Fish and Shellfish Immunology 86 (2019) 25–34

Contents lists available at ScienceDirect

Fish and Shellfish Immunology

journal homepage: www.elsevier.com/locate/fsi

Full length article

Local regulation of immune genes in rainbow trout (*Oncorhynchus mykiss*) naturally infected with *Flavobacterium psychrophilum*

Estefanía Muñoz-Atienza[a,1], Carlos Távara[b,1], Patricia Díaz-Rosales[a], Luis Llanco[b], Enrique Serrano-Martínez[b], Carolina Tafalla[a,*]

[a] Animal Health Research Centre (CISA-INIA), 28130, Valdeolmos, Madrid, Spain
[b] Veterinary Medicine and Zootechny Faculty, Universidad Peruana Cayetano Heredia (UPCH), Lima, Peru

ARTICLE INFO

Keywords:
Flavobacterium psychrophilum
Bacterial cold water disease (BCWD)
Rainbow trout
Skin
T cells
Immunoglobulin D (IgD)
Inflammatory response

ABSTRACT

Flavobacterium psychrophilum is the etiological agent of bacterial cold water disease (BCWD), also referred to as rainbow trout fry syndrome (RTFS), a disease with great economic impact in salmonid aquaculture. Despite this, to date, not many studies have analyzed in depth how the immune system is regulated during the course of the disease. In the current study, we have studied the transcription of several immune genes related to T and B cell activity in the skin of rainbow trout (*Oncorhynchus mykiss*) naturally infected with *F. psychrophilum* in a farm located in Lake Titicaca (Peru). The levels of expression of these genes were tested and compared to those obtained in asymptomatic and apparently healthy rainbow trout. In the case of symptomatic fish, skin samples containing characteristic ulcerative lesions were taken, as well as skin samples with no lesions. Our results pointed to a significant local up-regulation of IgD, CD4, CD8, perforin and IFNγ within the ulcerative lesions. On the other hand, no differences between the levels of expression of these genes were visible in the spleen. To confirm these results, the distribution of IgD^+ and $CD3^+$ cells was studied through immunohistochemical techniques in the ulcerative lesions. Our results demonstrate a strong local response to *F. psychrophilum* in rainbow trout in which IgD and T cells seem to play a major role.

1. Introduction

Bacterial cold water disease (BCWD), also referred to as rainbow trout fry syndrome (RTFS), is one of the most important diseases affecting the salmonid aquaculture industry worldwide. The disease is caused by a Gram-negative, rod-like, filamentous, and yellow-pigment-producing bacterium, *Flavobacterium psychrophilum* [1,2]. This pathogenic microorganism not only affects salmonid species such as rainbow trout (*Oncorhynchus mykiss*) or coho salmon (*Oncorhynchus kisutch*), but has also been reported to cause disease in non-salmonid species such as eel (*Anguilla japonica* and *Anguilla anguilla*), carp (*Cyprinus carpio* and *Carassius carassius*) and tench (*Tinca tinca*) [3,4]. The most commonly documented clinical signs of BCWD in adult fish include ulcerative lesions on the skin and the musculature, predominantly near the caudal peduncle and on the flank, followed by progressive tissue necrosis [1,5,6]. In fry, however, septicemia, anemia and lethargy have been reported without any external ulcers, with mortality rates ranging up to 90% in rainbow trout [2,6,7].

The first report of BCWD was described in rainbow trout in North America in 1941 [8]. Following this initial report, *F. psychrophilum* was reported from different geographical areas including Europe (Belgium, Denmark, France, Finland, Germany, Italy, and Spain), Asia (Japan and South Korea), Oceania (Australia) and South America (Chile and Peru) [2,9–12]. It has been revealed that culture density and poor water quality with systems lacking filtering devices can favor the permanence of the pathogen and the appearance of the disease. This is the case of some Chilean fish farms in which *F. psychrophilum* was declared as the etiological agent of BCWD outbreaks since 2013 [13]. In Peru, continuous mortalities in several rainbow trout farms have also been associated to the presence of *F. psychrophilum* [14,15]. Many of these *F. psychrophilum*-associated mortalities were reported in rainbow trout reared in cages in Lake Titicaca, within the region of Puno [14]. According to the Peruvian Ministry of Production, the region of Puno was the main producer of rainbow trout in 2016 with a production of 43,000 tonnes, representing 80% of the national production. Within the region of Puno, Lake Titicaca is the largest freshwater lake in South America localized amongst Peru and Bolivia at 3810 m above sea level with an area of 8559 km^2 [16].

* Corresponding author. Centro de Investigación en Sanidad Animal (CISA-INIA), Carretera de Algete a El Casar km. 8.1, Valdeolmos, 28130, Madrid, Spain.
E-mail address: tafalla@inia.es (C. Tafalla).
[1] These authors have contributed equally to this work.

https://doi.org/10.1016/j.fsi.2018.11.027
Received 21 September 2018; Received in revised form 7 November 2018; Accepted 11 November 2018
Available online 12 November 2018

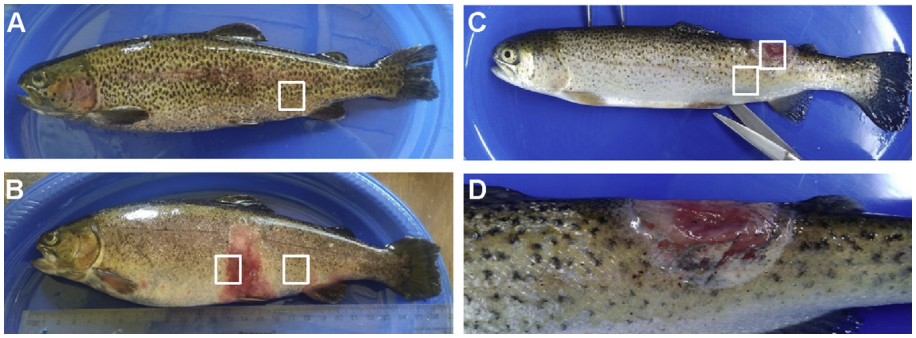

Fig. 1. Gross pathology in rainbow trout obtained from the region of Puno in Peru. (A) Appearance of fish lacking skin ulceration. (B) Ulcerative and hemorrhagic skin lesion on the flank. (C) Typical BCWD lesion in the caudal peduncle. (D) Amplification of the caudal lesion from image C. White boxes indicate sampled regions in both asymptomatic and symptomatic fish. In the case of symptomatic fish, two sections were obtained, one without lesion close to the caudal peduncle and a further section including injured skin.

Although several antigens have been identified as potential vaccine candidates and different serotypes of *F. psychrophilum* have been tested as inactivated vaccines, to our knowledge, at the moment, only one commercial vaccine is available to prevent BCWD (ALPHA JECT® IPNV-Flavo 0,025, Pharmaq) in Chile [6,13,17,18]. This scenario has promoted the use of antibiotics to reduce economic losses attributable to this disease in salmonid farming, with the consequent negative impact on the environment and the generation of antibiotic resistances [13,19]. To optimize an efficient control strategy, it seems key to have an in-depth understanding of the immune response elicited by the pathogen in the species targeted, especially at mucosal surfaces such as the skin, one of the tissues preferentially damaged by *F. psychrophilum*. Recently, our group has demonstrated that the rainbow trout skin is a T cell rich organ by means of transcriptomic analysis, immunohistochemistry and flow cytometry [20]. These T cells are not evenly distributed throughout the skin, but are preferentially accumulated in the most anterior area, close to the gills [20]. Furthermore, these skin T cell responses were locally regulated in response to a bath infection with hemorrhagic septicemia virus (VHSV), revealing a major role of skin T lymphocytes in the local response to pathogens [20]. In the same line, T cell functionality has been the focus of studies dealing with inflammatory skin disorders affecting rainbow trout such as red mark syndrome (RMS) or puffy skin disease (PSD) [21,22]. On the other hand, both IgM^+ and IgT^+ B cells are also known to be present in salmonid skin and respond to infections [23]. IgD, the other Ig isotype present in rainbow trout, is also actively transcribed in rainbow trout skin [20], however its precise role in immunity remains elusive both in fish and mammals. Despite all these previous data, so far, no studies have focused on analyzing the local transcription of genes related to T and B cells in the skin of rainbow trout infected with *F. psychrophilum* and this is what we have addressed in the current study. We have sampled skin and spleen from rainbow trout obtained from Lake Titicaca (Peru) with ulcerative lesions in which the presence of *F. psychrophilum* was confirmed. The levels of expression of several immune genes related to T and B cells were tested and compared to those obtained in asymptomatic and apparently healthy rainbow trout, namely CD4, CD8, Eomes, perforin, interleukin 4/13 (IL4/13), interferon γ (IFNγ), IgM, IgT, IgD and the CK11 chemokine, a chemokine strongly expressed in the skin with a major role in antimicrobial immunity [24]. Since our results pointed to a significant local up-regulation of IgD, CD4, CD8, perforin and IFNγ, the distribution of IgD^+ and $CD3^+$ cells was studied through immunohistochemical techniques in the ulcerative lesions. Our results reveal a strong local response to *F. psychrophilum* in rainbow trout in which IgD and T lymphocytes seem to play a major role.

2. Materials and methods

2.1. Fish sampling and description of the area where the outbreak occurred

Asymptomatic (showing no external signs of disease) and symptomatic adult rainbow trout (*Oncorhynchus mykiss*) with typical disease symptoms of BCWD and length of ~25 cm were obtained from Lake Titicaca in the region of Puno (Peru) between the months of December 2017 and February 2018, the summer season. The experiments described in this work comply with the Law for the Protection of Domestic and Wild Animals kept in captivity (Law 27265/Title 6/Art. 18) and were previously approved by the Institutional Committee on Ethics for the Use of Animals (CIEA) from the Universidad Peruana Cayetano Heredia (Peru).

2.2. Tissue collection from rainbow trout

Skin and spleen samples from 7 asymptomatic and apparently healthy rainbow trout and 13 symptomatic fish showing external ulcerative lesions on the flank and peduncle area were used in order to characterize the transcriptional regulation of immune genes and study the histological lesions. For this purpose, rainbow trout were killed by an overdose of benzocaine (Sigma-Aldrich Inc., 50 mg/L) and then dissected. Internally, a marked splenomegaly, liver anemia and kidney inflammation was clearly visible in fish showing external lesions. No internal clinical signs were observed in fish catalogued as asymptomatic. In all cases, one cm^2 skin samples were removed from the left side with a scalpel removing all the muscle tissue present. In asymptomatic fish (Fig. 1A), one section of healthy skin was collected near the area where the caudal peduncle is located. In fish showing wounds in their skin (Fig. 1B–D), two sections were obtained, one without lesion close to the caudal peduncle and a further section including injured skin (flank or caudal peduncle). Skin samples were immediately placed in RNA later (Ambion) for RNA extraction. In all these fish, the spleen was also removed and stored in RNA later for posterior RNA extraction. In some symptomatic fish showing external ulcerative lesions, skin samples from the lesions were taken and fixed in Bouin's solution for further immunohistological analysis. For comparative purposes, skin samples from apparently healthy fish were also sampled and fixed in parallel.

2.3. RNA extraction and cDNA preparation

Total RNA was extracted from skin and spleen samples using the SV Total RNA Isolation System (Promega) including a DNase treatment step (to remove genomic DNA) following the manufacturer's instructions. RNA was then quantified using a Nanodrop 1000 spectrophotometer (Thermo Scientific) and one μg of RNA and $Oligo(dT)_{15}$ was used to synthesize cDNA using the GoScript™ Reverse Transcription System (Promega) following the manufacturer's instructions. The resulting cDNA was diluted in nuclease-free water and stored at −20 °C until use.

2.4. PCR amplification and sequencing of bacterial 16S rDNA from rainbow trout skin

To verify the presence of *F. psychrophilum*, a pair of primers was designed in the hypervariable region of the *16S rDNA* gene (Table 1). Skin samples from asymptomatic rainbow trout as well as skin samples

Table 1
Primers used in this study.

Gene	Forward primer (5′-3′)	Reverse primer (5′-3′)	Application	Reference
EF-1α	GATCCAGAAGGAGGTCACCA	TTACGTTCGACCTTCCATCC	Real-time PCR	[50]
IgD	AGCTACATGGGAGTCAGTCAACT	CTTCGATCCTACCTCCAGTTCCT	Real-time PCR	[20]
Mb IgD	TGAACATATCCAAACCAGAGCTCC	GTCCTGAAGTCATCATTTTGTCTTGA	Real-time PCR	[51]
Sec IgD	TGAACATATCCAAACCAGGTGTCTG	GTCCTGAAGTCATCATTTTGTCTTGA	Real-time PCR	[51]
IgM	TGCGTGTTTGAGAACAAAGC	GACGGCTCGATGATCGTAAT	Real-time PCR	[20]
IgT	AACATCACCTGGCACATCAA	TTCAGGTTGCCCTTTGATTC	Real-time PCR	[20]
CK11	GAACATTCCTTTGAGCATACTAAT	TGCACAATACTTCCTCCCAT	Real-time PCR	[20]
CD4	CCTGCTCATCCACAGCCTAT	CTTCTCCTGGCTGTCTGACC	Real-time PCR	[52]
CD8	AGTCGTGCAAAGTGGGAAAG	GGTTGCAATGGCATACAGTG	Real-time PCR	[52]
EOMES	ACAACGTATTTGTTGAGGTCGTGTT	CATCTTGTTACCTTGCATGTTGTTG	Real-time PCR	[20]
Perforin	GGAACGACGACCTGTTAGGA	TCATAGGGGAGGGCACATAG	Real-time PCR	[20]
IL4/13	CAACCCAACCAAAGATGAAGACG	CAACGGTGCACTTCTGAAGTTTG	Real-time PCR	[20]
IFNγ	GAAGGCTCTGTCCGAGTTCA	TGTGTGATTTGAGCCTCTGG	Real-time PCR	[20]
FoxP3	CCAGAACCGAGGTGGAGTGT	TGACGGACAGCGTTCTTCCA	Real-time PCR	[20]
Gapdh	ATGTCAGACCTCTGTGTTGG	TCCTCGATGCCGAAGTTGTCG	Convectional PCR	[53]
16S rDNA	GCAATCTACCTTTTACAGAGGGATAGCCC	TCCTCACTGCTGCCTCCCGTAGGAG	Convectional PCR	This study

with ulcers from symptomatic of fish were chosen as a target organ for the bacterial identification by PCR. Samples were subjected to an initial cycle of denaturation (95 °C for 5 min), followed by 35 cycles of denaturation (95 °C for 30 s), annealing (58 °C for 30 s) and elongation (72 °C for 30 s), ending with a final extension step at 72 °C for 7 min in a T100 Thermal Cycler (Bio-Rad). In parallel, PCR analysis was undertaken to determine the levels of transcription of the rainbow trout glyceraldehyde-3-phosphate dehydrogenase (*gapdh*) gene, to be used as internal control. The amplification was performed following the protocol described above but increasing the elongation step (72 °C) to 60 s. PCR products were analyzed by electrophoresis on 1.5% (w/v) agarose (Pronadisa) gels stained with GelRed (Biotium), and visualized with the ChemiDoc Imaging System (Bio-Rad). DNA Ladder (100 bp, Promega) was used as molecular size marker. The intensities of the amplified products were quantified by optical densitometry using the ImageJ software (National Institutes of Health). Moreover, the *16S rDNA* amplicons were purified using the NucleoSpin gel and PCR clean up kit (Macherey & Nagel) and sequenced at our in-house sequencing unit (CISA, Madrid, Spain). The Chromas program (https://technelysium.com.au/wp/chromas/) and Clustal Omega software (https://www.ebi.ac.uk/Tools/msa/clustalo/) were used to build a consensus *16S rDNA* sequence. Analysis of the consensus sequence was performed with the BLAST software available at the National Center for Biotechnology Information (NCBI; https://blast.ncbi.nlm.nih.gov/Blast.cgi).

2.5. Evaluation of immune gene expression by real-time PCR

To evaluate the levels of transcription of immune genes, real-time PCRs were performed in a LightCycler 96 System instrument (Roche) using FastStart Essential DNA Green Master reagents (Roche) and specific primers (Table 1). Each sample was subjected, in duplicate, to an initial cycle of denaturation (95 °C for 10 min), followed by 40 amplification cycles (95 °C for 10 s, 60 °C for 10 s, and 72 °C for 10 s). A dissociation curve was determined by reading fluorescence every degree between 60 °C and 95 °C to ensure only a single product was amplified. Negative controls with no template and minus-reverse transcriptase controls (-RT) were included in all experiments. The expression of immune genes was normalized to the relative expression of rainbow trout elongation factor (EF-1α) gene amplified using specific primers previously described. Expression levels calculated using the $2^{-\Delta Ct}$ method, where ΔCt is determined by subtracting the EF-1α value from the target Ct as described previously [25].

2.6. Immunohistochemistry processing

In order to study pathological changes in fish showing symptoms of disease, sections of skin from asymptomatic fish and skin with ulcerative lesions from symptomatic rainbow trout were subjected to an indirect immunohistochemical method to detect rainbow trout IgD and CD3 [26]. Skin samples were fixed in Bouin's solution for 24 h, dehydrated with ethanol and xylol (Panreac Applichem), embedded in paraffin (Paraplast Plus, Sherwood Medical) and then sectioned at 5 μm. After dewaxing and rehydration, endogenous peroxidase was quenched by incubation in 3% H_2O_2 in methanol for 25 min and antigens retrieved by heating in Tris–EDTA buffer (pH 9) in a microwave oven (800 W for 5 min and 450 W for 5 min). Non-specific binding was blocked with 5% bovine serum albumin (BSA, Sigma-Aldrich) in Tris-buffered saline (TBS) at room temperature (RT) for 30 min. Then, sections were incubated with specific mouse monoclonal antibodies (mAbs) recognizing trout IgD and CD3. The incubation step with anti-trout IgD (4 μg/ml) [27] was carried out at RT for 1 h, while the incubation with the anti-trout CD3 (2.5 μg/ml) [28] was performed overnight at 4 °C. Unbound primary antibodies were then washed with TBS. Thereafter, a secondary anti-mouse IgG antibody conjugated with horseradish peroxidase (HRP) was added. The sections were incubated at RT for 30 min and visualized with 3,3′-diaminobenzidine tetrahydrochloride chromogen (EnVision + System/HRP, Dako). Harris hematoxylin was used as nuclear counter stain and mounting was conducted with DPX mounting medium (Casa Álvarez). Slides were examined with a Leica DM/LS optic microscope and images acquired with a Leica DFC320 digital camera.

2.7. Data analysis

Data handling, statistical analyses and graphic representation were performed using Office Excel 2010 (Microsoft Corporation) and GraphPad Prism version 6 (GraphPad Software Inc.). An unpaired two-tailed Student's *t*-test was used to determine statistically significant differences in gene expression between asymptomatic and symptomatic fish. When comparison was made amongst ulcerative and non-ulcerative sections of skin from the same fish, a paired two-tailed Student's *t*-test was used to determine statistically significant differences. The statistical significant level was accepted at $p < 0.05$.

3. Results

3.1. Identification of F. psychrophilum by sequencing

Conventional PCR and gel analysis showed that the primers designed to amplify the *16S rDNA* of *F. psychrophilum* amplified a fragment of 239 bp in all skin samples from the rainbow trout sampled (Fig. 2A). The sequencing of these PCR products obtained allowed us to build a consensus sequence and recognize the bacteria identified as *F. psychrophilum,* showing a 99% identity of this sequence and that of

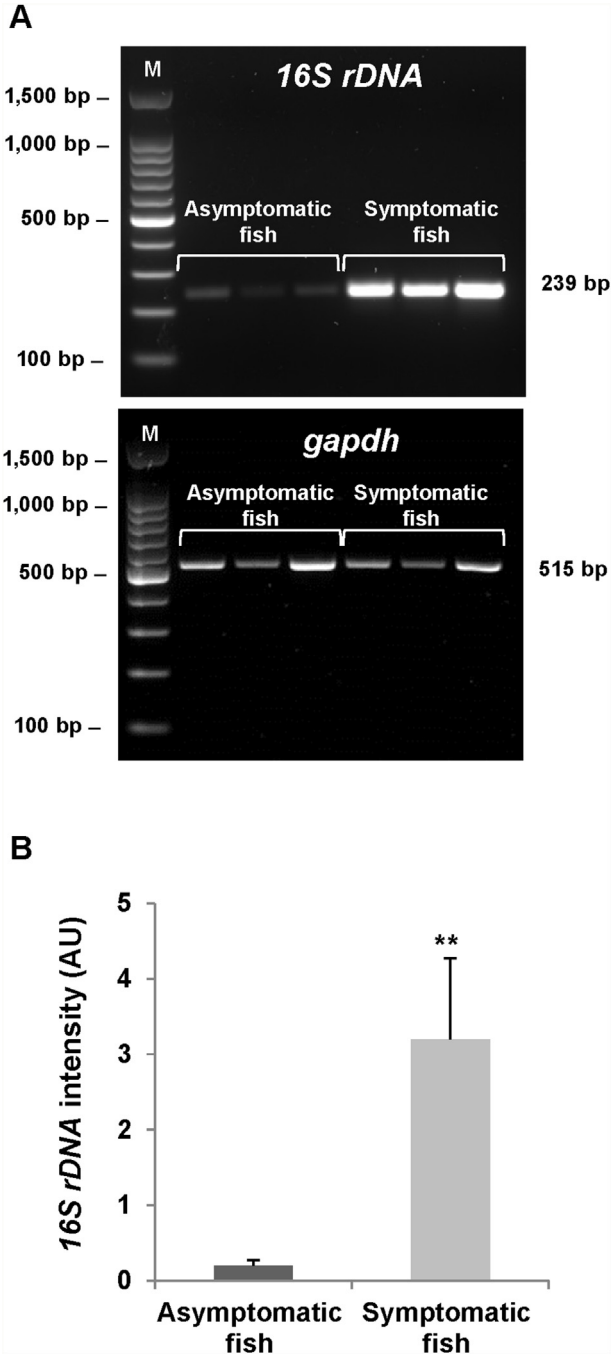

Fig. 2. Agarose gel electrophoresis of PCR products amplified in skin samples obtained from asymptomatic and symptomatic rainbow trout. (A) Agarose gels with amplified products corresponding to the *16S rDNA* gene and the rainbow trout *gapdh* house-keeping gene. Results from three representative individuals are shown in each group. M: DNA molecular weight marker. (B) The intensity of the *16S rDNA* gene amplicons was quantified by optical densitometry and normalized against rainbow trout *gapdh* products. Mean values in arbitrary units (AU) + SD are shown ($n = 3$). Asterisks denote significant differences between values obtained in skin samples from symptomatic and asymptomatic fish (**$p < 0.01$).

previously reported *F. psychrophilum* strains. Although *F. psychrophilum 16S rDNA* was amplified in skin samples from both apparently healthy and symptomatic fish, the levels of transcription of this gene were much higher in skin samples with ulcerative lesions in comparison to apparently healthy fish (Fig. 2B). The fact that *F. psychrophilum* is found in much higher numbers within the ulcerative lesions than in apparently healthy skin, strongly suggests that this pathogen is the causative agent of the lesions observed.

3.2. Symptomatic rainbow trout show high expression of IgD and immune genes related to T cell immunity in the skin

To expand our knowledge on the immune response elicited by *F. psychrophilum* at a local level, we studied the levels of transcription of immunoglobulins (IgM, IgD and IgT), the skin-related chemokine CK11, and immune genes related to T cell immunity in the skin of asymptomatic and symptomatic fish. In the case of the latter, we compared the levels of transcription of immune genes from skin sections containing the ulcerative lesion (symptomatic, ulcerative lesion) with those of skin sections obtained from these same fish but where no wounds were present (symptomatic, no lesion). While there were no significant differences among the levels of transcription of IgM and IgT in different skin samples from either symptomatic or asymptomatic fish, IgD mRNA levels were significantly higher in the injured skin of symptomatic fish in comparison to skin apparently healthy of both symptomatic and asymptomatic fish (Fig. 3). Thus, for this Ig, we performed additional real time PCRs to determine whether it was the membrane or the secreted form of IgD that was affected. We found that both the membrane and the secreted IgD mRNAs were significantly up-regulated in the injured skin of symptomatic fish in comparison to apparently healthy skin obtained from both symptomatic and asymptomatic fish (Fig. 3). No significant changes were observed however in CK11 mRNA levels (Fig. 3). Regarding the immune genes related to T cell immunity, symptomatic fish presented a significant increased level of transcription of CD4 in injured skin in comparison to the skin of asymptomatic fish, but these levels were not significantly different than those obtained in skin samples without lesion obtained from symptomatic fish (Fig. 3). Finally, the levels of transcription of CD8, perforin and IFNγ were significantly higher in damaged skin of symptomatic fish than those observed in areas of skin without lesions from the same fish as well as from asymptomatic rainbow trout (Fig. 3). However, no significant differences in the Eomes and IL3/14 transcription levels were detected among the skin samples included in this study (Fig. 3).

3.3. Asymptomatic and symptomatic rainbow trout show similar expression of immune genes in the spleen

For comparative purposes, we also studied the transcription of these immune genes in the spleen, the main secondary immune organ in teleost fish, comparing the levels of expression in asymptomatic and symptomatic fish. In this case, we also included the analysis of FoxP3 mRNA levels, since FoxP3 is expressed in $CD4^+$ regulatory T cells and involved in the maintenance of immunological self-tolerance [29]. Surprisingly, no significant differences were found among the levels of transcription of either of the genes tested (Fig. 4), suggesting that there are no systemic differences at a transcriptional level that condition that fish become more infected and show lesions as a consequence of *F. psychrophilum* infection.

3.4. IgD expression in ulcerative skin samples

To verify whether IgD was also up-regulated at protein level in the ulcerative lesions, we conducted an immunohistochemical study using a specific antibody against rainbow trout IgD. In ulcerative skin samples, an infiltration of mononuclear cells was observed in the epithelial basal cell layer and more strongly in the *stratum spongiosum* and inner *stratum compactum* of the dermis when compared to skin samples obtained from asymptomatic fish (Figs. 5 and 6). Moreover, immunohistochemical analysis showed a positive reactivity against the IgD antibody in cells within the dermis of ulcerative skin samples from symptomatic fish (Fig. 5). The presence of these IgD^+ cells was much lower in skin samples from rainbow trout without lesions (Fig. 5). These

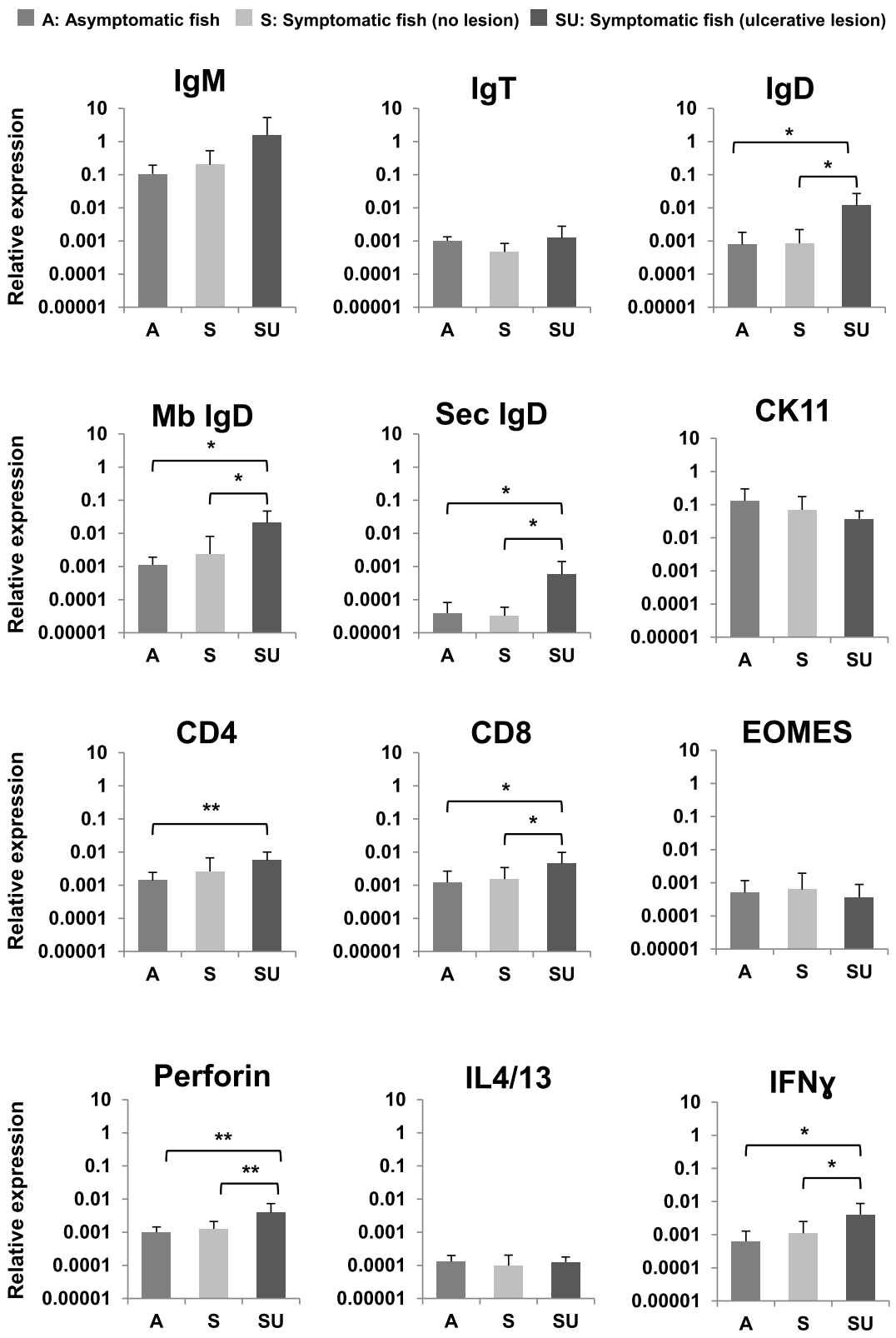

Fig. 3. Levels of transcription of immune genes in skin samples obtained from rainbow trout naturally infected with *F. psychrophilum*. Asymptomatic ($n = 7$) and symptomatic rainbow trout ($n = 13$) were killed and skin sampled to determine the levels of expression of a selection of immune genes by real-time PCR. Non-injured skin samples were taken from asymptomatic and symptomatic fish. Additionally, in the case of symptomatic fish, skin samples containing ulcerative lesions were also sampled and analyzed. Data are shown as the gene expression relative to the expression of endogenous control EF-1α (mean + SD). Asterisks denote statistical differences among groups as indicated (*$p < 0.05$; **$p < 0.01$).

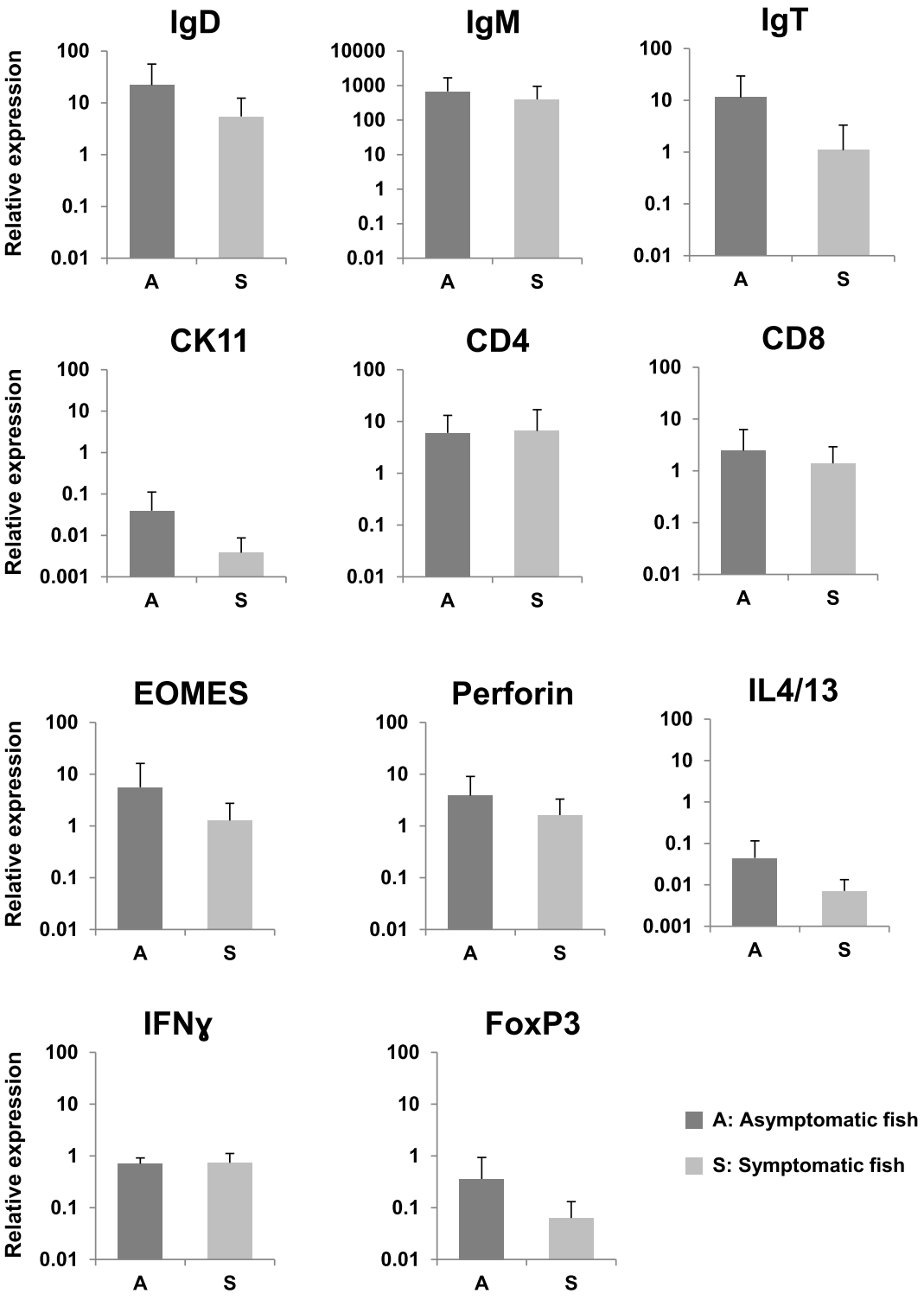

Fig. 4. Transcription levels of immune genes in spleen from rainbow trout naturally infected with *F. psychrophilum*. Asymptomatic (n = 7) and symptomatic rainbow trout (n = 13) were killed and spleen sampled to determine the levels of expression of a selection of immune genes by real-time PCR. Data are shown as the gene expression relative to the expression of endogenous control EF-1α (mean + SD).

results confirm the implication of IgD in the local immune response mounted against *F. psychrophilum*.

3.5. CD3 expression in ulcerative skin samples

The fact that both CD4 and CD8 mRNA levels were up-regulated in the ulcerative lesions strongly suggested an increased presence of T cells in the lesions, given that CD4 and CD8 mRNA are not transcriptionally regulated in individual T cells [30]. To verify this point, we also conducted an immunohistochemical study using a specific antibody against rainbow trout CD3, a pan T cell marker [28]. $CD3^+$ cells were identified in the epidermis of ulcerative skin samples from symptomatic fish (Fig. 6), whereas the presence of T cells was much lower in skin samples from rainbow trout without lesions (Fig. 6).

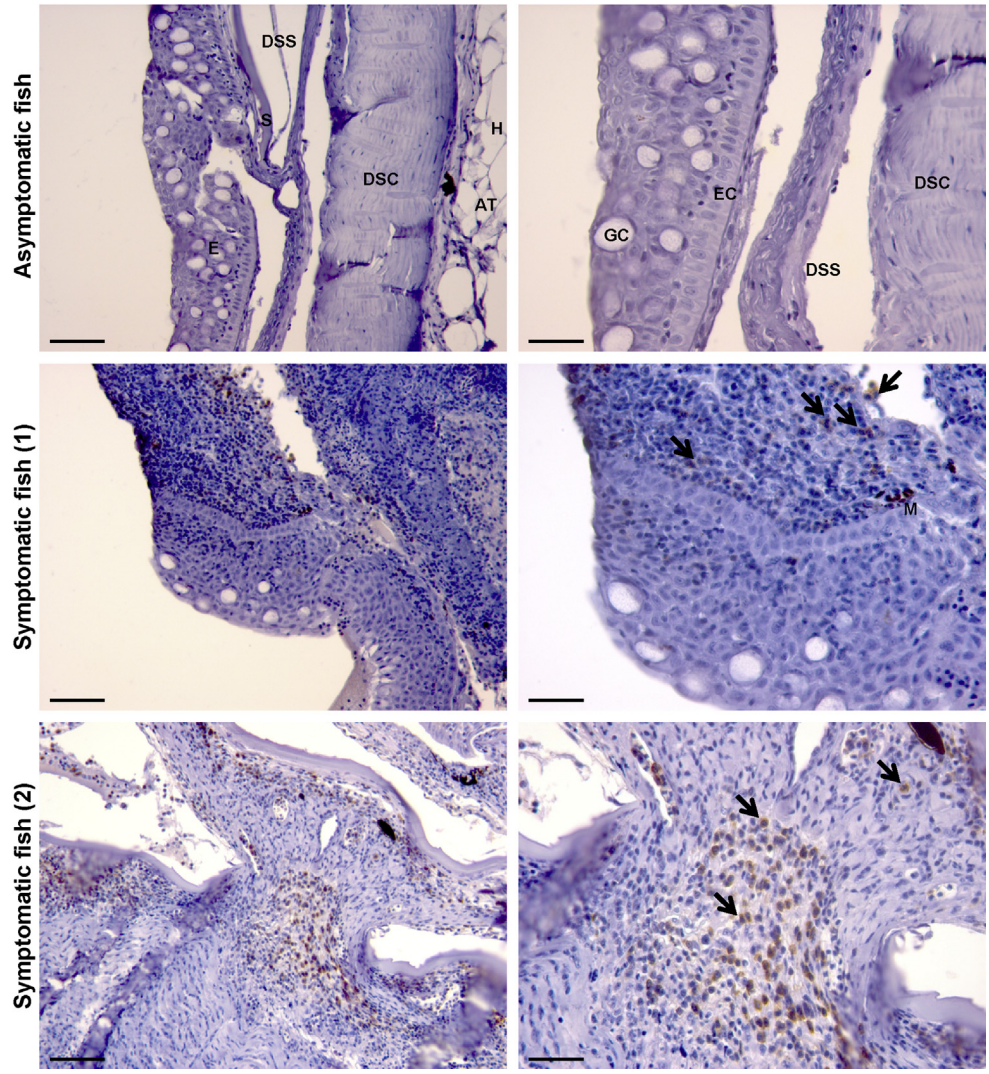

Fig. 5. Immunohistochemical detection of IgD in ulcerative skin sections from rainbow trout naturally infected with *F. psychrophilum*. Representative images for one asymptomatic and two symptomatic fish with ulcerative lesions are shown at 20× magnification (left images, scale bars = 40 μm) and at 40× magnification (right images, scale bars = 20 μm). Arrowheads indicate representative IgD^+ cells. E, epidermis; S, scale; DSS, dermis *stratum spongiosum*; DSC, dermis *stratum compactum*; H, hypodermis; AT, adipose tissue; GC, goblet cell; EC, epithelial cell; M, melanophores.

4. Discussion

Studying the immune response elicited during the course of an infection with a specific pathogen is key for the rational development of effective vaccines. In the case of *F. psychrophilum,* although some previous studies have focused on analyzing the transcriptional response to the pathogen after experimental infections [31,32], none of these studies were performed with naturally infected rainbow trout. Furthermore, these previous studies determined the transcriptional regulation of immune genes in central immune organs such as the anterior kidney [31,32], but did not determine the regulation of immune genes within the skin, the major adherence site for the bacteria, where it attaches and grows before entering the host and where major clinical signs are observed [33].

In fish, the skin is one of the first lines of defense against waterborne pathogens. Because in fish the skin is not keratinized, live cells in the epidermis are in direct contact with the water, and can immediately respond to pathogen exposure. Within the skin, both B and T cells are found scattered in a disorganized fashion, constituting what has been designated as the skin-associated lymphoid tissue (SALT) [34]. Thus, in the current work, we analyzed the levels of expression of different genes related to B and T cell function in the skin of rainbow trout showing clear clinical signs of BCWD obtained from Lake Titicaca in the region of Puno (Peru). As an initial step, we verified the presence of *F. psychrophilum* by PCR in skin samples, observing that even though *F. psychrophilum* could also be identified by PCR in the skin of apparently healthy fish, the levels of amplification of bacterial RNA were significantly lower than those found in fish with clear symptomatology. Similarly, Orieux *et al.* [35] detected *F. psychrophilum* by PCR in the skin of asymptomatic fish during the course of a BCWD outbreak, suggesting that apparently healthy fish act as reservoirs spreading the bacteria into the surrounding water. Interestingly, our group previously described that, in homeostasis, immune cells are not homogeneously distributed throughout the skin, but are more represented in anterior sections close to the gills [20]. This might explain why *F. psychrophilum* usually enters the host and produces damages in specific skin areas, such as the peduncle and flanks, described as body regions showing lower expression values for most immune genes [20].

The transcriptional study carried out in the skin revealed an up-regulation of the transcription levels of CD8 and perforin genes in ulcerative skin in comparison to those observed in skin samples without lesions from both asymptomatic and symptomatic fish. These results suggest that T cytotoxic cells play an important role in the response to *F. psychrophilum,* probably killing infected cells at the sites where

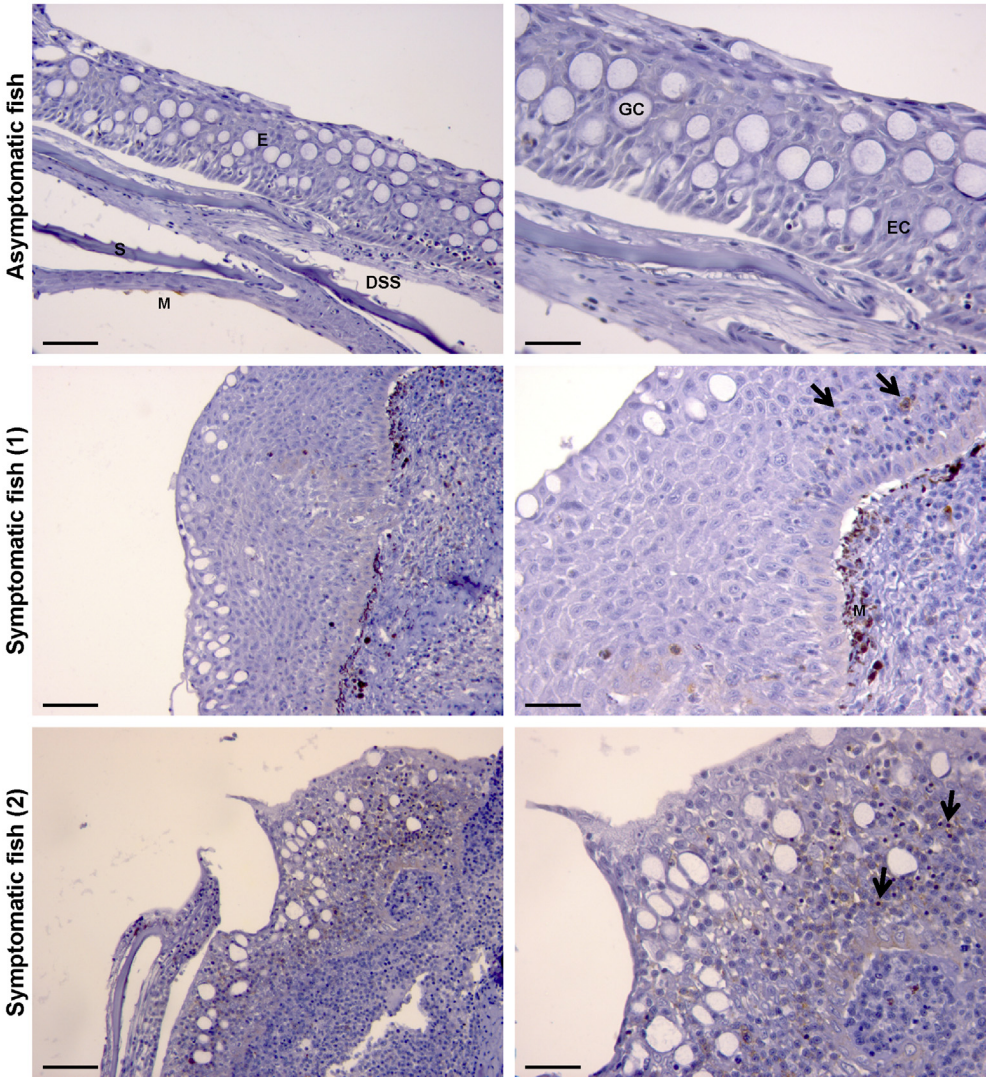

Fig. 6. Immunohistochemical detection of CD3 in ulcerative skin sections from rainbow trout naturally infected with *F. psychrophilum*. Representative images for one asymptomatic and two symptomatic fish with ulcerative lesions are shown at 20× magnification (left images, scale bars = 40 μm) and at 40× magnification (right images, scale bars = 20 μm). Arrowheads indicate representative $CD3^+$ T cells. E, epidermis; S, scale; DSS, dermis *stratum spongiosum*; GC, goblet cell; EC, epithelial cell; M, melanophores.

bacterial concentration is highest. Given that the capacity of *F. psychrophilum* to survive inside rainbow trout cells has already been demonstrated [36], our results seem to agree with those obtained in response to other intracellular microorganisms. For example, $CD8^+$ T cells were reported to play a major protective role against *Listeria monocytogenes* in mice though a perforin-dependent pathway [37]. Similarly, $CD8^+$ cytotoxic T cells that recognize *Salmonella*-infected cells were detected in mice challenged with a virulent strain of *S. typhimurium,* another intracellular pathogen [38].

IFNγ mRNA levels were also up-regulated in ulcerative skin sections when compared to those observed in skin samples without lesions from both asymptomatic and symptomatic fish. In homeostasis, the skin is believed to have a Th profile skewed towards Th2 [39]. Thus, our results point to a switch from Th2 to Th1 profiles in response to the bacterial infection. In this context, Th1 cells would secrete effector cytokines such as IFNγ and tumor necrosis factor α (TNF-α) to control intracellular infections, and interleukin 2 (IL-2) to induce lymphocyte proliferation [40]. In line with this hypothesis, CD4 mRNA levels were also up-regulated, although in this case the levels of expression in ulcerative lesions were higher than those observed in asymptomatic fish, but not when compared to skin sections with no lesions derived from symptomatic fish. On the other hand, $CD8^+$ T cells are also known to secrete IFNγ when activated. For example, in ginbuna crucian carp (*Carassius auratus langsdorfii*), the transfer of $CD8\alpha^+$ cells sensitized with *Edwardsiella tarda* to healthy recipients up-regulated the expression of IFNγ and perforin genes in kidney and spleen lymphocytes, thus revealing a major role of cell-mediated cytotoxicity and IFNγ-mediated responses against this intracellular pathogen [41]. Remarkably, these changes in the levels of expression of CD8, IFNγ or perforin were not visible in the spleen suggesting that local responses are quite important through the course of the disease. Of course, whether the activation of $CD8^+$ T cells at systemic or local levels correlate with protection against BCWD deserves further study, as this is an important issue to be taken into account for future vaccine design.

To further confirm the role of T cells in the local immune response to *F. psychrophilum*, we analyzed the distribution and abundance of $CD3^+$ cells in ulcerative and healthy skin. A perceptible inflammation with infiltration of mononuclear cells was observed in ulcers. Furthermore, $CD3^+$ T lymphocytes were identified within the epidermis layer, and were found to be in higher numbers within the inflamed area, than in healthy skin sections. This again strongly suggests a major role of skin $CD8^+$ T cells in the early immune response to

water-borne intracellular pathogens, as previously reported in rainbow trout challenged with VHSV [20].

Although the exact role of IgD is still unknown in mammals and other animal groups, knowing that it has been conserved throughout evolution from fish to mammals [42] has aroused a recent interest in elucidating its role [17,43,44]. In teleost, the majority of unstimulated B cells from central immune organs, co-express IgM and IgD on the cell membrane. When these cells are activated, as in occurs in mammals, IgD is lost from the cell membrane, giving rise to activated IgM^+IgD^- B cells that have a plasmablast/plasma cell profile. The presence of these cells has been reported in rainbow trout [45]. However, in addition to these populations, IgD^+IgM^- B cells have also been reported in channel catfish blood [46] and rainbow trout gills [47], as they have been described in specific mucosal areas in humans [43,48]. However the exact role of these cells and of the IgD they secrete has not yet been established. In our study, a significant up-regulation of membrane and secreted IgD mRNA was found in ulcerative skin when compared with skin sections without lesions. These results were confirmed by immunohistochemical techniques, through which we determined that the number of IgD^+ cells in the skin dermis was much higher within the ulcerative lesions. Whether IgD is implicated in the immune response of the skin to other pathogens is something that has still not been explored. But, interestingly, IgD was reported to be strongly regulated in response to a *F. psychrophilum* vaccine [17]. In that study, an increase of secretory IgD expression levels was detected in gills and intestine of rainbow trout immunized against *F. psychrophilum* through immersion or anal intubation, respectively. In the case of fish immunized through anal intubation, IgD levels were also significantly up-regulated in the skin [17]. All together, these results point to a major role of IgD in the response to *F. psychrophilum*, especially visible in mucosal compartments. In humans, IgM-to-IgD class switch has been reported in B cells from the respiratory mucosa, given rise to IgD-producing plasmablasts reactive to Gram-negative pathogenic bacteria such as *Moraxella catarrhalis* and *Haemophilus influenzae* and to their products [49]. Moreover, patients with auto-inflammatory disorders, including hyper-IgD syndrome, TNF receptor-associated periodic fever syndrome, Muckle-Wells syndrome, and periodic fever-aphthous stomatitis-pharyngitis-cervical adenitis syndrome, have shown to present more circulating and mucosal IgD^+IgM^- plasmablasts and more mucosal IgD-armed basophils [49].

In conclusion, we have demonstrated that upon a *F. psychrophilum* natural infection, the levels of transcription of IgD as well as those of genes related to T cell functionality (namely CD4, CD8, perforin and IFNγ) are up-regulated in the ulcerative lesions provoked by the bacteria. Similarly, the number of cells expressing IgD and CD3 was higher in ulcerative lesions, as determined through immunohistochemistry. These results point to a relevant role of IgD in skin immunity in rainbow trout, not previously described before. Additionally, it strongly suggests that T cells, and especially $CD8^+$ T cells, are activated to eliminate cells infected with this intracellular pathogen at the sites were bacterial presence is highest. These results will contribute to further understand how the rainbow trout immune system is regulated to combat *F. psychrophilum*.

Acknowledgements

The authors want to thank Lucía González and Laura Fernández for technical assistance. Dr. Erin Bromage (University of Massachusetts Dartmouth, USA) is also acknowledged for supplying the anti-IgD and anti-CD3 antibodies used in this study. This work was supported by the European Research Council (ERC Consolidator Grant 2016 725061 TEMUBLYM). Carlos Távara would like to acknowledge the financial support from the Master Program in Aquatic Animal Health of the UPCH subsidized by Cienciactiva of CONCYTEC (Management Agreement No. 230-2015- FONDECYT-DE-PROMOCION 2). Carlos Távara also would like to acknowledge Vitapro S.A. for the interest in this research and support in the development of this work. E. Muñoz-Atienza holds a Juan de la Cierva-Formación Postdoctoral Contract (FJCI-2015-24452) funded by the Spanish Ministry of Economy, Industry and Competitiveness.

References

[1] M.E. Barnes, M.L. Brown, A review of *Flavobacterium psychrophilum* biology, clinical signs, and bacterial cold water disease prevention and treatment, Open Fish Sci. J. 4 (2011) 40–48.
[2] A. Nematollahi, A. Decostere, F. Pasmans, F. Haesebrouck, *Flavobacterium psychrophilum* infections in salmonid fish, J. Fish. Dis. 26 (10) (2003) 563–574.
[3] S. Izumi, H. Liu, F. Aranishi, H. Wakabayashi, A novel serotype of *Flavobacterium psychrophilum* detected using antiserum against an isolate from amago, *Oncorhynchus masou rhodurus* Jordan & Gilbert, in Japan, J. Fish. Dis. 26 (11–12) (2003) 677–680.
[4] J. Lehmann, D. Mock, F.J. Stiirenberg, J.F. Bernardet, First isolation of *Cytophaga psychrophila* from a systemic disease in eel and cyprinids, Dis. Aquat. Org. 10 (1991) 217–220.
[5] R. Avendaño-Herrera, P. Ilardi, J. Fernández, Significance of *Flavobacterium* diseases on salmonid farming in Chile, Proceedings of the 2nd Conference on Members of the Genus *Flavobacterium*, 2009, pp. 21–23.
[6] E. Gómez, J. Méndez, D. Cascales, J.A. Guijarro, *Flavobacterium psychrophilum* vaccine development: a difficult task, Microb. Biotechnol. 7 (5) (2014) 414–423.
[7] H. Nilsen, A.B. Olsen, O. Vaagnes, H. Hellberg, K. Bottolfsen, H. Skjelstad, D.J. Colquhoun, Systemic *Flavobacterium psychrophilum* infection in rainbow trout, *Oncorhynchus mykiss* (Walbaum), farmed in fresh and brackish water in Norway, J. Fish. Dis. 34 (5) (2011) 403–408.
[8] H.S. Davis, Care and Diseases of Trout, Research Report, No. 12 Fish and Wildlife Service, US Government Printing Office, Washington, DC, USA, 1946.
[9] S. Hesami, K.J. Allen, D. Metcalf, V.E. Ostland, J.I. MacInnes, J.S. Lumsden, Phenotypic and genotypic analysis of *Flavobacterium psychrophilum* isolates from Ontario salmonids with bacterial coldwater disease, Can. J. Microbiol. 54 (8) (2008) 619–629.
[10] N.M. Lindstrom, D.R. Call, M.L. House, C.M. Moffitt, K.D. Cain, A quantitative enzyme-linked immunosorbent assay and filtration-based fluorescent antibody test as potential tools to screen broodstock for infection with *Flavobacterium psychrophilum*, J. Aquat. Anim. Health 21 (1) (2009) 43–56.
[11] N. Orieux, J.P. Bourdineaud, D.G. Douet, P. Daniel, M. Le Henaff, Quantification of *Flavobacterium psychrophilum* in rainbow trout, *Oncorhynchus mykiss* (Walbaum), tissues by qPCR, J. Fish. Dis. 34 (11) (2011) 811–821.
[12] C.E. Starliper, Bacterial coldwater disease of fishes caused by *Flavobacterium psychrophilum*, J. Adv. Res. 2 (2011) 97–108.
[13] R. Avendaño-Herrera, Proper antibiotics use in the Chilean salmon industry: policy and technology bottlenecks, Aquaculture 495 (2018) 803–805.
[14] J.G. Gonzáles Fernández, Flavobacteriosis on rainbow trout (*Oncorhynchus mykiss*) from Lake Titicaca, Puno, Peru 2009, The Biologist 11 (2) (2013) 205–215.
[15] E.C. Mateo, T. Castro, V. Sierralta, D.R. Mateo, Bacteriological and histopathological study of farmed rainbow trout, *Oncorhynchus mykiss*, in Peru, J. Fish. Dis. 40 (7) (2017) 941–946.
[16] M. Monroy, A. Maceda-Veiga, A. de Sostoa, Metal concentration in water, sediment and four fish species from Lake Titicaca reveals a large-scale environmental concern, Sci. Total Environ. 487 (2014) 233–244.
[17] M. Makesh, P.S. Sudheesh, K.D. Cain, Systemic and mucosal immune response of rainbow trout to immunization with an attenuated *Flavobacterium psychrophilum* vaccine strain by different routes, Fish Shellfish Immunol. 44 (1) (2015) 156–163.
[18] T.P.H. Ngo, K.L. Bartie, K.D. Thompson, D.W. Verner-Jeffreys, R. Hoare, A. Adams, Genetic and serological diversity of *Flavobacterium psychrophilum* isolates from salmonids in United Kingdom, Vet. Microbiol. 201 (2017) 216–224.
[19] T.P.H. Ngo, P. Smith, K.L. Bartie, K.D. Thompson, D.W. Verner-Jeffreys, Antimicrobial susceptibility of *Flavobacterium psychrophilum* isolates from the United Kingdom, J. Fish. Dis. 41 (2) (2018) 309–320.
[20] E. Leal, A.G. Granja, C. Zarza, C. Tafalla, Distribution of T cells in rainbow trout (*Oncorhynchus mykiss*) skin and responsiveness to viral infection, PLoS One 11 (1) (2016) e0147477.
[21] L. Christie, R. van Aerle, R.K. Paley, D.W. Verner-Jeffreys, H. Tidbury, M. Green, S.W. Feist, I. Cano, The skin immune response of rainbow trout, *Oncorhynchus mykiss* (Walbaum), associated with puffy skin disease (PSD), Fish Shellfish Immunol. 78 (2018) 355–363.
[22] U. McCarthy, E. Casadei, T. Wang, C.J. Secombes, Red mark syndrome in rainbow trout *Oncorhynchus mykiss*: investigation of immune responses in lesions using histology, immunohistochemistry and analysis of immune gene expression, Fish Shellfish Immunol. 34 (5) (2013) 1119–1130.
[23] Z. Xu, D. Parra, D. Gómez, I. Salinas, Y.A. Zhang, L. von Gersdorff Jorgensen, R.D. Heinecke, K. Buchmann, S. LaPatra, J.O. Sunyer, Teleost skin, an ancient mucosal surface that elicits gut-like immune responses, Proc. Natl. Acad. Sci. U.S.A. 110 (32) (2013) 13097–13102.
[24] E. Muñoz-Atienza, C. Aquilino, K. Syahputra, A. Al-Jubury, C. Araújo, J. Skov, P.W. Kania, P.E. Hernández, K. Buchmann, L.M. Cintas, T. Tafalla, CK11, a teleost chemokine with a potent antimicrobial activity, J. Immunol. (2018) (in press).
[25] N.A. Ballesteros, S. Rodríguez Saint-Jean, S.I. Pérez-Prieto, C. Aquilino, C. Tafalla, Modulation of genes related to the recruitment of immune cells in the digestive tract of trout experimentally infected with infectious pancreatic necrosis virus (IPNV) or orally vaccinated, Dev. Comp. Immunol. 44 (1) (2014) 195–205.

[26] B. Abós, I. Estensoro, P. Perdiguero, M. Faber, Y. Hu, P. Díaz-Rosales, A.G. Granja, C.J. Secombes, J.W. Holland, C. Tafalla, Dysregulation of B Cell activity during proliferative kidney disease in rainbow trout, Front. Immunol. 9 (2018) 1203.
[27] F. Ramírez-Gómez, W. Greene, K. Rego, J.D. Hansen, G. Costa, P. Kataria, E.S. Bromage, Discovery and characterization of secretory IgD in rainbow trout: secretory IgD is produced through a novel splicing mechanism, J. Immunol. 188 (3) (2012) 1341–1349.
[28] T. Boardman, C. Warner, F. Ramírez-Gómez, J. Matrisciano, E. Bromage, Characterization of an anti-rainbow trout (*Oncorhynchus mykiss*) CD3epsilon monoclonal antibody, Vet. Immunol. Immunopathol. 145 (1–2) (2012) 511–515.
[29] S. Hori, T. Nomura, S. Sakaguchi, Control of regulatory T cell development by the transcription factor Foxp3, Science 299 (5609) (2003) 1057–1061.
[30] J.M. Cliff, I.N. Andrade, R. Mistry, C.L. Clayton, M.G. Lennon, A.P. Lewis, K. Duncan, P.T. Lukey, H.M. Dockrell, Differential gene expression identifies novel markers of CD4+ and CD8+ T cell activation following stimulation by *Mycobacterium tuberculosis*, J. Immunol. 173 (1) (2004) 485–493.
[31] C. Langevin, M. Blanco, S.A. Martin, L. Jouneau, J.F. Bernardet, A. Houel, A. Lunazzi, E. Duchaud, C. Michel, E. Quillet, P. Boudinot, Transcriptional responses of resistant and susceptible fish clones to the bacterial pathogen *Flavobacterium psychrophilum*, PLoS One 7 (6) (2012) e39126.
[32] M.M. Henriksen, P.W. Kania, K. Buchmann, I. Dalsgaard, Evaluation of the immune response in rainbow trout fry, *Oncorhynchus mykiss* (Walbaum), after waterborne exposure to *Flavobacterium psychrophilum* and/or hydrogen peroxide, J. Fish. Dis. 38 (1) (2015) 55–66.
[33] A. Papadopoulou, I. Dalsgaard, A. Lindén, T. Wiklund, *In vivo* adherence of *Flavobacterium psychrophilum* to mucosal external surfaces of rainbow trout (*Oncorhynchus mykiss*) fry, J. Fish. Dis. 40 (10) (2017) 1309–1320.
[34] I. Salinas, The mucosal immune system of teleost fish, Biology (Basel) 4 (3) (2015) 525–539.
[35] N. Orieux, D.G. Douet, M. Le Henaff, J.P. Bourdineaud, Prevalence of *Flavobacterium psychrophilum* bacterial cells in farmed rainbow trout: characterization of metallothionein A and interleukin1-beta genes as markers overexpressed in spleen and kidney of diseased fish, Vet. Microbiol. 162 (1) (2013) 127–135.
[36] A. Decostere, E. D'Haese, M. Lammens, H. Nelis, F. Haesebrouck, *In vivo* study of phagocytosis, intracellular survival and multiplication of *Flavobacterium psychrophilum* in rainbow trout, *Oncorhynchus mykiss* (Walbaum), spleen phagocytes, J. Fish. Dis. 24 (8) (2001) 481–487.
[37] D. Kägi, B. Ledermann, K. Burki, H. Hengartner, R.M. Zinkernagel, CD8+ T cell-mediated protection against an intracellular bacterium by perforin-dependent cytotoxicity, Eur. J. Immunol. 24 (12) (1994) 3068–3072.
[38] W.F. Lo, H. Ong, E.S. Metcalf, M.J. Soloski, T cell responses to Gram-negative intracellular bacterial pathogens: a role for CD8+ T cells in immunity to *Salmonella* infection and the involvement of MHC class Ib molecules, J. Immunol. 162 (9) (1999) 5398–5406.
[39] F. Takizawa, E.O. Koppang, M. Ohtani, T. Nakanishi, K. Hashimoto, U. Fischer, J.M. Dijkstra, Constitutive high expression of interleukin-4/13A and GATA-3 in gill and skin of salmonid fishes suggests that these tissues form Th2-skewed immune environments, Mol. Immunol. 48 (12–13) (2011) 1360–1368.
[40] J.M. Damsker, A.M. Hansen, R.R. Caspi, Th1 and Th17 cells: adversaries and collaborators, Ann. NY Acad. Sci. 1183 (2010) 211–221.
[41] M. Yamasaki, K. Araki, T. Nakanishi, C. Nakayasu, A. Yamamoto, Role of CD4(+) and CD8alpha(+) T cells in protective immunity against *Edwardsiella tarda* infection of ginbuna crucian carp, *Carassius auratus langsdorfii*, Fish Shellfish Immunol. 36 (1) (2014) 299–304.
[42] M. Wilson, E. Bengtén, N.W. Miller, L.W. Clem, L. Du Pasquier, G.W. Warr, A novel chimeric Ig heavy chain from a teleost fish shares similarities to IgD, Proc. Natl. Acad. Sci. U. S. A 94 (9) (1997) 4593–4597.
[43] K. Chen, A. Cerutti, The function and regulation of immunoglobulin D, Curr. Opin. Immunol. 23 (3) (2011) 345–352.
[44] E.S. Edholm, E. Bengten, M. Wilson, Insights into the function of IgD, Dev. Comp. Immunol. 35 (12) (2011) 1309–1316.
[45] A.G. Granja, C. Tafalla, Different IgM(+) B cell subpopulations residing within the peritoneal cavity of vaccinated rainbow trout are differently regulated by BAFF, Fish Shellfish Immunol. (2017), https://doi.org/10.1016/j.fsi.2017.10.003 (in press).
[46] E.S. Edholm, E. Bengten, J.L. Stafford, M. Sahoo, E.B. Taylor, N.W. Miller, M. Wilson, Identification of two IgD+ B cell populations in channel catfish, *Ictalurus punctatus*, J. Immunol. 185 (7) (2010) 4082–4094.
[47] R. Castro, E. Bromage, B. Abós, J. Pignatelli, A. González Granja, A. Luque, C. Tafalla, CCR7 is mainly expressed in teleost gills, where it defines an IgD+IgM- B lymphocyte subset, J. Immunol. 192 (3) (2014) 1257–1266.
[48] A. Cerutti, K. Chen, A. Chorny, Immunoglobulin responses at the mucosal interface, Annu. Rev. Immunol. 29 (2011) 273–293.
[49] K. Chen, W. Xu, M. Wilson, B. He, N.W. Miller, E. Bengten, E.-S. Edholm, P.A. Santini, P. Rath, A. Chiu, M. Cattalini, J. Litzman, J. Bussel, B. Huang, A. Meini, K. Riesbeck, C. Cunningham-Rundles, A. Plebani, A. Cerutti, Immunoglobulin D enhances immune surveillance by activating antimicrobial, pro-inflammatory and B cell-stimulating programs in basophils, Nat. Immunol. 10 (8) (2009) 889–898.
[50] J. Montero, J. García, M.C. Ordás, I. Casanova, A. González, A. Villena, J. Coll, C. Tafalla, Specific regulation of the chemokine response to viral hemorrhagic septicemia virus at the entry site, J. Virol. 85 (9) (2011) 4046–4056.
[51] T. Wang, P. Johansson, B. Abós, A. Holt, C. Tafalla, Y. Jiang, A. Wang, Q. Xu, Z. Qi, W. Huang, M.M. Costa, P. Díaz-Rosales, J.W. Holland, C.J. Secombes, First in-depth analysis of the novel Th2-type cytokines in salmonid fish reveals distinct patterns of expression and modulation but overlapping bioactivities, Oncotarget 7 (10) (2016) 10917–10946.
[52] E. Chaves-Pozo, J. Montero, A. Cuesta, C. Tafalla, Viral hemorrhagic septicemia and infectious pancreatic necrosis viruses replicate differently in rainbow trout gonad and induce different chemokine transcription profiles, Dev. Comp. Immunol. 34 (6) (2010) 648–658.
[53] T. Wang, J. Zou, C. Cunningham, C.J. Secombes, Cloning and functional characterisation of the interleukin-1 beta 1 promoter of rainbow trout (*Oncorhynchus mykiss*), Biochim. Biophys. Acta 1575 (1–3) (2002) 108–116.