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Expression of genes related to the early immune response in rainbow trout (*Oncorhynchus mykiss*) after viral haemorrhagic septicemia virus (VHSV) infection

C. Tafalla[a,*], J. Coll[b], C.J. Secombes[c]

[a]*Centro de Investigación en Sanidad Animal (CISA-INIA), Carretera de Algete a El Casar km. 8.1, Valedeolmos, 28130 Madrid, Spain*
[b]*SGIT, INIA, Biotecnología, Carretera de La Coruña km. 7, 28040 Madrid, Spain*
[c]*Scottish Fish Immunology Research Centre, University of Aberdeen, Aberdeen AB24 2TZ, Scotland*

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Abstract

In the last few years, many cytokine and other immune related genes have been identified in different teleost species, thus allowing their study at a molecular level. However, very little is known about their effect on fish antiviral responses. In the current work, we have studied the effect of viral haemorrhagic septicemia virus (VHSV) infection on the expression of different immune genes in rainbow trout (*Oncorhynchus mykiss*) through semi-quantitative reverse transcription-polymerase chain reaction (RT-PCR). We have studied the effect of the viral infection on the expression of different cytokines such as interleukin 1β (IL-1β) and transforming growth factor β (TGF-β), the CXC chemokine IL-8, and other immune genes such as inducible nitric oxide synthase (iNOS) and the class II major histocompatibility complex (MHC II). The virus induced an increased transcription of IL-1β in the spleen, and to a lesser extent in the head kidney and liver at early times post-infection. IL-8 transcription was also significantly induced with the virus in the spleen at early times post-infection. TGF-β transcription was significantly induced in VHSV infection in the spleen and liver. In the spleen, a significant induction of TGF-β at day 1 post-infection was observed. A further significant increase occurred in the spleen and liver at day 7 post-infection. No effect of the virus on MHC II expression was ever observed while iNOS was induced in the spleen, head kidney and liver of VHSV-infected fish mostly at day 7 post-infection. These results constitute a first step towards the understanding of which molecules may have a role in antiviral defence in fish.

Keywords: Viral hemorrhagic septicemia virus (VHSV); Rainbow trout (*Oncorhynchus mykiss*); Cyto/chemokines; IL-1β; IL-8; TGF-β; iNOS; MHC-II

Abbreviations: dNTP, deoxynucleotide triphosphate; DTT, dithiothreitol; FCS, fetal calf serum; GADPH, glyceraldehyde 3-phosphate dehydrogenase; iNOS, inducible nitric oxide synthase; IHNV, infectious hematopoietic necrosis virus; IL-1β, interleukin 1β; IL-8, interleukin 8; L-15, Leibovitz medium; MHC, major histocompatibility complex; RT-PCR, reverse transcription-polymerase chain reaction; TGF-β, transforming growth factor β; VHSV, viral haemorrhagic septicemia virus.

* Corresponding author. Tel.: +34 916 202300; fax: +34 916 202247.
E-mail address: tafalla@inia.es (C. Tafalla).

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1. Introduction

Viral haemorrhagic septicemia is a severe fish disease caused by a rhabdovirus, viral haemorrhagic septicemia virus (VHSV), responsible for major losses in aquaculture production [1]. However, even though VHSV and another related rhabdovirus, infectious hematopoietic necrosis virus (IHNV) are the main cause of mortality in farmed rainbow trout (*Oncorhynchus mykiss*) worldwide, very little information is available concerning the immune response of fish against these viruses. These studies would be of importance to the understand of the mechanisms of viral resistance in fish. At the moment, there are no commercial treatments or vaccines available to control these viruses, even though very good results have been obtained with DNA vaccination experimentally [2–7]. However, the immune mechanisms through which resistance is conferred by these vaccines is unknown, since non-specific viral protection is conferred by the vaccine at early stages post-vaccination [8,9] and in some cases neutralizing antibodies do not correlate with protection [9]. It was suggested that non-specific defence mechanisms in fish play a crucial role in resistance to infection. It may be possible that long-time resistance is mediated by cellular factors more than antibodies, as the existence of specific cytotoxic T cells has now been demonstrated in Crucian carp hematopoietic necrosis virus (CHNV) and VHSV-infected fish [10,11]. However, it is also evident that non-specific defence mechanisms induced shortly after infection also determine the outcome of the infection. Therefore, it is important to determine which non-specific defence mechanisms are triggered in viral infections, in order to understand the importance of these factors in virus resistance.

In this work, the effect of VHSV infection in rainbow trout on the expression level of different cytokines and other immune regulated genes has been studied in the spleen, head kidney and liver at different times post-infection. Previous evidence in mammals has shown a role of these molecules in antiviral defence. In fish, the sequences of these genes have been recently published thus allowing the study of their regulation and role in antiviral defence at a molecular level.

2. Materials and methods

2.1. Experimental infection

Rainbow trout (*Oncorhynchus mykiss*) of approximately 8–10 cm (9–12 g; 7 month old) were obtained from Centro de Acuicultura El Molino (Madrid, Spain), located in a VHSV and IHNV-free zone. Fish were maintained at the Centro de Investigaciones en Sanidad Animal (CISA-INIA) laboratory at 14 °C and fed daily with a commercial diet (Trouw, Spain). Prior to the challenge experiments, fish were acclimatised to laboratory conditions for 2 weeks and during this period no clinical signs were ever observed.

VHSV (strain 0771) was propagated in the EPC cell line. Cells were cultured at 18 °C in Leibovitz medium (L-15, Invitrogen, Carlsbad CA, USA) supplemented with 10% fetal calf serum (FCS, Invitrogen), containing 100 I.U. penicillin and 100 μg streptomycin per ml. Virus was inoculated on EPC grown in L-15 with antibiotics and 2% FCS at 14 °C. When cytophatic effect was extensive, the supernatant was harvested and centrifuged to eliminate cell debris. Clarified supernatants were used for the experiments. The virus stock was titrated in 96-well plates according to Reed and Müench [12].

For the challenge experience with VHSV, trout were divided into two groups of 20 trout each. One group was infected by intraperitoneal injection with VHSV (100 μl of 1×10^8 $TCID_{50}$/ml per fish). The other group was mock-infected with the same volume of L-15 medium. At days 1, 2, 3 and 7 post-infection, two control and four VHSV infected trout were killed and head kidney, spleen and liver sampled. A further tank with 20 VHSV-infected trout, kept in the same conditions, was used to determine the mortality during 30 days.

2.2. cDNA synthesis

Total RNA of the different organs was extracted using Trizol (Invitrogen) as per manufacturer's instructions and stored at −80 °C until used. Five μg of RNA were used to obtain cDNA using the Superscript II reverse transcriptase (Invitrogen). Briefly, RNA was incubated with 1 μl of oligo (dT)12–18 (0.5 μg/ml) and 1 μl 10 mM deoxynucleotide

triphosphate (dNTP) mix for 5 min at 65 °C. After the incubation, 4 μl of 5× first strand buffer (250 mM Tris–HCl pH 8.3, 375 mM KCl, 15 mM $MgCl_2$) and 2 μl 0.1 M dithiothreitol (DTT) were added, mixed and incubated for 2 min at 42 °C. Then, 1 μl of Superscript II reverse transcriptase was added and the mixture incubated at 42 °C for 50 min. The reaction was stopped by heating at 70 °C for 15 min, and the resulting cDNA stored at −20 °C.

2.3. PCRs of immune genes

All amplification reactions contained 200 μM of each dNTP, 1 unit of Taq polymerase (Invitrogen), 20 mM Tris–HCl (pH 8.4), 50 mM KCl, 1.5 mM $MgCl_2$, 1 μM of each primer and 1 μl of cDNA in a final volume of 25 μl. First, a PCR with primers for glyceraldehyde 3-phosphate dehydrogenase (GADPH) was performed with all samples as a positive control for RT-PCR, since GADPH is constitutively expressed in all organs. These PCR products also allowed the verification that equivalent amounts of cDNA were present in the different samples and therefore amplifications of the different immune genes were comparable among samples.

Primers used for gene amplification, and the sizes of the different PCR products are shown in Table 1. All PCRs were carried out in a Perkin–Elmer 2400 cycler and all samples were amplified twice to verify the results. Amplification conditions always consisted in a denaturing step of 94 °C for 5 min followed by the different specific cycling conditions shown in Table 1 followed by a final extension of 7 min at 72 °C. These conditions were optimised for each gene following protocols described previously and referenced in Table 1. The PCR products (8 μl) were visualised on a 1.6% agarose gel stained with ethidium bromide. Samples that were to be compared were always run in the same agarose gel. A 100 bp ladder was used as a size marker. The intensity of the amplification bands was estimated using Image Gauge v. 4.0 software (Fujifilm). Semi-quantitative analysis of mRNA transcription for each gene was performed relative to the GADPH expression of the same sample using the formula: intensity of target gene band/intensity of its corresponding GADPH band. Data were then

Table 1
Primer sequences, sizes of PCR products and amplification conditions for the different genes studied

Gene	Primers	Size of PCR product (bp)	No. of cycles	Cycling conditions	Reference
GADPH	F: 5′ ATGTCAGACCTCTGTGTTGG 3′ R: 5′ TCCTCGATGCCGAAGTTGTCG 3′	514 bp	30	94 °C 30 s 58 °C 30 s 72 °C 1 min	[13]
IL-1β	F: 5′ AGGGAGGCAGCAGCTACCACAA 3′ R: 5′ GGGGGCTGCCTTCTGACACAT 3′	353 bp	28	94 °C 30 s 60 °C 30 s 72 °C 30 s	[14]
IL-8	F: 5′ GAATGTCAGCCAGCCTTGTC 3′ R: 5′ TCCAGACAAATCTCCTGACCG 3′	226 bp	25	94 °C 30 s 60 °C 30 s 72 °C 30 s	[15]
MHC II	F: 5′ ATGTCGATGCCAATTGCCTTCTA 3′ R: 5′ TGTCTTGTCCAGTATGGCGCT 3′	336 bp	27	94 °C 30 s 57 °C 30 s 72 °C 30 s	[16]
TGF-β	F: 5′ AGACTCTGAATGAGTGGCTGCAAG 3′ R: 5′ CTCCAAGACCTGTGGAACACAGCA 3′	482 bp	27	94 °C 30 s 60 °C 30 s 72 °C 1 min	[16]
iNOS	F: 5′ CATACGCCCCCAACAAACCAGTGC 3′ R: 5′ CCTCGCCTTCTCATCTCCAGTGTC 3′	746 bp	35	94 °C 1 min 62 °C 1 min 72 °C 2 min	[16]
VHSV N	F: 5′ GAAGATAGGAAGGTGATTGTGG 3′ R: 5′ GAGTTTCCTGATGGCTGCCTTG 3′	408 bp	35	94 °C 30 s 53 °C 30 s 72 °C 30 s	[17]

analysed using Student's *t*-test and differences were considered statistically significant at $P < 0.05$.

2.4. VHSV detection by PCR

The level of expression of the N gene of VHSV was estimated through RT-PCR in order to verify that the virus was replicating in these fish, and to determine if a correlation between the level of expression of the different cytokines and the capacity of the virus to replicate in each fish could be established. For this, PCRs were performed in cDNAs obtained from spleen as described above. Primers and amplification conditions are specified in Table 1.

3. Results

IL-1β was constitutively expressed in rainbow trout spleen, liver and head kidney, although a higher constitutive expression was observed in the spleen. The effect of VHSV infection on the expression of

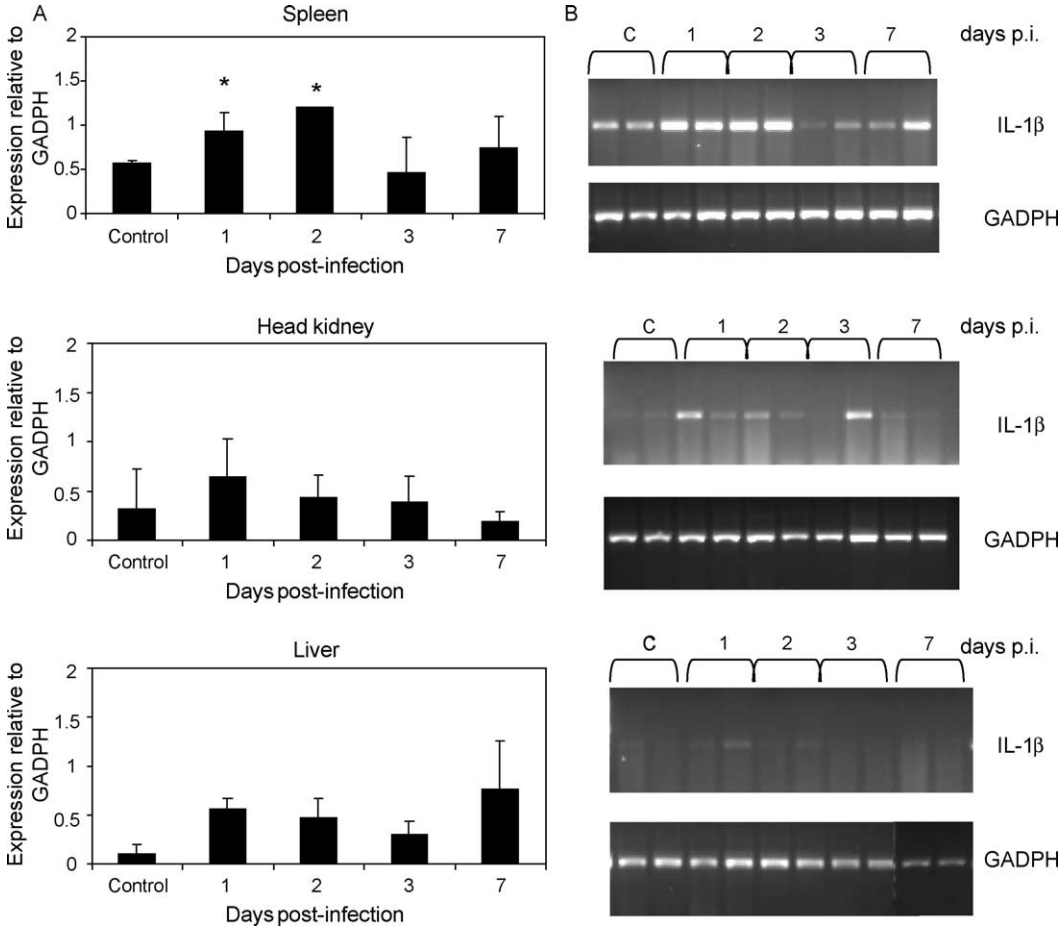

Fig. 1. IL-1β expression in response to VHSV. (A) Levels of expression in spleen, head kidney and liver of VSHV-infected rainbow trout compared to expression observed in uninfected controls. Data are presented as mean relative expression ± SD for four individuals at each time point. In the case of controls, mean relative expression for two samples from day 1 and two samples from day 3 are presented. (B) Stained gel showing two representative samples at each screening point, out of the four individuals studied. For the control samples (C), two representative bands were choosen from all the controls obtained at each time point, since no significant differences were observed among controls at different days post-infection. Corresponding GADPH bands for these samples are shown below. *Expression significantly higher than expression in the respective non-infected tissues.

IL-1β in these tissues is shown in Fig. 1. In the spleen, the virus induced a significant increase of IL-1β transcription at days 1 and 2 post-infection. After this time, IL-1β expression returned to levels in some cases even lower than those observed in control samples. In one out of four individuals screened, a strong IL-1β transcription was detected 7 days post-infection. In the head kidney, a VHSV induced expression of IL-1β was observed at days 1 and 2 post-infection in three out of four individuals, although results were not significant. In this case, one out of four fish studied showed a strong IL-1β expression 3 days post-infection. Very low levels of expression were observed in the liver of either uninfected or infected trout and only a slight induction of its expression was observed at day 1 post-infection.

IL-8 was constitutively expressed in all organs studied and its transcription was also induced in response to VHSV (Fig. 2). In the spleen, IL-8 expression was strongly induced in response to the virus at days 1 and 2 post-infection. After this time, results varied from fish to fish, and while some of them still had an increased IL-8 expression, other individuals had transcript levels similar to those in non-infected fish. In the head kidney, an increased expression was observed in three out of four VHSV infected animals 1 day post-infection. After this time, the induction remained in some individuals up to

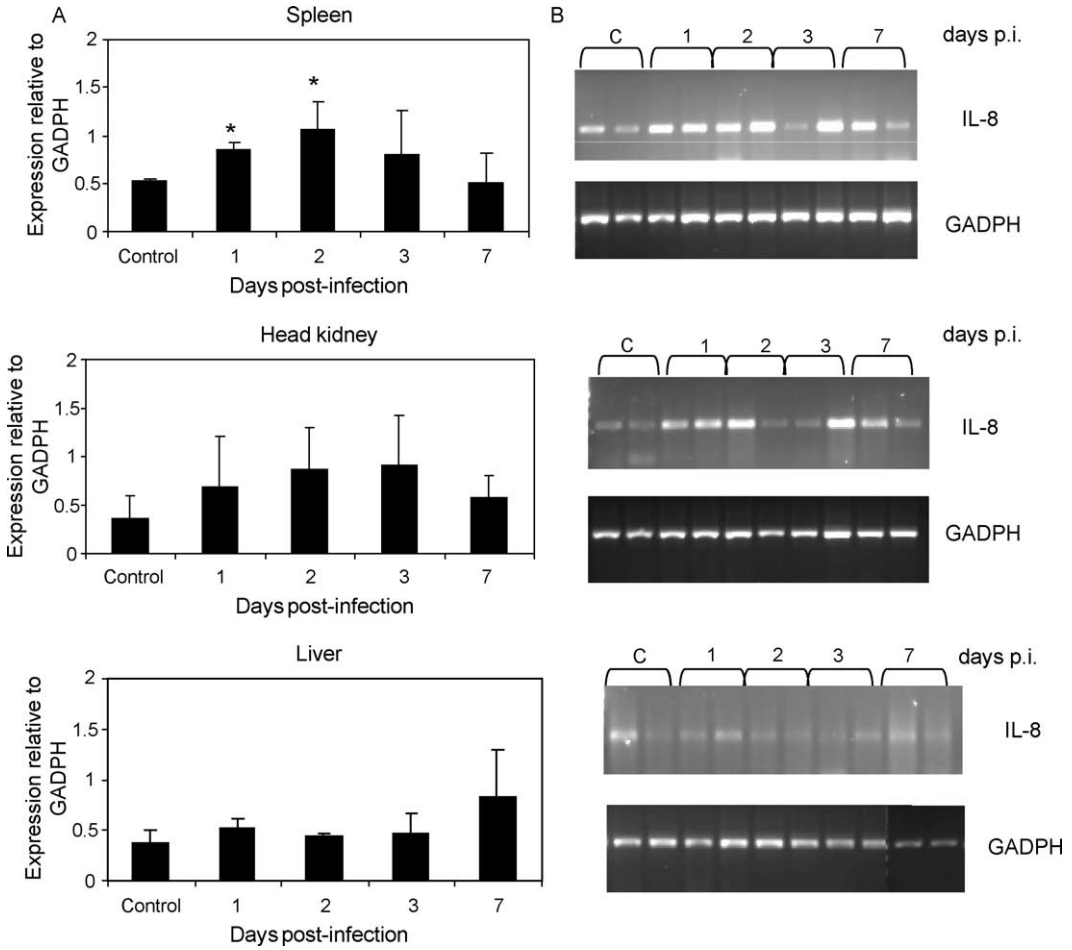

Fig. 2. Samples were assayed for IL-8 expression in response to VHSV as described in the Fig. 1. *Expression significantly higher than expression in the respective non-infected tissues.

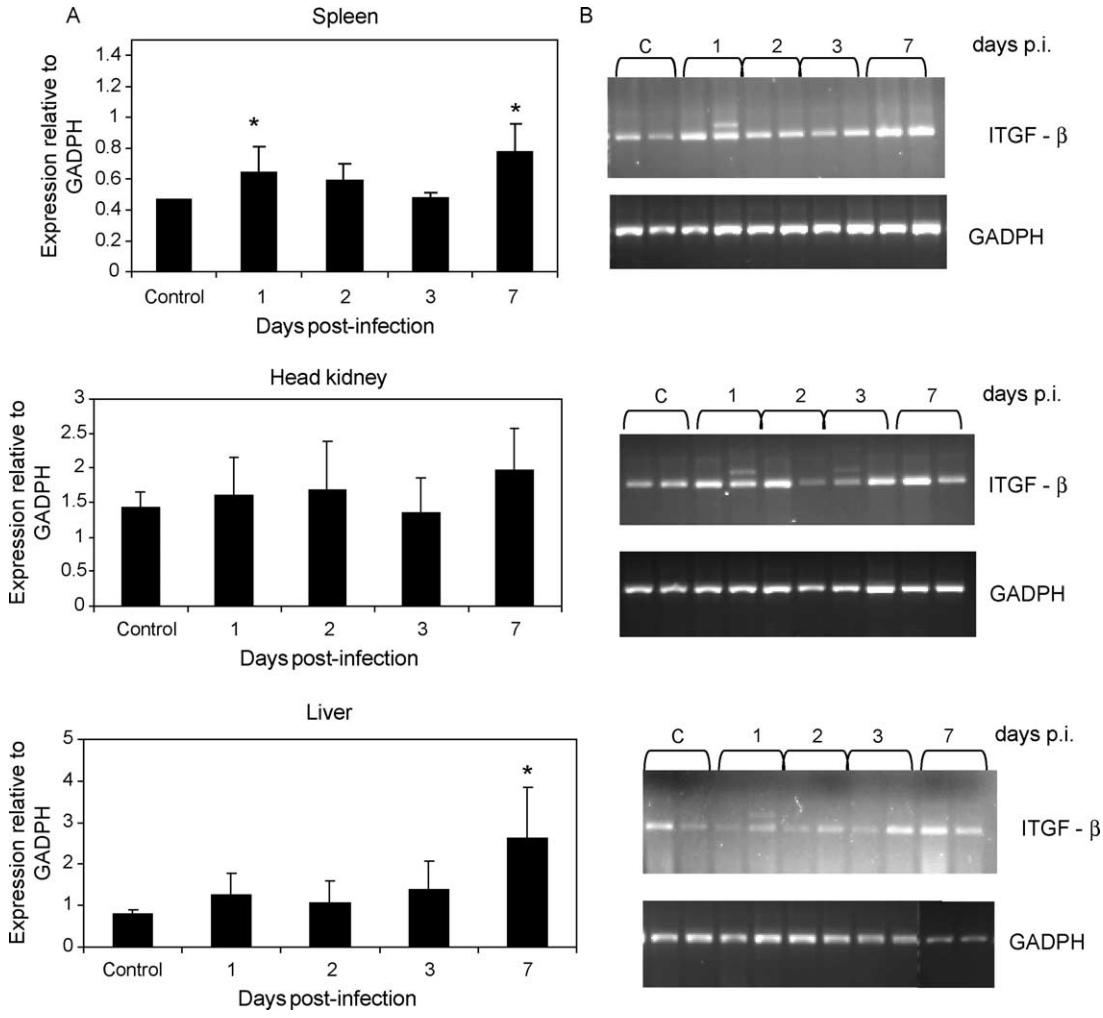

Fig. 3. Samples were assayed for TGF-β expression in response to VHSV as described in the Fig. 1. *Expression significantly higher than expression in the respective non-infected tissues.

7 days post-infection, but a high variation was observed among the different fish. No induction of IL-8 was observed in the liver.

TGF-β was constitutively expressed in all tissues studied (Fig. 3). In the spleen, VHSV induced a significantly increased expression of TGF-β after 1 day of infection, afterwards levels of expression returned to normal, and then at day 7 post-infection a strong transcription was again induced by the virus in all fish studied. In the head kidney, no significant effect of the virus was observed at any of the time points studied. However, an induction of TGF-β transcription was observed in two out of four fish after 1 day of infection, and one out of four fish on days 2 and 3 post-infection. After 7 days of infection, TGF-β transcript levels were strongly increased in the head kidney of three out of four fish studied, although in different degree among individuals. In the liver, VHSV induced a significantly increased expression of this cytokine at day 7 post-infection. In some cases, an induction was also observed after 3 days of infection, although the results were more variable from fish to fish, and therefore not significant.

No significant effect of VHSV on the levels of expression of MHC II was found in any of the organs studied at any time post-infection (Fig. 4).

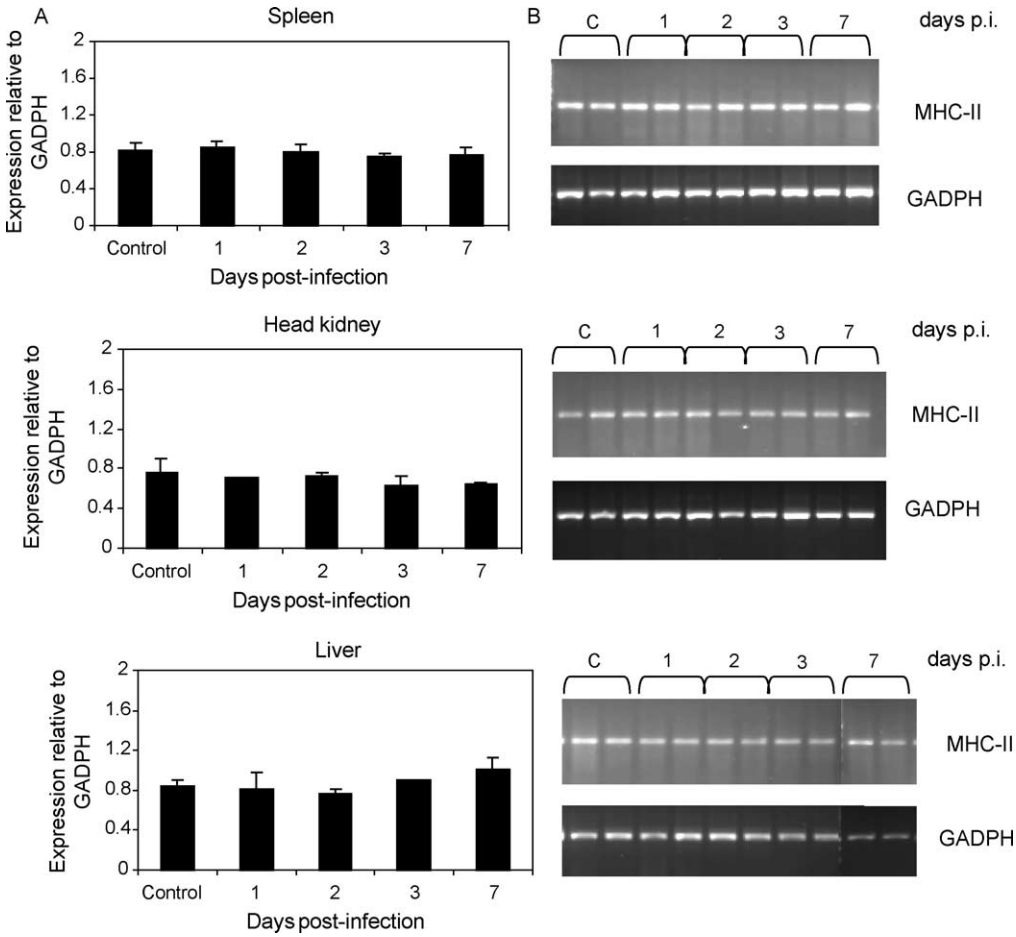

Fig. 4. Samples were assayed for MHC-II expression in response to VHSV as described in the Fig. 1.

Regarding the expression of iNOS, low constitutive expression was observed in all organs studied (Fig. 5). In the spleen, the infection induced a 5-fold increased expression of iNOS at day 1. Then, the level of expression decreased a little, but a significant induction was still observed at day 7 post-infection. In the head kidney, the virus induced the expression of iNOS at day 1 although results were not significant due to variations among fish, the transcription then decreased until a significant induction occurred at day 7 post-infection. In the case of the liver, VHSV augmented the transcription of iNOS, but the results were not significant and were variable from fish to fish until day 7 post-infection at which a 6–7 fold increase was observed.

The expression of the VHSV N gene was detected in the spleen of all infected fish screened. The level of expression of this viral gene in each fish did not correlate with the level of expression of any cytokine studied (data not shown).

4. Discussion

In the current work, we describe the effect of VHSV infection on the expression of different cytokines and other immune related genes in rainbow trout. Intraperitoneal injection of high dose of VHSV was used in the assay, in order to assure an immune response, however, due to the age and passage number

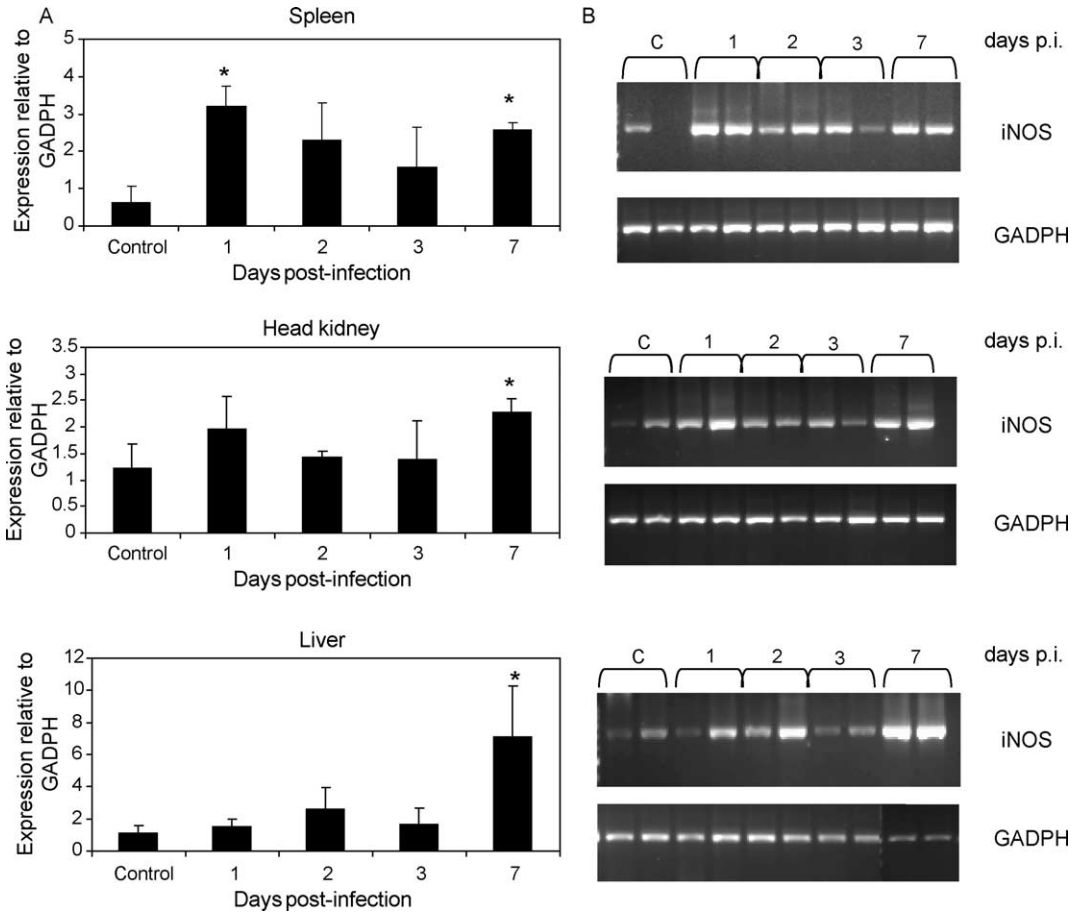

Fig. 5. Samples were assayed for iNOS expression in response to VHSV as described in the Fig. 1. *Expression significantly higher than expression in the respective non-infected tissues.

of the virus, this route of infection did not cause mortalities at any point, as determined in a parallel tank kept for 30 days. This constitutes one of the few reports of a viral immune effect in vivo in teleost fish, and constitutes a step towards the understanding of the viral immune role of these molecules in fish. The work presented here gives a preliminary view as to the role of these molecules in the immune defence against VHSV in rainbow trout.

First, the regulation of the cytokine IL-1β a typical pro-inflammatory cytokine, in response to VHSV was studied. The rainbow trout IL-1β sequence was first obtained by Zou et al. [18] and since then many studies have elucidated the bioactivity of this molecule, demonstrating for example its chemo-attractant capacity [19]. It has been demonstrated that the in vivo administration of IL-1β-derived peptides confers resistance to VHSV in rainbow trout 2 days post-administration [20], suggesting a role for IL-1β in antiviral defence. However, its role in viral infections has not been thoroughly studied. Previous work had determined that IL-1β was not constitutively expressed in rainbow trout [21], however, some degree of constitutive expression was observed in the macrophage/monocyte-like cell line RTS-11 obtained from rainbow trout spleen [22]. In this study, we did find a constitutive IL-1β expression in the spleen. Constitutive IL-1β expression was also observed in different organs of gilthead sea bream (*Sparus aurata*) [23]. It is very difficult to obtain fish that are not in contact with a stimulus capable of triggering a basal production of IL-1β, moreover as IL-1β is a pro-inflammatory

cytokine it is probably very sensitive to induction by any stimulus that comes in contact with the fish. This basal induction does not affect the capacity of a later induction, as observed in this study with VHSV. We found that the virus induced the expression of IL-1β in spleen and head kidney and to a lesser extent in the liver at early times post-infection, as would be expected from a pro-inflammatory cytokine. In the head kidney, although the results were not significant due to a high variation in the degree of induction among individuals, an increased transcription in response to VHSV was also observed in most individuals. These differences among individuals, could also be the result of differences in the time at which each fish is most stimulated, as they did not correlate with a decreased or increased replication, as determined by the level of expression of the VHSV N gene (data not shown). Purcell et al. [24] also described a high individual variation in the level of expression of different genes such as Mx, vig-8, TNF-α IL-1β and IL-8 in rainbow trout spleen. Further studies should be done to determine whether differences in the level of expression of different cytokines are determinant in viral resistance. It is known that VHSV is located in the spleen, head kidney and liver at early stages of an infection [1], so the lower effect on IL-1β expression observed in the liver may be due to the fact that it is not an hematopoietic organ. A recent work performed in rainbow trout with IHNV, also demonstrated that IL-1β is induced in the spleen in response to viral infection [24]. These results, together with the fact that IL-1β-derived peptides confer resistance to VHSV [20], indicate that IL-1β may be a good candidate for an adjuvant as has been previously demonstrated in other species [25,26] and suggested in fish [27].

The results presented here also strongly suggest a role for IL-8 in viral defence. IL-8 is a CXC chemokine, that in mammals is known to attract neutrophils, T lymphocytes and basophils but not macrophages or monocytes [28]. Its role in mammals is not only the recruitment of cells to the site of infection, but the triggering of a conformational change of intregins that allows stable binding to the site [29]. A fish IL-8 homologue was first described in *Lampetra fluviatilis* [30], and following this description, IL-8 molecules have also been identified in the Japanese flounder *Paralichthys olivaceous* [31] and rainbow trout *O. mykiss* [15]. An IL-8 receptor-like gene has also been described in rainbow trout [32]. Motifs similar to those present in IL-8 from mammals and found in the rainbow trout molecule suggest similar chemotactic activities [33], however, its immune role has not yet been elucidated. In the case of the Japanese flounder, no constitutive expression of IL-8 was observed and transcripts could only be detected in the spleen and head kidney of LPS-stimulated individuals [31]. In trout, however, as observed here, there is a strong constitutive expression of IL-8 in the spleen, while expression in the liver and head kidney is lower and more variable from fish to fish [15]. In this same study, no constitutive IL-8 expression was detected in the macrophage-like cell line RTS-11, although an induced transcription was observed in response to either LPS or to a lesser extent Poly I:C. However, VHSV in vivo induced a strong IL-8 response in the spleen, as was demonstrated for IHNV [24]. In the head kidney, although the results were not significant, an increased transcription in response to VHSV was also observed in most individuals. Therefore, since a strong IL-8 expression was induced in lymphoid organs in response to the virus in vivo, it may be possible that in vivo IL-8 expression is not only induced directly by the virus but through other factors or cytokines produced by cell types other than macrophages. This is confirmed by the fact that VHSV in vitro does not significantly stimulate head kidney macrophages for IL-8 production (data not shown). In mammals, IL-8 is known to be induced by pro-inflammatory cytokines such as IL-1, IL-6 or TNF [34]. In trout head kidney leukocytes, it has been demonstrated that IL-8 expression can be induced by a combination of LPS and TNF-α [35]. When subtractive suppressive hybridization was performed with VHSV-infected rainbow trout leukocytes, an homologue to a human CXC chemokine and to other chemoattractant molecules were obtained [36]. All these results give weight to the hypothesis that chemokines play an essential role in viral defence, as can be concluded from the fact that many viruses have created different strategies to inactivate chemokines in the host [37].

Transforming growth factors β (TGF-β) are a family of cytokines with pleiotropic effects on the immune system, and depending on the cell types involved and the surrounding conditions, they can

either have positive or negative effects on lymphocyte proliferation, cytokine responsiveness or cytokine expression [38]. Normally, TGF-β inhibits B and T cell proliferation and differentiation, antagonises pro-inflammatory cytokines such as IL-1β, TNF-α and IFN-γ [39], and blocks the expression of IL-1β and IL-2 receptors. TGF-β also has inhibitory effects on neutrophils as it has negative effects on the adhesion of these cells to the epithelium. In macrophages, the production of superoxide anion and nitric oxide is also inhibited by TGF-β [40], by interfering with the positive effect of interferon γ on the production of oxygen and nitrogen radicals by macrophages [41]. Although mainly inhibitory, it is known that TGF-β, at early stages of infection, can facilitate CD8+T responses such as differentiation [42] and IL-2 secretion [43]. Although it is unknown whether all these functions are true for fish TGF-β, it was demonstrated that bovine TGF-β inhibited the respiratory burst of rainbow trout macrophages [44]. Therefore, it may be possible that the induction of TGF-β that takes place in response to VHSV immediately after infection, mostly in the spleen, allows the virus to enter into macrophages, as it is known that VHSV replicates in rainbow trout macrophages [45]. In human macrophages, it has been demonstrated that through this mechanism TGF-β allows the replication of *Leishmania chagasi* [46,47]. However, it may also be possible that this increment of TGF-β at the early stages of the infection mediates positive effects on T cell responses. More work needs to be done to determine the significance of this early response. The late induction of TGF-β at day 7 post-infection, has a more clear function, in controlling the inflammatory immune response to the virus. IHNV, however, produced no effect on TGF-β expression in the spleen of rainbow trout when a lethal viral dose was used [24]. In our experiment, fish were not exposed to a lethal dose of VHSV, therefore the immune response must have been able to control the infection, and it is probable that at day 7 post-infection the response is turned-off through the action of TGF-β.

No effect of VHSV on MHC class II expression was observed. Hansen and LaPatra [48] described a shutdown of MHC class II and an up-regulation of MHC class I in the pronepros and spleen in response to IHNV. From these results it was concluded that rhabdoviral infections favoured an enhanced CD8+ CTL response via up-regulation of MHC class I, while they turned down T helper responses by lowering MHC II levels. Another explanations given for the decrease of MHC II expression was the decrease in the number of MHC II bearing cells by the virus or an induced migration to other places. In the current work, we did not observe a shutdown of MHC class II expression in response to VHSV. This strengthens the hypothesis that rhabdoviral infections act through an MHC class I pathway. However, DNA vaccines which effectively control rhabdoviral infections in fish are known to act through MHC class II [3,49]. The regulation of all of these immune related genes studied is expected to be interconnected. Thus, it is known that trout recombinant IL-1β can induce the expression of several immune genes such as IL-1β itself, COX-2 and MHC-II [50]. However, in these experiments, we detected an induction of IL-1β that did not correlate with an increase in MHC-II expression.

Lastly, we have demonstrated that VHSV infection also significantly induced the expression of iNOS in the spleen, head kidney and liver. NO is produced via the induction of iNOS by macrophages in response to different stimuli [51]. This NO is known to play an important role in cellular defence against specific viral infections [52–54], although there are some viruses against which NO has no effect [55,56]. In turbot, it was already demonstrated that NO is induced by VHSV in head kidney macrophages and that NO has antiviral activity against VHSV [57]. Through these new molecular methods, it is now possible to corroborate this production in rainbow trout in response to the virus by means of detection of iNOS transcript induction in spleen, liver and head kidney. TGF-β is thought to inhibit NO production, although this has not yet been demonstrated in fish. Surprisingly, we found a higher iNOS transcription at the same time points in which TGF-β expression was higher. This suggests that TGF-β does not have a significant effect on iNOS expression in trout.

In conclusion, we have demonstrated that VHSV infection in rainbow trout alters the levels of expression of IL-1β, IL-8, TGF-β?and iNOS genes, suggesting a role for these molecules in antiviral defence. More work should be done to study whether these molecules are determinant for viral resistance in fish.

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