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Trout oral VP2 DNA vaccination mimics transcriptional responses occurring after infection with infectious pancreatic necrosis virus (IPNV)

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ABSTRACT

Time-course and organ transcriptional response profiles in rainbow trout *Oncorhynchus mykiss* were studied after oral DNA-vaccination with the VP2 gene of the infectious pancreatic necrosis virus (IPNV) encapsulated in alginates. The profiles were also compared with those obtained after infection with IPNV. A group of immune-related genes (*stat1*, *ifn1*, *ifng*, *mx1*, *mx3*, *il8*, *il10*, *il11*, *il12b*, *tnf2*, *mhc1uda*, *igm* and *igt*) previously selected from microarray analysis of successful oral vaccination of rainbow trout, were used for the RTqPCR analysis. The results showed that oral VP2-vaccination qualitatively mimicked both the time-course and organ (head kidney, spleen, intestine, pyloric ceca, and thymus) transcriptional profiles obtained after IPNV-infection. Highest transcriptional differential expression levels after oral vaccination were obtained in thymus, suggesting those might be important for subsequent protection against IPNV challenges. However, transcriptional differential expression levels of most of the genes mentioned above were lower in VP2-vaccinated than in IPNV-infected trout, except for *ifn1* which were similar. Together all the results suggest that the oral-alginate VP2-vaccination procedure immunizes trout against IPNV in a similar way as IPNV-infection does while there is still room for additional improvements in the oral vaccination procedure. Some of the genes described here could be used as markers to further optimize the oral immunization method.

1. Introduction

Infectious pancreatic necrosis (IPN) is an economically important fish disease caused by a virus belonging to the *Birnaviridae* family which produces severe acute infections in young salmonids (i.e.: salmon and trout) and many other fish, resulting in the most widespread virus in aquaculture [1,2]. Furthermore, following a disease outbreak, surviving fish may become asymptomatic carriers contributing to the spreading of the disease. Therefore, broodstock carriage is considered a source of virus for the lethal infection of hatchery-reared fry. The development of an effective vaccine will be a convenient way to secure the future of fish farms and the health status of both inland and sea waters.

Because present oil-adjuvanted vaccines based on recombinant IPNV proteins delivered by intraperitoneal injection have important side effects on fish welfare [3–5], present licenced DNA vaccines still require fish-to-fish intramuscular injection [6] and small immunocompetent fish cannot be injected, oral vaccines are an important alternative to immunize against IPNV. However, oral vaccines have many requirements that are difficult to obtain, such as their need to be protected from stomach digestion, adhere to the fish guts, avoid induction of immune tolerance, induce the appropriated immune protective responses and be capable of being mixed with the feed. Thus although oral delivery has been considered the most desirable way to vaccinate both humans and animals [7,8], despite many research efforts there are yet few reports describing successful methods. Nevertheless, recent reports using pathogen recombinant proteins in salmon [9,10] and DNA in rainbow trout [11] or in Japanese flounder (*Paralichthys olivaceus*) [12], now suggest fish oral vaccination might be possible. However, the knowledge of immune-related responses to improve fish oral vaccines is still preliminary [13].

Intramuscular DNA vaccines can induce strong and long-lasting humoral and cell-mediated fish immune responses, resembling those induced by natural infection of intracellular pathogens [10,14]. In contrast, oral DNA vaccines are just beginning to be tested in fish in a similar way and therefore, little is known about the significance of the up or down regulation of the differential expression of genes related to antiviral activity [13]. Because, the knowledge of the transcriptional responses in salmonid fish to

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IPNV-infection is often limited by the small number of immune-related probes available [15], most of the studies have been developed in Atlantic salmon by RTqPCR demonstrating induced differential expression of several interferon-related genes [16]. More recently, up-regulation of *ifn* and *mx* genes together with inflammatory and proteolysis genes in fish survivors to IPNV lethal challenges have been also reported [17]. With regard to IPNV intramuscular DNA vaccines, the VP2 gene stimulation of some immune-related genes in rainbow trout has been described [18]. Furthermore, protection levels up to 83% RPS (relative percentage survival), neutralizing antibodies lasting for at least 60 days and early differential expression of *ifn* and *mx* transcripts was obtained by using VP2 complexed with alginates in orally vaccinated trout [11]. Finally, by combining EST-derived trout oligo-probes [19] with oligo-probes designed from selected GenBank gene entries, we used a newly designed oligo-microarray enriched in immune-related *Oncorhynchus mykiss* genes to study early transcript differential expression after oral vaccination with the VP2 gene [13]. Seven days after oral vaccination, a wide list of vaccine-upregulated genes in head kidney compared to pyloric ceca could be thus identified. A group of selected genes from that study was selected to carry out the present studies.

This work describes the time-course and organ profiles of selected transcriptional responses after oral DNA vaccination of rainbow trout *O. mykiss* and compares those to the corresponding profiles obtained after infection with IPNV. The IPNV VP2 gene encoded into a plasmid DNA and encapsulated in alginate microspheres [11] and a group of immune-related rainbow trout genes (*stat1*, *ifn1*, *ifng*, *mx1*, *mx3*, *il8*, *il10*, *il11*, *il12b*, *tnf2*, *mhc1uda*, *igm* and *igt*) selected as mentioned above [13] and their differential expression estimated by RTqPCR, were used for the comparisons. The results showed that the oral VP2-vaccine could mimic both the time-course (up to 7 days) and the organ (head kidney, spleen, intestine, pyloric ceca, and thymus) profiles of transcripts obtained after IPNV-infection. However, transcriptional levels were lower in VP2-vaccinated than in IPNV-infected trout, except for *ifn1* which were similar. The results obtained suggest that the mechanisms by which alginate-oral DNA vaccination induces protection are similar to the defense mechanisms induced by IPNV-infection. Furthermore, some of the genes described here could also be used as markers to follow up immunization procedures and/or to further optimize oral vaccination.

2. Materials and methods

2.1. Preparation of the IPNV VP2 DNA plasmid vector

The plasmid DNA was prepared as described previously [11]. Briefly, the IPNV VP2 gene was cloned into the pcDNA3.1/V5/His-TOPO (Invitrogen, Spain) plasmid vector under the control of the immediate-early CMV promoter (pDNA-VP2 or VP2-vaccine) and amplified in *Escherichia coli* TOP10. The plasmid DNA was isolated with the Endofree Plasmid Maxi purification Kit (Qiagen Iberia, S.L.-Spain) according to the manufacturer's instructions. The DNA concentration was measured in a spectrophotometer (NanoDrop 2000, Thermo scientific, Spain) before it was aliquoted and conserved at −20 °C. The pcDNA3.1/V5-His-TOPO plasmid (pDNA) was used as control plasmid.

2.2. Preparation of microspheres and formulation of the oral VP2-vaccine

The procedure was followed as previously described [11]. Briefly, 2.5 mL of 3% (w/v) of sodium alginate were mixed with 1.5 mL of 1 mg/mL of plasmid pDNA-VP2 and the mixture stirred at 500 rpm during 10 min. This solution was then added to an Erlenmeyer flask containing 100 mL of paraffin oil and 0.5 mL Span 80, and the mixture was emulsified for 30 min at 900 rpm. Microspheres were prepared by adding 2.5 mL of 0.15 M $CaCl_2$ to the emulsion drop by drop and stirring for 2 h at 900 rpm. Microspheres were collected by centrifugation at 1000 *g* for 10 min, and they were washed twice with 70% ethanol, lyophilized for 24 h and stored at 4 °C.

2.3. Experimental fish: rainbow trout

Rainbow trout (*O. mykiss*) of a mean weight of 1 g (mean size of 3.5 cm) were purchased from a spring water local farm with no history of viral disease. No fish showed any clinical signs. In addition, two pools of 5 fish each were tested by standard methods to confirm the absence of IPNV or any other salmonid virus by isolation using BF cells [20]. The trout were acclimatized for 2 weeks and kept under a 12/12 h light/dark regime at 15 °C in 350 l closed re-circulating water tanks (Living Stream, Frigid Units Inc, Ohio) at the "Centro de Investigaciones Biológicas" (CSIC, Madrid, Spain). Groups of trout were maintained in separate 45 l aquaria supplied with non-chlorinated water using exterior carbon filters (Eheim, Madrid, Spain) and additional aeration. The trout were fed daily with a diet of commercial pellets. The water-quality parameters were maintained at optimum levels for rainbow trout and the culturing conditions in all tanks were equal. Experimental protocols were performed with the approval of the CSIC ethical committee.

2.4. Oral VP2-vaccination

The trout were divided into three groups of 30 trout each. A group of trout was orally vaccinated with 10 μl of suspension of the vaccine microspheres each containing 10 μg of pDNA-VP2 diluted in 10 μl of PBS, while other group received similar microspheres suspension but with pDNA (empty plasmid). An untreated fish group was maintained unhandled. Vaccination was performed with an automatic pipette with a 20 μl tip which was introduced into the mouth of each trout, supporting the tip end at the entrance of the oesophagus. All fish were sacrificed by exposing to an overdose of tricaine methane sulphonate (MS-222, Sigma, Madrid, Spain) prior to tissue sampling. Head kidney, spleen, intestine, pyloric ceca and thymus from each trout were collected at 2, 3, 5, 7, 10 and 15 days post-vaccination and stored in TRIzol LS reagent (Invitrogen, Spain) until RNA isolation. There were 3 trout for each of 6 time points. In addition, 3 trout were sacrificed prior to IPNV immersion challenge, and their tissue samples collected, to serve as the time 0 h control. Because of their small size and similar location of the thymus and pseudobranch tissues, thymus harvested for the analysis was mixed with pseudobranch tissue. As estimated by fluocytometry (SSC/FSC) and confirmed by smear staining, the thymus tissue contained 19–20.6% of lymphocytes, the rest being red blood cells and large pseudobranch cells (i.e.: chloride cells) [21].

2.5. IPNV challenge by immersion

The trout were divided into 2 groups of 25 trout each. Group 1 was infected with IPNV in a reduced volume of water for 2 h with aeration (3×10^5 tissue culture infectious dose $TCID_{50}$/ml of IPNV). Group 2 was handled similarly but was mock infected. Head kidney (HK), spleen (S), intestine (IN), pyloric ceca (PC) and thymus (T) from each fish were collected at 2, 3, 5, and 7 days post-challenge. Since mortalities began after 7 days, there were no time points after 7 days. Samples were stored in TRIzol LS reagent until RNA isolation. There were 3 trout for each of the time points.

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2.6. Isolation of total RNA and cDNA synthesis

Organs were individually homogenized in 1 mL of TRIzol using the Tissue Lyser Cell Disruptor (Qiagen S.A, Spain) 5 min at 50 Hz with 2 mm glass beads. Total RNA was extracted by the TRIzol reagent according to the manufacturer's instructions, and the concentration and purity of the RNA obtained were measured in a NanoDrop™ spectrophotometer. RNA extracted was resuspended in pyrogen free DEPC treated water. RNA was treated with DNase I RNase free (Fermentas, Spain). The cDNA synthesis was performed with 5 μg of the RNA primed with oligo-d(T) (25 pmol/μl). The Super Script™ II kit (Invitrogen, Spain) was used for reverse transcription. The cDNA was diluted to 1:4 in DEPC treated water and 1 μl of the diluted cDNA was taken for each reaction in the real-time PCR assay.

2.7. Quantitative estimation of transcripts for selected immune-related genes by qPCR

The qPCRs (quantitative polymerase chain reactions) were performed by using the SYBR® green method, in an iQ5 iCycler thermal cycler (Bio-Rad Laboratories, Inc., Madrid, Spain). The qPCR amplifications were carried out in 96-well plates by mixing 1 μl of 4-fold diluted cDNA, 12.5 μl of 2× concentrated iQ SYBR® Green Super mix (Bio-Rad), 0.3 μM forward primer and 0.3 μM of reverse primer in a 25 μl reaction volume for each sample. The thermal profile was 10 min at 95 °C, followed by 40 amplification cycles of 10 s at 95 °C, 1 min at 60 °C and a dissociation cycle (1 min at 95 °C and 1 min at 60 °C). After the run, the melting curve of each amplicon was examined to determine the specificity of the amplification. No amplification product was observed in controls containing no RNA samples. The data obtained were analysed using the iQ5 optical system software version 2.0 (Bio-Rad) and the relative quantification of the amplified gene products was calculated by the comparative Ct method. The elongation factor 1α (*ef1a*) was used as the house keeping gene in each RNA sample in order to normalize the results by eliminating variation in mRNA and cDNA quantity and quality (normalized values). All the qPCR reactions were performed in duplicate (technical replicates) and their mean Ct values used for the calculations. First, the Ct for each gene was normalised to their corresponding *ef1a* Ct ($\Delta Ct_{gene} = Ct_{gene} - Ct_{ef1a}$). Second, mean control Ct values (mean $\Delta Ct_{control}$) from 3 trout immunized with empty plasmid for the VP2-vaccination or mock infected for the IPNV-infection were calculated. Third, folds were calculated by the $2^{-\Delta\Delta Ct}$ method [22], where $\Delta\Delta Ct_{gene} = \Delta Ct_{gene} -$ mean $\Delta Ct_{control}$. Finally, the mean and their standard deviations for each gene from 3 trout were calculated and represented. When ratios between IPNV-infected and VP2-vaccinated trout values were calculated (i.e.: Fig. 4), the corresponding standard deviations were derived by following the formula, square root of the sum of the squares of each of their standard deviations (User Bulletin #2: ABI PRISM 7700 sequence detection system, PE Applied Biosystems).

2.8. Statistical analysis

Prior to statistical analyses, the normal distribution of the data was checked and confirmed using the Shapiro–Wilk test. Data are presented as mean ± standard deviation of 3 trout. Factorial ANOVAs were run to determine if the differential expression gene differed between the replicates at an individual gene followed by Tukey's multiple comparison test for differences between the vaccinated group and IPNV-infected group. The Student *t* test was used also to compare some paired samples. All statistics were run in SPSS Version 15. The *p* value which was less than 0.05 was considered to be significant.

3. Results

3.1. Selection of trout genes

To compare the differential expression of transcripts induced by the oral VP2-vaccination with those induced by IPNV-infection, some of the genes previously found with differential expression >2-fold in oral VP2-vaccination and/or induced during IPNV-infection of trout were chosen. The genes selected could be distributed in 3 groups, (i) those belonging to interferon-related pathways such as *stat1* (an intermediary *ifn* responsive transcriptional protein) [23], *ifn1* [24], *ifng* [25], *mx1* [26] and *mx3* [27–29], (ii) those related to cytokines/interleukines such as *il8* [30], *il10* [31], *il11* [32], the p40 b chain of the *il12* heterodimer [33] and *tnf2* (similar to *tnfa*) [34] and (iii) those related to adaptive immune responses such as *mhc1uda* [35], *igm* [36] and *igt* [37]. The *mhc1* corresponding to the α chain sequence of the UDA-HC allele was chosen because previous microarray data showed to be regulated in most trout of the population used for the assay [13]. Table 1 shows the corresponding primer sequences designed for the RTqPCR analysis of those genes.

3.2. Time course of transcript differential expression after oral VP2-vaccination

To select for the optimal time to make the organ comparisons and to study whether the changes in differential transcript levels after VP2-vaccination were time-dependent, those were comparatively studied in head kidney (HK) and thymus (T) at different

Table 1
List of primer pairs designed for gene expression analysis by RTqPCR.

Genes	Primer sequence 5′–3′: forward reverse	Accession number
Interferon -related		
stat1	TTGAGAGCATCGACTGGGAAAA GGCTA GGAGGTCATGGAAACGT	U60332.1
ifn1	AAAACTGTTTGATGGGAATATGAAA CGTTTCAGTCTCCTCTCAGGTT	NM_001124531
ifng	CTGAAAGTCCACTATAAGATCTCCA CCCTGGACTGTGGTGTCAC	FM864345.1
mx1	AGCTCAAACGCCTGATGAAG ACCCCA CTGAAACACACCTG	NM_001171901
mx3	AGCTCAAACGCCTGATGAAG TGAATAT GTCTGTTATCCTCCCAAA	U47946.3
Cytokines /interleukines		
il8	GAATGTCAGCCAGCCTTGTC TCCAGAC AAATCTCCTGACCG	AJ279069
il10	CGACTTTAAATCTCCCATCGAC GCATT GGACGATCTCTTTCTT	AB118099
il11	TGCGCTGCAGAGGAGCAAGT TGCTGGA GACCCCAAGCACA	AJ535687.1
il12b	ATGTGGTTACGGGAGGC ATGTGGTTA CGGGAGGC	AJ548830.1
tnf2	TGCTGCTCCATGTGTGGTGC AGGGACG GGGAGCCTTGAT	DQ218473.1
Adaptive response		
mhc1uda-hc	GCAACCCAATTTCATGCAGG ACACTCA ATGCAGGTCTGGG	EU036638.1
igm	ACCTTAACCAGCCGAAAGGG TGTCCCA TTGCTCCAGTCC	X65263.1
igt	AGCACCAGGGTGAAACCA GCGGTGGG TTCAGAGTCA	AY870265
House keeping gene		
ef1a	GATCCAGAAGGAGGTCACCA TTACGT TCGACCTTCCATCC	AF498320

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times after vaccination. Fig. 1 shows that differential transcript levels in T were higher than in HK at all the time points studied, for most of the selected genes except for *ifn1* which showed a similar evolution in both organs.

Not only the magnitude but also the timing of transcript induction was different in HK and T. Thus, in HK, the VP2-vaccination induced significant increases in *ifn1* gene differential expression from the 3rd to the 10th day post-vaccination, peaking at day 7 with values of ~45 fold. The *ifn1*-related genes *mx1*, *mx3*, and *stat1* had smaller but significant increased differential expression levels, <10 fold and occurring between 5 and 7 days post-vaccination (Fig. 1A). Interestingly, differential expression of *ifng* was also detected early and up-regulated from 5 to 10 days post-vaccination although with ~8–9 folds. The interleukines and the rest of genes except *igt* were also detected and showed 4–6-fold increase of differential expression from 5 to 10 days post-vaccination. Fifteen days post-vaccination, nearly basal levels of differential expression were observed for all the genes studied.

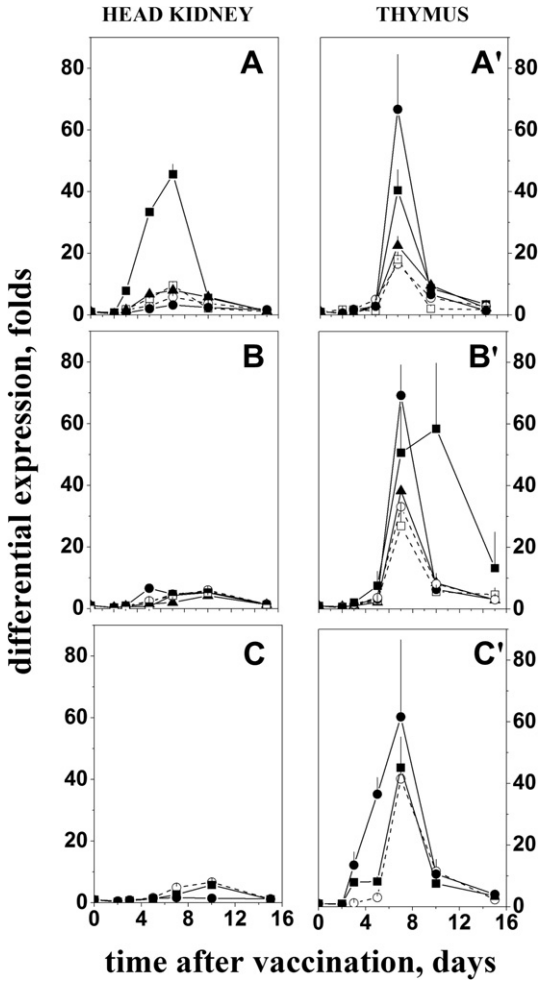

Fig. 1. Comparison of time-course of differential expression folds of selected genes in trout head kidney (HK) and thymus (T) after oral VP2-vaccination. Rainbow trout were vaccinated with pDNA-VP2 or mock vaccinated with pDNA (methods). The gene differential expression was evaluated in HK and T during 2, 3, 5, 7, 10 and 15 days after immunization. The primers described in Table 1 were used for the RTqPCR as described in methods. The differential expression of target genes was normalised to the endogenous *ef1a* gene and folds were then calculated by the $2^{-\Delta\Delta Ct}$ method. The figure shows the mean and standard deviations (sd) of folds from different trout ($n = 3$). Only the maximal sd was drawn for clarity. When compared to time 0, most of the data were significantly different at the $p < 0.05$ level, except for all genes after 2 and 15 days, *mx3* at day 3, *igt* at days 5 and 10 and *il11* and *il12b* at day 3 in HK or for most genes after 2 days and *ifn1* and *mx3* at day 3 in T. A, A′, interferon-related genes: ●, *stat1*; ■, *ifn1*; ▲, *ifng*; ○, *mx1*; □, *mx3*. B, B′, cytokines/interleukins-related genes: ●, *il8*; ■, *il10*; ▲, *il11*; ○, *il12b*; □, *tnf2*. C, C′, adaptive response-related genes: ●, *igt*; ■, *igm*; ○, *mhc*.

In T significant differences of transcript differential expression levels were recorded at the different time points for most of the genes. Differential expression of *ifn*-related genes began to increase at day 5 (2–5 fold), peaked on day 7 with values ranging from ~16 fold (*mx3*) to 67 fold (*stat1*) and declined at day 10 (2–8 fold, *mx3* and *ifn1*, respectively) (Fig. 1A′). The cytokines/interleukines-related genes showed high differential expression 7 days post-vaccination (27–60 fold), with changes elevating from the 5 day post-vaccination, declining at 10 day and remaining significantly increased by day 15 (3–4 fold). Exceptionally, only *il12b* had a higher fold increased differential expression on day 15 (~13 fold, Fig. 1B′ bold squares).

With respect to the adaptive response-related genes, their time-course differential expression was slightly different, as they began to increase earlier, at 3 days post-vaccination, peaked on day 7 (42- to 61-fold) and declined by day 15 to 3–4 fold values. The greatest up-regulation values recorded were 67-fold for *stat1* and 61-fold for *igt* (Fig. 1A′ and C′, respectively).

Maximal differential expression levels in both HK (Fig. 1A) and T (Fig. 1A′) were obtained 7 days post-vaccination for all the interferon-related transcripts. Maximal differential expression levels were obtained not only at 7 days post-vaccination for *il10* in HK and *il8*, *il11* and *tnf2* in T, but also at 10 days post-vaccination for *il8*, *il12b* and *tnf2* in HK (Fig. 1B) and *il12b* in T (Fig. 1B′). Finally, maximal differential expression levels were obtained 10 days post-vaccination for *mhc1uda*, *igm* and *igt* in HK (Fig. 1C) or 7 days post-vaccination for *igt*, *mx*, *ifn1*, *ifn g*, *stat* and *tnf* in T (Fig. 1C′). Therefore, 7 days post-vaccination was chosen as the optimal time to further study the organ differential expression profiles of the selected genes.

3.3. Differential expression of selected genes in trout organs 7 days post-vaccination

The distribution of differential transcript levels of the genes mentioned above was then studied in rainbow trout head kidney (HK), spleen (SP), intestine (IN), pyloric ceca (PC) and thymus (T), 7 days after oral VP2-vaccination.

The highest increased differential expression levels of all the organs studied were obtained in T in all the selected genes (16–70-fold) (Fig. 2, yellow bars). Only HK *ifn1* (Fig. 2, white bar) and IN *igt* (Fig. 2, hatched bar) showed >20-fold increased levels, while SP *stat1*, *il11*, *il12b*, *mhc1uda* and *igm* showed more than 10-fold increased levels. After the T, HK showed higher differential expression levels in most of the genes in the interferon group while SP showed higher differential expression levels in the cytokines/interleukines group. Most of the downregulated and/or unchanged transcripts (folds <2 and folds ±1.9, respectively), were found in either IN (*ifn1*, *ifng*, *mx1*, *mx3*, *il8*) or PC (*ifn1*, *ifng*, *mx1*, *mx3*, *il8*, *il10*, *tnf2*, *mhc1uda*) (Fig. 2).

3.4. Comparative kinetics of HK gene differential expression between IPNV-infected and VP2-vaccinated trout

To compare transcript differential expression levels in VP2-vaccinated trout with those in IPNV-infected trout, differential expression of the selected genes was first studied at different times after immunization. Because HK was a target organ of IPNV multiplication and also the main trout internal immunological responsive organ, it was chosen to comparatively optimize the time to study organ distribution of transcript levels up to 7 days (Fig. 3).

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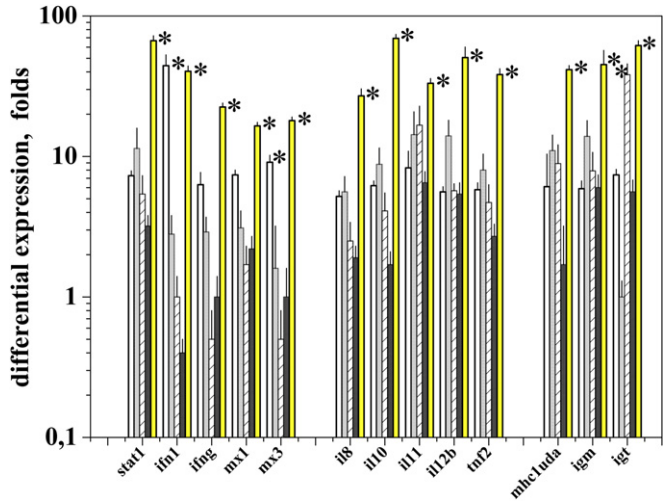

Fig. 2. Differential expression folds of selected genes in trout organs, 7 days after oral VP2-vaccination. Rainbow trout were vaccinated with pDNA-VP2 or mock vaccinated with pDNA. Organs were harvested 7 days after immunization. The trout selected primers described in Table 1 were used to estimate differential transcript levels in head kidney (HK) and spleen (SP) (internal organs) and pyloric ceca (PC), intestine (IN) and thymus (T) (surface exposed tissues). Assay conditions were as described in methods. The expression of target genes was normalised to the endogenous *ef1a* gene and folds were then calculated by the $2^{-\Delta\Delta Ct}$ method. The figure shows the mean and standard deviations of folds from different trout ($n = 3$). Only the maximal sd was drawn for clarity. HK, open bars. SP, light grey bars. PC, hatched bars. IN, black bars. T, yellow bars. *, gene folds >10 and significantly different to the rest of their corresponding organs at the $p < 0.05$ level.

Longer time points could not be studied because mortalities in the IPNV-infected group appeared after 7 days.

In HK from IPNV-infected trout, maximal levels increased with time up to more than 250-fold in *ifng* and *igm* transcripts while *ifn1*, *mx1*, *il8*, *il10*, *il12b*, and *igt* reached maximal levels in the 100–150-fold range. In the IPNV-infected trout, fold differential expression levels of around 10 or more were detected as early as 2 days for most of the selected genes, while in VP2-vaccinated trout, increased levels were most evident after 5 days. Although differences between transcript levels from IPNV-infected and VP2-vaccinated trout varied with time, differential expressions were generally 5–60-fold higher in IPNV-infected than in VP2-vaccinated trout except for *ifn1* which were only 2–3-fold higher, or for *igm/igt* which were 100–260/10–100-fold higher, respectively. Another exception was the peak differential expression of *il12b* in IPNV-infected trout after 5 days, which was almost 200-fold higher than in VP2-vaccinated fish. However, in all the genes studied, profiles after VP2-vaccination paraleled profiles after IPNV-infection. Because most of the data showed increasing levels of differential expression with time until reaching a plateau 5–7 days after immunization, 7 days was chosen to study their respective profiles of transcript levels in several organs.

3.5. Comparison of transcript expression of selected genes in different organs 7 days post-vaccination

Since we were interested in studying any differences between organs from either IPNV-infected or VP2-vaccinated trout, we first compared the levels of transcripts (values obtained after normalization) from either IPNV-infected or VP2-vaccinated trout. Since once normalized, the time profiles of HK expression levels (data not shown) were very similar to those of differential expression fold values (Fig. 3), we chose 7 days to make the comparison across different organs. Table 2 shows that IPNV-infected trout levels were up-regulated >6 fold in all studied organs for interferons (*ifn1* and *ifng*), *il8* and *igm*. Among the organs, HK showed the higher up-regulation, with normalized values >6 for *stat1*, *ifn1*, *ifng*, *mx1*, *il8*, *il10*, *il12b*, *mhc1uda*, *igm* and *igt* (all the studied genes except for *mx3*, *il11* and *tnf2*). Highest levels were 57.88–2304.12 for *il8* and up to 103.97 for *ifn1* or 236.39 for *igm*. In contrast, VP2-vaccinated trout transcript levels were less up-regulated than the corresponding IPNV-infected organs and only >30 for *il8* in all organs. Also, it was T, rather than HK, which showed levels >2 for *stat1*, *ifn1*, *ifng*, *mx1*, *il8*, *il12b*, *mhc1uda* and *igm* (all the studied genes except for *mx3*, *il11* and *tnf2* plus *il10* and *igt*). In this case, highest levels were 238.03 for *il8* and 45.25 for *ifn1* (Table 2).

We then compared the transcripts from IPNV-infected trout to those with mock-infected control on the one hand and from VP2-vaccinated trout with empty plasmid-vaccinated trout, on the other hand (differential transcripts or folds). Ratios between the folds from IPNV-infected and VP2-vaccinated trout were then calculated for each organ and plotted to make the comparison easier to follow. Thus, Fig. 4 shows that all transcript levels corresponding to interferon-related genes (*stat1*, *ifn1 ifng*, *mx1* and *mx3*) were 5–100-fold higher in IPNV-infected trout than in vaccinated trout in HK, SP or IN (all the internal organs). In contrast, all of those genes had lower or similar ratios in T or some of them in PC (external organs) (Fig. 4). Similarly, ratios of genes in the cytokines/interleukines group (*il8*, *il10*, *il11*, *il12b* and *tnf2*) or the adaptive response genes (*mhc1uda*, *igm* and *igt*) had higher ratios in HK, SP and IN and lower/similar ratios in T or some of them in PC (Fig. 4).

3.6. Transcript levels of VP2 in IPNV-infected and VP2-vaccinated trout

To help with the interpretation of the results obtained in IPNV-infected and VP2-vaccinated trout, the levels of the IPNV or the VP2-vaccine in HK were indirectly determined during several days by estimating the VP2 expression in each case. Time-course experiments revealed that in the IPNV-infected trout the level of VP2 peaked 7 days post-infection while in the VP2-vaccinated trout, the VP2 expression level was maintained from 2 to 15 days post immunization (results not shown). The VP2 expression induced by the IPNV-infection or the VP2-vaccine was also determined in organs 7 days after immunization or infection. Table 3 shows that depending on the organ, VP2 transcript expression was 10–100-fold higher in IPNV-infected trout than in VP2-vaccinated trout. Furthermore, VP2 transcript expression was highest in HK and IN than in the rest of the organs, while in VP2-vaccinated trout, VP2 levels were similar for all the organs.

4. Discussion

The results showed that in rainbow trout the oral VP2-vaccine encapsulated in alginate microspheres, could mimic most of the transcriptional profiles of differential expression induced by IPNV-infection both in organs and in timing. Not much was previously known concerning the organ distribution, degradation, persistence or responses after oral administration of pDNA since these vaccines are yet scarcely explored.

Several observations on the organ distribution of the plasmid DNA (pDNA) molecule had been reported for genetic vaccines after intramuscular administration. For instance, in Atlantic salmon, intramuscularly injected pDNA was transported into the blood plasma and was able to reach other organs (such as HK, SP and gills), suggesting that pDNA avoids degradation at the administration site and distributes through the internal and external organs [38]. It appeared that HK was preferentially acting as a scavenger tissue, clearing the pDNA from blood circulation. Our previous

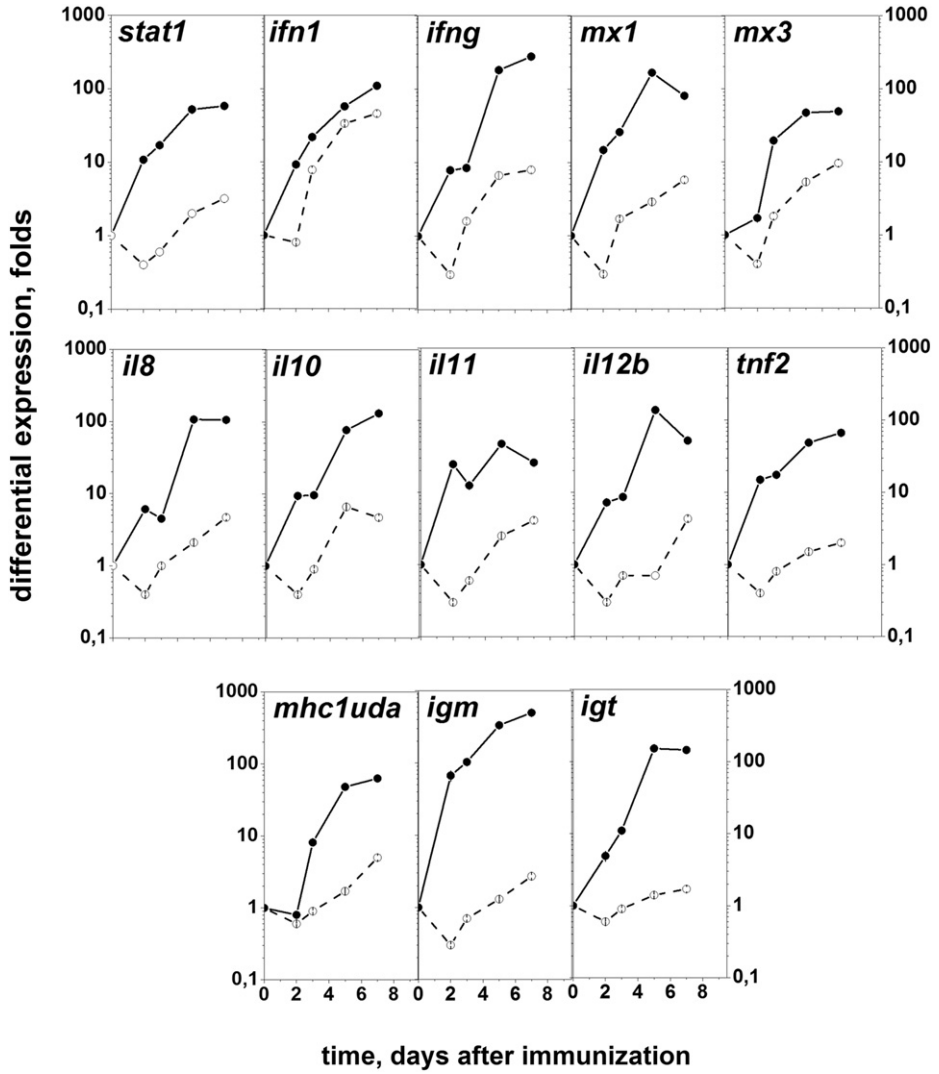

Fig. 3. Time course of differential expression folds of selected genes in head kidney (HK) from IPNV-infected and VP2-vaccinated trout. Assay conditions and analysis were as described in Fig. 1. The time course was followed up to 7 days, at which time mortalities appeared in the IPNV-infected group. The figure shows the mean and standard deviations of folds from different trout ($n = 3$). Only the maximal sd was drawn for clarity. ●, HK transcripts from IPNV-infected trout; ○, HK transcripts from VP2-vaccinated trout. When compared to time 0, most of the data were significantly different at the $p < 0.05$ level, except for *mhc1uda* at 2 days.

results demonstrated that VP2 transcripts were expressed in different organs early after oral vaccination of trout with pDNA-VP2 (VP2-vaccine) encapsulated in alginate microspheres [11]. This oral vaccine induced a wide range of innate and specific immune responses, as well as a high level of protection [11,39]. The VP2 transcript expressed in organs after their passing through the digestive tract was able to stimulate numerous immune-related genes in HK and less in PC. To further study those effects, we selected several representative trout genes from our previous work to be further studied (*stat1*, *ifn1*, *ifng*, *mx1*, *mx3*, *il8*, *il10*, *il11*, *il12b*, *tnf2*, *mhc1uda*, *igm* and *igt*) by RTqPCR analysis of their kinetics and transcript changes in several trout organs (head kidney, spleen, intestine, pyloric ceca, and thymus) after oral VP2-vaccination. Differences of magnitude and timing of the corresponding transcripts were also determined for IPNV-infected trout.

One of the most significant findings for most genes was the unexpected high levels of transcripts induced after VP2-vaccination in thymus (T) compared to the rest of the organs (Fig. 2). T highest transcript levels could be explained because T should be one of the first tissues to come in contact with the VP2-vaccine even when the DNA is still tightly packaged by the alginate microspheres. However, VP2 expression was higher in IN and PC than in T after VP2-vaccination (Table 3), making the above mentioned explanation less likely. Alternatively, IN could be one of the first target tissues for the VP2-vaccine. In this former case, the T high transcript levels could be explained as responses to signals released from other VP2-targeted internal organs, such as HK and SP. Such hypothesis seems to be confirmed by the earlier transcript differential expression in HK (5 days) than in T (7 days), at least for *ifn1* (Fig. 1A) and *il10* (Fig. 1B).

Another important observation was that the time-course differential expression of most of the genes in HK in VP2-vaccinated trout paraleled those from IPNV-infected trout, although at 10–100-fold lower levels (Fig. 3). An exception to those differences was *ifn1*, which showed similar levels in IPNV-infected and VP2-vaccinated trout at all the time points. Would those *ifn1* levels be sufficient to get the high protection obtained by VP2-vaccination? Since ~80% relative percent survival (rps) after 30 days of oral VP2-vaccination of both brown and rainbow trout was described before [11], the increase of the differential expression levels of some or all of the genes studied might be still required to obtain either higher rps and/or duration of the protection. Further

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Table 2
Normalized values of selected genes in IPNV-infected and VP2-vaccinated rainbow trout 7 days after immunization by RTqPCR.

Gen	Organ	IPNV-infected	VP2-vaccinated
		Mean ± sd	Mean ± sd
stat1	Head Kidney	14.98 ± 0.44	*2.16 ± 0.01
	Spleen	1.33 ± 0.01	0.77 ± 0.12
	Intestine	3.75 ± 0.03	*0.71 ± 0.08
	Pyloric ceca	0.13 ± 0.00	0.36 ± 0.05
	Thymus	0.28 ± 0.00	*3.58 ± 0.03
ifn1	Head Kidney	30.17 ± 1.68	*9.99 ± 0.19
	Spleen	45.89 ± 0.17	*1.02 ± 0.16
	Intestine	103.97 ± 0.57	*2.16 ± 0.26
	Pyloric ceca	6.30 ± 0.02	*0.91 ± 0.13
	Thymus	11.20 ± 0.24	*45.25 ± 0.42
ifng	Head Kidney	74.29 ± 0.15	*1.66 ± 0.09
	Spleen	35.51 ± 0.38	*1.02 ± 0.16
	Intestine	100.80 ± 1.32	*0.51 ± 0.06
	Pyloric ceca	7.59 ± 0.02	*0.80 ± 0.11
	Thymus	9.92 ± 0.07	9.22 ± 0.06
mx1	Head Kidney	35.88 ± 0.36	*3.23 ± 0.02
	Spleen	2.41 ± 0.01	*0.33 ± 0.05
	Intestine	5.66 ± 0.03	*0.64 ± 0.07
	Pyloric ceca	0.75 ± 0.01	0.88 ± 0.12
	Thymus	0.56 ± 0.00	*2.79 ± 0.01
mx3	Head Kidney	0.67 ± 0.77	0.12 ± 0.15
	Spleen	0.20 ± 0.00	*0.01 ± 0.00
	Intestine	0.48 ± 0.00	*0.01 ± 0.00
	Pyloric ceca	0.79 ± 0.00	*0.13 ± 0.00
	Thymus	0.07 ± 0.00	0.15 ± 0.00
il8	Head Kidney	2304.12 ± 61.84	*117.38 ± 1.19
	Spleen	186.75 ± 0.79	*37.27 ± 5.83
	Intestine	621.67 ± 7.76	*44.94 ± 5.42
	Pyloric ceca	29.75 ± 0.10	33.13 ± 4.76
	Thymus	57.88 ± 1.07	*238.03 ± 2.90
il10	Head Kidney	7.39 ± 0.10	*0.33 ± 0.00
	Spleen	0.42 ± 0.00	*0.05 ± 0.00
	Intestine	1.40 ± 0.03	0.27 ± 0.03
	Pyloric ceca	0.05 ± 0.00	0.10 ± 0.01
	Thymus	0.15 ± 0.00	*0.57 ± 0.00
il11	Head Kidney	0.33 ± 0.01	0.03 ± 0.00
	Spleen	0.01 ± 0.00	0.00 ± 0.00
	Intestine	0.04 ± 0.00	0.01 ± 0.00
	Pyloric ceca	0.00 ± 0.00	0.01 ± 0.00
	Thymus	0.00 ± 0.00	0.01 ± 0.00
il12b	Head Kidney	15.30 ± 0.46	*1.82 ± 0.01
	Spleen	0.50 ± 0.00	0.50 ± 0.07
	Intestine	1.16 ± 0.07	0.56 ± 0.06
	Pyloric ceca	0.38 ± 0.00	0.41 ± 0.06
	Thymus	1.07 ± 0.02	*3.20 ± 0.04
tnf2	Head Kidney	2.02 ± 0.24	*0.18 ± 0.00
	Spleen	0.02 ± 0.00	0.02 ± 0.00
	Intestine	0.06 ± 0.00	0.03 ± 0.00
	Pyloric ceca	0.07 ± 0.00	0.02 ± 0.00
	Thymus	0.01 ± 0.00	0.11 ± 0.00
mhc1uda	Head Kidney	16.17 ± 0.15	*1.56 ± 0.11
	Spleen	2.50 ± 0.01	*0.53 ± 0.08
	Intestine	6.48 ± 0.04	*1.06 ± 0.12
	Pyloric ceca	0.45 ± 0.00	*0.18 ± 0.03
	Thymus	0.69 ± 0.00	*2.72 ± 0.01
igm	Head Kidney	236.39 ± 4.20	*6.08 ± 0.08
	Spleen	98.70 ± 0.57	*12.42 ± 1.94
	Intestine	228.33 ± 2.23	*2.44 ± 0.29
	Pyloric ceca	19.84 ± 0.07	*1.78 ± 0.25
	Thymus	21.04 ± 0.33	*7.94 ± 0.20
igt	Head Kidney	54.38 ± 1.47	*3.02 ± 0.02
	Spleen	0.72 ± 0.01	*0.05 ± 0.00
	Intestine	4.03 ± 0.07	*0.26 ± 0.00
	Pyloric ceca	0.35 ± 0.01	0.03 ± 0.00
	Thymus	0.21 ± 0.00	0.31 ± 0.00

The normalized values to *ef1a* were calculated by the $2^{-\Delta CT}$ method, according to the formula, $\Delta Ct = Ct\ gene\text{-}Ct_{ef1a}$. 0.00 < 0.01.
*, significantly different when compared to their corresponding infected organ at the $p < 0.05$ level.

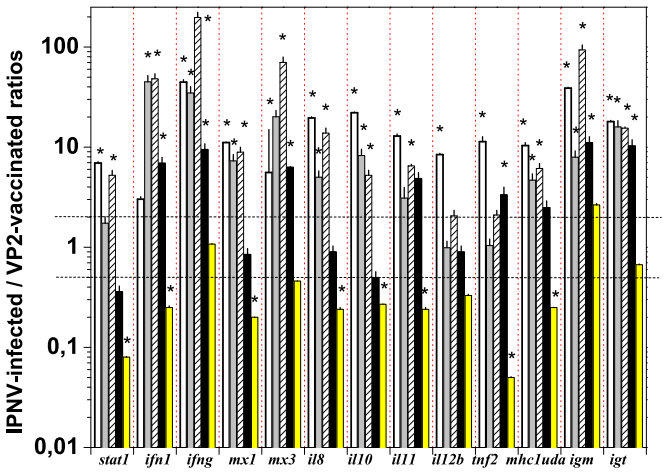

Fig. 4. Comparison of differential expression folds of selected genes in organs from IPNV-infected and VP2-vaccinated trout. Rainbow trout were infected with IPNV by 2 h immersion in 3×10^5 TCID/ml (mock infected with PBS) or orally vaccinated with pDNA-VP2 (mock vaccinated with pDNA) and their corresponding organs/tissues harvested 7 days later. The trout selected primers described in Table 1 were used to estimate differential transcript levels in different organs. Means of differential expression folds and their standard deviations were calculated for each of the organs from either IPNV-infected or VP2-vaccinated trout groups ($n = 3$, for each group) as indicated in methods. Ratios represented in the figure were calculated by the formula, folds from IPNV-infected trout/folds from VP2-vaccinated trout. Standard deviations (sd) were calculated as indicated in methods. Only the maximal sd were drawn for clarity. *, ratios significantly different from 2 or 0.5 at the $p < 0.05$ level. Horizontal dashed lines were drawn for ratios = 2 or 0.5. Red vertical dashed lines were drawn to separate genes. HK, open bars. SP, light grey bars. PC, hatched bars. IN, black bars. T, yellow bars.

work will clarify that possibility. It was also observed than a small but significant down-regulation occurred 2 days after oral vaccination in most of the studied genes (Fig. 3). It is most probable that the stress caused by the manipulation of trout during the artificial oral delivery method (a 20 µl tip introduced into the mouth–oesophagus of each trout) might have caused those effects.

Among the responses studied, several genes belonged to the IFN system. It is well known that the type I IFN system is involved in the first line of defense against viral infections and many reports have described its multiple functions in innate and adaptive immune responses. Their antiviral effect is acting through specific cell-surface receptors which trigger the JAK–STAT signal transduction pathway and a large number of genes known as IFN-stimulated genes (ISGs). Some of these genes encode antiviral proteins, such as Mx [40]. Thus, the salmon Mx protein has been shown to directly inhibit IPNV protein synthesis [29] or decrease viral infective titres

Table 3
Distribution of VP2 transcript relative values in organs from IPNV-infected and VP2-vaccinated rainbow trout.

Organ/tissue	IPNV-infected	VP2-vaccinated
	Mean relative value ± sd	Mean relative value ± sd
Head kidney	1961.46 ± 45.25	0.97 ± 0.08*
Spleen	80.32 ± 3.48	1.47 ± 0.03*
Intestine	865.69 ± 148.79	7.74 ± 0.33*
Pyloric ceca	47.27 ± 5.85	5.99 ± 0.32*
Thymus	24.30 ± 2.29	3.30 ± 0.04*

Rainbow trout were infected with IPNV by 2 h immersion in 3×10^5 $TCID_{50}$/ml. Relative values ($2^{-\Delta Ct}$) were multiplied by 10,000 to facilitate tabulation of the data. Means and standard deviations of VP2 relative values from different trout ($n = 3$) are represented.
*, significantly different when compared to their corresponding infected organ at the $p < 0.05$ level.

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of either IPNV or IHNV [41]. In the present work, both *ifn1* and *ifng* were induced at high levels in SP, IN and PC in IPNV-infected trout while levels were also higher in T upon VP2-vaccination (Table 2). Furthermore, all the levels of IFN-related genes (*stat1*, *ifn1*, *ifng*, *mx1* and *mx3*) could be mimicked (ratios of IPNV-infected/VP2-vaccinated <1) in T by VP2-vaccination (Fig. 4), thus suggesting that the success of oral VP2-vaccination might be due to its capacity to induce such a response.

On the other hand, differential expression of IFNs and their induced genes have been reported in Atlantic salmon or trout [42,43] upon infection with IPNV. However, the virus could counteract those innate host defences as infection progressed. Thus, Skjesol et al. [44] reported on the ability of IPNV to redirect those initial cellular processes in favour of virus propagation while avoiding cellular antiviral responses and suggested VP4 and VP5 as candidate molecules to counteract the IFN response. These two proteins reduced the initial IFN-induced differential expression by acting on the *mx* promoter. The question might be relevant for the next generation of IPNV vaccines. Thus, in the light of our results with the oral VP2-vaccine, would it be better to avoid combination of VP2 with plasmids containing VP3 or VP4 to maintain an efficient immune defence system? Alternatively, would it be necessary to assay new vaccines with other immune genes looking for synergistic effects with the VP2?

Kinetic data reported after intraperitoneally injected IPNV showed an initial *stat1* up-regulation up to 4 days, as similarly estimated in this work (Fig. 3), which was later downregulated when the IPNV began to replicate [23]. High levels could be also induced in HK and T by VP2-vaccination when compared to IPNV-infection, suggesting that such an increase in this transcriptional regulator of the type I IFN system could be important to induce VP2-vaccine protection.

There is little information regarding pro- and anti-inflammatory cytokines on fish and their relation to viral infection and/or vaccination. It is known that recombinant trout *il8* attracts neutrophils [45] and it was induced by injected rhabdoviral DNA vaccines [30], while *il10* is a Th2-cytokine [25] suppressing excessive inflammatory responses [46] in response to *tnfa* [47]. Of all the genes examined in this work, *il8* showed the highest differential expression levels in all the organs, both in the IPNV-infected and the VP2-vaccinated trout (Table 2). Thus, up-regulated levels expressed as normalised relative values were from 57-fold in T to 2304-fold in HK in IPNV-infected trout, corresponding to 238-fold in T or 117-fold in HK in VP2-vaccinated trout (Table 2). In contrast, the anti-inflammatory *il10* had low differential expression levels in both the IPNV-infected (except in HK) and VP2-vaccinated trout. Reyes-Cerpa et al. [48] studying several interleukines during acute and persistent IPNV-infection of Atlantic salmon, found that the increased differential expression of *il10*, accompanied by the absence of induction of *il1b* and *il8*, indicates that IPNV triggers an anti-inflammatory response that may be part of the mechanisms to establish their persistence. According to our results, the levels of *il8* and *il10* in IPNV-infected fish were mimicked in VP2-vaccinated trout, although at lower levels and therefore in either case persistence seems to be avoided. Further studies are required to elucidate if up-regulation of *il8* and down regulation of *il10* could inhibit IPNV persistence and thus inhibit the establishment of an IPNV carrier state. This information would be also valuable for improvement of future vaccines against IPNV.

Bartee et al. [49] reported that synergy between *tnf* and *ifn* occurs mainly at the level of gene transcription, suggesting they could activate signal transduction pathways and transcription factors. *Tnf2* is an important pro-inflammatory cytokine, similar to *tnfa* [34] which is induced after viral infection [50], but not by killed virus or isolated viral proteins unless *ifng* is also provided [51]. If important for protection, however only in SP and T the *tnf2* levels obtained upon IPNV-infection could be mimicked by VP2-vaccination, perhaps showing a point in which oral vaccination could be improved.

Increased *il11* and *il12b* transcripts were found in the HK upon IPNV-infection. *Il12b* is one of the heterodimer chains of a Th1-cytokine also known in mammals as natural killer cell stimulatory factor 2 produced in macrophages, monocytes, dendritic cells and B lymphocytes in response to intracellular pathogens [52]. *Il12* has been used as vaccine adjuvant to increase protective mucosal immunity [53,54], but it has not been characterized nor tested in fish. Only in T could the transcript levels of *il11/il12b* be induced by the VP2-vaccine to higher fold levels than in IPNV-infection and therefore these interleukines might deserve further studies.

MHC-I molecules are involved in cell-mediated immunity by participating in antigen processing and presentation after viral infection and they are important for host antiviral immunity by regulating natural killer cell activity [55]. *Mhc1* was up-regulated after intramuscular injection of DNA vaccines to IPNV [56] and also in T after oral VP2-vaccination (Fig. 2C′). The levels of both *igm* and *igt* after IPNV-infection were 100–300-fold higher than the levels induced by the VP2-vaccine in HK (Fig. 3). However it is interesting to point out that *igm* and *igt* showed high levels of differential expression in IN and T after 7 days of vaccination (Fig. 2). The discovery of *igt* in trout [57] has changed the paradigm that *igm* was the only immunoglobulin class responding to antigenic challenge both in systemic and mucosal compartments. More recent work reveals that rainbow trout *igt* is an immunoglobulin specialized in mucosal immune responses [37]. With respect to the DNA vaccines many intriguing questions remain to be answered such as the immunoglobulin repertoires in systemic and mucosal sites of naïve and vaccinated fish or how *igt* and *igm* induction may be modulated by oral or intramuscular delivery. These deeper studies on mucosal responses are being designed for future work. In the rest of the organs similar levels of *igm*/*igt* were obtained (Fig. 4). Although IPNV neutralizing antibodies were demonstrated in the sera of VP2-vaccinated trout [11], whether those transcript levels correspond to specific antibodies or not will have to be demonstrated.

Differential expression of VP2 transcripts induced by VP2-vaccination was high and mimicked those caused by IPNV-infection even thought their levels of differential expression were lower. Thus, there is still room for improvement of oral VP2-vaccines by investigating ways to increase the present VP2 expression levels. Perhaps such an improvement could raise the efficacy of the VP2-vaccine in terms of both survival (>80%) and duration (>30 days). In this regard, we have defined some of the gene markers (i.e.: *ifn1*, *mx1*, *mx3*, *tnf2*, *igm*, *igt*) whose expression could be followed during future oral vaccine formulation development (DNA-vaccine dose, inclusion of other DNA delivery agents, synergy with plasmid adjuvants, etc).

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