Immune-relevant genes expressed in rainbow trout following immunisation with a live vaccine against *Ichthyophthirius multifiliis*

Louise von Gersdorff Jørgensen*, Egemen Nemli, Rasmus D. Heinecke, Martin K. Raida, Kurt Buchmann

Department of Veterinary Pathobiology, Faculty of Life Sciences, University of Copenhagen, Stigbøjlen 7, 1870 Frederiksberg C., Denmark

ABSTRACT: Rainbow trout *Oncorhynchus mykiss* were immunised by intra-peritoneal injection using a live vaccine based on *Ichthyophthirius multifiliis* (Ich) theronts, which previously has shown protection against white spot disease. Samples were taken pre-vaccination and on Day 1, 7, 21 and 28 post-immunisation (p.i.). Expression of immune relevant genes in the liver, spleen and head kidney was monitored by qPCR. To describe the immune reaction following this immunisation, a series of genes encoding cytokines, complement factors, immunoglobulins and acute phase reactants were studied. Genes encoding acute phase reactants in the liver were up-regulated with serum amyloid A (SAA) as the most pronounced with a 2299-fold increase at 24 h p.i. Hepcidin and pre-cerebellin were also up-regulated in the liver 24 h p.i., by 7- and 4-fold, respectively. Complement factors C3, C5 and factor B (Bf) were up-regulated in the spleen and the head kidney 24 h and 28 d p.i. Genes encoding immunoglobulins were not up-regulated, but a specific low titer IgM response (titer 25) against parasite antigens was detected by a modified ELISA 4 wk p.i.

KEY WORDS: Vaccine · White spot disease · Fish · Immune response

INTRODUCTION

The skin-parasitic ciliate *Ichthyophthirius multifiliis* (Ich) is a widespread pathogen in wild and cultured freshwater fish populations (Matthews 2005). Control methods are highly needed against this parasitosis, known as white spot disease, as the main chemical and medical treatments are environmentally questionable (Dickerson & Clark 1996, Matthews 2005) or labour intensive (Buchmann et al. 2003, Dickerson 2006). Vaccination strategies may prove to be a sustainable way to move toward large-scale control of the disease. Investigations have clearly shown that protection can be conferred following a sublethal infection or vaccination using a live theront preparation (Burkhart et al. 1990, Alishahi & Buchmann 2006).

Development of immunity towards Ich was reported early in the 20th century (Buschkiel 1910, Bauer 1953). Several different experimental vaccines have been developed and tested, but only a few of those confer good protection. Experimental trials with formalin killed theronts, trophonts and purified cilia conferred only limited protection (Goven et al. 1980, Burkart et al. 1990, Dalgaard et al. 2002, Sigh & Buchmann 2002), and experiments with recombinant vaccines including DNA vaccinations have thus far not resulted in a high degree of protection (He et al. 1997, Lin et al. 2002, Sigh 2003). Vaccine preparations including 1 or more species within the genus *Tetrahymena*, a closely related ciliate to Ich, have also been used in experimental vaccination trials but conferred only limited protection (Dickerson et al. 1984, Houghton et al. 1992, Sigh & Buchmann 2002).

A number of investigations have been conducted to elucidate the causative mechanisms involved in protection (Hines & Spira 1974, Clark et al. 1988, Sigh & Buchmann 2001, Wang et al. 2002, Sigh et al. 2004a,b, Xu et al. 2004). Although specific antibodies may play a role in immunity, it is clear that several other immune factors determine the protective state of the fish against this skin parasite (Sigh et al. 2004a). However, the different factors involved in protection, and their relative importance, following vaccination are unknown. The development of a live vaccine conferring high protection towards Ich in rainbow trout (Alishahi & Buchmann 2006) has made it feasible to study immune genes activated upon protective immunisation. Here we describe vaccination of rainbow trout using such a live vaccine and report on the quantitative expression of genes encoding complement factors (C3, C5, Bf), immunoglobulins (IgM, IgT), cytokines (IL-1β(1), IL-6, IFN-γ, TNF-α) and acute phase reactants (transferrin, serum amyloid protein [SAP] hepcidin, trout C-polysaccharide-binding protein [Tcbp], serum amyloid A [SAA], and pre-cerebellin) during development of protection. The measured genes were selected both on account of availability and their possible involvement in an immune response against Ich. Complement factors have previously been shown to play a role in immobilisation of theronts using trout serum *in vitro* (Buchmann et al. 1999, Sigh & Buchmann 2001). Genes encoding immunoglobulins were chosen in order to detect antibody responses against Ich (Xu et al. 2004). IgT was recently found to be upregulated in a study using a bath vaccination with *Yersinia ruckeri* (M. Raida & K. Buchmann unpubl.) and could also be involved in responses against Ich. Genes encoding 4 cytokines were selected due to previous records of their important roles in the innate immune response (Gonzalez et al. 2007a, M. Raida & K. Buchmann unpubl.). The SAA gene was chosen based on a report on the up-regulation of this gene during an Ich infection in carp (Gonzalez et al. 2007b). Genes encoding other acute phase proteins (APPs), including Tcbp and SAP (Bayne & Gerwick 2001) and hepcidin and pre-cerebellin (Gerwick et al. 2007), become up-regulated during infections and inflammation. Hepcidin and transferrin regulations are associated with regulation of available iron during bacterial infections (Bayne & Gerwick 2001, Gerwick et al. 2007) but may exert additional antimicrobial effects, which could play a role against Ich infections.

MATERIALS AND METHODS

Out-bred rainbow trout *Oncorhynchus mykiss* reared under pathogen-free conditions (Danish Centre for Wild Salmon, Randers, Denmark) with a weight of 20 g were transferred to aerated aquaria with internal biofilters (Eheim) and acclimatised for 15 d at 18°C with a 12:12 h light:dark cycle. The fish were fed pelleted dry feed of 1% biomass d^{-1} (Biomar). In total, 96 fish were used. The control and the vaccinated group each included 48 fish reared in 120 l aquaria. All experiments were conducted at 18°C.

Vaccine. A live vaccine was prepared as described previously (Alishahi & Buchmann 2006). In brief, a laboratory stock of Ich (originally isolated from Kongeåen trout farm, Jutland, Denmark) was used for the vaccine preparation. One specimen of rainbow trout infected with Ich was killed with an overdose of MS222 (Sigma-Aldrich) and kept in a container with 2 l of water for 4 h at 20°C, which allowed tomonts to leave the host. The fish was removed and the container was left for 24 h at 20°C resulting in tomocyst formation and theront release. Theronts were concentrated by centrifugation and filtration. The centrifugation was done at 160 × *g* on a Universal 320R centrifuge (Hettich) in 50 ml Greiner tubes (In Vitro). After centrifugation, the supernatant was discharged. The filtration was done with Minisart syringe end filters (Sartorius) fitted on a 5 ml omnifix syringe (B Braun). These procedures were repeated until the fluid contained 6000 live theronts ml^{-1}.

Immunisation. Forty-eight fish were individually anaesthetised with MS222 (80 mg l^{-1}) and were i.p. injected with approximately 600 live theronts per fish in 0.1 ml water. The concentration of the theronts was determined in a Sedgewick Rafter counting cell (100 mm^3 of liquid 1 mm deep over an area of 50 × 20 mm). Immediately thereafter, the fish were immersed in sodium percarbonate (Biomar, 10 mg l^{-1}) to avoid infection in the aquaria. The 48 control fish were injected in the same way but with 0.1 ml of sterile water with bovine serum albumin (2 mg ml^{-1}). These fish were also immersed in sodium percarbonate (10 mg l^{-1}).

Sampling. Eight fish from the vaccinated and control groups were sampled at 24 h, 7 d, 21 d and 28 d following immunisation. Additionally, 8 fish were sampled at 0 h. Fish were anaesthetised with MS222 (80 mg l^{-1}), and blood samples were taken by caudal vein puncture using heparinised syringes (1 ml). Thereafter, the fish were killed by cervical incision. Blood samples were kept on ice and centrifuged for 3 min at 5000 × *g* immediately after sampling, and plasma was transferred to sterile tubes and stored at –20°C. The head kidney, liver and spleen were aseptically removed and immediately transferred to RNAlater (Sigma Aldrich). These samples were pre-stored at 4°C for 24 h and then stored at –20°C until RNA purification.

Challenge. The remaining fish, i.e. 9 control and 9 immunised fish, were challenged on Day 32 p.i. with

live theronts (1750 fish^{-1}) in a 100 l aquarium. Nine days post challenge the fish were anaesthetised with MS222 (60 mg l^{-1}), and the number of white spots on the skin was counted.

Antibody detection. To investigate the presence of Ich-antigen antibodies in the trout plasma from the various sampling points, a modified ELISA was performed. Plasma samples from 2×4 fish in each group at each sample point were pooled and diluted 1:1, 1:5, 1:25, 1:125, 1:625. For this analysis, 2 µl of an antigen solution (sonicated tomonts, 415 µg protein ml^{-1}) in water were applied to nitrocellulose membrane pieces (0.7×0.7 cm, Millipore) in 48 well Nunc multiwell trays with lids (Fisher Scientific). The membranes were air-dried for 5 min. Trout plasma (2 µl) was applied to 2 nitrocellulose pieces as a positive control. The nitrocellulose membranes were blocked in 1% dilution buffer (1% skimmed milk powder in PBS) for 30 min, after which the membrane (except the 2 positive controls) were incubated with trout plasma samples from each time point (for 1 h at room temperature [RT]). This was followed by 3×5 min in washing buffer (0.05% Tween 20 [Sigma-Aldrich] in PBS). The membranes were incubated (1 h) in peroxidase conjugated anti-trout IgM Mab (Aquatic Diagnostics) and subsequently washed for 3×5 min in washing buffer. Positive samples developed dark-brown colouring following addition of di-amino-benzidine (DAB) substrate (Sigma-Aldrich).

Total RNA isolation for qPCR. Tissues were homogenised by sonication on ice (Sonicator Ultrasonic Liquid Processor Model XL 2020, Heat Systems). Total RNA was isolated using RTN350 (Sigma-Aldrich) and subsequently DNase treated (DNase I). The quantity of the RNA was tested by optical density (OD) at 260/280 nm (BIORAD SmartSpec^{TM} 3000). The concentration of the RNA was adjusted to 200 ng in all samples using MilliQ H$_2$O.

cDNA synthesis. cDNA was synthesised using Taq-Man$^{®}$ Reverse Transcription reaction with random hexamers following the manufacturer's instruction (Applied Biosystems). A 10 µl setup was used. A negative control contained RT-reagents without reverse transcriptase (RT minus). The cDNA was diluted 1:10 in MilliQ H$_2$O.

Real-time PCR. Real-time quantitative assays were performed using a Stratagene MX3000PTM real-time PCR system. Primers and probes are shown in Table 1. The reactions were optimised with MgCl$_2$ (Sigma-Aldrich). The cycling conditions were 94°C for 2 min followed by 40 cycles of 94°C for 30 s and 60°C for 1 min. A 12.5 µl setup was used: 6.25 µl $2 \times$ Jump-Start^{TM} Taq ReadyMix^{TM} (Sigma-Aldrich), 0.5 µl forward primer (10 µM), 0.5 µl reverse primer (10 µM), 2.5 µl cDNA template DNA, 0.5 µl TaqMan$^{®}$ probe

(5 µM) and 2.25 µl MilliQ H$_2$O. Negative controls included RT minus and mastermix with water instead of cDNA template. Negative controls were included on every plate setup. Elongation factor (EF 1-α) was used as a housekeeping gene (Olsvik et al. 2005, Ingerslev et al. 2006, Raida & Buchmann 2007). When the real-time curves did not reach the threshold after 40 cycles, a value of 40 cycle threshold (C$_t$) was used.

Data analyses. C$_t$ was determined at the lowest value possible on the linear slope in a log fluorescence/C$_t$ plot. The expression data were determined according to the $2^{-\Delta\Delta Ct}$ method (Livak & Schmittgen 2001). The data are presented as the fold increase/decrease of the vaccinated fish (n = 8) compared to non-vaccinated control fish (n = 8) at each sample point. The $\Delta\Delta C_t$ value of the 8 vaccinated fish was calculated from the mean ΔC_t value of the controls. Subsequently, the mean of the $\Delta\Delta C_t$ was calculated for the vaccinated fish followed by the calculation of the fold increase/decrease. Data were tested for normality (Sigma Stat 3.0 and SPSS) and evaluated by t-test (p < 0.05). The Mann-Whitney rank-sum test was applied (p < 0.05) if data did not follow a normal distribution (Sigma Stat 3.0). The data are presented as the mean value of the fold change of the 8 fish at each sampling point. Significance intervals were calculated by adding and subtracting the standard error of the mean (SEM) from the $\Delta\Delta C_t$ values followed by a calculation of the fold change.

A significance level of 0.05 was chosen, and the minimum acceptable fold change was set at 3-fold to eliminate biological variation. Regulation was considered when both settings were satisfied.

RESULTS

The 9 vaccinated fish showed significant protection (t-test, p < 0.05) against Ich 9 d after challenge. The control fish exhibited a mean of 18.7 (SD ± 8.9) white spots per fish, whereas the vaccinated fish showed 4.0 (SD ± 2.5) white spots per fish.

Expression data were obtained mainly from the liver, but the spleen and head kidney were also investigated with regard to certain relevant genes (Tables 2 & 3). The examined genes showed various levels of constitutive expression (Table 3), but some genes became significantly up- or down-regulated following vaccination (Table 2).

Complement factors. The genes for complement factors (C3, C5 and Bf) were not regulated in the liver (Table 4). In the spleen, an up-regulation of 3-fold was observed for C5 24 h after vaccination, and an up-regulation of C5 of 4 (not significant) was observed 1 wk post-injection. Up-regulations were seen 4 wk after

Table 1. *Oncorhynchus mykiss*. Primers and probes (Fwd: forward, Rev: reverse) and their GenBank accession no. or source, sequence and product size. EF: elongation factor, Bf: factor B, Ig: immunoglobulin, IL: interleukin, IFN: interferon, TNF: tumor necrosis factor, SAP: serum amyloid protein, Tcbp: trout C-polysaccharide-binding protein, SAA: serum amyloid A

Gene	Acc. no.	Product size (bp)	Primer	Probe
Housekeeping gene				
EF1-α	AF498320	63	Fwd: ACCCTCCTCTTGGTCGTTTC	
			Rev: TGATGACACCAACAGCAACA	GCTGTGCGTGACATGAGGCA
Complement factor				
C3	U61753[a]	85	Fwd: ATTGGCCTGTCCAAAACACA	
			Rev: AGCTTCAGATCAAGGAAGAAGTTC	TGGAATCTGTGTGTCTGAACCCC
C5	AF349001[a]	64	Fwd: TGGCAAGGACTTTTTCTGCT	
			Rev: AGCACAGGTATCCAGGGTTG	CTGGCAGGGATTGCATCAAATC
Bf	AB044939[a]	66	Fwd: AGAGACTGGCAGCGTTTGAT	
			Rev: AGAGCAGCGTACAGGTTGGT	CACCGAAGCCACGGGAACAA
Immunoglobulin				
IgM	S63348	72	Fwd: CTTGGCTTGTTGACGATGAG	
			Rev: GGCTAGTGGTGTTGAATTGG	TGGAGAGAACGAGCAGTTCAGCA
IgT	AY870265	72	Fwd: AGCACCAGGGTGAAACCA	
			Rev: GCGGTGGGTTCAGAGTCA	AGCAAGACGACCTCCAAAACAGAAC
Cytokine				
IL-1β(1)	AJ223954	91	Fwd: ACATTGCCAACCTCATCATCG	
			Rev: TTGAGCAGGTCCTTGTCCTTG	CATGGAGAGGTTAAAGGGTGGC
IL-6	DQ866150[a]	91	Fwd: ACTCCCCTCTGTCACACACC	
			Rev: GGCAGACAGGTCCTCCACTA	CCACTGTGCTGATAGGGCTGG
IFN-γ	AY795563	68	Fwd: AAGGGCTGTGATGTGTTTCTG	
			Rev: TGTACTGAGCGGCATTACTCC	TTGATGGGCTGGATGACTTTAGGA
TNF-α	AJ277604[a]	75	Fwd: GGGGACAAACTGTGGACTGA	
			Rev: GAAGTTCTTGCCCTGCTCTG	GACCAATCGACTGACCGACGTGGA
Acute phase protein				
Transferrin	D89083[a]	66	Fwd: TTCACTGCTGGAAAATGTGG	
			Rev: GCTGCACTGAACTGCATCAT	TGGTCCCTGTCATGGTGGAGCA
SAP	X99385[a]	67	Fwd: CAACGTCTCAAAGCCCATTT	
			Rev: GCCTCGTTCTTGCTCAGAGT	CTGCTATGACCATGTGTCAGAGGTTC
Hepcidin	AF281354.1[a]	95	Fwd: GAGGAGGTTGGAAGCATTGA	
			Rev: TGACGCTTGAACCTGAAATG	AGTCCAGTTGGGGAACATCAACAG
Tcbp	AF281345[a]	65	Fwd: TCCAGGACAATGAGGGCTAC	
			Rev: TGACGCTTGAACCTGAAATG	AGTCCAGTTGGGGAACATCAACAG
SAA	X99387[a]	79	Fwd: GGGAGATGATTCAGGGTTCCA	
			Rev: TTACGTCCCCAGTGGTTAGC	TCGAGGACACGAGGACTCAGCA
Pre-cerebellin	AF192969[a]	112	Fwd: TGGTGTTGCTTTGCTGTTGT	
			Rev: GCCACTTTTGGTTTGCTCTC	ATGGTTGAGACTCAGACGGAGAGTG

[a]From M. Raida & K. Buchmann (unpubl.)

vaccination of C3 (4-fold) and Bf (22-fold). In the head kidney, both C3 (9-fold) and C5 (21-fold) were up-regulated after 24 h, and Bf was non-significantly up-regulated 5-fold at this sample point. At 3 wk, both C5 and Bf were down-regulated 6- and 7-fold, respectively (Table 4).

Immunoglobulins. No regulation of immunoglobulins was observed for any of the 3 organs (Tables 2 & 4).

Cytokines. No significant regulation more than 3-fold of genes encoding cytokines in the liver or the spleen was observed. However, a weak but non-significant regulation of all 4 genes was observed in the liver. A down-regulation of IFN-γ of 3-fold was seen 1 wk p.i. in the head kidney (Tables 2 & 4).

Acute phase proteins in the liver. Hepcidin, SAA and pre-cerebellin were up-regulated 7, 2299 and 4 times, respectively, 24 h after injection. SAA was also up-regulated at 1 wk post-vaccination (65 times; Table 4).

Antibody detection. The overall transcription of the genes for IgM and IgT was not up-regulated after 4 wk according to the gene expression studies. However, the modified ELISA showed an antibody titer (IgM) of 25 from both sub-groups of vaccinated fish 4 wk p.i.

Table 2. *Oncorhynchus mykiss*. An overview of gene regulations. ↑* = significant up or down regulation. ↑ = non-significant up or down regulation, X: less than 3-fold change between controls and vaccinated fish (significant or non-significant). Abbreviations as in Table 1

Gene	Liver 24 h	1 wk	3 wk	4 wk	Spleen 24 h	1 wk	3 wk	4 wk	Head kidney 24 h	1 wk	3 wk	4 wk
Housekeeping gene												
EF1-α	X	X	X	X	X	X	X	X	X	X	X	X
Complement factor												
C3	X	X	X	X	X	X	X	↑*	↑*	X	X	X
C5	X	X	X	X	↑*	↑	X	X	↑*	X	↓*	X
Bf	X	X	X	X	X	X	X	↑*	↑	X	↓*	X
Immunoglobulin												
IgM	X	X	X	X	X	X	X	X	X	X	X	X
IgT	X	X	↓	X	X	X	↓	X	X	↑	X	X
Cytokine												
IL-1β(1)	X	↑	X	X	X	X	X	X	X	X	X	X
IL-6	X	X	X	↑	X	X	X	X	X	X	X	X
IFN-γ	X	X	↓	↑	X	X	X	X	X	↓*	X	X
TNF-α (1 and 2)	X	X	X	↑	X	X	X	X	X	X	X	X
Acute phase protein												
Transferrin	X	X	X	X	–	–	–	–	–	–	–	–
SAP	X	X	X	X	–	–	–	–	–	–	–	–
Hepcidin	↑*	X	X	X	–	–	–	–	–	–	–	–
Tcbp	X	X	X	X	–	–	–	–	–	–	–	–
SAA	↑*	↑*	X	X	–	–	–	–	–	–	–	–
Pre-cerebellin	↑*	X	X	X	–	–	–	–	–	–	–	–

DISCUSSION

Our experiments have clearly shown that injections of live theronts confer partial protection against white spot disease caused by *Ichthyophthirius multifiliis* as described previously for channel catfish *Ictalurus punctatus* and rainbow trout *Oncorhynchus mykiss* (Burkart et al. 1990, Alishahi & Buchmann 2006). The protected fish still carry a few trophonts following challenge, but the parasite load is clearly decreased. This experimental model facilitates the study of mechanisms involved in protective immunity in trout against Ich. By conducting qPCR, we were able to pin-point a series of genes that are specifically up-regulated following immunisation.

The observation that some APPs were highly up-regulated in the liver indicates that innate immunity is a main player in the response. SAA is a conserved apolipoprotein and is one of the major APPs in mammals (Uhlar & Whitehead 1999). This protein has several important functions, including a binding capacity to a range of Gram-negative bacteria (Hari-Dass et al. 2005). This could indicate that it is a pattern recognition protein. In our study, the constitutive expression of SAA in the liver was very low (Table 3). However, it was highly up-regulated (2299-fold) following immunisation, and it cannot be excluded that SAA has a direct influence on the parasite as suggested by Gonzalez et al. (2007b). Future studies should be conducted to

Table 3. *Oncorhynchus mykiss*. Real time cycle threshold (C_t) values for various genes in liver, spleen and head kidney before i.p. injection of live theronts. SEM in parentheses. Abbreviations as in Table 1

Gene	Liver	Spleen	Head kidney
Housekeeping gene			
EF1-α	21.18 (±0.17)	20.37 (±0.12)	22.05 (±0.06)
Complement factor			
C3	23.05 (±0.27)	34.82 (±0.39)	31.96 (±0.43)
C5	22.87 (±0.09)	31.97 (±0.34)	30.76 (±0.53)
Bf	23.72 (±0.04)	37.99 (±0.98)	32.95 (±0.88)
Immunoglobulin			
IgM	27.38 (±0.19)	19.27 (±0.12)	19.57 (±0.27)
IgT	32.26 (±0.59)	27.33 (±0.39)	26.68 (±0.29)
Cytokine			
IL-1β(1)	33.95 (±0.53)	30.56 (±0.05)	32.56 (±0.26)
IL-6	37.29 (±0.61)	33.69 (±0.28)	37.66 (±0.54)
IFN-γ	34.31 (±0.40)	32.09 (±0.24)	32.28 (±0.40)
TNF-α (1 and 2)	33.26 (±0.17)	33.81 (±0.33)	31.29 (±0.20)
Acute phase protein			
Transferrin	17.74 (±0.13)	–	–
SAP	23.71 (±0.05)	–	–
Hepcidin	25.53 (±0.42)	–	–
Tcbp	24.97 (±0.98)	–	–
SAA	39.99 (±0.11)	–	–
Pre-cerebellin	25.90 (±0.31)	–	–

Table 4. *Oncorhynchus mykiss*. Real time quantitative expression of complement factors, immunoglobulins and cytokines in liver spleen and head kidney plus acute phase proteins in liver following an i.p. injection of live theronts. Expression was compared to controls injected with bovine serum albumin, and values are fold increase or decrease in the target genes relative to EF1-α. Confidence intervals are indicated in parentheses. *p < 0.05 and more than 3-fold change. Abbreviations as in Table 1

Gene	24 h	1 wk	3 wk	4 wk
Liver				
Complement factor				
C3	1.39 (1.12;1.71)	1.35 (1.18;1.54)	1.04 (–114;124)	–1.25 (–1.60;1.03)
C5	1.39 (1.21;1.59)	1.10 (–1.02;1.24)	–1.17 (–1.28;–1.06)	1.08 (–1.01;1.19)
Bf	–1.12 (–1.33;1.06)	1.15 (1.06;1.26)	–1.31 (–1.44;–1.18)	1.03 (–1.06;1.13)
Immunoglobulin				
IgM	1.18 (–1.02;1.43)	1.17 (1.02;1.33)	–1.63 (–1.78;–1.49)	1.79 (1.09;2.94)
IgT	–1.88 (–3.50;–1.01)	–1.90 (–4.27;1.18)	–4.06 (–8.75;–1.89)	2.45 (1.53;3.93)
Cytokine				
IL-1β(1)	1.96 (1.58;2.44)	4.88 (2.73;8.72)	–1.37 (–2.10;1.12)	1.67 (–2.35;6.57)
IL-6	2.95 (1.69;5.14)	1.66 (1.06;2;58)	–1.40 (–2.04;1.03)	3.14 (–1.12;11.05)
IFN-γ	2.22 (1.67;2.97)	2.93 (1.83;4.70)	–3.52 (–4.31;–2.87)	3.86 (1.07;13.94)
TNF-α (1 and 2)	2.78 (2.36;3.29)	1.76 (1.16;2.66)	–2.35 (–2.75;–2.01)	6.55 (2.39;17.97)
Acute phase protein				
Transferrin	1.57 (1.39;1.79)	1.79 (1.52;2.10)	1.04 (–1.14;1.23)	–1.05 (–1.27;1.16)
SAP	–1.82 (–2.46;–1.35)	1.28 (1.05;1.56)	–1.28 (–1.50;–1.09)	1.04 (–1.12;1.21)
Hepcidin	7.34 (4.29;12.55)*	1.92 (1.62;2.28)	–2.35 (–3.91;–1.41)	–1.53 (–1.85;–1.27)
Tcbp	–2.13 (–3.83;–1.19)	1.04 (–1.65;1.79)	–1.06 (–1.66;1.47)	–2.05 (–4.33;1.03)
SAA	2298.99 (913.64;5784.92)*	65.33 (25.65;167.90)*	1.23 (–1.57;2.38)	1.29 (–1.66;2.77)
Pre-cerebellin	3.76 (2.91;4.85)*	1.46 (1.06;2.00)	–1.02 (–1.26;1.22)	–1.53 (–2.47;1.06)
Spleen				
Complement factor				
C3	–1.61 (–3.58;1.39)	1.42 (–1.01;2.02)	–1.37 (3.37;1.79)	4.16 (2.64;6.53)*
C5	3.14 (1.64;5.99)*	4.29 (2.77;6.64)	–1.38 (–2.39;1.25)	1.44 (–1.02;2.12)
Bf	–1.11 (–1.97;1.60)	2.68 (1.49;4.81)	–1.35 (–2.80;1.54)	22.14 (15.04;32.59)*
Immunoglobulin				
IgM	–1.14 (–1.33;1.03)	–1.16 (–1.26;–1.07)	–1.12 (–1.32;1.05)	1.02 (–1.08;1.12)
IgT	–2.86 (–7.24;–1.13)	–1.12 (–1.36;1.08)	–3.84 (–11.39;–1.29)	1.09 (–1.24;1.46)
Cytokine				
IL-1β(1)	1.62 (1.10;2.39)	1.18 (–1.08;1.51)	–1.04 (–1.69;1.56)	–1.84 (–2.45;1.38)
IL-6	–1.98 (–2.30;–1.71)	–1.29 (–1.51;–1.10)	1.35 (–1.14;2.09)	–1.36 (–1.62;–1.15)
IFN-γ	1.30 (–1.02;1.71)	1.03 (–1.13;1.21)	1.50 (–1.00;2.25)	–1.43 (–1.67;–1.23)
TNF-α (1 and 2)	1.29 (–1.04;1.73)	1.12 (–1.03;1.29)	1.32 (–1.10;1.91)	–1.30 (–1.57;–1.08)
Head kidney				
Complement factor				
C3	8.97 (5.84;13.78)*	–2.84 (–4.51;–1.78)	–2.04 (–2.83;–1.47)	–1.34 (–2.48;1.38)
C5	21.24 (12.58;35.86)*	1.27 (–1.62;2.63)	–5.87 (–9.01;–3.82)*	1.16 (–1.42;1.92)
Bf	4.74 (2.13;10.57)	1.14 (–1.98;2.57)	–6.84 (–12.55;–3.73)*	–1.81 (–4.32;1.32)
Immunoglobulin				
IgM	1.27 (1.13;1.42)	–1.61 (–1.89;–1.37)	–1.61 (–1.86;–1.39)	–1.02 (–1.17;1.13)
IgT	–1.51 (–3.36;1.47)	–4.40 (–12.23;–1.59)	–2.42 (–2.95;–1.99)	1.07 (–1.20;1.37)
Cytokine				
IL-1β(1)	1.97 (1.36;2.86)	–1.06 (–1.33;1.19)	–1.38 (–1.96;1.03)	–1.30 (–1.63;–1.03)
IL-6	1.44 (–1.06;2.20)	–1.22 (–1.71;1.15)	1.02 (–1.25;1.29)	–1.42 (1.75;1.16)
IFN-γ	2.51 (2.06;3.05)	–3.14 (–3.49;–2.83)*	–1.08 (–1.28;1.10)	1.51 (1.12;2.04)
TNF-α (1 and 2)	1.68 (1.31;2.15)	1.36 (1.25;1.48)	–1.07 (–1.35;1.19)	1.27 (–1.08;1.76)

investigate SAA binding to and its effect on the parasite. The expression of pre-cerebellin, which could be part of the innate immune response (Gerwick et al. 2000), was also increased. An increased level of hepcidin is normally observed during bacterial infections and influences the iron stores (Gerwick et al. 2007), but

functional studies on this molecule should be performed in order to elucidate its immunological role.

Complement factors has been shown to play a part in immobilisation of theronts (Buchmann et al. 1999). In the present investigation, only extrahepatic regulation of complement genes was observed. An up-regulation

of the genes for C3 (9-fold), C5 (21-fold) and Bf (5-fold, though not significant) was observed 24 h post vaccination in the head kidney, which corresponds well with a possible activation of the alternative pathway of the complement cascade, which occurs in trout (Jensen & Koch 1992, Boshra et al. 2004). The subsequent down-regulation of C5 and Bf after 3 wk could possibly represent some kind of regulatory mechanism. Likewise, expression of C5 was increased in the spleen 24 h p.i. Increased expression levels of C3 (4-fold) and Bf (22-fold) were observed 4 wk p.i., which could indicate that the alternative complement pathway still plays a role at this late stage. Interestingly, no up-regulations of complement factor genes were observed for the liver, but this lack of regulation is probably due to the high constitutive expression of these genes in the organ (Table 3; Løvoll et al. 2007b). Alternatively, the absent regulation of C3 in the liver could perhaps be assigned to counteracting regulations of the 3 C3 subtypes. This mechanism could secure essential homeostasis as suggested by Løvoll et al. (2007a). Unfortunately, our C3 primers targeted only 2 C3 subtypes. The complement factors investigated here are present as pro-enzymes in the blood; therefore, up-regulation or activation of other regulatory factors could induce activation of these enzymes. The cytokines IL-1β(1), IL-6, TNF-α and IFN-γ were not significantly up-regulated in any of the sampled organs. This indicates that protective immune mechanisms can be activated without a continuous excessive expression of cytokines. This is in line with Løvoll et al. (2007a), who showed that an increase of C3 is not necessarily associated with an increase of these cytokines in rainbow trout and Atlantic salmon head kidney macrophages.

We merely found a non-significantly increased level in the liver of IL-1β(1) and TNF-α at 1 and 4 wk post-vaccination, respectively. The fate of the theronts in the peritoneal cavity is not known; therefore, it is possible that they exert an effect on the fish after 4 wk and possibly influence the regulation of several genes. Thereby, the weak regulation of the genes encoding the 4 cytokines could be due to prolonged survival of injected theronts. We did expect a significant up-regulation at 24 h for IL-1β(1), IL-6, IFN-γ and TNF-α in the liver, but the expression level was only between 2- and 3-fold. However, the maximum production of cytokines may have taken place between 4 and 12 h after vaccination as has been observed in other pathogen/host systems (Fast et al. 2007, Raida & Buchmann 2007).

Our results did not show an up-regulation of genes encoding immunoglobulins. However, this could be due to the low amount of specific antibodies against Ich compared to the total amount of Igs produced in all 3 organs. This is supported by the result from the mod-ified ELISA trials that showed a weak increase of the specific IgM antibody titer 4 wk post vaccination, indicating that immunoglobulins could participate in the protection against Ich. Several experiments have already shown that antibodies could play a role in the defence against this parasite (Clark et al. 1988, Clark & Dickerson 1997, Sigh & Buchmann 2001, Xu et al. 2004). Nonetheless, our investigations indicate that other factors are involved in protection, which was also hypothesised by Sigh & Buchmann (2001). In this context, it is worth noting that immunised fish of different species only sustain immunity for 3 mo in a sterile environment and for 8 mo in continued exposure to the parasite (Hines & Spira 1974, Burkart et al. 1990, Matthews 1994). This could indicate that Ich is less effective in triggering a prolonged immunological memory as seen for VHS and red mouth disease (Ellis 1988, Lorenzen & Einer-Jensen 2000).

Additional information about the specific gene regulations related to Ich could have been obtained by including an additional control group with fish injected with *Tetrahymena* spp., which survive for a period of time in the peritoneal cavity, as does Ich. By comparing this particular control group to the target group injected with live Ich theronts, it could be possible to pinpoint specific responses and exclude non-specific reactions associated with protection against Ich. However, some cross-protection against Ich occurs when fish have been immunised with *Tetrahymena* spp. (Goven et al. 1980, Wolf & Markiw 1982, Ling et al. 1993, Sigh & Buchmann 2002). Further studies on surface molecules (phosphatidylinositol [GPI]-anchored cell surface proteins) that induce cross-protection of *Tetrahymena* spp. and *Ichthyophthirius multifiliis* should be conducted.

Recent studies on rainbow trout naturally infected with Ich (Sigh et al. 2004a,b) showed regulations of a few genes in the spleen, head kidney and skin, organs that were also studied in our investigation. Similarities and differences were observed between the 2 studies. In the head kidney, Sigh et al. (2004a) did not see a regulation of C3 at any time, and in the spleen they saw approximately the same up-regulation after approximately 4 wk that we observed. IgM gene regulations were not found in the head kidney or spleen in any of the studies. In our study, the cytokine IL-1β(1) (Zou et al. 2000) was not regulated at any time point, whereas Sigh et al. (2004b) saw a 3-fold up-regulation after 24 h (head kidney) and 26 d (spleen and head kidney) post-infection. The differences between the 2 studies could partly be due to differences between the semi-quantitative RT-PCR method and the more sensitive real-time PCR method used in our study. Another possibility is that the differences in gene expression represent the actual differences between a natural Ich

infection in the skin and an i.p. injection using live theronts.

The present study showed that immunity against the parasite *Ichthyophthirius multifiliis* in rainbow trout can be obtained with a vaccine containing live theronts injected i.p. It is noteworthy that this immunisation induces a systemic reaction that affects the number of parasites in the skin during challenge. During this process a huge production of SAA is immediately triggered and to some extent hepcidin and pre-cerebellin in the liver. Furthermore, an up-regulation of the genes associated with the alternative pathway in the complement cascade is observed in the spleen and the head kidney. Future work should elucidate the antigens associated with this protective innate immune response. Moreover, less labour-intensive immunisation procedures should be developed that could replace the live vaccine.

LITERATURE CITED

Alishahi M, Buchmann K (2006) Temperature-dependent protection against *Ichthyophthirius multifiliis* following immunisation of rainbow trout using live theronts. Dis Aquat Org 72:269–273

Bauer ON (1953) Immunity in fish infected by *Ichthyophthirius multifiliis* Fouquet. Dokl Akad Nauk SSSR 93:377–379

Bayne CJ, Gerwick L (2001) The acute phase response and innate immunity of fish. Dev Comp Immunol 25:725–743

Boshra H, Gelman AE, Sunyer JO (2004) Structural and functional characterization of complement C4 and C1s-like molecules in teleost fish: insights into the evolution of classical and alternative pathways. J Immunol 173:349–359

Buchmann K, Lindenstrøm T, Sigh J (1999) Partial cross protection against *Ichthyophthirius multifiliis* in *Gyrodactylus derjavini* immunized rainbow trout. J Helminthol 73:189–195

Buchmann K, Jensen PB, Kruse KD (2003) Effects of sodium percarbonate and garlic extract on *Ichthyophthirius multifiliis* theronts and tomocysts: in vitro experiments. N Am J Aquac 65:21–24

Burkart MA, Clark TG, Dickerson HW (1990) Immunization of channel catfish, *Ictalurus punctatus* Rafinesque, against *Ichthyophthirius multifiliis* (Fouquet): killed versus live vaccines. J Fish Dis 13:401–410

Buschkiel AL (1910) Beiträge zur Kenntnis des *Ichthyophthirius multifiliis* Fouquet. Arch Protistenkd 21:61–102

Clark TG, Dickerson HW (1997) Antibody-mediated effects on parasite behaviour: evidence of a novel mechanism of immunity against a parasitic protist. Parasitol Today 13:477–480

Clark TG, Dickerson HW, Findly RC (1988) Immune response of channel catfish to ciliary antigens of *Ichthyophthirius multifiliis.* Dev Comp Immunol 12:581–594

Dalgaard M, Buchmann K, Li AH (2002) Immunization of rainbow trout fry with *Ichthyophthirius multifiliis* sonicate: protection of host and immunological changes. Bull Eur Assoc Fish Pathol 22:288–297

Dickerson HW (2006) *Ichthyophthirius multifiliis* and *Cryptocaryon irritans* (Phylum Ciliophora). In: Woo PTK (ed) Fish diseases and disorders,Vol 1: protozoan and metazoan infections. CAB International, Wallingford, p 116–153

Dickerson HW, Clark TG (1996) Immune response of fishes to ciliates. Annu Rev Fish Dis 6:107–120

Dickerson HW, Brown J, Dawe DL, Gratzek JB (1984) *Tetrahymena pyriformis* as a protective antigen against *Ichthyophthirius multifiliis* infection: comparisons between isolates and ciliary preparations. J Fish Biol 24:523–528

Ellis AE (1988) Vaccination against enteric red mouth (ERM). In: Ellis AE (ed) Fish vaccination. Academic Press, London, p 85–92

Fast MD, Johnson SC, Jones SRM (2007) Differential expression of the pro-inflammatory cytokines IL-1β, TNFα-1 and IL-8 in vaccinated pink (*Oncorhynchus gorbuscha*) and chum (*Oncorhynchus keta*) salmon juveniles. Fish Shellfish Immunol 22:403–407

Gerwick L, Wendy SR, Bayne CJ (2000) A precerebellin-like protein is part of the acute phase response in rainbow trout, *Oncorhynchus mykiss.* Dev Comp Immunol 24:597–607

Gerwick L, Corley-Smith G, Bayne CJ (2007) Gene transcript changes in individual rainbow trout livers following an inflammatory stimulus. Fish Shellfish Immunol 22:157–171

Gonzalez SF, Buchmann K, Nielsen ME (2007a) Real-time gene expression analysis in carp (*Cyprinus carpio* L.) skin: inflammatory responses caused by the ectoparasite *Ichthyophthirius multifiliis.* Fish Shellfish Immunol 22:641–650

Gonzalez SF, Buchmann K, Nielsen ME (2007b) *Ichthyophthirius multifiliis* infection induces massive up-regulation of serum amyloid A in carp (*Cyprinus carpio*). Vet Immunol Immunopathol 115:172–178

Goven BA, Dawe DL, Gratzek JB (1980) Protection of channel catfish *Ictalurus punctatus* Rafinesque, against *Ichthyophthirius multifiliis* Fouquet by immunisation. J Fish Biol 17:311–316

Hari-Dass R, Shah C, Meyer DJ, Raynes JG (2005) Serum amyloid A protein binds to outer membrane protein A of Gram-negative bacteria. J Biol Chem 280:18562–18567

He J, Yin Z, Xu G, Gong Z, Lam TJ, Sin YM (1997) Protection of goldfish against *Ichthyophthirius multifiliis* by immunization with a recombinant vaccine. Aquaculture 158:1–10

Hines RS, Spira DT (1974) Ichthyophthiriasis in mirror carp *Cyprinus carpio* (L.). III. Pathology. J Fish Biol 6:189–196

Houghton G, Healey LJ, Matthews RA (1992) The cellular proliferative response, humoral antibody response, and cross reactivity studies of *Tetrahymena pyriformis* with *Ichthyophthirius multifiliis* in juvenile carp (*Cyprinus carpio* L.). Dev Comp Immunol 16:301–312

Ingerslev HC, Pettersen EF, Jakobsen RA, Petersen CB, Wergeland HI (2006) Expression profiling and validation of reference gene candidates in immune relevant tissues and cells from Atlantic salmon (*Salmo salar* L.). Mol Immunol 43:1194–1201

Jensen LB, Koch C (1992) Use of monoclonal and polyclonal antibodies to analyse the degradation of rainbow trout C3 in inulin-activated serum. Fish Shellfish Immunol 2:241–249

Lin Y, Chen G, Wang X, Clark TG (2002) The use of synthetic genes for the expression of ciliate proteins in heterologous systems. Gene 288:85–94

Ling KH, Sin YM, Lam TJ (1993) Protection of goldfish against some common ectoparasitic protozoans using *Ichthyo-*

phthirius multifiliis and *Tetrahymena pyriformis* for vaccination. Aquaculture 116:303–314

Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2^{-DDC_T}. Methods 25:402–408

Lorenzen E, Einer-Jensen K (2000) DNA vaccination of rainbow trout against viral hemorrhagic septicaemia virus: a dose-response and time-course study. J Aquat Anim Health 12:167–180

Løvoll M, Fischer U, Mathisen GS, Bøgwald J, Orotake M, Dalmo RA (2007a) The C3 subtypes are differentially regulated after immunostimulation in rainbow trout, but head kidney macrophages do not contribute to C3 transcription. Vet Immunol Immunopathol 117:284–295

Løvoll M, Dalmo RA, Bøgwald J (2007b) Extrahepatic synthesis of complement components in the rainbow trout (*Oncorhynchus mykiss*). Fish Shellfish Immunol 23:721–731

Matthews RA (1994) *Ichthyophthirius multifiliis* Fouquet, 1876: infection and protective response within the fish host. In: Pike AW, Lewis JW (eds) Parasitic diseases of fish, 1st edn. Samara, Tresaith, p 17–42

Matthews RA (2005) *Ichthyophthirius multifiliis* Fouquet and ichthyopthiriosis in freshwater teleosts. Adv Parasitol 59: 159–241

Olsvik PA, Lie KK, Jordal AE, Nilsen TO, Hordvik I (2005) Evaluation of potential reference genes in real-time RT-PCR studies of Atlantic salmon. BMC Mol Biol 17: 6–21

Raida MK, Buchmann K (2007) Temperature-dependent expression of immune-relevant genes in rainbow trout following *Yersinia ruckeri* vaccination. Dis Aquat Org 77: 41–52

Sigh J (2003) The immune response in rainbow trout (*Oncorhynchus mykiss*) against the ciliate *Ichthyophthirius multifiliis* Fouquet, 1876 — selected immune mechanisms and testing of DNA vaccination. PhD dissertation, The Royal Veterinary and Agricultural University, Frederiksberg

Sigh J, Buchmann K (2001) Comparison of immobilization assays and enzyme linked immunosorbent assays for detection of rainbow trout antibody-titres against *Ichthyophthirius multifiliis* Fouquet, 1876. J Fish Dis 24: 49–51

Sigh J, Buchmann K (2002) Comparative analysis of cross-reactivity between *Ichthyophthirius* and *Tetrahymena.* Bull Eur Assoc Fish Pathol 22:37–44

Sigh J, Lindenstrøm T, Buchmann K (2004a) The parasite ciliate *Ichthyophthirius multifiliis* induces expression of immune relevant genes in rainbow trout, *Oncorhynchus mykiss* (Walbaum). J Fish Dis 27:409–417

Sigh J, Lindenstrøm T, Buchmann K (2004b) Expression of pro-inflammatory cytokines in rainbow trout (*Oncorhynchus mykiss*) during an infection with *Ichthyophthirius multifiliis.* Fish Shellfish Immunol 17:75–86

Uhlar CM, Whitehead AS (1999) Serum amyloid A, the major vertebrate acute-phase reactant. Eur J Biochem 265: 501–523

Wang X, Clark TG, Noe J, Dickerson H (2002) Immunisation of channel catfish, *Ictalurus punctatus*, with *Ichthyophthirius multifiliis* immobilisation antigens elicits serotype-specific protection. Fish Shellfish Immunol 13: 337–350

Wolf K, Markiw ME (1982) Ichthyophthiriases: immersion immunization of rainbow trout (*Salmo gairdneri*) using *Tetrahymena thermophila* as a protective immunogen. Can J Fish Aquat Sci 39:1722–1725

Xu DH, Klesius PH, Shelby RA (2004) Immune responses and host protection of channel catfish, *Ictalurus punctatus* (Rafinesque), against *Ichthyophthirius multifiliis* after immunization with live theronts and sonicated trophonts. J Fish Dis 27:135–141

Zou J, Holland J, Pleguezuelos O, Cunningham C, Secombes C (2000) Factors influencing the expression of interleukin-1, in cultured rainbow trout (*Oncorhynchus mykiss*) leucocytes. Dev Comp Immunol 24:575–582