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Genome-wide survey of the gene expression response to saprolegniasis in Atlantic salmon

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Abstract

Pathogenic saprolegniaceae species are among the major disease-causing agents in farmed salmonids and in freshwater fish in general. Recent studies have used high-throughput cDNA-based methods to identify new potential actors of fish defence systems against various bacteria and viruses. However, the response of fish to fungal or fungus-like pathogens is still poorly documented. Here, we used a 16,006-gene salmonid cDNA microarray to identify genes which transcription levels are modified in juvenile Atlantic salmon (*Salmo salar*) affected with saprolegniasis compared to healthy fish from the same families. Our results confirmed the importance of non-specific immunity in the response of fish to saprolegniaceae infections and identified both similarities and differences in their genome-wide transcriptional response to oomycetes compared with their responses to bacterial or viral infections. Moreover, several clones with no known homologues were shown to be over-transcribed in infected fish. These may represent as yet unidentified immune-relevant genes in fish.

Keywords: Atlantic salmon; Genome-wide transcription profiles; *Saprolegniacea*; Saprolegniasis; Oomycetes

1. Introduction

The fish farming industry as well as the interest for comparative and evolutionary immunology has motivated the edification of a substantial body of knowledge in fish immunology (Iwama and Nakanishi, 1996). Hence, the activity of non-specific immunity, and in particular that of the alternative complement pathway (Yano, 1996), was found to be extremely high in fish compared to mammals (Bayne and Gerwick, 2001; Ellis, 2001). Various high-throughput methods, including the creation of EST libraries enriched in immune relevant transcripts (Nam et al., 2000; Kono et al., 2004; Kono and Sakai, 2001; Savan and Sakai, 2002; Kocabas et al., 2002), subtractive EST libraries (Tsoi et al., 2004; Bayne et al., 2001; Fujiki et al., 2003, 2001) and microarrays (Ewart et al., 2005; Meijer et al., 2005; Rise et al., 2004; Kurobe et al., 2005), have recently been employed in attempts to identify new molecular actors of the immune response in various teleost fish and clarify host–pathogen interactions. Microarrays are particularly well suited for this task (Meijer et al., 2005) and have been employed to characterize fish responses to various bacteria (*Piscirickettsia salmonalis*, *Aeromonas salmonicida*, *Mycobacterium marinum*) and viruses (viral hemorrhagic septicemia virus, hirame rhabdovirus). However, no such study has assessed the overall gene expression response when fishes are exposed to fungi or fungus-like protists, though various lines of evidence suggest that fish, like humans, show distinct acute phase responses to viral, bacterial and fungal agents (Ellis, 2001; Bayne et al., 2001).

The saprolegniaceae family comprises closely related water moulds ubiquitous in freshwater and usually associated with dead tissues and fish eggs (Hart and Reynolds, 2002). This family belongs to the oomycetes, a group of fungus-like protists sharing similarities with fungi and brown algae. Other members of this group are well known plant and mammal pathogens (Kamoun, 2003). Phylogenetically very different from eumycotan fungi, oomycetes likely evolved different mechanisms for interaction with plants and animals (Kamoun, 2003; Torto-Alalibo et al., 2005). Furthermore, some plant pathogenic oomycetes suppress host defence (Kamoun, 2003), a property that has been suggested to be shared by *Saprolegnia* (Alvarez et al., 1995, 1988).

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Pathogenic saprolegniaceae species are known to cause saprolegniasis, a fish tegumentary mycosis characterized by visible patches of filamentous mycelium covering the epidermal tissues (Beakes et al., 1994). If untreated, saprolegniasis leads to death, presumably by heamodilution (Hatai and Hoshia, 1994). Saprolegniasis is one of the most important freshwater fish diseases, both in nature and aquaculture, where it causes economically important losses worldwide (Tampieri et al., 2003; Hughes, 1994). This problem has achieved new heights recently (Torto-Alalibo et al., 2005) since malachite green, a triarylmethane dye efficiently used to control saprolegniasis since the mid 1930' (Alderman, 1994), was prohibited in most producer countries because of its suspected teratogenicity and toxicity (Meyer and Jorgenson, 1983; Alderman and Polglase, 1984). Pathogenic saprolegniacea can act either as secondary or as primary pathogens (Neish and Green, 1977; Whisler, 1996). Fish stressed by diverse factors including handling, other infections, mechanical damage, sexual maturity, temperature changes, poor hygiene or social interaction are more susceptible to develop saprolegniasis (Pickering, 1994), and salmonids are known to be particularly vulnerable (Hughes, 1994). Here, we used a 16,006-gene salmonid cDNA microarray to identify genes which transcription levels are modified in juvenile Atlantic salmon (*Salmo salar*) affected with saprolegniasis compared with healthy fish.

2. Methods

2.1. Fish samples

Six months post-hatching, Atlantic salmon juveniles grown from eggs in controlled conditions were collected from freshwater laboratory holding tanks during a non-provoked episode of saprolegniasis. Five alevins from four distinct full-sib families showing clear symptoms of the disease were collected and frozen along with five healthy alevins from the same families. Saprolegniasis was confirmed by a fish veterinarian and hypha were observed both under optical and electron microscopy (Fig. 1).

2.2. RNA extraction, labelling and cDNA hybridisation

Juvenile salmons that had been frozen at −80 °C were homogenised individually in 10 ml TRIZOL@Reagent (Invitrogen), and total RNA was extracted in 10 separate assays as

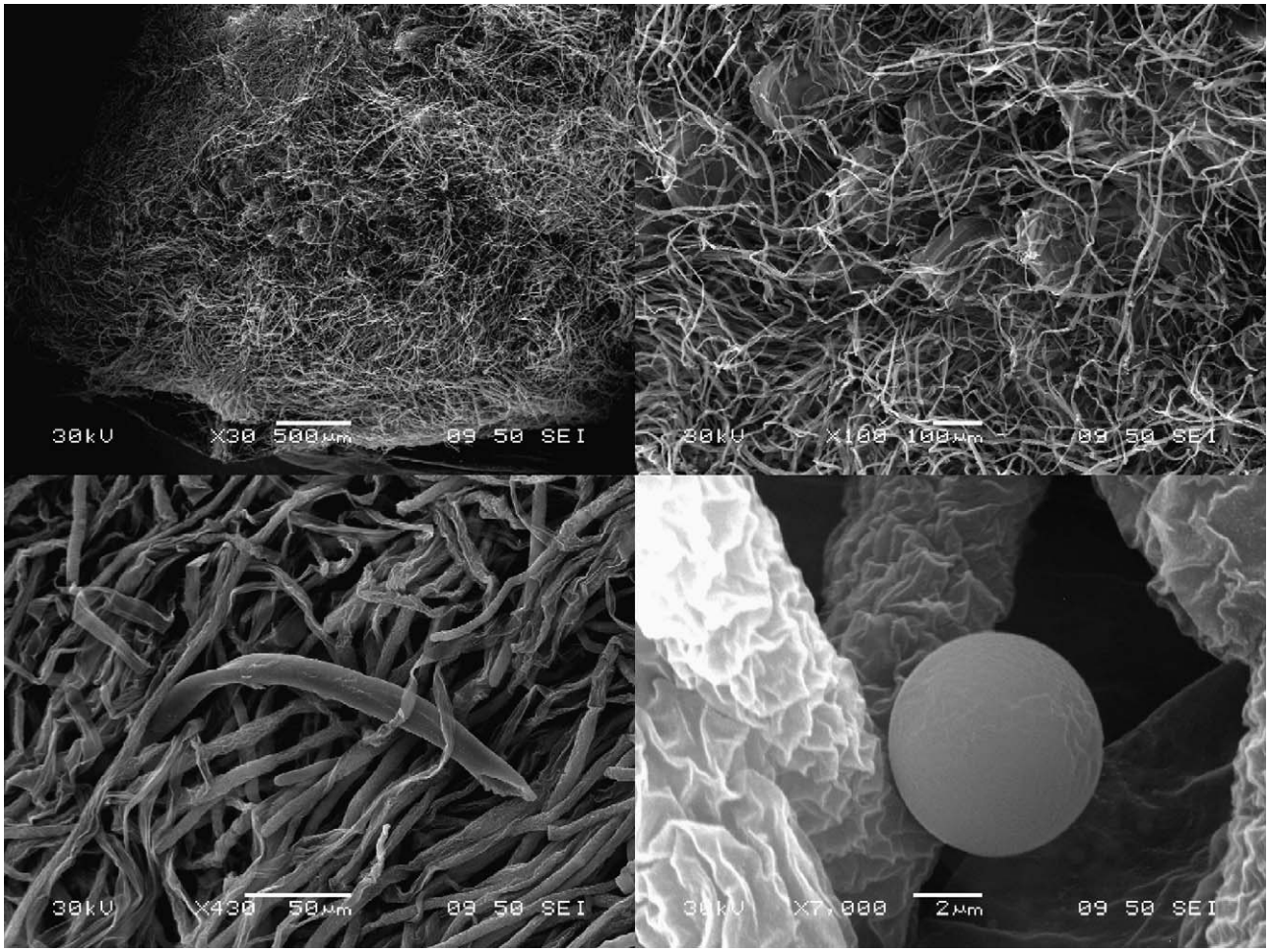

Fig. 1. Scanning electron micrographs of the skin surface of a fish severely affected with saprolegniasis. The top pictures are general views of the mycelium-infected epidermis. Bottom left is an empty zoosporangium among hypha and bottom right is a cyst. Scale, acceleration voltage and magnification are indicated on each image. Samples were fixed, dehydrated and gold stained prior to observation.

previously described (Roberge et al., 2006). For each sample, 15 μg of the pooled RNA from the 10 separate extractions was then retro-transcribed and labelled (Roberge et al., 2006). Transcription profiles of five healthy and five saprolegniasis-affected fish were contrasted on five microarrays, always comparing an infected fish to a healthy fish of the same family on a given array. Dye-sample coupling was flipped between biological replicates. The cDNA microarrays used here were obtained through the Genomic Research on Atlantic Salmon Project (GRASP, available from Ben F. Koop, bkoop@uvic.ca) and comprise 16,006 salmonid cDNA clones (von Schalburg et al., 2005).

2.3. Signal detection, data preparation and statistical analysis

Signal detection and data preparation was as previously reported (Roberge et al., 2006). Genes with mean intensities smaller than the mean intensity of control empty spots plus twice its standard deviation in both channels were removed from the analysis, leaving 7987 detected clones. To assess differences between healthy and saprolegniasis-affected fish, data were analysed using a mixed model of ANOVA (Wolfinger et al., 2001) and the MAANOVA R package (Kerr et al., 2002, 2000). The model included the "array" term as a random term and the "sample type" (infected or healthy) and "dye" terms as fixed terms. A permutation-based F-test (Fs, with 1000 sample ID permutations) was then performed, and restricted maximum likelihood was used to solve the mixed model equations. Hierarchical clustering analysis of Pearson correlation distance between genes and between treatments was run using the GeneSight 3.5 software (BioDiscovery).

3. Results

At a significance threshold of $P < 0.005$, the transcription level of genes corresponding to 430 cDNA clones printed on the array differed significantly between saprolegniasis-affected and healthy fish. Among the 430 significant clones, 144 (33%) were marked as "unknown" on the microarray used in this study, meaning that they did not generate any

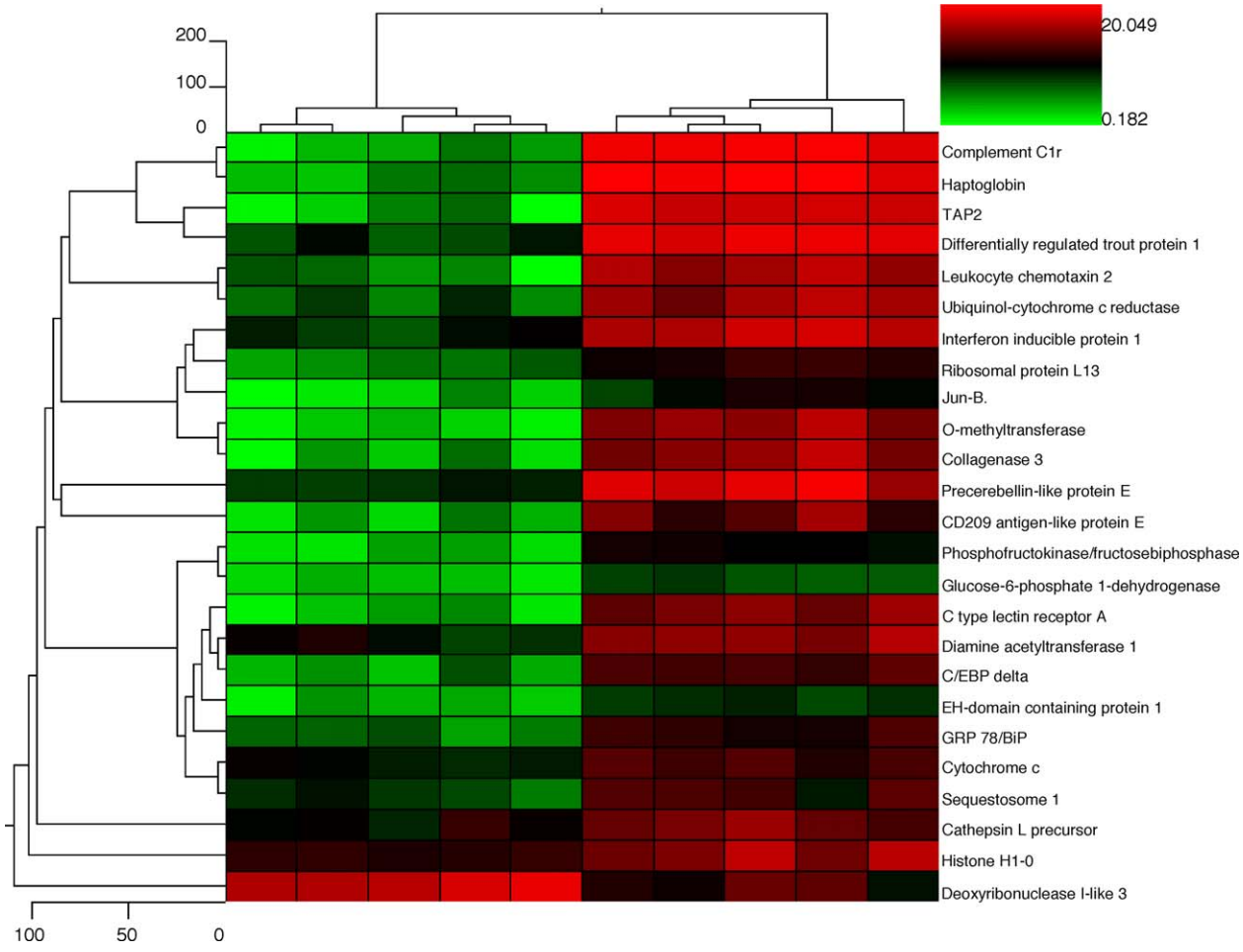

Fig. 2. Graphical representation of the differences in transcript abundance between five saprolegniasis-affected (right) and five healthy (left) fish. The normalized expression level is represented for the 25 most significantly differentially expressed genes by a coloured box. The palette of colours symbolizing low to high normalized expression levels is shown on the upper right part of the figure (green = low, red = high). "Unknown" genes where not considered and only the data from the most significant clone was considered in the case of genes represented by several significant cDNA clones. Hierarchical clustering of gene expression data by gene and by experiment is shown as a horizontal and a vertical tree, respectively. The trees represent relationships in expression patterns, with branch lengths indicative of the magnitude of the differences between gene expression patterns.

BLAST hits with *e*-values less than 1×10^{-15} and an informative name (for a detailed description of the annotation process, see http://web.uvic.ca/cbr/grasp). Each of these represents a potential unidentified molecular actor of immunity in fish (Supplementary Table 6). The sequences of most of these cDNA clones are available in GeneBank. To correct for multiple testing, *q*-values were calculated from the *p*-values with the *Q*-value R package (Storey, 2002). The number of significant genes increased to 1158 when applying the $Q < 0.05$ significance threshold on the obtained *q*-values.

Hierarchical clustering analysis was run using the normalized gene expression data from the 25 genes for which the transcription level differences between sick and healthy fish were the most significant, excluding the unknown genes (Fig. 2). Clustering analysis on experimental conditions (Fig. 2, columns) showed a clear dichotomy between healthy (left vertical cluster) and infected (right vertical cluster) fish. Moreover, 24 of these 25 genes were over-transcribed in diseased fish and most significant cDNA clones at $P < 0.005$ were also over- (67%) rather than under- (33%) transcribed in infected fish, which echoed previous observations with different infectious agents (Ewart et al., 2005).

Genes coding for several acute phase proteins and, in particular, complement proteins were over-transcribed in infected fish (Table 1). Significant over-transcription was detected from a number of different cDNA clones corresponding to the same genes for C1r, C3, mannose-binding protein C, transferrin (serotransferrin) as well as for the fish APPs precerebellin-like protein and differentially regulated trout protein 1 (a snake neurotoxin homologue). The average fold induction (9.7-fold) was very high for the genes in Table 1, some such as haptoglobin (38-fold) and the complement component C1r (32-fold) reaching particularly high values. Numerous additional cDNA clones corresponding to genes shown to be over-expressed in fish infections in previous studies were also over-expressed in saprolegniasis-affected fish (Table 2). Again, many genes were represented by more than one significant clone and most of these genes have well-characterized immune functions. Table 3 presents cDNA clones for genes previously reported as under-transcribed in diseased fish and which also showed under-expression in saprolegniasis-affected fish. These include 17 clones corresponding to the collagen alpha 1 coding gene. Table 4 presents a subset of genes, previously observed as differentially expressed in a given direction in other studies, but that were differentially expressed in a different direction in this study. Finally, an important corpus of cDNA clones corresponding to genes whose expression has not previously been reported to change in immuno-stimulated fish were differentially expressed in this study (Table 5). While some of these have characterized roles in immunity, several other functional groups not directly related to the immune system were also represented.

4. Discussion

Various histological and physiological observations were made in previous studies following infection of different fish species with *Saprolegnia*. Histopathological changes beneath the superficial mycelia include dermal necrosis and oedema dur-

Table 1
Complement proteins as well as other acute phase protein coding genes that were significantly over-transcribed in saprolegniasis-affected fish

Gene product	*P*-value[†]	cDNA clone	Fold change	Reference
Complement C1r	1.60E−05	3	31.98	
Complement C2	1.01E−03	1	1.79	
Complement C3	2.00E−03	7	2.38	Tsoi et al. (2004), Baynes (2001), Meijer et al. (2005)
Complement C6	2.68E−03	1	2.25	Meijer et al. (2005)
Haptoglobin	5.32E−05	1	37.96	Tsoi et al. (2004), Baynes (2001), Ewart et al. (2005), Rise et al. (2004)
Precerebellin-like protein	2.24E−04	2	11.42	Tsoi et al. (2004), Baynes (2001), Ewart et al. (2005), Rise et al. (2004)
Differentially regulated trout protein 1	1.60E−04	4	14.24	Tsoi et al. (2004), Baynes (2001), Ewart et al. (2005), Rise et al. (2004)
Hemopexin	4.63E−03	1	2.80	Baynes (2001), Rise et al. (2004)
Ceruloplasmin	4.33E−03	1	2.36	Baynes (2001), Rise et al. (2004)
Serotransferrin	3.19E−03	3	2.90	Baynes (2001), Rise et al. (2004)
Mannose-binding protein C	1.72E−03	2	2.19	Tsoi et al. (2004), Baynes (2001), Ewart et al. (2005), Rise et al. (2004)
C type lectin receptor A	7.45E−05	2	14.40	
CD209 antigen-like protein E	6.29E−04	1	8.31	

P-values from the ANOVA permutation-based *F*-test, the number of significant cDNA clones, the average fold change (expression level in infected fish over that in healthy fish) for all clones and references in which the gene differential expression was previously observed in fish infected with other agents are presented. A supplementary online table (Supplementary Table 1) presents this information in a more detailed form, with individual fold changes, *P*-values and *Q*-values for each cDNA clone as well as GeneBank accessions.

[†] In the case of genes with multiple significant cDNA clones, only the smallest observed *P*-value is presented.

Table 2
Genes which were significantly over-transcribed in saprolegniasis-affected juvenile salmon and that were observed as over-transcribed in fish infected with other agents in previous studies

Gene product	*P*-value[†]	cDNA clone	Fold change	Reference
Cathepsin L	9.58E−05	6	2.22	Baynes (2001), Meijer et al. (2005)
Cathepsin D	3.52E−03		1.37	Baynes (2001), Meijer et al. (2005)
Collagenase 3	3.09E−04	2	12.92	Meijer et al. (2005), Kurobe et al. (2005)
Matrix metalloproteinase	1.01E−03	1	4.27	Tsoi et al. (2004), Ewart et al. (2005), Meijer et al. (2005), Rise et al. (2004)
CXCR4	4.80E−03	1	1.54	Meijer et al. (2005)
NADPH oxidase flavocytochrome *b* small subunit	4.45E−03	2	1.44	Tsoi et al. (2004), Rise et al. (2004)
6-phosphogluconate dehydrogenase	3.39E−03	1	2.17	Rise et al. (2004)
Interferon inducible protein 1	7.45E−05	3	5.80	Byon et al. (2005)
Phosphofructokinase/fructosebiphosphatase 2	3.19E−05	1	3.79	Tsoi et al. (2004)
Leukocyte chemotaxin 2	3.62E−04	2	9.45	Tsoi et al. (2004), Baynes (2001), Ewart et al. (2005)
Jun-B	5.22E−04	3	4.27	Tsoi et al. (2004), Kurobe et al. (2005)
CCAAT/enhancer binding protein delta	5.01E−04	2	5.22	Meijer et al. (2005)
Biotinidase	8.84E−04	1	3.90	Baynes (2001)
O-methyltransferase	1.92E−04	1	15.52	Tsoi et al. (2004), Ewart et al. (2005), Rise et al. (2004)
Plasma glutathione peroxidase	4.36E−03	1	1.89	Tsoi et al. (2004), Baynes (2001), Ewart et al. (2005), Rise et al. (2004)
Agglutination-aggregation factor 18K-LAF	1.06E−03	2	3.36	Tsoi et al. (2004), Ewart et al. (2005), Rise et al. (2004)
TAP2	9.58E−05	2	13.45	Ewart et al. (2005), Rise et al. (2004)
S-100/ICaBP-like	6.71E−04	1	2.43	Rise et al. (2004)
Ribosomal protein L13	4.16E−04	1	3.59	Baynes (2001), Ewart et al. (2005)
Ribosomal protein S27-like	4.50E−03	1	1.78	Tsoi et al. (2004)
Vacuolar ATP synthase 16 kDa proteolipid subunit	2.34E−03	5	1.72	Tsoi et al. (2004), Meijer et al. (2005), Rise et al. (2004)
Thioredoxin interacting protein	6.29E−04	2	2.62	Kurobe et al. (2005)
Thioredoxin	1.35E−03	2	2.20	Tsoi et al. (2004), Ewart et al. (2005), Meijer et al. (2005)
Solute carrier family 31, member 1	7.99E−04	1	5.06	Meijer et al. (2005)
Solute carrier family 37, member 4	8.63E−04	1	1.94	Meijer et al. (2005)
Solute carrier family 25, member 5	2.72E−03	4	1.42	Meijer et al. (2005)
Sequestosome 1	5.22E−04	2	2.51	Ewart et al. (2005)
Thrombospondin-4	9.53E−04	1	4.12	Meijer et al. (2005)
Fibronectin	3.05E−03	1	2.20	Meijer et al. (2005)
26S proteasome regulatory subunit p27	1.02E−03	1	1.97	Meijer et al. (2005)
26S proteasome regulatory subunit p44.5	3.21E−03	1	1.60	Meijer et al. (2005)
Endoplasmin	1.26E−03	5	2.56	Tsoi et al. (2004)
Plasma retinol-binding protein	1.42E−03	4	2.04	Ewart et al. (2005)
Polyposis locus protein 1 homolog	1.86E−03	2	1.99	Tsoi et al. (2004)
Translation initiation factor 4E binding protein 3	2.53E−03	1	1.76	Meijer et al. (2005)
Probable RNA-dependent helicase p68	4.95E−03	1	1.79	Rise et al. (2004)
Heat shock protein 90-beta	2.55E−03	1	1.39	Kurobe et al. (2005), Rise et al. (2004)
CD63	3.84E−03	2	1.57	Meijer et al. (2005)
Placental thrombin inhibitor	3.61E−03	1	1.78	Rise et al. (2004)
Insulin-like growth factor I	4.76E−03	1	1.96	Rise et al. (2004)
Histone H1-0	2.45E−04	5	2.51	Byon et al. (2005)[a]
Histone H1.3	2.63E−03	1	0.65[a]	
Histone H2A.X	3.72E−03	1	0.60[a]	
H1fx-prov protein	2.38E−03	1	1.92	

P-values from the ANOVA permutation-based *F*-test, the number of significant cDNA clones, the average fold change (expression level in infected fish over that in healthy fish) and references in which the gene differential expression was previously observed in fish infected with other agents are presented. A supplementary online table (Supplementary Table 2) presents this information in a more detailed form, with individual fold changes, *P*-values and *Q*-values for each cDNA clone as well as GeneBank accessions.

[a] The observed histone H1 subtype was not specified in Byon et al. (2005). We presented all subtypes in this table for easier comparison.

[†] In the case of genes with multiple significant cDNA clones, only the smallest observed *P*-value is presented.

ing early stages with deeper myofibrillar necrosis and extensive haemorrhage in the more progressive lesions (Beakes et al., 1994). Other studies reported signs of inflammation, lethargy and paralysis in severely infected fish (Hughes, 1994). Also, data suggested damage to the liver (Richards and Pickering, 1979) and thymus (Alvarez et al., 1995). While some authors have suggested that *Saprolegnia* could exert immunosuppression on infected fish (Alvarez et al., 1995; Bly et al., 1992), Bly et al. (1993) observed an acute phase response in *Saprolegnia*-infected channel catfish.

Here, we investigated for the first time the genome-wide transcriptional response of fish to saprolegniasis. Over-transcription of several acute phase protein coding genes (Table 1) suggests that saprolegniasis-affected salmon undergo an acute phase

Table 3
Genes that were significantly under-transcribed in saprolegniasis-affected fish and which were observed as under-transcribed in immuno-stimulated fish in previous studies

Gene product	*P*-value[†]	cDNA clone	Fold change	Reference
Selenoprotein P, plasma, 1b	4.32E−03	1	0.68	Ewart et al. (2005)
Deoxyribonuclease I-like 3	2.13E−04	2	0.25	Meijer et al. (2005)
Collagen alpha 1(I)	1.09E−03	17	0.33	Ewart et al. (2005), Meijer et al. (2005)
UDP-glucuronosyltransferase 2B5	1.80E−03	2	0.66	Tsoi et al. (2004)
Ependymin	2.66E−03	3	0.63	Ewart et al. (2005)
Myosin regulatory light chain 2	4.98E−03	1	0.76	Ewart et al. (2005)
Troponin I	3.70E−03	1	0.58	Meijer et al. (2005)
Type II keratin E2	1.52E−03	2	0.38	Meijer et al. (2005)[a]
Keratin 13	1.60E−03	1	0.49	
Keratin, type II cytoskeletal 6A	1.83E−03	3	0.71	
Keratin 12	1.87E−03	2	0.51	
Keratin, type I cytoskeletal 14	2.47E−03	1	0.54	
Keratin, type I cytoskeletal 13	3.49E−03	1	0.45	

P-values from the ANOVA permutation-based *F*-test, the number of significant cDNA clones on the array, the average fold change (expression level in infected fish over that in healthy fish) and references in which the gene differential expression was previously are presented. A supplementary online table (Supplementary Table 3) presents this information in a more detailed form.

[a] Keratin type was not specified in Meijer et al. (2005).

[†] In the case of genes with multiple significant cDNA clones, only the smallest observed *P*-value is presented.

response, providing molecular support to the results of Bly et al. (1993) on catfish. Among these genes, C1r and C2 were not observed as over-transcribed in previous studies assessing genome-wide transcriptional responses of fish to various infectious agents. The very strong over-transcription of the C1r coding gene in this study might reflect activation of the classical complement pathway in infected fish. The over-transcription of the mannose-binding lectin (Table 1) suggests that the MB-lectin pathway is also vitalized. Considering the results for C3 and C6 (Table 1), all three complement activation pathways may be more active in saprolegniasis-affected fish. The major positive APP serum amyloid A and C-reactive proteins were not represented on the microarray we used. Serum amyloid P was represented by four cDNA clones, but none was significantly differentially expressed in infected fish. Serum amyloid P is a major positive mammalian APP but was shown to be under-transcribed in *A. salmonicida* infected Artic charr (Jensen et al., 1997); however, this result was unclear and contested (Bayne and Gerwick, 2001). Jensen et al. (1997) suggested that serum amyloid P transcriptional activation might be sex-specific; this could explain why it was not significantly over-expressed in our samples, which were all sexually immature.

The transcription level of most genes identified as differentially expressed both in this and in previous studies assessing the global gene expression response to immune challenges in fish changed in the same direction (Table 2). Among the genes represented in Table 2, several genes over-transcribed in infected fish encode proteases with a strong collagenolytic activity. Over-transcription of these genes may facilitate leucocyte transmigration. Other induced immunity-related genes include the ATP-binding cassette transporter TAP, which is essential to the MHC class I antigen presenting system, the cytokine receptor CXCR4, CD63, leucocyte chemotaxin 2, glutathione peroxidase, an agglutination and aggregation factor and the interferon inducible protein 1. It is noteworthy that the transcription factors JunB and CCAAT/enhancer binding protein coding genes, both over-transcribed in this study, transactivate APP-coding genes through cytokine and glucocorticoid response elements (Baumann et al., 1991). More surprising is the induction of some histone-coding genes, also observed by Byon et al. (2005). How-

Table 4
Genes observed as differentially expressed in different directions in this study and in previous studies

Gene product	*P*-value[†]	cDNA clone	Fold change	Reference
Tob1 protein	2.28E−03		2.41	Under-transcribed in Ewart et al. (2005)
B-cell translocation gene 1	2.28E−03	2	1.78	Under-transcribed in Ewart et al. (2005)
Prostaglandine D synthase	1.26E−03	2	0.40	Over-transcribed in Baynes (2001), Ewart et al. (2005)
Glutathione *S*-transferase kappa 1	2.57E−03	1	0.70	Over-transcribed in Ewart et al. (2005), Rise et al. (2004)
Microsomal glutathione *S*-transferase 3	2.93E−03	2	0.80	Over-transcribed in Ewart et al. (2005), Rise et al. (2004)
High mobility group protein 1	2.89E−03	1	0.70	Over-transcribed in Rise et al. (2004)
Putative steroid dehydrogenase KIK-I	4.85E−03	1	0.65	Over-transcribed in Rise et al. (2004)
ATP synthase alpha chain	1.32E−03	1	0.25	Over-transcribed in Rise et al. (2004)
Proteasome subunit alpha type 2	1.21E−03	1	0.25	Over-transcribed in Rise et al. (2004)

P-values from the ANOVA permutation-based *F*-test, the number of significant cDNA clones on the array, the average fold change (expression level in infected fish over that in healthy fish) and references in which the gene differential expression was previously observed in fish infected with other agents are presented. A supplementary online table (Supplementary Table 4) presents this information in a more detailed form.

[†] In the case of genes with multiple significant cDNA clones, only the smallest observed *P*-value is presented.

Table 5
Genes which were significantly differently transcribed in whole saprolegniasis-affected juvenile salmon and that had not been observed as so in fish immuno-challenged with other agents in previous studies

Gene product and functional class	*P*-value[†]	cDNA clone	Fold change
Immunity-related gene			
Prostaglandin-H2 D-isomerase precursor	2.97E−03	1	0.49
Acidic chitinase L	2.33E−03	1	0.55
Calreticulin	2.42E−03	2	2.45
G protein-coupled receptor 2	2.92E−03	1	1.76
FYN-binding protein	3.37E−03	1	1.51
Small inducible cytokine	9.69E−04	2	1.78
Myeloperoxidase	1.48E−03	1	0.45
Glucose-6-phosphate 1-dehydrogenase X	3.20E−04	1	1.98
Immunoglobulin heavy chain binding protein	3.52E−04	1	3.57
Immunophilin FKBP12.6	1.46E−03	1	1.64
Extracelluar matrix components			
Collagen alpha 1(X)	7.46E−04	3	0.29
Collagen alpha 2(I)	1.21E−03	7	0.31
Collagen a3(I)	3.75E−03	1	0.35
SPARC	3.96E−03	1	0.60
Electron transport chain			
Cytochrome *c*	4.58E−04	2	2.24
Ubiquinol-cytochrome *c* reductase complex 7.2 kDa protein	4.05E−04	1	8.47
Cytochrome *c* oxidase subunit IV isoform 1	2.62E−03	1	0.68
Cytochrome *c* oxidase polypeptide VIIa	4.95E−03	1	0.74
NADH-ubiquinone oxidoreductase 30 kDa subunit	2.00E−03	2	0.81
NADH-ubiquinone oxidoreductase B18 subunit	4.75E−03	1	0.91
ATP synthase e chain	1.32E−03	1	0.74
ATP synthase D chain	3.35E−03	1	1.95
ATP synthase coupling factor 6	4.18E−03	1	0.40
Ion transport			
Na+/K+ ATPase 1	1.88E−03	1	1.66
Na+/K+ ATPase 3	3.32E−03	1	1.75
Na+/K+ ATPase alpha subunit isoform 1b/ii	4.91E−03	2	1.08
Signal transduction			
Mcl-1a	1.11E−03	1	2.45
Tumor-associated calcium signal transducer	1.65E−03	1	2.14
Centd2	4.68E−03	1	1.50
Transcription/transcription activation			
40S ribosomal protein S16.	3.42E−03	2	2.78
RNA polymerase II transcriptional coactivator p15	7.14E−04	3	0.65
Zinc finger A20 domain containing 2, like	9.27E−04	1	3.63
Zinc finger protein 330	1.14E−03	1	2.06
XP8	1.36E−03	3	2.76
Aryl hydrocarbon receptor 2b	8.95E−04	2	2.38
RNA-binding region containing protein 2	3.16E−03	1	0.79
Similar to thyroid hormone receptor interactor 3	4.70E−03	1	0.55
Metal ion binding protein			
Selenoprotein W	1.04E−03	1	0.77
Metallothionein B	1.75E−03	1	1.75
Metallothionein-I	2.82E−03	1	2.05
Pyrimidine biosynthesis			
CTP synthase	1.12E−03	1	2.09
Enhancer of rudimentary homolog	1.15E−03	1	2.29
Protein degradation			
Similar to Ubiquitin binding enzyme	1.75E−03	1	1.90
Ubiquitin-conjugating enzyme E2 A	3.25E−03	1	2.09
Ubiquitin-conjugating enzyme E2 D2	4.00E−03	1	1.60
F-box only protein 2	2.86E−03	1	2.39
Calpain small subunit 1	3.63E−03	1	1.65
His-tagged cytosolic leucine aminopeptidase	4.13E−03	1	0.86
Meprin A alpha-subunit	3.66E−03	1	0.53

Table 5 (*Continued*)

Gene product and functional class	*P*-value[†]	cDNA clone	Fold change
Protein localisation/folding			
Signal recognition particle 9 kDa protein	4.21E−03	1	0.65
SSR alpha subunit	3.89E−03	1	0.57
Protein disulfide-isomerase A3	1.77E−03	4	1.89
DNA replication/repair			
DNA polymerase delta subunit 4	1.81E−03	1	0.67
Proliferating cell nuclear antigen	4.05E−03	1	0.70
Cell structure and adhesion/cellular junction			
Actin-5C.	1.35E−03	2	2.03
Beta-actin	4.09E−03	1	1.30
Periostin	1.96E−03	1	0.39
Neural-cadherin	2.11E−03	1	2.13
Epithelial-cadherin	3.42E−03	1	1.77
Claudin-3	2.39E−03	1	2.21
Integrin, beta-like 1	2.64E−03	1	2.16
Nicotinamide riboside kinase 2	2.45E−03	2	1.79
Miscellanous			
Delta-6 fatty acyl desaturase	2.04E−03	1	0.79
Diamine acetyltransferase 1	3.20E−04	4	2.94
ALDH class 2	1.18E−03	1	0.64
Transposase	1.67E−03	1	2.45
Nucleophosmin	3.56E−03	1	2.58
EH-domain containing protein 1	4.69E−04	1	2.33
Uncoupling protein 2	1.49E−03	2	2.22
Fatty acid-binding protein, intestinal	2.09E−03	5	0.40
Glutamine synthetase	4.94E−03	2	1.82
Protein translation factor SUI1 homolog GC20	3.32E−03	3	1.77
Small nuclear ribonucleoprotein E	2.59E−03	1	2.57
Mid1 interacting protein 1	2.78E−03	1	0.61
Transport protein SEC61 gamma subunit	3.22E−03	1	1.89
Gelsolin	3.82E−03	1	0.62

P-values from the ANOVA permutation-based *F*-test, the number of significant cDNA clones on the array and the average fold change (expression level in infected fish over that in healthy fish) are presented. A supplementary online table (Supplementary Table 5) presents this information in a more detailed form, with individual fold changes, *P*-values and *Q*-values for each cDNA clone, GeneBank accessions, as well as other significant genes with unknown function.

[†] In the case of genes with multiple significant cDNA clones, only the smallest observed *P*-value is presented.

ever, recent data suggest that histones may play a role in innate immunity in a wide range of animal species (Silphaduang et al., 2006). Here and in Meijer et al. (2005), under-expression of cyto-structural keratin-coding genes was observed, as well as that of collagen alpha 1-coding gene. We also observed down-regulation of four other genes coding for collagens (Table 5).

In a few cases, the direction of the infection-driven expression changes did not correspond between this and previous studies (Table 4). Also, some expression changes detected in other studies (e.g. alpha-1-microglobulin down-regulation) were not detected here as significant. This could be due to many differences in the experimental protocols, including different infection protocols, microarray platforms, statistical treatment of the data, timing of sampling, water temperatures and the fact that different tissues were sampled and different infectious agents were involved. Different host fish species were also used in some of these studies. The tissue-specificity of the response and the timing of sampling likely explain most of the observed lacks of correspondence. Hence, even within a study, results between tissues are not always consistent (see, for instance, Rise et al., 2004). Alternatively, some of these differences may reflect specificity of the response to saprolegniaceae infection. While saprolegnaceae are suspected to exert immunosuppressive actions (Alvarez et al., 1995), *Tob-1* and the *B-cell translocation gene 1* were both under-transcribed instead of over-transcribed in infected fish, as previously reported. *Tob-1* is known to maintain T cells in a quiescent state and has to be down-regulated for T cells to proliferate and release cytokins (Tzachanis et al., 2001), and the *B-cell translocation gene 1* also negatively regulates cell proliferation (Berthet et al., 2002).

Finally, expression differences for several genes between infected and healthy fish was evidenced here for the first time. The fact that all previous microarray studies except that of Meijer et al. (2005) merely used a fold change criterion for identifying differentially expressed genes probably explains that many differences with a smaller than 2-fold change in Table 5 were not previously detected (Jin et al., 2001). As stated earlier, some of the changes listed in Table 5 might also reflect specificities in our experimental settings; others may represent infection stage-specific or saprolegniaceae-specific response genes. Nonetheless, many functional classes are represented in Table 5. Several genes encoding proteins implicated in cellular structure and cellular junctions appear over-transcribed in infected fish. Also, G protein-coupled receptor 2, which is involved

in T cell-mediated skin inflammation processes (Homey et al., 2002), was induced in infected fish. More surprisingly, the prostaglandin-H2 D-isomerase, acidic mammalian chitinase and myeloperoxidase-coding genes, three genes with known immune functions, were under-expressed in infected fish, which may reflect late infection-stage specific changes.

Potential caveats of this study include the fact that some of the observed differences may reflect the lower condition of the infected fish (lower social status, stress, other infections). Also, infection may not have been at identical stages in the different fish sampled, though it was always advanced and all infected samples displayed similar moribund behaviours. In some cases, observed changes might also reflect pathological or morbid changes instead of the immune response itself, since, as Meijer et al. (2005), we sampled heavily infected fish. Two inconsistencies were also observed in our data: two different cDNA clones representing small inducible cytokine and Na+/K+ ATPase alpha subunit isoform 1b/ii were observed as significantly differentially expressed in different directions. The same situation occurred in Rise et al. (2004), which suggested that it might be due to a misidentified EST. Alternatively, one of the two clones of each pair may be a false positive, or the two clones may represent isoforms regulated in opposite directions.

Several authors argue that innate ("non-specific") immunity might be more important than adaptive ("specific") immunity in fish. In most animals, it would also be a more efficient response to fungus-like pathogen driven infections (Ellis, 2001). Indeed, many of the genes observed as differentially expressed in saprolegniasis-affected fish are associated with innate immunity. Yet evidence suggests that fish, as humans, show substantially distinct APR to viral, fungal and microbial agents (Ellis, 2001; Bayne and Gerwick, 2001). Here, we characterized for the first time the genome-wide transcriptional response of fish to saprolegniasis and identified several differentially expressed genes, many of which were not previously observed. Some of these might represent elements of a saprolegniaceae-specific immune response. Further studies comparing the immune response of fish to various infectious agents in controlled conditions could reveal whether the non-specific immune response of fish to diverse pathogens differs. Such differences could be used for molecular diagnosis of fish condition using dendograms similar to Fig. 2 illustrating the expression of a set of genes with pathogen-specific patterns. In addition, several cDNA clones with unknown functions were differentially expressed in saprolegniasis-affected fish. Each of these represents a potential uncharacterized molecular actor of immunity in fish, and perhaps in other taxonomic groups.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.molimm.2006.05.005.

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