BACTERIAL RICKETTSIAL DISEASES are common and important human and veterinary pathogens (93, 94). In fish, rickettsial-like organisms affect numerous species from different geographical regions and diverse aquatic environments (26). Piscirickettsia salmonis is the etiological agent of piscirickettsiosis, or salmonid rickettsial septicemia (SRS) (59). P. salmonis is a Gram-negative intracellular nonmotile, nonencapsulated, bacterial pathogen isolated from fish (6, 39) and is considered to be an intracellular facultative bacterium (61) showing a close relationship with the facultative intracellular gamma-proteobacteria (25). In addition, structural studies of lipid A have demonstrated a high similarity to endotoxic enterobacteria lipid A, indicating high endotoxicity of P. salmonis (86).

The transmission of P. salmonis is believed to be by horizontal infection with no requirement for a vector (25). The main agents of transmission of the disease are feces and urine of infected fish, and the infection route is through the gills and wounds in the skin (79).

P. salmonis is responsible for a systemic infection in fish, targeting the cytoplasmic vacuoles of hepatocytes and liver-associated macrophages, as well as kidney, spleen, and peripheral blood macrophages (1, 5, 17). The systemic spread and replication of the pathogen cause severe anemia in fish (17), resulting in pale gill filaments, liver and kidney hemorrhages, and ulcerations in the fins (16) during later stages of infection.

This aggressive disease has affected salmonid aquaculture since the first reported outbreak in Chile in 1989 (6, 27, 55), causing major economic losses to the industry (25). Atlantic salmon generally exhibit symptoms of the disease 6–12 wk postseawater transfer (17); the infection can be present in freshwater but normally does not manifest as disease. Since the pathogen’s discovery, P. salmonis have been detected in a variety of cultured salmonid species (7, 15) as well as in farmed white sea bass (Atractoscion nobilis) (2), European sea bass (Dicentrarchus labrax) (62, 81), and tilapia (Oreochromis niloticus, O. mossambicus, and Sarotherodon melanotheron) (60), demonstrating a diverse host range of the pathogen. Efficient control and treatment of SRS have been difficult (42), since there have been no efficient commercial vaccines and antibiotics also have limited effect on the disease (9). Management strategies including periods of fallowing have been one approach to limit the spread of the pathogen (69). Recently there have been a number of experimental vaccines that show promise for the future (92).

Little is known about the infection strategy of P. salmonis due to the experimental difficulties associated with the intracellular nature of the pathogen (76). Recent studies have shown that P. salmonis can infect, survive within, and replicate inside salmonid macrophages (63) and monocyte-like cells without cytopathic effect (76). Moreover it has been proposed that P. salmonis promotes apoptosis in salmon phagocytes (75); these properties may reflect bacterial strategies for survival and evasion of the host immune response (75).

Even less is known about the early response to this pathogen; however, a previous study of Rise et al. 2004 (73) showed changes in redox status and a general downregulation of genes involved in the adaptive immune response in primary cultured head kidney macrophage-like cells and in the head kidney of Atlantic salmon 24 h and 14 days, respectively, postinfection.

In this study we examined the early transcriptional response to an experimental P. salmonis infection in Atlantic salmon.
We used a microarray-based approach to examine differential gene expression in three tissues, liver, head kidney, and muscle. The results obtained show how the fish respond to the infection and how the pathogen potentially modulates the host’s immune response. The results of this research can help the identification and development of gene expression markers relating to early stage *P. salmonis* infection.

**METHODS**

Fish maintenance and feeding trial. Postsmolt Atlantic salmon (n = 150, *Salmo salar*) of mixed sex (mean weight 237 g, <2% CV) were maintained in triplicate 1 m³ tanks at SGS Chile, Puerto Montt, Chile, and shared the same nutritional and environmental background. The fish had previously been grown for 68 days to achieve a ~150% weight gain by feeding to apparent satiation with commercial formulated feed (CPK 100, 4 mm, 24/44 lipid: protein, Biomar Chile) at 10.1 °C water temperature, 27.6% salinity.

Prior to being handled the fish were anesthetized with benzocaine (20% wt/vol; 50 mg/l), and then 90 fish per tank were injected with 0.2 ml ip *P. salmonis* (PS14LT8) (1 × 10⁶ plaque-forming units/ml) in minimal essential media (MEM) into the intraperitoneal cavity. *P. salmonis* inoculum was grown in CHSE-214 cell culture (CHSE-T-39) with 80–90% cell confluence, according to standard operating procedures (SGS, Chile). Inoculated fish were transferred to triplicate 1 m³ tanks in the biosecurity section of the challenge facility. An additional 12 naive fish, comprising four fish from each of the originating triplicate tanks, were injected with 0.2 ml ip of MEM and transferred into a new tank to serve as noninfected control fish. Feeding of fish resumed following the challenge with the same feed. On the second day postchallenge five fish per *P. salmonis* challenge tank and the 12 control fish were killed by percussive stunning before white muscle, liver, and head kidney tissue were excised by aseptic technique and stabilized in RNAlater (Ambion) at 4°C for 24 h then stored at ~80°C until RNA extraction. Mortality was monitored in the *P. salmonis* challenge tanks until no further increase in mortality was recorded for 30 consecutive days. No control fish died during the trial. The cause of mortality observed as SRS was confirmed by postmortem autopsy and standard immunohistochemistry techniques; immunofluorescence analysis kit SRS-Flurotest (Biogroup Chile) was used to determine the level of *P. salmonis* infection.

RNA isolation. RNA was extracted from ~100 mg of tissue that had been stored in RNAlater. Tissues were homogenized in TRIzol (Invitrogen) with tungsten carbide beads (3 mm, Qiagen) for 3 min (Retsch MM300 ball mill homogenizer) at 300 shakes/min. Following homogenization, RNA was extracted according to manufacturer’s instructions. The RNA pellet was washed in 500 µl of RNase-free Tris Buffered Saline (Ambion) at 10 °C water temperature, 27.6% salinity. The RNA pellet was washed in 500 µl of RNase-free Tris Buffered Saline (Ambion) at 10 °C water temperature, 27.6% salinity. The results obtained show how the fish respond to the infection and how the pathogen potentially modulates the host’s immune response. The results of this research can help the identification and development of gene expression markers relating to early stage *P. salmonis* infection.

**Microarray hybridization and analysis.** For microarray analysis, for each tissue examined four pools of RNA (biological replicates) were made from SRS-challenged fish and from control fish. Each pool comprised an equal quantity of RNA from four different fish chosen randomly from each experimental group. For microarray hybridization a common reference design was employed. For this, a reference RNA sample was produced, which comprised an equimolar mix of RNAs extracted from all individual fish and tissue samples. Each experimental sample (Cy3 labeled) was hybridized against this reference sample (Cy5 labeled) in a two-color experiment. To generate fluorescently labeled RNA for hybridizations, a MessageAMP aRNA Amplification Kit (Ambion) was used for initial amplification of mRNA. Briefly, 2 µg total RNA was reverse transcribed and the cdNA was used as a template for in vitro transcription in the presence of amino allyl modified dUTP, which generated amplified antisense RNA (aRNA). For labeling, aRNA (3 µg) was denatured at 70°C for 2 min in a volume of 10 µl to which 3 µl of 0.5 M NaHCO₃ and 2 µl Cy dye (dye Cy3 or Cy5 mono-reactive dye pack, GE Healthcare) was added. Incorporation of dyes was performed for 1 h in the dark and the excess label was removed by DyeEx 2.0 spin column purification (Qiagen). The level of dye incorporation was checked by spectrophotometry (Nanodrop ND1000, LabTech). Prior to hybridization, 825 ng of each labeled template was fragmented in the presence of 11 µl of 10× blocking agent, 2.2 µl of 25× fragmentation buffer (Agilent), and made up to a final volume of 20 µl with nuclelease-free dH₂O. Fragmentation progressed in the dark at 60°C for 30 min and then 57 µl of 2× GE hybridization buffer (Agilent) was added to each sample, briefly mixed, spun down, and stored on ice until ready to load (~103 µl) onto each microarray. The hybridizations were performed in a rotisserie-style Microarray Hybridization Oven (Agilent) overnight (18 h) at 65°C. Following hybridization, the slides were rinsed in gene expression wash buffers 1 and 2 (Agilent) following the manufacturer’s instructions. The slides were scanned using a GenePix personal 4100A Scanner (Axon Instruments) at a resolution of 5 µm. Images, saved as *.TIF files, were extracted, and initial analysis were performed with Feature Extraction software v9.5.3 (Agilent). Background correction of feature intensities was performed within this software. Statistical analysis of the arrays was performed using the GeneSpring GX analysis platform (version 9.5, Agilent Technologies). A Lowess normalization of background-corrected data was conducted, and all intensity values <1.0 were set to 1.0. All quality control features were excluded from subsequent analyses. Further data filtering involved removal of saturated probe features, nonuniform features, population outliers, and those features showing intensities not significantly different from background in Cy3 or Cy5 channels. After these relatively stringent procedures 20,130 of the original 43,850 array features progressed to subsequent analyses. The experimental hybridizations are at European Bioinformatics Institute archived under accession number E-MEXP: E-MTAB-685.

Significant differential expression between challenged fish and control fish was established by t-test analysis (P < 0.05). Further filtering on fold change was conducted, with only transcripts showing more than twofold change in expression being further characterized. **Analysis of GO.** Enrichment for specific GO categories was performed on all features that had associated GO identifiers using the GOEAST program (95). Fisher’s exact test was used within the GOEAST program to determine if GO identifiers occurred significantly more often in one biological condition than another.
SRS-Flurotest (Biogroup Chile), which utilizes a mix of mono-
P. salmonis
the total mortalities, were selected at random and analyzed for
mortem autopsy. Additionally 100 fish, representing 16% of
in a final cumulative mortality of 30.7
days postchallenge, reaching a plateau from
sign of disease. An increase in mortality was observed from 10
fish during the first 48 h, no control fish died or showed any

RESULTS
One mortality was recorded for the P. salmonis challenged
fish during the first 48 h, no control fish died or showed any
sign of disease. An increase in mortality was observed from 10
days postchallenge, reaching a plateau from day 25, resulting in
a final cumulative mortality of 30.7 ± 5.3% at day 30. The
cause of mortality observed as SRS was confirmed by post-
mortem autopsy. Additionally 100 fish, representing 16% of
the total mortalities, were selected at random and analyzed for
P. salmonis in liver tissue by immunofluorescence analysis kit
SGS (Biogroup Chile), which utilizes a mix of mono-
clonal antibodies bound to FITC specific to P. salmonis; 88%
of selected fish were positive for P. salmonis (SGS Chile, Puerto Varas, Chile).

For the gene expression studies we chose to examine the
transcriptional response to an experimental infection of P. salmonis at 48 h postinfection. This time point was chosen to
highlight early host responses at a point before any physiologi-
ical stress from the disease occurs that could potentially con-
found analysis of the early immune response. We also chose to
perform an intraperitoneal injection of the pathogen so that all
animals would receive the same initial dose of the bacterial
pathogen, as a natural infection always results in a gradation of
infection levels due to natural variation in load and immune
response/capacity between individuals. The P. salmonis infec-
tion led to transcriptional responses in all three tissues exami-
ned, with limited correlation of transcripts expressed between
tissues, indicative of differential tissue responses. The liver
showed the greatest differential transcript expression with 886
transcripts significantly (P < 0.05) differentially expressed
greater than twofold. Of these 503 were found to have in-
creased in expression following the P. salmonis challenge, and
383 transcripts were found to have decreased in expression. In
the head kidney 207 transcripts were found to show significant
differential expression, with 87 up- and 120 genes downregu-
lated, respectively. Muscle showed the least number of trans-
scripts altered, with 153 transcripts differentially expressed,
and 34 of these transcripts showing increased and 119 de-
creased expression. Only four transcripts showed differential
expression (three downregulated and one upregulated) com-
mon to all examined tissues, demonstrating the lack of a
systemic response to the pathogen at this time point. The
greatest number of transcripts showing correlated expression
was found in liver and head kidney, with six transcripts
increased and 23 decreased in common (Fig. 1 Venn diagram).
The full list of differentially expressed genes is given in
Supplementary Table S1.1

To further investigate the biological significance of the
differentially expressed transcripts, we used GO analysis to
help identify biological processes that were altered in tissues
following the P. salmonis infection. This allowed statistical
analysis for enrichment for GO biological processes to help

1 The online version of this article contains supplemental material.
interpret the changes in the transcriptome following the pathogen challenge (Fig. 2).

Transcripts modified in head kidney. The GO analysis showed that a number of biological processes were significantly altered in the head kidney following the infection (Table 1). The head kidney is the major site of hematopoiesis in teleost fish and is very rich in many different types of leukocytes. Early immune responses are often found in this tissue, which may then help coordinate the immune response. The largest group of genes increased in expression can be regarded as being involved in the

Fig. 2. Gene Ontology (GO) (biological processes) found to be significantly enriched in head kidney (A), liver (B), and muscle (C) following 48 h infection with *P. salmonis* in Atlantic salmon. Only GO categories for which >3 genes were represented are included.
Table 1. Genes altered significantly in head kidney, liver, and muscle by Piscirickettsia salmonis infection

<table>
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<th>Probe Name</th>
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<th>FC ± SE</th>
<th>Identity</th>
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Table 1.—Continued

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**Energy metabolism**

- **Omy#S15335104 BX309746 2.1**
- **Ssa#STIR10973 TC106938 4.2 ± 1.8** Snrnp70 protein
- **Omy#CR372924 CR372924 2.8 ± 1.1** topoisomerase I alpha
- **Omy#S15286226 BX072594 2.4 ± 1.3** DEAD (Asp-Glu-Ala-Asp) box polypeptide 27

**Cell proliferation**

- **Omy#S15335104 BX309746 2.1**
- **Ssa#STIR10973 TC106938 4.2 ± 1.8** Snrnp70 protein
- **Omy#CR372924 CR372924 2.8 ± 1.1** topoisomerase I alpha
- **Omy#S15286226 BX072594 2.4 ± 1.3** DEAD (Asp-Glu-Ala-Asp) box polypeptide 27

**Apoptosis**

- **Omy#S15335104 BX309746 2.1**
- **Ssa#STIR10973 TC106938 4.2 ± 1.8** Snrnp70 protein
- **Omy#CR372924 CR372924 2.8 ± 1.1** topoisomerase I alpha
- **Omy#S15286226 BX072594 2.4 ± 1.3** DEAD (Asp-Glu-Ala-Asp) box polypeptide 27

**Lipid metabolism**

- **Ssa#S35700291 EG935009 2.9 ± 1.0** heat shock protein 30
- **Ssa#S35700291 EG935009 2.3 ± 1.0** heat shock protein 30
- **Ssa#S35700291 EG935009 2.1 ± 1.0** heat shock protein 30
- **Ssa#S35700291 EG935009 2.0 ± 1.0** heat shock protein 30

**Stress and Immune related**

- **Ssa#S35700291 EG935009 2.9 ± 1.1** heat shock protein 30
- **Ssa#S35700291 EG935009 2.2 ± 1.1** heat shock protein 30
- **Ssa#S35700291 EG935009 2.0 ± 1.0** heat shock protein 30
- **Ssa#S35700291 EG935009 1.9 ± 1.0** heat shock protein 30

**G protein signaling**

- **Ssa#S35700291 EG935009 2.3 ± 1.1** heat shock protein 30
- **Ssa#S35700291 EG935009 1.9 ± 1.0** heat shock protein 30
- **Ssa#S35700291 EG935009 1.8 ± 1.0** heat shock protein 30
- **Ssa#S35700291 EG935009 1.7 ± 0.9** heat shock protein 30

**Protein metabolism and apoptosis**

- **Ssa#S35700291 EG935009 2.2 ± 1.1** heat shock protein 30
- **Ssa#S35700291 EG935009 2.1 ± 1.0** heat shock protein 30
- **Ssa#S35700291 EG935009 2.0 ± 1.0** heat shock protein 30
- **Ssa#S35700291 EG935009 1.9 ± 1.0** heat shock protein 30

List of selected mRNAs, grouped according to functional classes (shown in bold), found to be up- and downregulated after 48 h of *P. salmonis* infection in Atlantic salmon. The selection was based on manual assignment of function and genes with greatest fold differences in expression are presented, the genes that are down-regulated are denoted by (−) value. In this table the genes within each group are ordered by mean fold-change (FC) expression level following infection. The genes shown were significant at *P* < 0.05 following t-tests and >2-fold change. Probe name indicates the unique code for the feature on the microarray, ACC, accession number of the cDNA sequence. FC ± SE, for genes increased in expression following *P. salmonis* infection. Identity, identity of the probe target as determined by BLASTX and BLASTN searches. The complete list of informative transcripts can be found online in Supplemental Table S1.
innate immune response, with several proinflammatory cytokines being upregulated. For example, transcripts encoding interleukin 8 (IL-8), IL-11, and TNF-α were increased, as were several other CC and CXC chemokines and chemokine receptors. Interferon-γ (IFN-γ) was also found to be significantly increased, this being involved in the activation of macrophages and antiviral defenses. Several immune-related mRNAs were also depressed in expression following the challenge, with the anti-inflammatory cytokine IL-10, the transcriptional regulator TSC22D3, the transcriptional activator CCAAT/EBP-δ, and a gene encoding SMAD4, the latter a transforming growth factor (TGFi)-β-induced transcript, which may also be related to damping the inflammatory response. Interestingly there were a number of genes decreased related to the adaptive immune response such as IL-4 receptor alpha chain, IL4, which is a pivotal cytokine in directing cells toward a T helper (Th) 2 phenotype and subsequently immunoglobulin production by B cells. T cell receptors (TCR-α and TCR-β and CD8β, which forms heterodimers with CD8α and interacting with the α3 domain of the class I MHC molecule, were also downregulated following pathogen challenge. A Krüppel-like protein (BTG1), pigment epithelium-derived factor and mitogen-activated protein kinase 15, DNA damage-inducible transcript 4, and unc-51-like kinase 1 were all decreased following the challenge, suggesting a general directional response toward a proliferative state.


### Table 2. Primers for expression analysis

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Ann. T, annealing temperature.
Genes modified in liver. The key biological processes that were found to be significantly modulated in the liver were related to cellular metabolic processes. These included processes such as intracellular transport, protein metabolism, cell proliferation, and biosynthesis. Immune response genes and genes involved in responses to stress were also significantly altered. Some key genes related to these groups are shown in Table 2; the full list is given in Supplementary Table S1. Genes related to immune function both increased and decreased. Two complement components (component C3-3 and component C1q) were increased, indicating there may have been a stimulation of innate responses. Two transcription factors IRF3 and IRF7 were also both increased, which may indicate an ongoing IFN response (as also indicated by IFN-γ in head kidney). However, in general there was not a dramatic increase in immune-responsive genes in the liver following the P. salmonis challenge. Genes related to immune function that were downregulated included chemokines (CCL8 and 13, CXCL10), two chemokine receptors CXCR 4 and CCR9, and an inhibitor of NF-κB, which is a central transcription factor for inflammatory response proteins. A second transcription factor CCAAT/EBP-β, which is a major factor in driving expression of immune related genes, was also reduced in expression. In addition, the tripartite motif-containing protein 25 gene, which is involved in innate immune defense against viruses, was also found to be downregulated. Genes encoding adaptive immune proteins were also decreased in expression following the P. salmonis infection, as with the TCR-α, TCR-γ, T-cell activation Rho GTPase-activating protein, and CD80, a protein associated with lymphocyte activation. Related to the early immune response in the liver, stress-associated genes including a number of heat shock proteins such as DnaJ (Hsp40) homolog subfamily B member 9, DnaJ homolog subfamily C member 3, DnaJ-like subfamily B member 11, Hsp 90, and hypoxia-upregulated protein 1 were upregulated.

Another biological process enriched in the liver after infection was cell proliferation, with expression of ornithine decarboxylase 1, embryonic nuclear protein 1, nucleostemin, and proliferating cell nuclear antigen, among others found to be increased. Genes involved in apoptosis, however, were found to be downregulated (such as Bcl10-interacting CARD protein, cell death activator CIDE-B, and cyclin-dependent kinase inhibitor 1c). Additionally, genes involved in the TGF-β pathway were also decreased in expression, including TSC22D3, TGF-β-inducible early protein and connective tissue factor.

The liver has a high but variable rate of protein turnover, and in the challenged fish genes involved in both protein synthesis (ribosomal protein S6 kinase, 40S ribosomal protein S11, 40S ribosomal protein S26, and 40S ribosomal protein S27) and protein degradation (E3 ubiquitin-protein ligase RNF8, UBX domain protein 6, and ubiquitin-specific protease 16) were increased in expression (with COP9 signalosome complex subunit 8 downregulated), suggesting a general increase in both pathways. Protein turnover is energy consuming, and genes involved in energy metabolism (NADPH oxidase cytosolic protein, ATPase type 13A, cytochrome c, 4-hydroxyphenylpyruvate dioxygenase, mevalonate kinase, ADP-ribose/CDP-alcohol pyrophosphatase, isopentenyl-diphosphate delta isomerise) were increased in expression in challenged fish, while genes related to gluconeogenesis (fructose-bisphosphate aldolase B and phosphoenolpyruvate carboxykinase) were markedly decreased. The major changes in gene expression following the pathogen challenge may be associated with a large increase of transcription factors, but interestingly there is a parallel decrease in genes involved in cell signaling mediated by G proteins, including regulator of G protein signaling 1 and 21, guanine nucleotide binding protein, and G protein-coupled receptor 137b, among others. Lipid and cholesterol metabolism-related genes were also increased in expression following the challenge, the latter including lanosterol 14-alpha demethylase, isopentenyl-diphosphate delta-isomerase 1, and hydroxymethylglutaryl-CoA synthase. Related to these processes, fatty acid-modifying genes were also increased in expression, including elongation of very long chain fatty acids-like 6, endothelial lipase, and fat-inducing transcript 2, whereas in contrast a phospholipase A2 was decreased in expression.

Genes modified in muscle. The general transcriptional response in muscle showed more transcripts being reduced in expression than increased, suggesting that at 48 h postinfection limited transcriptional alterations in muscle were occurring. The GO analysis found a number of biological processes to be significantly modulated (Fig. 2). Transcripts involved in protein metabolism (synthesis and degradation) were generally found to be decreased following the P. salmonis challenge, including ubiquitin-conjugating enzyme E2 R2, cathepsin D, and UBTF, a transcription factor required for expression of ribosomal RNAs and related to synthesis of ribosomal protein S25. Associated with the presumed reduced protein turnover was a decrease in expression of growth hormone receptor that may indicate a reduced level of protein deposition. The reduced protein metabolism may decrease energy demand in the muscle tissue, and this is reflected in downregulation of both glyceraldehyde phosphate dehydrogenase and adenylyl kinase, both central to cellular energy metabolism. Key genes altered are presented in Table 1 and Supplementary Table S1.

Although there was an apparent reduction in protein turnover and cellular energy metabolism, several genes associated with cell proliferation were found to be increased in expression: unc-51-like kinase 1, the transcription factor v-maf (musculoaponeurotic fibrosarcoma oncogene), a protein related to cellular proliferation in tumors and coatomer subunit alpha (which mediates biosynthetic protein transport from the endoplasmic reticulum). Genes encoding apoptotic proteins were decreased in expression, including THAP domain apoptosis-associated protein 1 and Wilms tumor-associated protein. Together these may indicate an increase of cell proliferation and a decrease of apoptosis in muscle of fish infected with P. salmonis.

Although general immune function transcripts in muscle were not found to be significantly altered by GO analysis, a number of immune and stress-related genes were found to be differentially expressed. These genes included an IFN-γ-inducible-lysosomal thiol reductase, indicating there may be an early IFN response, heat shock protein 30 (HSP 30), ETS domain-containing protein Elk-3, and FKBP12-rapamycin complex-associated protein, and protein-histidine 6-oxidase, which were all increased in expression. In contrast, MyD88, CCAAT/EBP-β, IFN-1, SMAD 3, TSC22D3, cathelicidin I, imp family member 8, nuclear factor interleukin-3-regulated protein, and the B cell receptor CD22 molecule showed a significant downregulation. Finally several genes encoding proteins involved in cell signaling were altered, with erythroblast membrane-assoc-
ciated protein upregulated and oxysterol binding protein-like 1, SVOP-like isofrom 2, regulator of G protein signaling 21, regulator of G protein signaling 14, and proline-serine-threonine phosphatase interacting protein 1 all downregulated.

Transcripts altered in common between tissues. Only a small number of transcripts showed correlated expression between tissues following the *P. salmonis* infection (Fig. 1). Three genes were significantly downregulated in all tissues in this experiment (CCAAT/EBP-δ, regulator of G protein signaling 21, TSC22D3) and one gene upregulated (coatomer subunit alpha). The downregulated genes are all involved in the immune response; indeed, CCAAT/EBP-δ is an important transcriptional activator in the regulation of genes involved in immune and inflammatory responses, while G protein signaling 21 plays a central role in switching off G protein-coupled receptor signaling pathways and is responsible for the suppression of IL-8. TSC22D3 plays a role in the anti-inflammatory and immunosuppressive effects of glucocorticoids and IL10 and in apoptosis. The only upregulated gene, coatomer subunit alpha, is a cytosolic protein complex that binds to dilyseine motifs and reversibly associates with Golgi nonclathrin-coated vesicles, which further mediate biosynthetic protein transport from the endoplasmic reticulum, via the Golgi up to the trans-Golgi network.

Six transcripts were upregulated in both head kidney and liver, and 23 transcripts were decreased in expression in these two tissues. Transcripts that were increased in expression play a role in cell proliferation and organization of the cytoskeleton; these included ornithine decarboxylase 1, which is central in the synthesis of polyamines, which are important for stabilizing DNA structure; Snrnp70 protein, which mediates the splicing of pre-mRNA via spliceosomes; and tubulin beta-2A, which is the major component of microtubules and has a role in protein polymerization. Genes that showed a decrease in expression play a pivotal role in the immune response and cell proliferation/apoptosis. Indeed, COP9 signalosome complex subunit 8 is an important regulator of the ubiquitin conjugation pathway, decreasing the ubiquitin ligease activity of the SCF complex; BTG1 and DNA-damage-inducible transcript 4 protein are, respectively, an antiproliferative protein and a promoter of apoptosis.

Confirmation of expression by qPCR. qPCR analysis was performed on a number of genes for each tissue to confirm microarray data (Table 2, Fig. 3). The transcripts selected for the head kidney were: IFN-γ, thymosin beta12 (TSMB12), TNF-α, TSC22 domain family protein 3, TCR-α, and TCR-β. The transcripts chosen for the liver were: isopentenyl-diphosphate delta-isomerase 1 (IDI1), proliferating cell nuclear antigen (PCNA), ornithine decarboxylase 1 (ODC1), TSC22 domain family protein 3 (TSC22D3), tripartite motif-containing protein 25 (TRIM25), and fructose-bisphosphate aldolase B (ALDOB). The transcripts chosen for muscle were: ets domain-containing protein elk-3 (ELK3), heat shock protein 30 (HSP30), protein-lysine 6-oxidase (LOX), immunoglobulin G-binding protein A (spa), GAPDH, and TSC22 domain family protein 3. To assess the presence of CD3 T cells in the head kidney, the expression of two markers such as CD3γδ and CD3 zeta was studied (Fig. 4).

The qPCR expression was normalized to that of EF-1α, as the expression of this housekeeping gene was not modulated by treatment in the microarray analysis. For all genes the expres-
Transcriptional responses in all three tissues examined. All gene expression related to protein deposition and has recently been modified during an immune response (83). The muscle is the main tissue that may give an indication of the production of acute phase response proteins (82) and possesses extracellular bacteria and viruses. We have used a model infection approach to examine differential transcriptomic responses in head kidney, liver, and muscle by microarray analysis. In recent years cDNA (84, 89) microarrays have been used to examine immune (52, 57, 58) and physiological responses (28) and developmental processes (77) in Atlantic salmon, including the response to *P. salmonis* at late (14 days) stage of infection (73), where a downregulation of adaptive immune responses in head kidney tissue was found (73). In the present study we expand on these previous studies and examined tissues that have different roles in fish immune responses and metabolism. The head kidney is a major lymphoid tissue and rich in macrophages and is often one of the first tissues to exhibit an immune response. The liver also regulates energy metabolism, synthesis and secretion of serum proteins (88), and toxins and also has important roles in the immune response (56). During an infection the liver is responsible for the production of acute phase response proteins (82) and possesses specialized liver-specific macrophages, the Kupffer cells (48). The muscle is the main tissue that may give an indication of gene expression related to protein deposition and has recently been shown in fish to have protein degradation pathways altered during an immune response (83).

This study demonstrated that the infection led to large transcriptional responses in all three tissues examined. All gene expression data presented were significant at *P* < 0.05, and only genes that had a greater than twofold differences in expression were considered for interpretation of the results. Below we discuss how the different processes are altered following the *P. salmonis* infection and how these responses vary between tissues.

**Immune response.** Transcripts related to immune responses were modified in all three tissues studied. The head kidney had the greatest increase of expression of immune-related transcripts. The expression pattern suggested an inflammatory response was being initiated; two TNF-α transcripts 1 and 2, IL-8, several chemokines, and their receptors were all increased in expression. IL-10 was also decreased in expression, a gene often associated with dampening inflammation to reduce host tissue damage (64, 97). TNF-α is a proinflammatory cytokine produced by monocytes/macrophages in response to antigen exposure (45, 74, 98). In addition, three different chemokine-related genes were also upregulated: CCL13, CXCR3, and IL-8. Chemokines are small secreted cytokines that play an essential role in inflammation and host responses controlling the attraction of leukocytes to sites of infection (40, 51). IL-8 is produced by macrophages in response to a variety of stimuli (e.g., LPS, viruses, cytokines) (85), and in salmonid fish it has been demonstrated to be upregulated in many infection and immune models (41). Moreover, IL-10 was found to be downregulated in head kidney following the *P. salmonis* infection. In monocytes immune stimulated, IL-10 inhibits nuclear stimulation by NF-κB, a transcription factor involved in the expression of inflammatory cytokine genes (91). Together the transcriptional response in the head kidney tissue indicates that genes that could induce an inflammatory response were affected. In the head kidney IFN-γ was also upregulated. IFN-γ activates macrophages, enhances antigen presentation, and induces T cell differentiation, coordinating host defense against intracellular pathogens. It also stimulates macrophages to produce toxic oxygen intermediates and reactive nitrogen intermediates to kill intracellular bacteria. It has been shown that this cytokine is induced in trout head kidney leukocytes by stimulation with phytohemagglutinin or poly I:C (96).

In liver two transcription factors that activate the interferon response (IRF3 and 7) during viral infection (29–31) were increased in expression. In addition, two complement components, complement C3-3 and C1q, were found to be upregulated in liver. Complement C3 is one of the components of the complement system and plays a role in host immune surveillance and immune response against infection (50, 53). Complement C1q is the ligand recognition subcomponent of the classical complement pathway and is considered as a bridge between innate and adaptive immunity (4, 66).

In the liver, several transcripts related to immune function were also decreased, as seen with the major transcription factor CCAAT/EBP-β, chemokines CXCL10, CCL13, and chemokine receptors CXCR4 and CCR9, which may indicate a suppression of the inflammatory response in this tissue. The ontology and functions of the fish chemokine repertoire are not yet fully determined, but current nomenclature of salmonid chemokines is described by Ref. 40. In muscle the innate immune regulatory gene, ETS domain-containing protein Elk-3, which interacts with a variety of transcription factors, including AP-1 and NF-κB, to regulate gene expression of genes that participate in the propagation of the inflammatory response (14), was also increased, while the expression of MyD88, IFN type, and cathelicidin I was found to be downregulated. MyD88 is an adaptor protein that is central in vertebrate innate immunity, interacting directly with TLRs (71) and protecting against viruses (78). The antimicrobial peptide
cathelicidin I, which is one of the first lines of defense against microbial pathogens and protects fish against bacterial infections (11, 12, 54), was decreased in expression.

Several other transcripts involved in adaptive immune responses were also found to be significantly downregulated in *P. salmonis* infected head kidney. Both TCR-α and -β, TSC22 domain member 3 (TSC22D3), the cell adhesion molecule CD2, CD8 and a putative IL-4 receptor were decreased in expression. TCR-α and -β are the two peptide chains that form the TCR (32), responsible for recognizing antigens bound to major histocompatibility complex (MHC) molecules. TSC22D3 is induced by glucocorticoids and has an important role in the regulation of TCR-mediated cell death (18–20, 72). CD2 enhances antigen-specific functions by acting as a cell adhesion molecule (80). CD8 is a T cell-specific cell surface marker that encodes one of the most important receptors on T lymphocytes, stabilizing TCR with MHC antigen-expressing cells (65). CD3 molecules are essential components of the TCR complex and have a key role in signal transduction (49). Interestingly CD3γδ was found to be upregulated as determined by qPCR analysis. The induction of this gene may indicate that αβ T cells are still present in the head kidney at this stage of the infection, even if expressed at lower levels. The decrease in the IL-4 receptor like gene may be related to controlling IL-4 activity. IL-4 is a key regulator in humoral and adaptive immunity secreted by activated T lymphocytes, basophils, and mast cells (33). It plays an important role in modulating the balance of Th cell subsets, favoring expansion of the Th2 lineage relative to Th1 or Th17 (33). In the liver genes involved in the adaptive immune response (as CD80-like protein and B-cell lymphoma 6 protein homolog) were also downregulated, as well as genes involved in the TGF-β pathway. TGF-β is a pleiotropic cytokine involved in tissue remodeling, wound repair, development, and hematopoiesis (43, 46, 47). Its principal actions are to inhibit cell growth and proliferation and to deactivate macrophages (21). The downregulation of the TGF-β pathway may indicate reduced apoptosis and suppression of immunity mediated by this molecule.

Similarly, several muscle genes involved in adaptive immunity were found to be downregulated, including TSC22D3 (also downregulated in both head kidney and liver), imap family member 8, nuclear factor IL-3-regulated protein, and the membrane immunoglobulin (mlg)-associated protein of B cells CD22 (67).

Overall these data indicate that *P. salmonis* affects the immune system of the host, activating the innate immune response in head kidney, muscle, and liver, potentially inducing inflammatory responses in head kidney and an IFN-mediated response in liver. Moreover this pathogen may compromise the adaptive immune response in infected fish as a mechanism to escape host defenses.

**Cell signaling.** Transcripts involved in G protein pathways were downregulated in all three tissues examined in the challenged fish and in particular regulators of G protein signaling (RGS) were found to be decreased in all the tissues. RGS proteins are responsible for the rapid switching off of G protein-coupled receptor signaling pathways (90). RGS1, downregulated in the head kidney, inhibits IL-8-induced MAPK activation (which may dampen the inflammatory response) (23), while RGS2 is a protein that upregulates T-cell proliferation and IL-2 production, and downregulation of this gene in mouse leads to impaired antiviral immunity (38).

Likewise, RGS14, downregulated in muscle, attenuates IL-8 receptor-mediated MAPK activation (13). A further uncharacterized RGS21 was also found to be downregulated in all the three tissues, demonstrating that this process is consistently altered in a directional manner. Such results suggest that suppression of G protein signaling may be a part of the mechanism used by *P. salmonis* to evade host antimicrobial defenses.

**Response to stress.** Responses to stress, which are associated with the early immune response, were also modulated in this study. The host cell protects itself from oxidative damage by synthesizing the strong antioxidant glutathione (87). The expression of genes involved in the response to oxidative stress was found to be upregulated in all tissues examined. NAPDH oxidase was found to have increased expression in liver, indicating that fish were likely undergoing oxidative stress. Activated macrophages respond to bacterial infection by reducing oxygen to superoxide anion (O2−) and single oxygen using NAPDH oxidase. These free radicals create a toxic intracellular environment that protects the host against invading microbes. Interestingly, glutathione-S-transferase activity was downregulated in liver. This resulted in the upregulation of catalase in head kidney, which is in accordance with the 2004 study of Rise et al. (73) of head kidney infected with *P. salmonis*. In addition, a decreased level of glutathione-S-transferase and increased peroxide levels have been correlated with signs of cell damage due to the rickettsial agent of Rocky Mountain spotted fever in humans.

Genes encoding for heat shock proteins were also induced following infection of the pathogen. It is well known that these proteins are able to induce cellular and humeral immune responses (36, 37) in infectious diseases caused by bacteria, protozoa, fungi and nematodes (99). Both HSP-90α and -β were also upregulated in head kidney and liver, respectively. These proteins mediate macrophage activation during bacterial infection (8). In liver, three members of DnaJ (Hsp40) were significantly upregulated. This family of proteins functions as cochaperones in stimulating the ATP-dependent activity of HSP70 (68), upregulated in this tissue, which protects cells during LPS infection, upregulating the expression of sphingosine kinase 1 (SK1) (22).

All these results suggest that *P. salmonis* infection may affect the host antioxidant system, eventually causing the cell death and necrosis as observed in several tissues of moribund fish infected by this bacterium (1).  

**Protein metabolism.** Following infection there was an increase in genes related to protein metabolism in the liver, with genes involved in both protein synthesis and degradation being upregulated. The liver is a tissue with very high and variable demand for ATP production to respond to the growing demand for free amino acids as substrates for protein synthesis. Indeed, in the liver, genes involved in energy metabolism were increased in expression in challenged fish, whereas genes related to gluconeogenesis were markedly decreased. Control of amino acid biosynthesis and oxidation can be severely altered by infection (44) in mammals, with redistribution of amino acids.
acids, in particular those required for synthesis of inflammatory response proteins, which have a different amino acid composition to proteins synthesized during normal conditions.

In contrast to liver, in muscle genes involved in both protein synthesis [RNA polymerase I (UBTF) and ribosomal protein s25] and degradation (cathepsin D and ubiquitin-conjugating enzyme E2 R2), were found to be decreased in diseased fish. This result suggests that *P. salmonis* may induce a decreased protein turnover in this tissue, reflecting a decrease in both anabolic and catabolic pathways in muscle at this time following the infection. These finding demonstrate how control of similar functional pathways can be regulated in a differential way between muscle and liver tissues.

**Cell proliferation and apoptosis.** During the complex interaction between a pathogen and host organism, induction or prevention of apoptosis may play a critical role in the outcome of infection. The obligate intracellular bacterium *Rickettsia rickettsii*, the etiologic agent of Rocky Mountain spotted fever, is capable of suppressing host cell apoptosis through regulation of levels of apoptotic and antiapoptotic proteins (35). Our results suggest *P. salmonis* may also inhibit apoptosis by downregulating genes encoding proapoptotic proteins (Becl10-interacting CARD protein and gsk-3-binding protein in liver; that apoptosis associated protein 1) and inducing cell proliferation-related genes in both liver and head kidney. This is in agreement with Rise et al. (73), who also found a decrease in expression of apoptosis-related genes, while genes related to cell proliferation and the cell cycle were upregulated.

Modulation of the host cells’ apoptotic response and proliferation could be a mechanism evolved by *P. salmonis* to ensure the maintenance of host cells and to allow them to continue as the site of infection.

In conclusion, the present study demonstrates that *P. salmonis* infection has profound effects on transcription in the head kidney, liver, and muscle of salmon. Each tissue showed common biological processes being altered, as well as tissue-specific changes. Head kidney, liver, and muscle tissue showed a decrease in expression of mRNAs related to acquired immune function that may indicate the pathogen is downregulating this response, controlled in part by the inhibition of the G protein signaling pathway. Within head kidney there was a marked increase in innate immune encoding genes, which likely reflects this tissue as being the major hemopoietic tissue in fish, rich in macrophages and coordinating the early response. The liver showed increases in cell proliferation and decreased apoptosis, a possible mechanism that allows intracellular pathogen survival. Liver functions including oxidative stress genes were increased as were genes related to lipid mobilization and protein metabolism; together these pathways suggest increased energy reallocation during the pathogen response. Finally muscle demonstrated a general decrease in both innate immune-related genes and a decrease in protein metabolism, suggesting this could be an energy conserving mechanism in this tissue. These results improve understanding of the mechanisms by which this bacterium survives and replicates within host cells and may assist selection of molecular biomarkers useful for the development of a diagnostic tools, vaccines, and therapeutics for the aquaculture industry.

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