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Oral immunization of rainbow trout to infectious pancreatic necrosis virus (Ipnv) induces different immune gene expression profiles in head kidney and pyloric ceca

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ABSTRACT

Induction of neutralizing antibodies and protection by oral vaccination with DNA-alginates of rainbow trout *Oncorhynchus mykiss* against infectious pancreatic necrosis virus (IPNV) was recently reported [1]. Because orally induced immune response transcript gene profiles had not been described yet neither in fish, nor after IPNV vaccination, we studied them in head kidney (an immune response internal organ) and a vaccine entry tissue (pyloric ceca). By using an oligo microarray enriched in immune-related genes validated by RTqPCR, the number of increased transcripts in head kidney was higher than in pyloric ceca while the number of decreased transcripts was higher in pyloric ceca than in head kidney. Confirming previous reports on intramuscular DNA vaccination or viral infection, *mx* genes increased their transcription in head kidney. Other transcript responses such as those corresponding to interferons, their receptors and induced proteins ($n = 91$ genes), VHSV-induced genes ($n = 25$), macrophage-related genes ($n = 125$), complement component genes ($n = 176$), toll-like receptors ($n = 31$), tumor necrosis factors ($n = 32$), chemokines and their receptors ($n = 121$), interleukines and their receptors ($n = 119$), antimicrobial peptides ($n = 59$), and cluster differentiation antigens ($n = 58$) showed a contrasting and often complementary behavior when head kidney and pyloric ceca were compared. For instance, classical complement component transcripts increased in head kidney while only alternative pathway transcripts increased in pyloric ceca, different β-defensins increased in head kidney but remained constant in pyloric ceca. The identification of new gene markers on head kidney/pyloric ceca could be used to follow up and/or to improve immunity during fish oral vaccination.

1. Introduction

Oral delivery is considered the most desirable way to vaccinate both humans and animals [2,3]. Nevertheless, despite many efforts to find suitable fish oral vaccination methods, there are yet few reports describing their successful use. However, recent reports using either pathogen-coding DNA in trout [1] and in Japanese flounder (*Paralichthys olivaceus*) [4] or pathogen recombinant proteins in salmon [5,6], suggest fish oral vaccination might be possible in the future.

Oral vaccination methods are needed because present oil-adjuvanted vaccines delivered by intraperitoneal injection have important side effects on fish welfare [7–9], while the present licensed DNA salmon vaccines in Canada still require fish-to-fish intramuscular injection [10]. Furthermore, small immunocompetent fish to be vaccinated cannot be injected. However, oral vaccines have many difficult-to-obtain requirements such as to be protected from stomach digestion by some antigen-encapsulation method, adhere to fish guts, avoid induction of immune tolerance or induce immune responses in both local epithelial surfaces and internal organs. Because most of those necessary immune responses are not yet known, we have focused this study on trying to clarify those. In order to do that, the recently reported successful oral immunization of rainbow trout *Oncorhynchus mykiss* against infectious pancreatic necrosis virus (IPNV) with a DNA vector coding for the VP2 capsid gene of infectious pancreatic necrosis virus (IPNV), has been used. Alginate microspheres protected the DNA, which was expressed early and late in different organs of the vaccinated trout, induced neutralizing antibodies and protected 80% of the vaccinated trout [1,11].

IPNV is an economically important *Birnaviridae* which causes severe acute lethal infections in young salmonid fish, remaining one of the most widespread causing-disease virus in aquaculture

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[12,13]. The IPNV genome consists of two double-stranded RNA segments (A and B) that encode VP1 (a RNA-dependent RNA polymerase), VP2 (the major antigenic capsid protein and type-specific antigen with a great antigenic diversity), VP3 (an internal capsid protein and group-specific antigen), NS and VP5 (non structural proteins). The accepted serotyping of IPNV includes two serogroups, the first containing nine serotypes from fish (A_1–A_9) and the second containing a single serotype (B_1) [14]. Because following a disease outbreak, surviving fish may become asymptomatic carriers for life; broodstock carriage is considered an important source of IPNV for lethal infection of hatchery-reared fry. The development of an effective vaccine is a necessity to secure the future of salmonid (salmon and trout) farms.

To study the transcriptional profile of rainbow trout organs after successful oral immunization, we used a newly designed 60-mer oligo microarray enriched in immune-related genes. We studied not only one of the most important fish internal organs involved in fish responses to infection (head kidney) but also one of the entry sites of the vaccine: the pyloric ceca (mucosal immunity). The head kidney was selected as the target internal organ because other reports have demonstrated the presence of DNA at different times after oral vaccination in Atlantic salmon [15] or brown and rainbow trout [1,11]. On the other hand, pyloric ceca could offer a novel approach to study the immunity response at the gut mucosa, which might be especially important for oral vaccination. Results confirmed the induction of *mx1* transcripts in head kidney reported earlier for this and for other immunization methods. More immune-related transcripts increased in head kidney than in pyloric ceca after oral immunization. The study contributed to clarify the immune response to oral vaccination and allowed the identification of novel genes which can be used as markers to improve oral vaccination in fish.

2. Materials and methods

2.1. Preparation of the IPNV-VP2 DNA vector

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

2.2. Preparation of microspheres and formulation of the oral vaccine

The procedure for the preparation of the microspheres was described previously [1]. Briefly, 2.5 mL of 3% (w/v) of sodium alginate were mixed with 1.5 mL of 1 mg/ml of pcDNA-VP2 and the mixture stirred at 500 rpm for 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 drop-by-drop adding 2.5 ml of 0.15 M $CaCl_2$ to the emulsion and stirring for 2 h at 900 rpm. Microspheres were then collected by centrifugation at 1000*g* for 10 min, and were washed twice with 70% ethanol, lyophilized and stored at 4 °C.

2.3. Oral vaccination of 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. Two pools of 5 fish were tested by standard methods to confirm the absence of IPNV or any other salmonid virus by isolation using BF cells [16]. 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 20 trout were maintained in separate 45 L tanks supplied with non-chlorinated water using exterior carbon filters (Eheim) and additional aeration. The trout were fed daily with a diet of commercial pellets. Trout were obtained from a unique farm but from 4 different trout populations, one population per group. Each of the 4 groups was divided into 2 subgroups of 6 trout each. First subgroup was orally vaccinated with 10 μl of suspension of the vaccine microspheres each containing 10 μg of pcDNA-VP2 diluted in 10 μl of PBS, while second subgroup received similar amounts of microspheres with pcDNA. 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 esophagus. The water-quality parameters were maintained at optimum levels and the conditions in all tanks were equal.

The trout were anaesthetized by immersing in 50 mg/mL tricaine-ethanesulfonate (MS-222, Sigma, Madrid, Spain) buffered in PBS prior to handling. After decapitation, the head kidney and pyloric ceca were harvested from each trout. The organs were immediately immersed in RNAlater (Ambion, Austin, USA) and kept at 4 °C overnight before being frozen at −70 °C until processed. Experimental protocols were performed with the approval of the CSIC ethical committee.

2.4. RNA extraction of trout head kidney and pyloric ceca

RNA was extracted from each individual trout head kidney and pyloric ceca after sonication (1 min × 3 times at 40 W in ice) in the RTL buffer and by using the RNAeasyPlus kit (Qiagen, Hilden, Germany). RNA concentrations were estimated by nanodrop and the presence of 18 and 28 S bands confirmed by denaturing RNA agar electrophoresis (Sigma, Che.Co, MS, USA). Stringent RNA quality control was performed prior to hybridization. For each group, 4 of the best quality RNA per group were pooled and further analyzed.

2.5. Design of oligo microarrays enriched in rainbow trout immune-related genes

To design the immune-related gene enriched microarray used in these experiments, rainbow trout sequences were selected from both GenBank and Agilent's EST-derived oligo microarray (ID16271) [17]. The immune-related genes were retrieved by using the following keywords: interferon, chemokine, interleukin, cytokine, defensin, macrophage, lymphocyte, antimicrobial, neutrophil, leukocyte, cytotoxic, natural killer, antiviral, antibacterial, LPS, Vig, antigen, histocompatibility, phagocyte, viral, Mx, complement, immunoglobulin, hepcidin, IgG, IgM, Toll, T cell, B cell, dendritic, presenting, TNF, perforin, MHC, NK, transcription, chaperone, stress, Hsp, Hsp70, Hsp90, tlr, flagellin, keratinocyte, cathepsin, NOD, IRF, IKK, JBK1, TRAM, TAK, TAB, JNK, P38, AP-1, TIRAP, IgT, IgH and high mobility. To simplify the analysis of results, the probes were classified according to 16 groups: AM, antimicrobial peptides; C, complement components; CD, cluster differentiation antigens; CK, chemokines; HSP, heat shock proteins; IFN, interferons; IG, immunoglobulins; IL, interleukins; MA, macrophage; MHC, major histocompatibility; MX, interferon-inducible proteins mx; TCR, T cell receptors; TLR, toll-like receptors; TNF, tumor necrosis factor; TR, transcription factors; and VIG, VHSV-induced genes. The resulting list of retrieved gene accession numbers and/or genes and sequences was formatted in excel, duplicates eliminated and the

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final list submitted to Agilent's microarray design tool (eArray application https://earray.chem.agilent.com/earray/search.do?search1/4arrayDesign). The final 8x15K Agilent design called mini-trout12.8 (ID032303) contains 6442 unique 60-mer oligo sequences each in duplicate randomly arranged in the microarray. The microarrays were synthesized *in situ* by using Agilent's non-contact inkjet technology. The final design is available for review free of charge for registered users of the Agilent eArray application (Table 1). A complete list of gene IDs and gene names is included in Gene Expression Omnibus (GEO) platform submission number GPL14155. The microarrays are available from Agilent Technologies.

2.6. Quantitation of immune-related rainbow trout transcripts by microarray hybridization

Two rainbow trout 60-mer oligo 8x15K format custom microarrays (ID032303) containing 6442 60-mer oligo sequences was obtained from Agilent. RNA was kept frozen at −80 °C until all the experiments were hybridized and processed simultaneously. Labeling of 2 μg of RNA (~50 μg/ml) and hybridization to the microarrays were performed by the NimGenetics company (Madrid, Spain) complying with the Minimum Information About a Microarray Experiment (MIAME) standards. Briefly, high quality RNA were labeled with Cy3 (Amersham Pharmacia) by using SuperScript III reverse transcriptase (InVitroGen) and oligo(dT) primer, and the resulting cDNA was purified with Microcon YM30 (Millipore). The slides were pre-treated with 1% BSA, fraction V, 5 × SSC, 0.1% SDS (30 min at 50 °C) and washed with 2 × SSC (3 min) and 0.2 × SSC (3 min) and hybridized overnight in cocktail buffer containing 1.3 × Denhardt's, 3 × SSC 0.3% SDS, 2.1 μg/μl polyadenylate and 1 μg/μl yeast tRNA. The fluorescence signal was captured, processed and segmented using an Agilent scanner (G2565B, AgilentTechnologies) by using the Agilent Feature ExtractionSoftware (v9.5) with the protocol GE1-v5_95, extended dynamic range and preprocessing by the Agilent feature extraction. Normalization within each microarray was carried out by using the sum of all the fluorescences and results deposited in the GEO bank (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE31591 and 31557). The gProccesedSignal was chosen for statistical analysis. Data was first filtered by non-uniform pixel distributed outliers and other replicate outliers (glsFeatNonUnifOL, glsBGNonUnifOL, glsFeatPopnOL and glsBGPopnOL) according to the default Agilent feature extraction criteria; ratio between processed signal and its error <2; differentiation from background signal; linear relationship between concentration and/or intensity below limits according to Spike-In information. Then biological replicate outliers were defined as values outside the mean ± standard deviation. They were masked from calculations by using a homemade program in Origin vs8.5 (Northampton, USA). After removing outliers, control values from trout immunized with control pcDNA were averaged and fold calculated by the following formula, values from trout immunized with pcDNA-VP2/mean values from trout immunized with pcDNA, $n = 4$. Outliers in the calculated folds were again eliminated before calculating the final mean and standard deviations. The Student's t one tail statistic associated p was computed from outlier-free values obtained from trout immunized with pcDNA-VP2 and trout immunized with control pcDNA for each immune-related gene. A double simultaneous criterion was used to identify differentially expressed genes: (i) genes with ratios IPNV-infected/non-infected >2 and (ii) genes which deviated from the null hypothesis using the t-test at $p < 0.05$. Calculations were made from four biological replicates of pcDNA-VP2 and pcDNA each and by two independent researchers using Origin pro vs 8.5 (Northampton, USA) and their results confronted until all discrepancies were solved.

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

RNA was extracted as described above. For RT quantitative real time PCR (RTqPCR), we primed 5 μg of total RNA with 25 pmol/μl Oligo-d(T) and used the Super Script™ II kit (Invitrogen, USA) for reverse transcription. qPCRs were then performed by using SYBR green, in an iQ5 iCycler thermal cycler (Bio-Rad Laboratories, Inc., Richmond, CA, USA). The qPCR amplifications were carried out in 96-well plates by mixing 5 μL of 20-fold diluted cDNA, 12.5 μL of 2× concentrated iQ SYBR® Green Supermix (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 and 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. The elongation factor 1α (*EF-1α*) gene was used as house keeping gene in each RNA sample in order to normalize the results to eliminate variation in mRNA/cDNA quantity and quality. No amplification product was observed in controls containing no RNA samples. All qPCR reactions were performed in triplicate and the results were expressed as mean ± standard deviation. The data obtained were analyzed using the iQ5 optical system software version 2.0 (Bio-Rad) and the

Table 1
Number of *Oncorhynchus mykiss* immune-related gene sequences in GenBank (list of probes selected by the eArray program of Agilent) and in the ID16271 microarray [17].

Short name	Groups of genes	GenBank sequences	Agilent eArray list	Previous ID16271	Final design ID32303
AM	Antimicrobials and apoptosis	44	28	30	58
C	Complement components	69	46	130	176
CD	Cluster differentiation	50	37	21	58
CK	Chemokines and receptors	92	80	41	121
HSP	Heat shock proteins	107	88	159	247
IFN	Interferons and receptors	56	46	45	91
IG	Immunoglobulins and receptors	901	814	100	914
IL	Interleukins and receptors	94	75	44	119
MA	Macrophages	41	30	95	125
MHC	Major histocompatibility complex	272	252	68	320
MX	Interferon-inducible Mx proteins	6	6	1	7
TCR	T cell receptors	119	114	6	120
TLR	Toll-like receptors	26	16	15	31
TNF	Tumor necrosis factor	39	25	7	32
TR	Transcription factors	289	210	461	671
VIG	VHSV-induced genes	37	25	1	26
0.5					
Total		2242	1892	1214	3106

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expression of target genes was calculated as relative folds of the expression of pcDNA controls according to the $2^{-\Delta\Delta CT}$ method [18].

3. Results

3.1. Design of oligo microarrays enriched in rainbow trout O. mykiss immune-related genes

The GenBank immune-related genes retrieved by using selected keywords were cured by eliminating duplicates and non-appropriated sequences to a total of 2242 sequence entries (Table 1). Those were submitted to the Agilent eArray design application to recover 1892 (84.3%) unique 60-mer oligo probes. The same keywords were also used to extract 1214 immune-related probes from the previously published EST-derived oligo microarray (ID16271) [17]. The final ID32303 design contained a total of 3106 probes, which were randomly distributed in the microarray in duplicates. Table 1 shows the number of probes classified in groups of genes obtained in the intermediate steps to get to the final version of the microarray. The probes retrieved from the GenBank resulted in an overall 2.5-fold enrichment in immune-related genes of the previous ID16271 trout microarray with only 2.4 % of repeated probes between both designs. There were a higher number of probes in the IG and TCR gene groups due to the abundant number of sequences from the variable regions which were in the GenBank. The CD, CK, IL, MX, TNF and VIG groups of genes were the groups where the enrichment was higher (Table 1).

3.2. Genes identified by hybridization to rainbow trout O. mykiss microarrays

Fig. 1 shows a comparison of the distribution of the mean of fluorescence signals reflecting transcript expression levels obtained from trout immunized with pcDNA-VP2 and pcDNA and distributed among the different gene groups in head kidney and pyloric ceca. The signal intensities ranged from 10 to 1,000,000 fluorescent relative units. The distribution of signal intensities shows that more fluorescences from head kidney were >1-fold (above the white line in the figure), than for pyloric ceca.

Fig. 2 shows the distribution of the differential expression with folds >2 (pcDNA-VP2/pcDNA) of transcripts classified by gene groups (Table 1) in both head kidney and pyloric ceca. In the head kidney, the percentage of transcripts with increased expressions was higher than the percentage of genes with decreased expressions while in pyloric ceca the percentage of transcripts with increased expressions was lower than the percentage of genes with decreased expressions except for the IFN and IL groups.

Expression levels of genes from head kidney or pyloric ceca were first selected and tabulated under highly restrictive conditions such as folds >4 and $p < 0.05$. Then, those folds of the corresponding genes of pyloric ceca or kidney, respectively, were also tabulated for comparison. The increased (+) or decreased (−) differential expression of transcripts from genes under those highly restrictive conditions is shown in the Supplementary data. Again, most of the increasing differential expression genes were found in head kidney when compared to pyloric ceca (68.8% compared to 7.8%, $n = 74$) and most of the decreasing differential expression genes were found in pyloric ceca when compared to head kidney (24.6% compared to 3.9%, $n = 74$), confirming the general tendency of the transcriptional data commented above. The genes showing more than 9-fold differential expression in head kidney were the antimicrobial peptide *leap2a*, chemokine *cxcd1*, *procathepsin B*, *il11*, transcription factor *dermo1* and the acute phase protein *pentraxin* (increased) and the chemokine *scya113* and transcription factors *jnk2* (decreased), while in pyloric ceca were the *il10* and transcription factors *lectin* and *zn503* (decreased).

A more detailed analysis of the differential expression of transcripts was undertaken group by group. Thus, Fig. 3 (interferons, their receptors and induced proteins, $n = 91$ genes), Fig. 4 (*mx*, $n = 6$ and VHSV-induced genes, $n = 25$), Fig. 5 (macrophage-related genes, $n = 125$ and complement component genes, $n = 176$), Fig. 6 (toll-like receptors, $n = 31$ and tumor necrosis factors, $n = 32$), Fig. 7 (chemokines and their receptors, $n = 121$ and interleukines and their receptors, $n = 119$), and Fig. 8 (antimicrobial peptides, $n = 59$ and cluster differentiation antigens, $n = 58$) show the main results of those groups of immune-related genes.

Head kidney 9 *ifn* gamma-inducible transcripts corresponding to 7 different genes (*i-p30*, *i-gig2*, *i-hep*, *i-p35*, *i-p58*, *i-p2*, and *i-gtp*), *ifn* gamma receptor (*ifn gr1*) and *type 1 ifn* were increased while *type 1 ifn* a, 3 and 4 were decreased. In contrast, pyloric ceca 2 *ifn* gamma-inducible and 4 *ifn* gamma transcripts (corresponding to genes *ifng1* and *ifng2*) were increased while an *ifn*-inducible protein 30 (*i-p30*) and interferon regulatory factor (*irf1*) transcripts were decreased (Fig. 3).

Head kidney, *mx1*, *2* and *3* transcripts were increased while in pyloric ceca only *mx3* was increased to a similar level than in head kidney (Fig. 4 up). No *mx*-related transcripts were decreased in any of the organs tested.

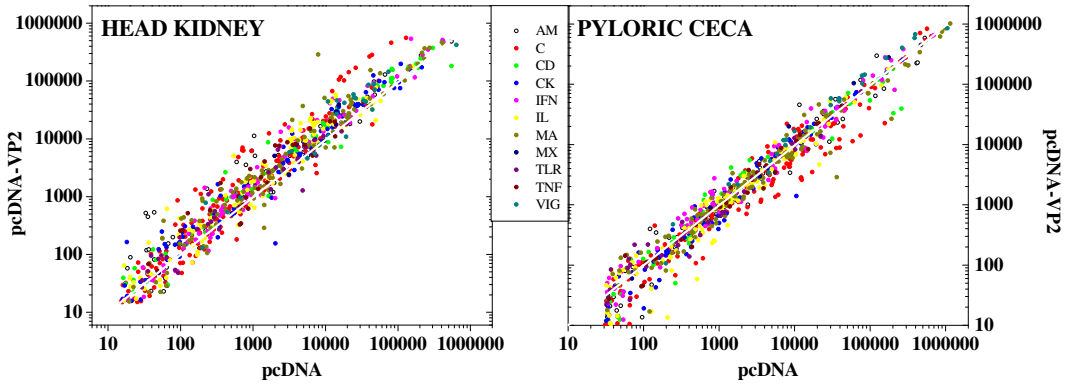

Fig. 1. Comparison of fluorescence intensities in genes from head kidney or pyloric ceca obtained by hybridization to microarrays of transcripts from pcDNA-VP2- and pcDNA-orally vaccinated trout. The trout ID32303 microarray described in Table 1 was used to estimate differential transcript levels in head kidney or pyloric ceca in orally vaccinated rainbow trout. Assay conditions and analysis were as described in Section 2 and Supplementary data. The figure shows the range of mean fluorescences obtained from 4 different pools of trout ($n = 6$ per pool) classified in the gene groups described in Table 1, except those corresponding to the most abundant IG, MHC, TCR, HSP and TR groups which were omitted for clarity. A white straight line has been drawn to show the fold =1.

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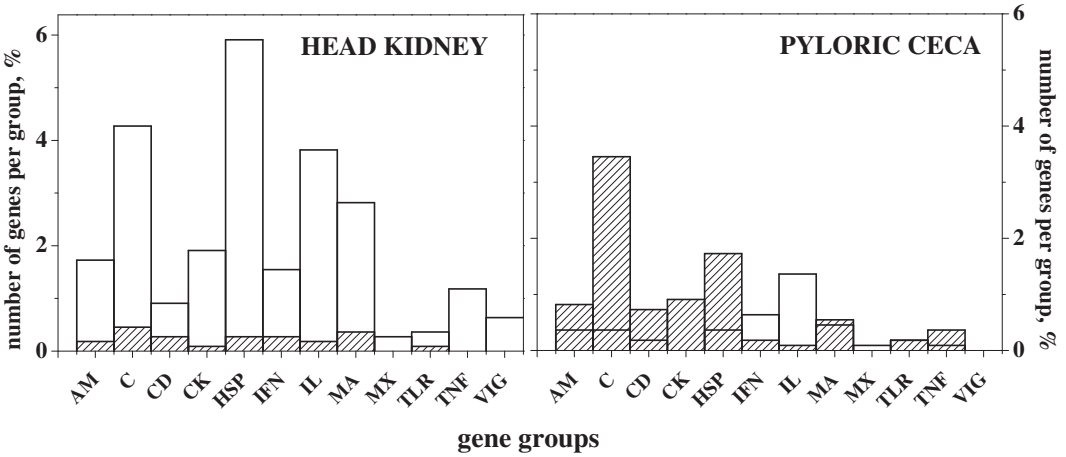

Fig. 2. Distribution of percentages of grouped genes showing differential expression with folds >2 in head kidney and pyloric ceca after oral vaccination. Assay conditions and analysis were as described in Section 2. For each of the gene groups, the percentages of genes showing differential expression were calculated by the formula, 100 × number of genes in the group with folds >2/total number of genes (Table 1). Up: genes showing increased folds (open bars). Down: genes showing decreasing folds (hatched bars). The IG, MHC and TCR groups have been omitted for clarity.

Head kidney *Vig* transcripts *b191*, *b88*, *4*, *5* and *1* were increased, however, neither head kidney nor pyloric ceca *vig* genes decreased (Fig. 4 down).

Among the 125 gene probes classified as macrophage-related, transcripts from the head kidney pentraxin (an acute phase response protein similar to C-reactive protein) was increased >30-fold. In addition, 3 probes of *cd11* (a dendritic cell marker), 3 of macrophage stimulating factor (*csf*) probes, and one each of stabilin (scavenger receptor), cadherin (an adhesive molecule involved in calcium-dependent cell to cell adhesion) and leucocyte immune-type receptor detected increased transcripts. In pyloric ceca, only the 3 *cd11* transcripts were also increased while *stabilin* and *lect2* were decreased (Fig. 5 up).

Head kidney transcripts corresponding to the classic complement pathway (i.e.: *c1r/cls*, *c3*, *c5*, *bf2*) and to the downstream membrane attack complex (*c6*, *c7*, *c8*, *c9*) were increased including one complement receptor (*cr*). Both precursor and mature *properdin* (main actor in the alternative pathway) transcripts and factor *H* were also increased. In the pyloric ceca, in contrast, only the *properdin* and *perforin* transcripts remained increased while those corresponding to the factor *H* gene together with the *c3*, *bf2*, *cfh*, and *cr* were decreased (Fig. 5 down).

In both head kidney and pyloric ceca, differential expression changes in transcripts of pattern recognition toll-like receptor *tlr* members were detected only in a few gene transcripts and with small increments (Fig. 6 up). Thus, in head kidney, *tlr2* (recognizing bacterial lipoproteins and glycolipids), *tlr5m* (located in the membrane of dendritic cells recognizing bacterial flagellins) and an unknown *tlr* and *tlr5m* and *tlr9* (recognizing bacterial CpG DNA) in pyloric ceca, were only slightly increased. Soluble *tlr5* and *tlr8* in pyloric ceca were decreased (Fig. 6 up).

Among the inducers of systemic inflammation of the tumor necrosis factor *tnf* family, main changes in head kidney transcripts with <3-fold increasing changes included 3 probes of *tnf10* (apoptosis inducer), 2 of *tnf11* (dendritic survival factor), *tnf14* (stimulator of T cells), *tnf c* and *tnf a* (most important inducers of systemic inflammation) and *tnfr*. In contrast, in pyloric ceca, only *tnf13* (involved in B-cell development) was increased and *tnf a*, an unknown *trf* and *tnfr* were decreased (Fig. 6 down).

Chemo attractant cytokines (chemokines, *ck*) and their receptors (number of probes tested $n = 121$) showing differential gene expression after VP2 oral vaccination, could be grouped in: *scya* (small inducible cytokine A family-like genes), C, CXC and *socs* (suppressor of cytokines signaling) (Fig. 7 up). In the head kidney, transcripts corresponding to genes similar to *scya109* were increased while those of *scya113* were decreased. In the head kidney, CC chemokines such as *ck3*, *ck5*, *ck9*, *ck10* and *ck12* were also increased while in pyloric ceca, *ck2* and *ck11* were decreased. In

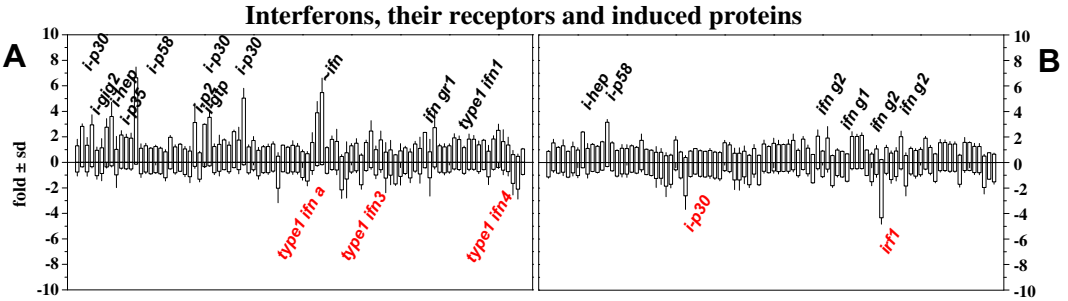

Fig. 3. Hybridization of head kidney (A) and pyloric ceca (B) transcripts to immune-gene enriched oligo microarray. Differential transcript expression of interferons, their receptors and induced proteins. Trout were vaccinated with 10 μg of pcDNA-VP2 or pcDNA ($n = 4$). RNA was extracted from the head kidney and pyloric ceca 7 days after vaccination, labeled with Cy3 and hybridized to the microarrays. After normalization, the mean and standard deviations were represented. Short names of the genes with differential expression folds >2 are to the right of the corresponding bars. Fold, expression level in pcDNA-VP2-vaccinated samples/expression level in pcDNA-vaccinated samples. +, increased folds in black; and −, decreased folds in red. *i*, induced; *r*, receptor; *ifn*, interferon; *g*, gamma; and *p*, protein. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

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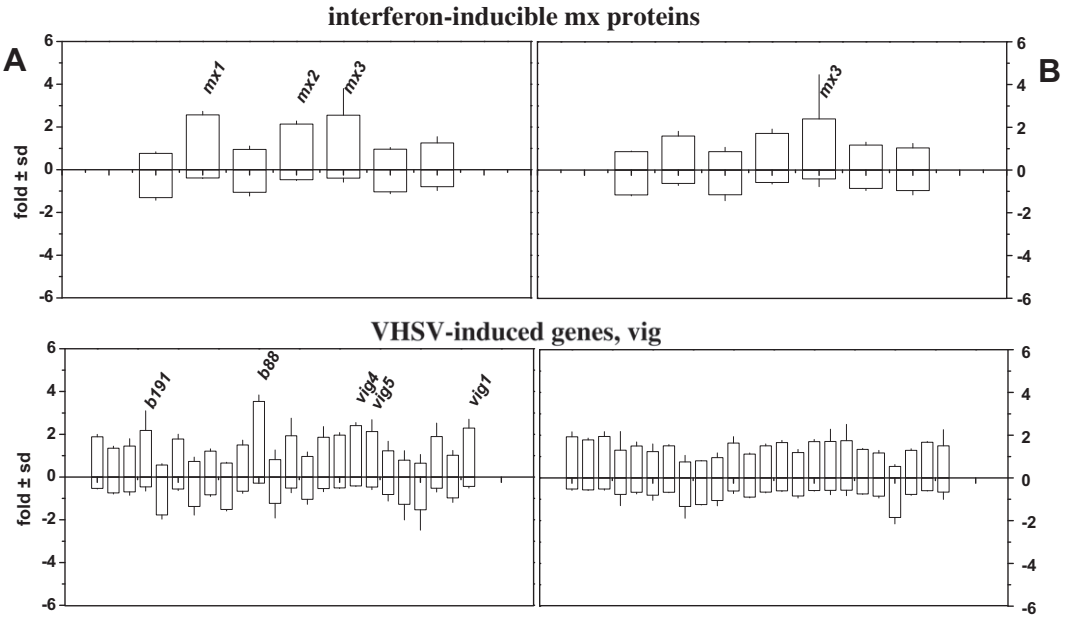

Fig. 4. Hybridization of head kidney (A) and pyloric ceca (B) transcripts to immune-gene enriched oligo microarray. Differential transcript expression of mx (up) and VHSV-induced genes (down). Details as described in the legend of Fig. 3. *vig*, VHSV-induced genes.

the head kidney, the CXC chemokines *cxcd1*, *cxc*, *cxc*r and *il8/il8r* (a CXC chemokine involved in neutrophil chemotaxis, whose results are shown in the interleukines gene group, Fig. 7 down) were also increased while in pyloric ceca, *cxcd1* and *ccr5/cxcr3a* receptors were decreased. Only in head kidney the *socs3* and *socs5* were increased, most probably to control excessive expression levels of other cytokines. In contrast, in pyloric ceca all the differential expression changes detected in chemokine transcripts were decreased. Thus, not only the *cxcd1* (upregulated in head kidney) but also some chemokine receptors (*ccr5, cxcr3* and *r duffy*), *ikhlaii* (a downregulator of *hlaii*), *ck2*, *ck11* and *iNOS* (signaling molecule participating in the oxidative burst of macrophages, among other functions) were decreased.

In the head kidney, of the 119 interleukin and receptors tested, *il10* (inhibits cytokines such as *ifng*, *il2*, *il3*, *tnf* and *csf*), *il12β* (maintains T helper 1 cellular responses to intracellular pathogens), *il17* (pro-inflammatory cytokine produced by activated memory T cells) and *il20* (regulates proliferation and differentiation of keratinocytes) transcripts were increased. In contrast, in pyloric ceca most of the detected transcripts with differential expression levels were decreased (Fig. 7 down). Also increased in head kidney were the *il8* and its receptor (chemokine discussed above), *il11* (inhibitor of apoptosis) and *il13* (pleitropic cytokine involved in the regulation of *ifng*) and the receptors corresponding to *il8*, *il13*, *il20* and *il4* (*il4* is an interleukin produced by *cd4+* cells for B-cell responses). In the pyloric ceca, 4 probes of *il1β* (a lymphocyte mitogen produced by

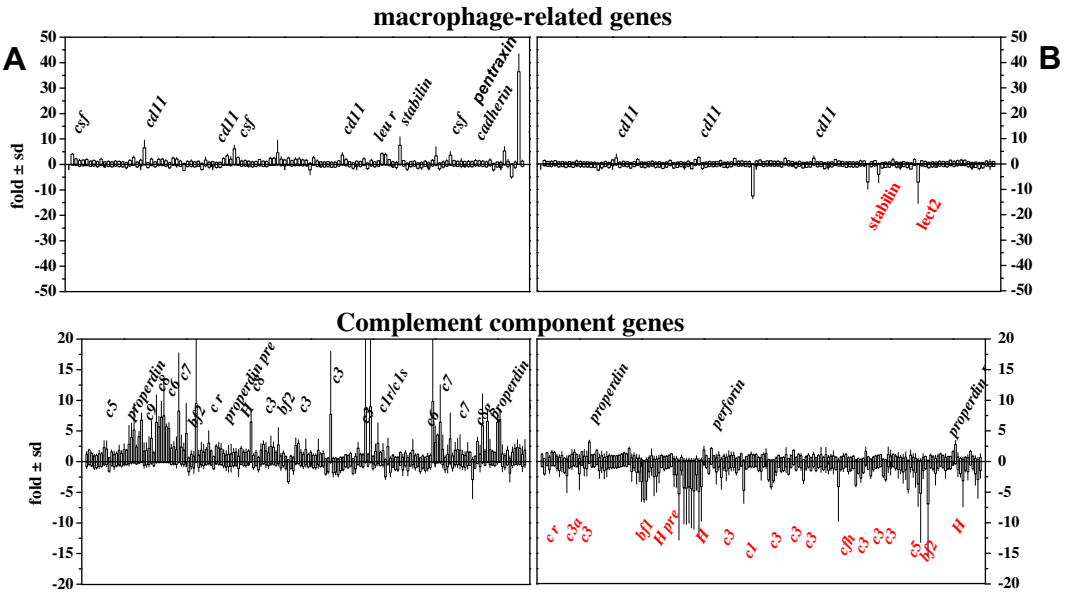

Fig. 5. Hybridization of head kidney (A) and pyloric ceca (B) transcripts to immune-gene enriched oligo microarray. Differential transcript expression of macrophage-related genes (up) and complement component genes (down). Details as described in the legend of Fig. 3. *cd*, cluster differentiation antigens. *r*, Receptor; *c*, complement component; and *H*, factor H.

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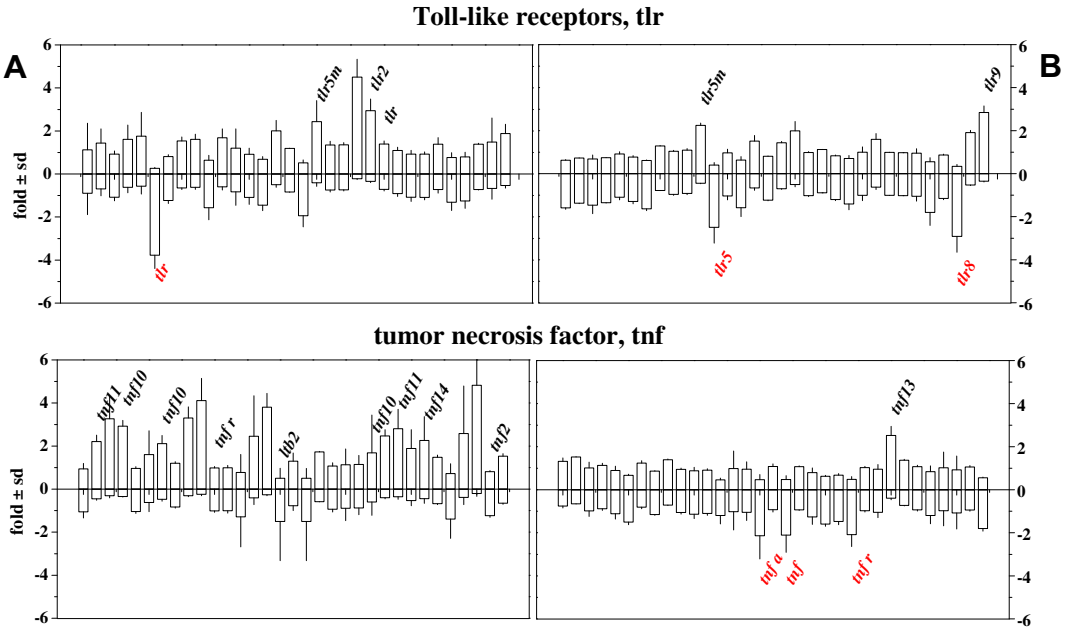

Fig. 6. Hybridization of head kidney (A) and pyloric ceca (B) transcripts to immune-gene enriched oligo microarray. Differential transcript expression of toll-like receptors (up) and tumor necrosis factors (down). Details as described in the legend of Fig. 3. *tlr*, toll-like; *r*, receptor; *m*, membrane; *tnf*, tumor necrosis factor; and *a*, alpha.

macrophages and an important mediator of inflammatory responses), *il6* (B-cell stimulating factor important in the differentiation of B cells into secretory cells) and *il22* (increasing the acute phase proteins) were also decreased.

Fig. 8 (up) shows that in the transcripts grouped in the antimicrobial peptide genes, all the folds were increased much more in the head kidney than in the pyloric ceca. Thus, in the head kidney, transcripts incremented included *leap2a* (2 probes) and *βdef1* (5 probes), *βdef2* and *βdef4* (2 probes), while in pyloric ceca only the *leap2a* and *βdef4* were slightly incremented.

In the head kidney, the *cd* transcripts present in B, T and macrophage cells were increased (Fig. 8 down). Thus, *cd79* (protein associated with membrane-bound immunoglobulin in B-cells) and *cd80/86m* (primordial molecule existing in membrane-bound B-cells), transcripts corresponding to *cd4* (a typical marker of T helper cells) (4 probes), *cd36* and *cd163* precursor (scavenger receptors of activated monocytes and macrophages) and *cd166* and *cd97* (activated leukocyte cell adhesion molecules) were increased. In head kidney, *cd209* (c-type lectin marker of dendritic cells) and the secreted form of *cd80/86* were decreased. In pyloric ceca, only the

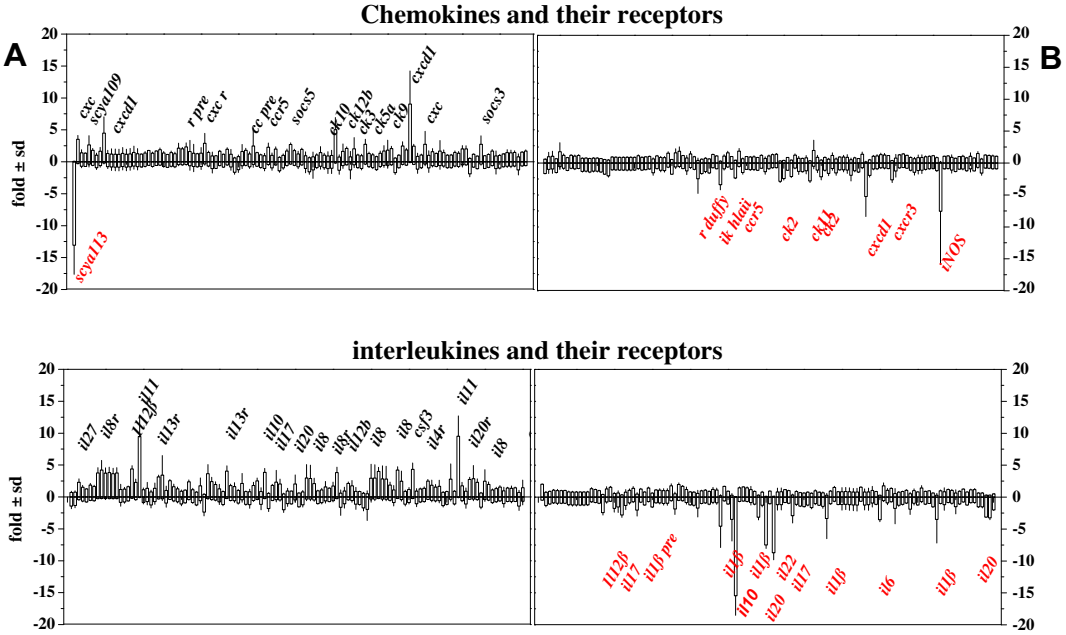

Fig. 7. Hybridization of head kidney (A) and pyloric ceca (B) transcripts to immune-gene enriched oligo microarray. Differential transcript expression of chemokines and their receptors (up) and interleukines and their receptors (down). Details as described in the legend of Fig. 3. *ck*, chemokine; *scya*, small inducible cytokine A; *socs*, suppressor of cytokine signaling; *pre*, precursor; *r*, receptor; and *il*, interleukin.

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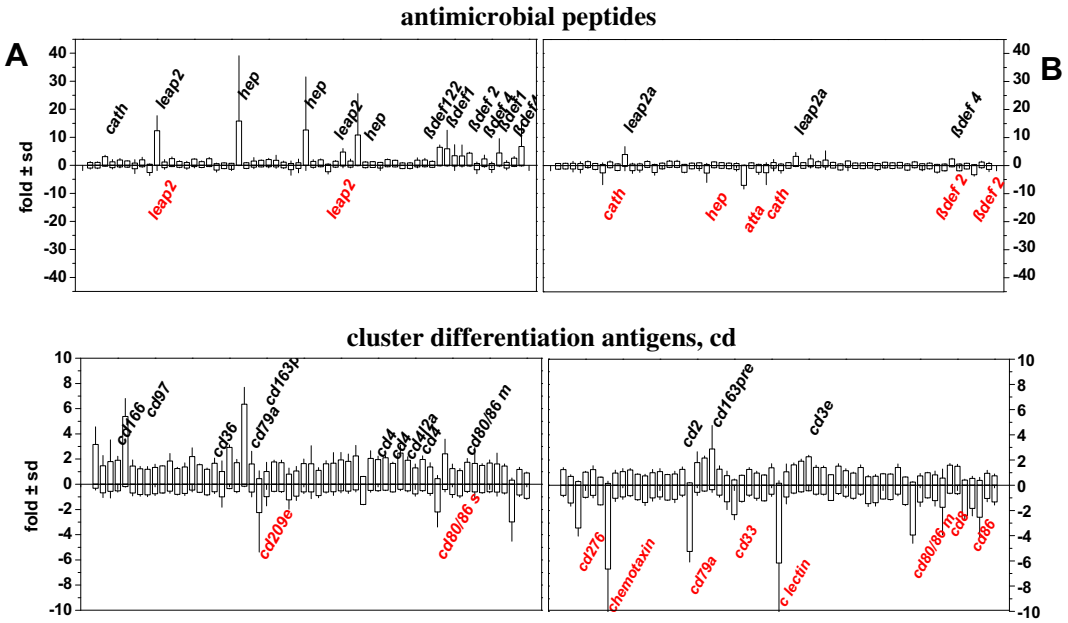

Fig. 8. Hybridization of head kidney (A) and pyloric ceca (B) transcripts to immune-gene enriched oligo microarray. Differential transcript expression of antimicrobial peptides (up) and cluster differentiation antigens (down). Details as described in the legend of Fig. 3. *cath*, cathepsin; *leap*, liver-expressed antimicrobial peptide; *def*, defensin; *cd*, cluster differentiation antigen; and *pre*, precursor.

cd163 precursor was increased together with *cd2* (thymocytes) and *cd3* (signaling component of the T-cell receptor complex) while, chemotaxin (a chemoattractant of leukocytes), *cd8* (marker of cytotoxic lymphocytes), *cd79*, *cd33* (marker of immature myeloid cells), *c-lectin* (cell adhesion and immune response to pathogens), *cd276* (costimulatory molecule of *cd80/86*) and the membrane-bound form of cd80/86 were decreased.

In the head kidney, increased differential transcripts were detected with 3 probes of *hsp70* (folding and unfolding), 3 of *hsp40* (a cofactor of hsp70), 2 of *hsp90* (maintenance of transcription factors) and 3 of prefoldin (*pfd*) (implicated in the correct folding of nascent proteins). Furthermore, different cathepsin (*cath*) proteases were also increased (12 probes). In pyloric ceca, on the contrary, *hsp70* (2 probes), *hsp40* (1 probe) and *hsp90* (2 probes) and *cath* (6 probes), were all decreased while *pfds* were maintained (Supplementary data and results not shown).

There were no detectable transcript changes in the secreted mu chain of *igm*, nor in the *igt*, nor in the *tcr* in any of the organs studied 7 days after vaccination, except for a decreased level of *tcrb* in pyloric ceca (not shown).

In the head kidney there were transcript increases as detected by multiple probes corresponding to the MHC class I and II precursors, MHC class Ia and Ib (UA, ZE, UBA, UEA and UAA), MHC class II antigen β (DAB, DBB and DAA) and β2 microglobulin. In the pyloric ceca most of those remained unchanged, except MHC class II antigen β which was down-regulated (4 probes).

In the head kidney there were many transcription factors whose transcripts were increased. Thus, general transcription was activated as shown by the increasing transcription levels of genes corresponding to transcription initiation (*tfiid, tfiia* and *iifa*) and elongation (*siiip15*, *siii*, and *spt4*) factors (as detected by several different probes for most of them), several transcription-related-like factors (i.e.: *iib*, *iih*, *lipl21*), CCAAT enhancer binding protein β*c/ebpb* (6 probes) and RNA polymerase-related molecules such as RNA polymerase I cofactor (*rrn3*) and termination factor (*ttf1*) and RNA polymerase III polypeptide (*rpc11*). In pyloric ceca although a few general transcription factors decreased (i.e.: *iif* and *tfiih*), most of them remained unchanged. Among the *sox* family (a family of transcription factors containing an hmg DNA-binding domain), the *sox21* (counteracts *sox1*, *2*, *3*), the *sox30* and the *sox*-related *sry* were increased. In pyloric ceca most of the *sox* transcripts were decreased except the *sox21*. Other transcription factors increased in head kidney were *ap1* (stimulated by different cytokines and viral infections), which remained increased in pyloric ceca, and the *g0s2*, also called the lymphocyte G0/G1 switch protein (whose expression is required to commit lymphocytes to pass from the G0 to enter the G1 phase of the cell cycle to start division). Strikingly the *jnk2* N-terminal protein kinase was >10-fold decreased in head kidney while it was 8-fold increased in pyloric ceca (Supplementary data). The *jnk2* has been linked to *tnf* levels and apoptosis [19,20], but it is not known why it is expressed so differently in the studied organs.

3.3. Selected gene expression by RTqPCR

To validate the results of the microarray analysis, some of the genes commented above were selected for RTqPCR analysis. Genes previously described as induced in IPNV infected fish were also included. The selected genes were: *ifn1* [21], *ifng* [22], *mx1* [23] and *3* [24–26], *stat1* [27], *mhc1* [28], *tnf2* [29], *il8* [30], *1l10* [31], *1l11* [32], *1l12* [33], *βdef1* [34], *igm* [35], and *igt* [36]. Table 2 represents the selected genes, their accession numbers and the corresponding primer sequences used for the RTqPCR analysis. Fig. 9 shows the comparison between the folds of transcripts obtained by microarray and by RTqPCR corresponding to either head kidney (A) or pyloric ceca (B). The folds were similar for the microarray and the RTqPCR for the majority of each of the gene data, except one data >3.5-fold in head kidney and two data >5-fold in pyloric ceca. RTqPCR results show that in head kidney *stat1a*, *il11*, *il12* and *il10* were more increased than in pyloric ceca while *ifn1*, *ifng*, *mx3*, *igm*, and *igt* were more increased in pyloric ceca than in head kidney.

4. Discussion

To the authors' knowledge, this is the first report describing the extensive transcript profile induced by an oral DNA vaccine [1] in any fish and against the infectious pancreatic necrosis virus (IPNV).

Table 2
List of primer pairs designed for gene expression analysis by RTqPCR.

Genes	Primer name	Primer sequence 5′–3′	Accession number
ifn1	IFN-1F	AAAACTGTTTGATGGGAATATGAAA	NM_001124531
	IFN-1R	CGTTTCAGTCTCCTCTCAGGTT	
ifng	IFNG-F	CTGAAAGTCCACTATAAGATCTCCA	FM864345.1
	IFNG-R	CCCTGGACTGTGGTGTCAC	
mx1	Mx1-F	AGCTCAAACGCCTGATGAAG	NM_001171901
	Mx1-R	ACCCCACTGAAACACACCTG	
mx3	Mx3-F	AGCTCAAACGCCTGATGAAG	U47946.3
	Mx3-R	TGAATATGTCTGTTATCCTCCCAAA	
stat1	stat1-F	TTGAGAGCATCGACTGGGAAAA	U60332.1
	stat1-R	GGCTAGGAGGTCATGGAAACGT	
mhc1	mhc-F	GCAACCCAATTTCATGCAGG	EU036638.1
	mhc-R	ACACTCAATGCAGGTCTGGG	
tnf2	tnf-F	TGCTGCTCCATGTGTGGTGC	DQ218473.1
	tnf-R	AGGGACGGGGAGCCTTGAT	
il8	IL-8F	GAATGTCAGCCAGCCTTGTC	AJ279069
	IL-8R	TCCAGACAAATCTCCTGACCG	
il10	IL-10F	CGACTTTAAATCTCCCATCGAC	AB118099
	IL-10R	GCATTGGACGATCTCTTTCTT	
il11	IL-11F	TGCGCTGCAGAGGAGCAAGT	AJ535687.1
	IL-11R	TGCTGGAGACCCCAAGCACA	
il12	IL-12F	ATGTGGTTACGGGAGGC	AJ548830.1
	IL-12R	ATGTGGTTACGGGAGGC	
bdef1	db-1F	GTTGTGGAGGATGAGGCTGC	FN545575.1
	db-1R	TGGCAGGCAAAGTTTTCGG	
igm	IgM-F	ACCTTAACCAGCCGAAAGGG	X65263.1
	IgM-R	TGTCCCATTGCTCCAGTCC	
igt	IgT-F	AGCACCAGGGTGAAACCA	AY870265
	IgT-R	GCGGTGGGTTCAGAGTCA	
ef1	EF-F	GATCCAGAAGGAGGTCACCA	AF498320
	EF-R	TTACGTTCGACCTTCCATCC	

Both internal immune responsive organs (head kidney) as well as some of the tissue first exposed to the vaccine (pyloric ceca) were analyzed. A 60-mer oligo microarray enriched in rainbow trout immune-related genes (2.5-fold with respect to previous designs) was used. The use of a microarray enriched in immune-related genes present in the GenBank, in addition to those probes previously obtained from EST sequences [17], not only allowed the study of a larger number of rainbow trout immune-related genes but also a high reliability of the data because of their exact annotation. Similar designs could be made with any other fish species for which sufficient gene numbers are deposited in the GenBanks (i.e. salmon, zebrafish, etc).

Seven days after oral vaccination was chosen because previous evidence of VP2 transcript expression in head kidney, pyloric ceca or other organs after oral [1] or intramuscular [37] DNA vaccination. That allows us to evaluate trout transcript expression during VP2 expression.

In orally vaccinated trout, the number of increased immune-related transcripts differentially expressed was higher in head kidney than in pyloric ceca. In contrast, the number of differentially decreased transcripts was higher in pyloric ceca than in head kidney. Those levels were confirmed by comparing absolute immunofluorescence measurements (Fig. 1), by comparing differentially expressed transcripts with folds >2 (Fig. 2), by individually studying the genes with folds >4 and $p < 0.05$ (Supplementary data) or by analysis of the individual gen groups with folds >2 (Figs. 3–8). All those results demonstrate that 7 days after vaccination the transcript response is more intense in head kidney (an internal immune response organ) than in pyloric ceca (one of the hypothetical entrance sites of the oral vaccine). Most probably some molecular signals had traveled from pyloric ceca to head kidney through the blood to induce such a response. Alternatively, pyloric ceca immune responsive cells might have migrated to the internal organ, thus causing a decreasing level of most transcripts in pyloric ceca. We have yet no evidence for either case.

Most of the studies of transcriptional responses in salmonid fish to IPNV infection that have been made by RTqPCR in Atlantic salmon [38–41] or cell culture [42] demonstrated induced expression of *ifn-mx*-related genes. For instance, both *type 1 ifn* and *ifng* have been detected and shown antiviral activity on IPNV [43]. With regard to trout intramuscular DNA vaccines to IPNV, we have previously described the induction of *in vitro* antiviral activity of pcDNA-VP2 and the *in vivo* stimulation of some immune-related genes [11]. Similar results were described for expression of *mx* and *ifn* genes in orally vaccinated trout [1] and confirmed in the present work with more genes. Thus, VP2 orally vaccinated trout increased several *ifng*-inducible genes in head kidney or pyloric ceca probably as a result of the increased *ifns*, as suggested in head kidney by *type 1 ifn*1 and in pyloric ceca by several *ifng* (*type 2 ifn*) transcript levels (Fig. 3). Other *type 1 ifns* (a, 3 and 4) were inhibited in head kidney, but their functional characterization is not yet available to make a possible interpretation [44,45]. Recent results

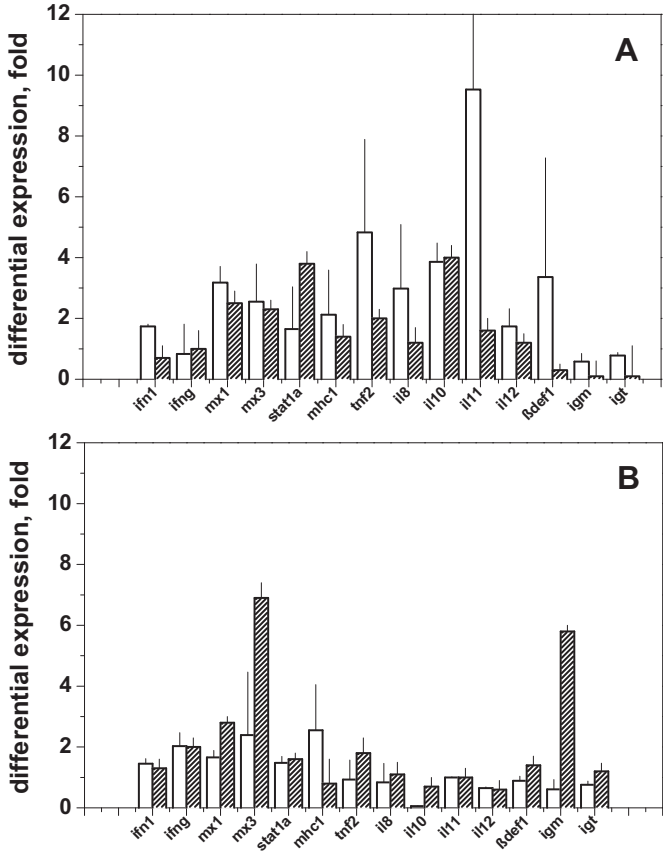

Fig. 9. Comparison between differential expression levels of selected transcripts obtained by hybridization to microarray (empty bars) and RTqPCR (hatched bars) in head kidney (A) and pyloric ceca (B). The RNA samples were the same than those used for the microarray. The cDNA were obtained, and qPCR performed as indicated in Section 2 by using SYBR green. The elongation factor 1α (*ef-1α*) gene was used to normalize the results. All qPCR reactions were performed in triplicate and the results were expressed as mean ± standard deviation. The expression levels were calculated as relative folds of the expression of pcDNA controls according to the $2^{-\Delta\Delta CT}$ method [18]. *ifn1*, type 1 interferon 1; *ifng*, type II interferon gamma; *mx1* and 3, interferon-inducible mx proteins 1 and 3; *stat1*, transcription factor participating in interferon pathways; *mhc1*, major histocompatibility complex I molecules; *tnf2*, tumor necrosis factor 2; *il8*, CXC chemokine involved in neutrophil chemotaxis; *1l10*, interleukin which inhibits cytokines such as *ifng*, *il2*, *il3*, *tnf* and *csf*; *1l11*, interleukin inhibitor of apoptosis; *1l12*, interleukin that has been tested as vaccine adjuvant; *βdef1*, beta defensin 1 antimicrobial peptide; *igm*, serum immunoglobulin M; and *igt*, mucosal immunoglobulin T.

suggest complementary roles of all viral-induced *ifns* [46], which are in the same direction than the results commented above.

All the *ifn*-inducible *mx1*, *mx3* (cytoplasmic localization) and *mx2* (nuclear localization) transcripts characterized in rainbow trout [25,47] increased in head kidney while only *mx3* increased (Fig. 4 up) in pyloric ceca (the site of delivery of the oral vaccine). Similar results were obtained after intramuscular vaccination. Thus, expression of the 3 *mx* isoforms were obtained in head kidney while only *mx3* increased in muscle (the site of delivery of the DNA vaccine) [48]. All these results suggest that at least in head kidney, the expression of all 3 *mx* isoforms might be required for their antiviral effects, as also described after rhabdoviral or IPNV infection of rainbow trout [48] or sea bream [49], respectively. Thus, the 3 fish *mx* isoforms might have complementary mechanisms to inhibit viral replication in internal organs. The differential expression levels induced in internal versus local organs, however, suggest different but yet difficult-to-understand functions for fish *mx* isoforms.

Pentraxin (an acute phase protein related to C-reactive protein) showed the highest elevated transcript levels (>30-fold) (Fig. 5A). Similar increased pentraxin transcript levels were reported before only after bacterial infections [50]. However, it is not known what component in the present DNA vaccine formulation triggers such an increased level of pentraxin and whether or not it is important for the vaccine success. On the other hand, it might be relevant that 3 different probes of *cd11* (a marker of dendritic cells) were increased in both head kidney and pyloric ceca, suggesting a role of dendritic cells (antigen-presenting cells) in the responses induced by oral vaccination. At this respect, other increased dendritic cell-associated transcripts were also detected: *tlr5m* (located in the membrane of dendritic cells recognizing bacterial flagellins), *tnf11* (dendritic survival factor), *cd209* (c-type lectin marker of dendritic cells), *vig1* (VHSV-induced gene in dendritic cells), and *il12* (produced in dendritic cells in response to intracellular pathogens).

Head kidney genes corresponding to the classic complement pathway and its membrane attack complex were increased, suggesting an activation of the classic complement pathway. Although both precursor and mature properdin transcripts (main actors of the alternative pathway) were also increased, the simultaneous upregulation of factor *H* suggests a possible inhibition of the alternative pathway in this organ. In contrast, properdin and perforin genes remained increased while the factor *H* gene together with the *c3*, *bf2*, *cfh*, and *cr* genes were decreased in pyloric ceca, thus suggesting that the alternative pathway might be more active in this organ (Fig. 5 down).

The small fold changes detected in both head kidney and pyloric ceca, on *tlr* members, suggested that those genes do not participate on the response to VP2 oral vaccination. Nevertheless, the membrane-bound *tlr5m* was induced in both organs and showed an opposite behavior to soluble *tlr5*. The possible functional meaning of those results is not yet known. With respect to the tumor necrosis factor (*tnf*) family of pro-inflammatory cytokines, many changes were induced in *tnf*-related gene transcripts (9 probes with folds >2) in head kidney during oral VP2 vaccination (Fig. 6 down), similarly to what has been described for *tnfα* and *tnfβ* during IPNV infection in fish cells [40].

Catfish-like chemokine *scya109* was increased after VP2 vaccination in head kidney while *scya113* was decreased. Both *scya109* [51] and *scya113*[52] increased after bacterial infection in catfish but there is no more functional information available in any other fish. Similarly to IPNV infection [53,54] and rhabdoviral DNA vaccination [30], VP2 oral immunization also increased other chemokines such as *ck5*, *ck6*, *ck7*, *ck9* and *ck12*.

The high number of interleukins (*ils*) showing increased transcription in head kidney suggests that this organ is at its optimum immune response 7 days after vaccination (Fig. 7 down). Thus, increased expression of pro-inflammatory cytokines such as *il8*, *il11* and *il12* was observed in head kidney although *il1β* transcripts were down-regulated in pyloric ceca. IPNV/IHNV infections increased expression of *il1β* and *il18* during single infections but decreased *il1β* expression after co-infection in brown trout (*Salmo trutta*) [55,56]. Down-regulation of *il1β* has also being correlated with chronic inflammation in salmon [57]. The suppression of *il1β* expression observed in pyloric ceca in this work may, therefore, indicate an inhibition of earlier inflammatory processes.

Increased *il12* transcripts were found in head kidney (Fig. 9A). Mammalian *Il12* stimulates *tnfa*, and several *tnf* were also increased in head kidney in orally vaccinated trout (Fig. 6 down). *Il12* has been described as a viral vaccine adjuvant for mucosal immunity [58] but it has not been tested in fish. Other transcripts, such as *il17* (Fig. 7 down) also increased their levels in head kidney. Since *il17* helps to induce antimicrobial peptides [59,60] that might explain why antimicrobial peptides were also increased in head kidney (*leapa2* and several *βdefs*) (Fig. 8 up) and pyloric ceca (*βdef*/*βdef*). Because previous findings have implicated *βdef* in both rhabdoviral blocking and activation of trout immune defense genes [61,62], antimicrobial peptides such as *leapa2* and *βdefs* might deserve further studies.

The increase in differential transcription of several heat shock proteins (*hsp70*, *hsp40* and *hsp90)* together with prefoldin (*pfd*) in head kidney indicated that some new protein(s) were being translated after vaccination. The increase in 12 different probes of cathepsin (*cath*) proteases (family of related lysosomal proteases implicated in antigen processing) also suggests an activation of antigen processing due to the appearance of new protein(s) such as those probably derived from the transcription of the pcDNA-VP2 plasmid [1].

The increased *igm* and *igt* transcript expression levels detected in pyloric ceca compared to kidneys (Fig. 9B), might be important because of their potential implication on mucosal immunity, however, those increased levels were only detected by RTqPCR.

The data obtained is very consistent because it was obtained from four different populations of rainbow trout, and each population sample was obtained by pooling 6 trout. These results obtained 7 days after oral vaccination, demonstrate that the 30-day protection induced by these vaccines [1] was preceded by a complex and specific immune response in internal as well as in local organs. However, to understand the factors affecting expression of these different genes requires further work. Our present results indicated that series of time-course experiments in different organs focused in some of the immune-related genes identified in this work could be the next step to provide a more detailed view of these processes.

GenBank nucleotide sequences were searched with the following keywords: interferon, chemokine, interleukin, cytokine, defensin, macrophage, lymphocyte, antimicrobial, neutrophil, leukocyte, cytotoxic, natural killer, antiviral, antibacterial, LPS, Vig, antigen, histocompatibility, phagocyte, viral, Mx, complement, immunoglobulin, hepcidin, IgG, IgM, Toll, T cell, B cell, dendritic, presenting, TNF, perforin, MHC, NK, transcription, chaperone, stress, Hsp, Hsp70, Hsp90, tlr, flagellin, keratinocyte, cathepsin, NOD, IRF, IKK, JBK1, TRAM, TAK, TAB, JNK, P38, AP-1, TIRAP, IgT, IgH, high mobility. The resulting list of genes (GenBank sequences) was submitted to Agilent's eArray designing tool (https://earray.chem.agilent.com/earray/search.do?search1/4arrayDesign) to retrieve the Agilent list. The unique 60-mer probes were then added to the immune-related probes similarly extracted from the previously published EST-derived oligo array plus 3336 unknowns (Previous ID16271) [17] and classified into 16 groups to facilitate analysis. The final randomly arranged 8x15K Agilent's design (ID32303) was 2.5-

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fold enriched in immune-related sequences, with a total of 6442 different sequences each by duplicate.

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Appendix A. Supplementary material

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.fsi.2012.03.016.

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