. A more tolerant animal will be less affected by the

parasite in many ways, and therefore it is logical to find genes involved in different

functions correlated with our definition of tolerance. Several immune related genes were

positively correlated with weight gain, such as C-X-C chemokine receptor type 4,

interferon regulatory factor 2, caspase-8 or TNF receptor-associated factor 5. Very

different immune responses for IPNV resistant and IPNV susceptible Atlantic salmon

families have been observed [50], so it is well possible that Atlantic salmon can also

elicit different immune responses against sea lice, leading to different outcomes.

However, while IPNV resistance is mainly explained by a single locus [51], sea lice

tolerance is most likely highly polygenic, with probably different mechanisms operating

in the different families, which hinders the understanding of tolerance (and resistance)

mechanisms.

Signatures of differentiation

A total of 126,741 SNPs were identified from the RNA-Seq of the 21 individual fish. These were used to compute Fst values between the resistant and susceptible samples, and between tolerant and not tolerant samples, through a sliding window approach (100 Kb windows every 50 Kb). Only windows with ≥ 5 SNPs were retained for further analysis. Genomic windows with Fst values ≥ 0.20 were considered to show putative signs of differentiation between both groups (35 out of 15,321 total windows, 99.8 percentile). 35 differential genomic regions were found to be associated with resistance and 28 with tolerance (Additional file 5). While the tolerance results did not overlap with differentially expressed genes, genomic regions showing signs of differentiation between resistant and susceptible sample did highlight some interesting candidate genes (Figure 5). The genomic region showing the clearest sign of differentiation between resistant and susceptible samples was found in chromosome 5 between 18.50 and 18.75 Mb, with four consecutive windows with Fst values between 0.237 and 0.306. There are 29 SNPs and 7 genes in this genomic region (Figure 5B). One of these genes, Neurexophilin And PC-Esterase Domain Family Member 3 (NXPE3), was also differentially expressed between resistant and susceptible samples (p-val = 0.02, FC = -0.92). One of the SNPs in the region (Fst = 0.333) codes for a non-synonymous mutation in NXPE3, and four additional SNPs (Fst = 0.421-0.454) could fall within coding regions of NXPE3 as well (these SNPs fall in a transcribed region without annotation in the official salmon annotation; blast searches against NCBI's nr database match NXPE3). Despite being conserved in vertebrates there is not much information about this gene. In humans it binds alpha neurexins, a group of presynaptic transmembrane receptors that promote adhesion between dendrites and axons [52]. Neurexins have been linked to behavioural response to parasites in bees [53] and in host

response to parasite infection in hard clam, *Mercenaria mercenaria* [54]. While not an

obvious functional candidate, NXPE3 is worthy of further investigation in host

resistance to sea lice due to its differential expression and overlap with a genomic

region showing putative differentiation between resistant and susceptible fish. Another

interesting gene in the same region of chromosome 5 is COP9 signalosome complex

subunit 6 (COPS6). This gene is highly expressed in our samples and contains three

SNPs, the one with the highest Fst (0.312) codes for a synonymous mutation. This gene

is part of the COP9 signalosome complex, which is a regulator of the ubiquitin

conjugation pathway but is also involved in phosphorylation-mediated regulation of

various immune-related genes, such as NF-kappa-B-inhibitor alpha or IRF8.

CONCLUSIONS

The results of this study highlight that the early gene expression response of Atlantic

salmon to sea lice involves up-regulation of many different components of the immune

system (inflammatory response, cytokine production, TNF and NF-kappa B signalling

and complement activation) along with tissue repair activation. The comparison of

resistant versus susceptible animals highlighted enrichment of pathways related to fish

activity, iron availability and receptors modulating pathogen recognition and immune

response, and identified a few candidate genomic regions potentially involved in genetic

resistance. Overall, this study contributes to an improved understanding of Atlantic

salmon early response to sea lice in skin, and into the gene expression profiles

underpinning genetic resistance to sea lice in salmon. The identified pathways and

genes may be targets for future studies aimed at development of new treatments,

vaccines or prevention strategies. The data can also be cross-referenced with high power

genome-wide association studies to help prioritise putative causative genes and variants

that have potential to improve genomic selection programs for genetic improvement of

resistance to this industry's most serious disease.

METHODS

Experimental design

2,668 Atlantic salmon (*Salmo salar*) pre-smolts (average weight 136 g) from 104

families from the breeding population of Salmones Chaicas, Xth Region, Chile, were

experimentally challenged with *Caligus rogercresseyi* (chalimus II-III). Briefly,

infestation with the parasite was carried out by using 13 to 24 copepodids per fish and

stopping the water flow for 6 h after infestation. Eight days after the infestation fish

were euthanized and fins from each fish were collected and fixed for processing and lice

counting.

42 samples from 21 fish from six different families (2 to 5 fish per family) were

selected for RNA sequencing (Additional file 6) based on the traits of interest (number

of sea lice attached to their fins and growth during challenge). Skin samples (both from

attachment sites and health skin) were obtained from each animal and stored in

RNAlater at 4 ºC for 24 h, and then at -20ºC until RNA extraction for sequencing.

RNA extraction and sequencing

For all the 42 samples a standard TRI Reagent RNA extraction protocol was followed.

Briefly, approximately 50 mg of skin was homogenized in 1 ml of TRI Reagent (Sigma,

St. Louis, MO) by shaking using 1.4 mm silica beads, then 100 µl of 1-bromo-3-

chloropropane (BCP) was added for phase separation. This was followed by

precipitation with 500 µl of isopropanol and posterior washes with 65-75 % ethanol.

The RNA was then resuspended in RNAse-free water and treated with Turbo DNAse

(Ambion). Samples were then cleaned up using Qiagen RNeasy Mini kit columns and

their integrity was checked on Agilent 2200 Bioanalyzer (Agilent Technologies, USA).

Thereafter, the Illumina Truseq mRNA stranded RNA-Seq Library Prep Kit protocol

was followed directly. Libraries were checked for quality and quantified using the

Bioanalyzer 2100 (Agilent), before being sequenced on three lanes of the Illumina

Hiseq 4000 instrument using 75 base paired-end sequencing at Edinburgh Genomics,

UK. Raw reads have been deposited in NCBI's Sequence Read Archive (SRA) under

accession number SRP100978.

Read mapping

The quality of the sequencing output was assessed using FastQC v.0.11.5

(http://www.bioinformatics.babraham.ac.uk/projects/fastqc/). Quality filtering and

removal of residual adaptor sequences was conducted on read pairs using Trimmomatic

v.0.32 [55]. Specifically, Illumina specific adaptors were clipped from the reads, leading

and trailing bases with a Phred score less than 20 were removed and the read trimmed if

the sliding window average Phred score over four bases was less than 20. Only reads

where both pairs were longer than 36 bp post-filtering were retained. Filtered reads were

mapped to the most recent Atlantic salmon genome assembly (ICSASG_v2; Genbank

accession GCF_000233375.1 [23]) using STAR v.2.5.2b [56], the maximum number of

mistmatches for each read pair was set to 10 % of trimmed read length, and minimum

and maximum intron lengths were set to 20 bases and 1 Mb respectively. Uniquely

mapped paired-reads were counted and assigned to genes (NCBI *Salmo salar*

Annotation Release 100) using FeatureCounts [57], included in the SourceForge

Subread package v.1.5.0. Only reads with both ends mapped to the same gene were

considered in downstream analyses.

SNP identification

SNPs were identified and genotypes for individual samples were called using samtools v1.2 [58]. Reads with mapping quality < 30 and bases with phred quality scores < 30 were excluded. To call a SNP, a read depth ≥ 10 and ≥ 3 reads for the alternative allele were required. Only SNPs called in > 75% of the samples were retained for downstream analyses. The putative effect of the SNPs was assessed using the official salmon genome annotation (NCBI *Salmo salar* Annotation Release 100) and the SnpEff v.4.2 software [59].

Differential Expression

Statistical analyses related to differential expression and pathway enrichment were performed using R v.3.3.1 [60]. Gene count data were used to estimate differential gene expression using the Bioconductor package DESeq2 v.3.4 [61]. The Benjamini-Hochberg false discovery rate (FDR) was applied, and transcripts with corrected p-values < 0.05 and absolute $\log_2$ fold change values (FC) > 0.5 were considered differentially expressed genes. Samples were hierarchically clustered according to gene read counts after a variance stabilizing transformation, using Euclidean as the distance measure and complete-linkage as the agglomeration method (R package flashClust [62]). Heatmaps of gene expression were created using the R package gplots v3.0.1 heatmap.2 function, using read counts after regularized log transformation (DESeq2 [61]).

Pathway Enrichment

Gene Ontology (GO) enrichment analyses were performed using Blast2GO v.4.1 [63]. Briefly, genes showing > 10 reads in > 90 % of the samples were annotated against the manually curated protein database Swiss-Prot [64] and GO terms were assigned to them

using Blast2GO. GO enrichment for specific genes lists was tested against the whole set

of expressed genes using Fisher's Exact Test. GO terms with ≥ 5 DE genes assigned and

showing a Benjamini-Hochberg FDR corrected p-value < 0.05 were considered

enriched. Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analyses

were performed using KOBAS v3.0.3 [65]. Briefly, genes showing > 10 reads in > 90 %

of the samples were annotated against KEGG protein database [66] to determine KEGG

Orthology (KO). KEGG enrichment for specific gene lists was tested by comparison to

the whole set of expressed genes using Fisher's Exact Test. KEGG pathways with ≥ 5

DE genes assigned and showing a Benjamini-Hochberg FDR corrected p-value < 0.05

were considered enriched.

Network Correlation Analysis

Gene expression network correlation analyses were performed using the R package

WGCNA [67]. Read counts after variance stabilizing transformation were used as a

measure of gene expression. The genes were clustered using a power of 6 (scale free

topology index > 0.9), a minimum of 30 genes per cluster and merging those clusters

with a distance < 0.25 between them. Correlation between network summary profiles

and external traits was quantified, and network-trait association showing p-values <

0.05 were considered significant. Genes showing network membership significance

values < 0.05 and | r | > 0.75 were considered to be correlated with the trait of interest.

Fst Analyses

Weir and Cockerham's Fst statistics were calculated using VCFtools v0.1.14 [68].

Mean Fst values were calculated using a sliding window approach with 100 Kb

windows and a step of 50 Kb. Only those windows containing at least 5 SNPs were

considered.

DECLARATIONS

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Authors´ contributions

RDH, JMY and DR were responsible for the concept and design of this work. AB managed the collection of the samples. AP performed the molecular biology experiments. DR performed bioinformatic and statistical analyses. DR, RDH and JMY drafted the manuscript. All authors read and approved the final manuscript.

Competing interests

The authors declare that they have no competing interests.

Ethics approval and consent to participate

The lice challenge experiments were performed under local and national regulatory systems and were approved by the Animal Bioethics Committee (ABC) of the Faculty of Veterinary and Animal Sciences of the University of Chile (Santiago, Chile), Certificate N° 01-2016, which based its decision on the Council for International Organizations of Medical Sciences (CIOMS) standards, in accordance with the Chilean standard NCh-324-2011.

Consent to publish

Not applicable

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Availability of data and materials

The datasets generated during the current study are available in the NCBI's Sequence Read Archive (SRA) repository under accession number SRP100978 [https://www.ncbi.nlm.nih.gov/sra/?term=SRP100978].

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FIGURES

Figure 1. Sample clustering and differential expression. A) Volcano plot showing

the result of differential expression analysis between healthy and injured skin (all

samples). Level of expression is shown in the x-axis and $\log_2$ fold change in y-axis,

genes with false discovery rate corrected p-values < 0.05 are shown in red. B) Heatmap

showing the gene expression of the most significant 156 DE genes (p < 0.001) between

healthy (blue) and injured (red) skin. C) Volcano plot showing the result of differential

expression analysis between healthy and injured skin using those animals whose injured

skin shows some sort of response to *Caligus*. Level of expression is shown in the x-axis

and $\log_2$ fold change in y-axis, genes with false discovery rate corrected p-values < 0.05

are shown in red.

Figure 2. Healthy vs injured skin. A) Important immune-related genes showing

differential expression between healthy and injured skin. B) Selection of GO terms

enriched amongst DE genes between healthy and injured skin.

Figure 3. Up-regulation in susceptible fish. A) Genes DE between resistant and

susceptible fish, being up-regulated in the latter. B) Enriched GO terms amongst DE

genes up-regulated in susceptible fish.

Figure 4. Network correlation analyses. Correlation between gene networks and

different traits of interest in healthy and injured skin.

Figure 5. Fst between resistant and susceptible fish. A) Manhattan plot showing the

chromosomes and position in the x-axis and Fst in the y-axis. B) High Fst region in

chromosome 5.

ADDITIONAL FILES

Additional file 1.

PNG (.png)

Hierarchical cluster of the whole dataset.

Hierarchical clustering for all the samples using the expression of all the genes in our

dataset. Healthy (blue) and injured (red) sample phenotypes are shown using a white

(minimum) to red (maximum) color code.

Additional file 2.

Excel (.xlsx)

Comparison between healthy and injured skin.

Differentially expressed genes (1st sheet), GO term and KEGG pathway enrichment (2nd

sheet) are shown for the comparison between healthy and injured skin.

Additional file 3.

Excel (.xlsx)

733 Comparison between resistant and susceptible samples.

734 Differentially expressed genes (1st sheet), GO term and KEGG pathway enrichment (2nd

735 sheet), and network analysis results (3rd sheet) are shown for the comparison between

736 resistance and susceptible samples.

738 **Additional file 4.**

739 Excel (.xlsx)

740 Comparison between tolerant and not tolerant samples.

741 Differentially expressed genes (1st sheet) and network analysis (2nd sheet) are shown for

742 the comparison between tolerant and not tolerant samples.

744 **Additional file 5.**

745 Excel (.xlsx)

746 Fst values for resistance and tolerance comparisons.

747 Genomic regions showing Fst values > 1 for resistance (1st sheet) and tolerance (2nd

748 sheet) comparisons.

750 **Additional file 6.**

751 Excel (.xlsx)

752 RNA-seq samples

753   List of samples sequenced in this study and their phenotypes.

A)

B)

Up-regulated in susceptible fish

A) Differentially expressed genes

B) Enriched GO terms