ORIGINAL ARTICLE

Immune gene expression in gilthead seabream (*Sparus aurata*) after Lymphocystis disease virus (LCDV-Sa) challenge resulting in asymptomatic infection

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Abstract

Aims: To determine the immune gene expression response of gilthead seabream (*Sparus aurata*) that is experimentally infected with the lymphocystivirus LCDV-Sa.

Methods and Results: Viral DNA and transcripts were detected by qPCR in all samples from fish injected with LCDV-Sa, demonstrating that the virus establish a systemic and asymptomatic infection. The expression of 23 immune-related genes was also analysed by RT-qPCR in the head kidney (HK) and intestine at several times post-infection (dpi). In HK, the expression of five type I interferon (IFN)-related genes (*ifn*, *irf3*, *mx2*, *mx3* and *isg15*), *il10* and *ck10* was upregulated at 1–3 dpi, while genes related to the inflammation process (*tnfa*, *il1ß*, *il6*, *casp1*) were not differentially expressed or even downregulated. The expression profile in the intestine was different regarding type I INF-related genes. An upregulated *c3* and *ighm* expression was observed in both HK and intestine at 3–8 dpi. Finally, the transcription of *nccrp1* and *mhcIIa* was induced in HK, whereas *tcrb* expression was downregulated in both organs.

Conclusions: LCDV-Sa seems to trigger an immune response in gilthead seabream characterized by a partial activation of type I IFN system and a lack of systemic inflammatory response which may be related to viral persistence.

Significance and Impact of the Study: The immune response observed in gilthead seabream infected by LCDV-Sa could be implicated in the establishment of an asymptomatic persistent infection.

Introduction

Members of the genus *Lymphocystivirus*, family Iridoviridae, are the causative agents of the lymphocystis disease (LCD), a pathology that affects more than 150 fish species from both marine and freshwater environments (Borrego *et al.* 2017). To date, three species have been recognized in the genus, *Lymphocystis disease virus 1* (LCDV1) isolated from European flounder (*Platichthys flesus*), *Lymphocystis disease virus 2* (LCDV-C) isolated from Japanese flounder (*Paralichthys olivaceus*) and *Lymphocystis disease virus 3* (LCDV-Sa) isolated from gilthead seabream (*Sparus aurata*) (Chinchar *et al.* 2017). LCDV-Sa has also been detected in Senegalese sole (*Solea senegalensis*), both diseased and asymptomatic (Cano *et al.* 2010; Valverde *et al.* 2017b).

The LCD is characterized by the appearance of tumour-like lesions on the skin and fins that may cover the entire body in heavily affected individuals (Smail and Munro 2012). Although it is rarely fatal, fish showing these lesions cannot be commercialized, causing important economic losses to the aquaculture sector (Masoero *et al.* 1986). LCD is the viral infection most frequently reported in gilthead seabream, one of the main species in the Mediterranean and South-Atlantic marine aquaculture (Borrego *et al.* 2001; Colorni and Padro´s 2011). In this fish, lesions associated with LCD usually resolve within 20–45 days depending on water temperature

(Colorni and Padro´s 2011), but the virus establish an asymptomatic carrier state which may extend for at least 2 months in recovered individuals, with viral DNA and transcripts detected in the skin and other organs including gills, intestine, kidney, liver or spleen (Cano *et al.* 2009; Valverde *et al.* 2017a). In addition, asymptomatic LCDV-Sa infections are frequently detected in gilthead seabream farms, even in those farms where LCD outbreaks have not been reported, which indicates that this fish is a common LCDV-carrier (Cano *et al.* 2007, 2013; Valverde *et al.* 2016, 2017a).

The immune defensive mechanisms of fish against LCDV are still unclear. Very limited number of studies have deepened on the subject in different fish species (Carballo *et al.* 2017; Wu *et al.* 2018), and only one study in gilthead seabream has been carried out, analysing fish from a natural LCD outbreak (Cordero *et al.* 2016). To this end, the present study evaluated the immune response of gilthead seabream during the course of an experimental asymptomatic infection with LCDV-Sa, quantifying the expression of 23 immune-related genes in the head kidney (HK), a primary immune organ in fish, and intestine. The results obtained will allow us to understand which genes are essential in this host–pathogen interaction and could be used as molecular markers for vaccine efficacy evaluation.

Materials and methods

Virus and cell culture

The LCDV-Sa isolate used in this study was obtained from diseased gilthead seabream specimens collected at a local farm (South-western Spain), using skin and fin lesions which were homogenized (20% w/v) in Leibovitz L-15 medium (Gibco, Life Technologies, Carlsbad, CA). Cell suspension was sonicated at 40 W for 20 min and centrifuged (1000 g, 5 min, 4°C). The supernatant was recovered and incubated with 10% penicillin–streptomycin overnight at 4°C and maintained at −80°C. The viral stock was titrated in SAF-1 cells using the 50% cell culture infectious dose ($TCID_{50}$) endpoint dilution assay as previously described (Alonso *et al.* 2005).

Fish maintenance and experimental design

Gilthead seabream specimens (70–100 g weight) were obtained from IFAPA centre El Toruño (El Puerto de Santa Mar´ıa, Spain). Prior to the experiment, 10 fish were analysed by real-time PCR (qPCR) (Valverde *et al.* 2016) and found LCDV-negative. Fish were acclimated for a week before starting the experiment. The water temperature and salinity were 22 1°C and 35–37 g l^{-1} respectively. The fish were maintained under natural photoperiod conditions and fed a commercial pellet diet (Skretting, Burgos, Spain) administrated once per day at a rate of 1% fish biomass.

Fish were separated into two groups, anesthetized with MS-222 (Sigma-Aldrich, St. Louis, MO) and intramuscularly injected with 100 μl of the LCDV-Sa stock diluted in phosphate-buffered saline (PBS) (10^5 $TCID_{50}$ per fish) for the virus-infected group, or with 100 μl of PBS for the control group. Five fish per group were randomly selected at 1, 3 and 8 days post-inoculation (pi). Animals were euthanized by a MS-222 overdose. Samples from caudal fin, HK and intestine were aseptically collected and immediately frozen in liquid nitrogen and stored at −80°C until used.

All procedures were carried out under the Spanish directive (RD 1201/2005) for the protection of animals used in scientific experiments, and given the registration number 10-06-2016-102 by the Spanish authorities for the regulation of animal care and experimentation.

DNA and RNA extraction and cDNA synthesis

DNA was extracted from the organs collected (30–50 mg) using E.Z.N.A. Tissue DNA Kit (Omega Bio-Tek Inc., Norcross, GA). The DNA samples were quantified by spectrophotometry at 260 nm using a NanoDrop 1000 Spectrophotometer (Thermo Scientific, West Palm Beach, FL) and stored at −20°C until used.

For total RNA extraction, pieces (30–70 mg) of the collected organs were processed following the procedure specified by Labella *et al.* (2018). RNA samples were resuspended in nuclease-free water (Qiagen Inc., Valencia, CA), treated with RNase-free DNase I (Sigma-Aldrich) according to the manufacturer's instructions and quantified by spectrophotometry. The cDNA was synthesized from 2 μg of total RNA using the High Capacity cDNA Reverse Transcription kit (Applied Biosystems, Life Technologies, Carlsbad, CA) and stored at −20°C. After DNase treatment, total RNA (100 ng) was used as template for real-time PCR (qPCR) using the primers for the beta-actin (*actb*) gene (Table 1), in order to confirm the absence of residual contaminating DNA.

LCDV-Sa DNA quantification and gene expression

Viral DNA quantification was carried out by qPCR according to the procedure described by Valverde *et al.* (2016). The assays were performed in a LightCycler® 96 Instrument (Roche Life Science, Indianapolis, IN) using a 25-μl final volume reaction containing 12·5 μl of FastStart Essential DNA Green Master (Roche Life Science), 300 nmol l^{-1} of each primer and 200 ng of DNA. The

Table 1 Primers used for gene expression analysis by real-time PCR

Symbol	Gene name	Sequence (5^0–3^0)	Amplicon size	References/Genbank accession number*
mcp	LCDV-Sa major capsid protein	F: ACGTTTCTCGAGGCGGAGAT R: CGGACGTTTGCTTGACCAA	150	Valverde *et al*. (2016)
tlr5	Toll-like receptor 5	F: CTTCCTGAGCCAACTTGAGG R: TCAGCTTCTGGACGTCCTTT	128	KF857324
tlr9	Toll-like receptor 9	F: CATCCTTTGCCAGCTTCTTC R: GGGACACGTTTGAGTGGTCT	117	AY751798
ifn	Type I interferon	F: ATGGGAGGAGAACACAGTGG R: GGCTGGACAGTCTCTGGAAG	244	Valero *et al*. (2015)
*irf*1	Interferon regulatory factor 1	F: GAGGAAGAGGACAGCGACAC R: CGGAGCCACAAATGAGAAAT	123	AY962254
irf3	Interferon regulatory factor 3	F: TCAGAATGCCCCAAGAGATT R: AGAGTCTCCGCCTTCAGATG	107	Valero *et al*. (2015)
irf9	Interferon regulatory factor 9	F: GTTTTTAACAGTCATGGCAGGAG R: GCGGAACATGGTTTTAGCAT	130	AM920661
pkr	RNA-dependent protein kinase	F: TCCTTTGGAACCTCCCTACC R: TCGAGGGGGAAATGTTGTAA	100	Valero *et al*. (2015)
mx1	Interferon-induced GTP-binding protein Mx 1	F: AGGAGACGGTGGTTGACATC R: TCTCTTTCCGTTGCCTCAGT	127	FJ490556
mx2	Interferon-induced GTP-binding protein Mx 2	F: AAGAGGAGGACGAGGAGGAG R: TTCAGGTGCAGCATCAACTC	148	FJ490555
mx3	Interferon-induced GTP-binding protein Mx 3	F: GGTGATCCGCTACCAGATGT R: TCTGGGTGCCGATATCAAA	125	FJ652200
isg15	Interferon-stimulated gene 15	F: GTGAGCTCCCTGAAGCAACT R: GACCGTTTACAAACACCAGC	79	Álvarez-Torres *et al*. (2018)
tnfa	Tumour necrosis factor alpha	F: TTCCGACTGGTGGACAATAAG R: GAGATCCTGTGGCTGAGAGG	143	AJ413189
*il1*b	Interleukin 1 beta	F: AGCGCAGTAGAAGAGCGAAC R: CACTCGGACTAAGTGCCTCTG	117	AJ277166
il6	Interleukin 6	F: CCAGATCCCCTCAAGATTCA R: AAGGTGTCGGAGCTGTCG	144	EU244588
il10	Interleukin 10	F: CAGGCCATGAACAACATCC R: TGGACGTCAGATTTGAGCTG	143	JX976621
casp1	Caspase 1	F: TCGAAGAGACGGACAGTGTG R: CGTTGATGGGGAACTCATCT	123	AM490060
ck3	CC chemokine 3	F: AAAAGACCCTCTGAAACATCTCAC R: ACGATGCTGACCTCAGTGC	149	GU181394
ck10	CC chemokine 10	F: TGTCGGTCAAAAACCAAACA R: AGTCACTCCTGGTCACCAAAAT	111	GU181390
c3	Complement component C3	F: AAAGGCCGATACCACACAAG R: TTGACATCCACCCAAACAGA	146	HM543456
nccrp1	Nonspecific cytotoxic cell receptor protein 1	F: ACCGCAACTGTCTGATGTACC R: CTGAGGCAGTTGACAGACCA	145	AY651258
tcrb	T-cell receptor beta	F: AAGTGCATTGCCAGCTTCTT R: TTGGCGGTCTGACTTCTCTT	131	AM261209
ighm	Immunoglobulin mu heavy chain	F: AGCCTCGAGAAGTGGAAACA R: TACCCGATGGACCTGACAA	101	AM493677
mhcIIa	Major histocompatibility complex class II alpha	F: AGATGATGGTGGTCCTCGTC R: TTCACCATCCAGACCGTACA	125	DQ019401
b actin	Beta-actin	F: ATCACCATCGGCAATGAGA R: ACAGGTCCTTACGGATGTCG	139	AF384096
ef1a	Elongation factor 1 alpha	F: ATTGTCAAACTGCACCCACA R: GCTCAACAGCCTTGATGACA	127	AF184170

*Primer3web version 4.1.0 (http://bioinfo.ut.ee/primer3/) was used to design primers in this study (Koressaar and Remm 2007; Untergasser *et al*. 2012).

amplification conditions were: initial denaturation at 95°C for 10 min, followed by 42 amplification cycles at 95°C for 15 s, 62°C for 10 s and 72°C for 10 s. Dissociation curve analyses were carried out in order to detect nonspecific amplification products, using the following thermal profile: 95°C for 10 s, 65°C for 60 s and 97°C for 1 s. The qPCR were carried out in triplicate. The number of copies of LCDV DNA was calculated by interpolation in a standard curve, and viral loads were expressed as viral DNA copies per milligram of tissue.

Viral *mcp* gene expression was analysed as an indicator of productive infection. Relative quantification was carried out by RT-qPCR, following the protocol above-mentioned but using 20-μl final volume reactions and cDNA generated from 100 ng of the original RNA template. These qPCR were carried out in duplicate. Relative *mcp* expression levels were calculated for each sample using the comparative delta Ct method (Pfaffl 2004) with two reference genes, the elongation factor 1 alpha (*ef1a*) and *actb*, used for normalization (see below).

Host gene expression analysis

The expression of 23 host immune-related genes was analysed by qPCR using the primers shown in Table 1. The qPCR were performed as previously specified with a 20-μl final volume and cDNA generated from 100 ng of the original RNA template. The amplification conditions were as follows: 95°C for 10 min and 42 cycles of 95°C for 10 s, 58°C for 10 s, and 72°C for 10 s. The samples were run in duplicate. Relative mRNA expression was determined using the 2^{-DDCt} method (Schmittgen and Livak 2008), and data were expressed as fold change (mean SEM). Two reference genes, *ef1a* and *actb*, were used to normalize gene expression. No significant differences in Ct values were observed for the reference genes between infected and control groups during the course of the infection in the two organs analysed (Kruskal–Wallis test, $P > 0{\cdot}11$ and $P > 0{\cdot}13$ for *ef1a* and *actb* respectively). Samples from the control group (non-infected) were used as calibrator. Standard curves were performed for each reference and target gene based on 10-fold serial dilutions corresponding to cDNA transcribed from 100 to 0·1 ng total RNA (three replica per dilution). In all cases, the efficiency was over 95%.

A cluster analysis of the samples, based on the $\log_2$ fold change of the host genes, was conducted using the Expression Heat Map option on the web server Heatmapper (http://www2.heatmapper.ca/) (Babicki *et al.* 2016) with Euclidean as distance measurement method and complete linkage as clustering method. In addition, the expressed genes were also clustered using the same parameters.

To identify differentially expressed genes (DEGs) involved in gilthead seabream response against LCDV infection, and determine whether those genes were up- or downregulated in both organs analysed and at different times pi, volcano plots were constructed using the online software ShinyVolcanoPlot (https://paolo.shinyapps.io/ShinyVolcanoPlot/). A $P < 0{\cdot}05$ and $|\log_2$ fold change$| > 0{\cdot}5$ were set as threshold for statistically significant DEGs.

Statistical analysis

The qPCR data were log-transformed to get normality and homogeneity of variance, and normality of the data was analysed using a Shapiro–Wilk test. To determine significant differences in gene expression among organs and time points, a two-way ANOVA followed by Bonferroni *post hoc* test was carried out. When comparisons were carried out for a specific organ through time, or for different organs at a time point, a one-way ANOVA followed by Fisher's LSD test was used. Differences were considered significant when $P < 0{\cdot}05$. The statistical tests were carried out using xlstat software.

Results

Viral load and gene expression

To study the time course of LCDV-Sa infection in gilthead seabream after intramuscular injection, the number of viral DNA copies and the expression of the *mcp* gene were determined. No mortality or signs of disease were registered during the experimental period (8 dpi). LCDV-Sa was detected by qPCR in all the samples from inoculated fish analysed, whereas no amplification was obtained in samples from the control group. The viral load in the organs examined at different times pi is shown in Fig. 1a. In caudal fin samples, the amount of viral DNA remained stable over the experimental period, with an estimated viral load of 9·5 0·9 copies of viral DNA mg^{-1} of tissue. The viral load in HK was not significantly different ($P < 0{\cdot}05$) to that obtained in caudal fin at 1 and 3 dpi, whereas it was significantly lower ($P < 0{\cdot}05$) in the intestine at any time point analysed. At the end of the experiment, viral loads in HK and intestine were lower than in caudal fin, with no significant differences ($P < 0{\cdot}05$) between both organs.

Viral transcripts were detected in four of five fish analysed at 1 dpi and in all fish analysed at 3 and 8 dpi, although not in all the organs tested in each fish (Fig. 1b). The relative *mcp* expression in caudal fin was significantly lower than in HK at 1 dpi, with a slight but statistically significant ($P < 0{\cdot}05$) increase at 3 dpi.

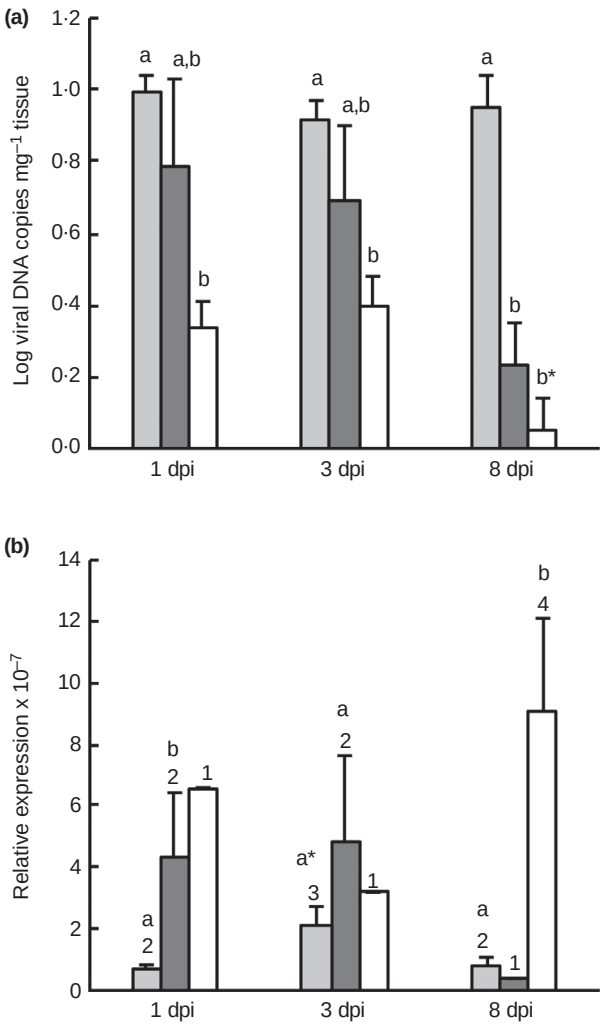

Figure 1 Viral loads (a) and relative *mcp* gene expression values (2^{-DCt}) (b) in gilthead seabreams infected with LCDV-Sa determined by qPCR. Data are expressed as mean SEM (total number of fish analysed per sample time, $n = 5$). In (b), the number of positive samples per organ and time point analysed is given above each column. Different letters denote significant differences between organs at a time point. Significant differences in an organ analysed at different times post-inoculation are indicated by an asterisk ($P < 0{\cdot}05$). Caudal fin (light grey bars), head kidney (grey bars), intestine (white bars).

Interestingly, the highest relative expression value was detected in the intestine at 8 dpi, while the viral load in those samples was the lowest.

Expression of immune-related genes

To evaluate the immune response against LCDV-Sa, a set of 23 genes were analysed both in HK and intestine samples at different times pi. The clustering analysis exhibit organ-specific expression profiles that change over the course of the infection (Fig. 2). In the HK, there is a clear difference in the expression profile at 1–3 dpi *vs* 8 dpi, whereas in the intestine the profiles are time-specific.

The *pkr* was the only gene not differentially expressed in any of the samples from LCDV-Sa-infected fish analysed. HK and intestine samples shared 14 DEGs in common. In addition, four genes were exclusively up- or downregulated in the HK (*il10*, *nccrp1*, *mhcIIa* and *tnfa*) or the intestine (*mx1*, *casp1*, *ck3* and *tlr9*) (Fig. 3).

Regarding HK samples, 18 genes were differentially expressed including *ifn*, *irf3*, *isg15*, *mx3, il10*, *ck10* and *ighm* that were upregulated at 1 dpi (3 dpi in the case of *ighm*) but appeared downregulated at 8 dpi. The expression of *mx2* and *mhcIIa* was also upregulated at 1 dpi, although their expression levels were not significantly different ($P < 0{\cdot}05$) to the control group at 3 and 8 dpi. The only transcripts upregulated at the end of the experimental period were *nccrp1* (also upregulated at 1 dpi), *tlr5* and *c3* (its expression was downregulated at 1 dpi). The other DEGs showed a downregulation, *il6* at 3 dpi, and *irf1*, *irf9*, *il1b*, *tnfa* and *tcrb* later on (Fig. 3a).

The number of DEGs in the intestine in response to LCDV-Sa infection was also 18, but the direction and/or the temporality of the expression were different in both organs (Fig. 3b). At 1 dpi, only *mx3* and *ck3* were upregulated, whereas *irf3*, *isg15*, *mx1*, *mx2* and *casp1* appeared downregulated. At 3 dpi, the downregulation of *irf3*, *isg15* and *mx1* was maintained and extended to *tlr9*, *mx3*, *ighm* and *tcrb*. As observed in HK, *irf3*, *il1b*, *ck10* and *tcrb* were downregulated at 8 dpi. The expression of *tlr5*, *irf1*, *irf9*, *il6* and *c3* appeared upregulated at 3 dpi. Finally, at 8 dpi the upregulation of *tlr5*, *irf9* and *il6* was maintained together with a delayed, compared to HK, upregulation of *ifn* and *ighm*.

In addition, a comparative analysis of the transcriptional level over time in both organs studied was carried out. Five genes, *tlr9*, *tnfa*, *casp1*, *ck3* and *mhcIIa*, were differentially expressed in one of the organs at a single time point and they had not been included in the analysis. Only two DEGs were upregulated in both organs, *tlr5* at 3 and 8 dpi (no significant differences were observed in the expression levels), and *ifn* at 1 and 3 dpi in the HK (mean transcription level of 2·7-fold change) and at 8 dpi in the intestine (5·8-fold) (Fig. 4). The IRF-encoding transcripts analysed showed an organ-specific regulation, with *irf1* and *irf9* upregulated at 3 dpi in the intestine (2·9- and 3·3-fold respectively), but significantly downregulated at 8 dpi in the HK, whereas *irf3* appeared upregulated only in HK samples at 1 dpi (1·7-fold), being downregulated in HK at 8 dpi and in all intestine samples analysed, with nonsignificant differences in the fold change (Fig. 4). In HK, *isg15*, *mx2* and *mx3* were differentially overexpressed early in the infection (with fold changes ranging from 1·8 to 3·6), with transcripts

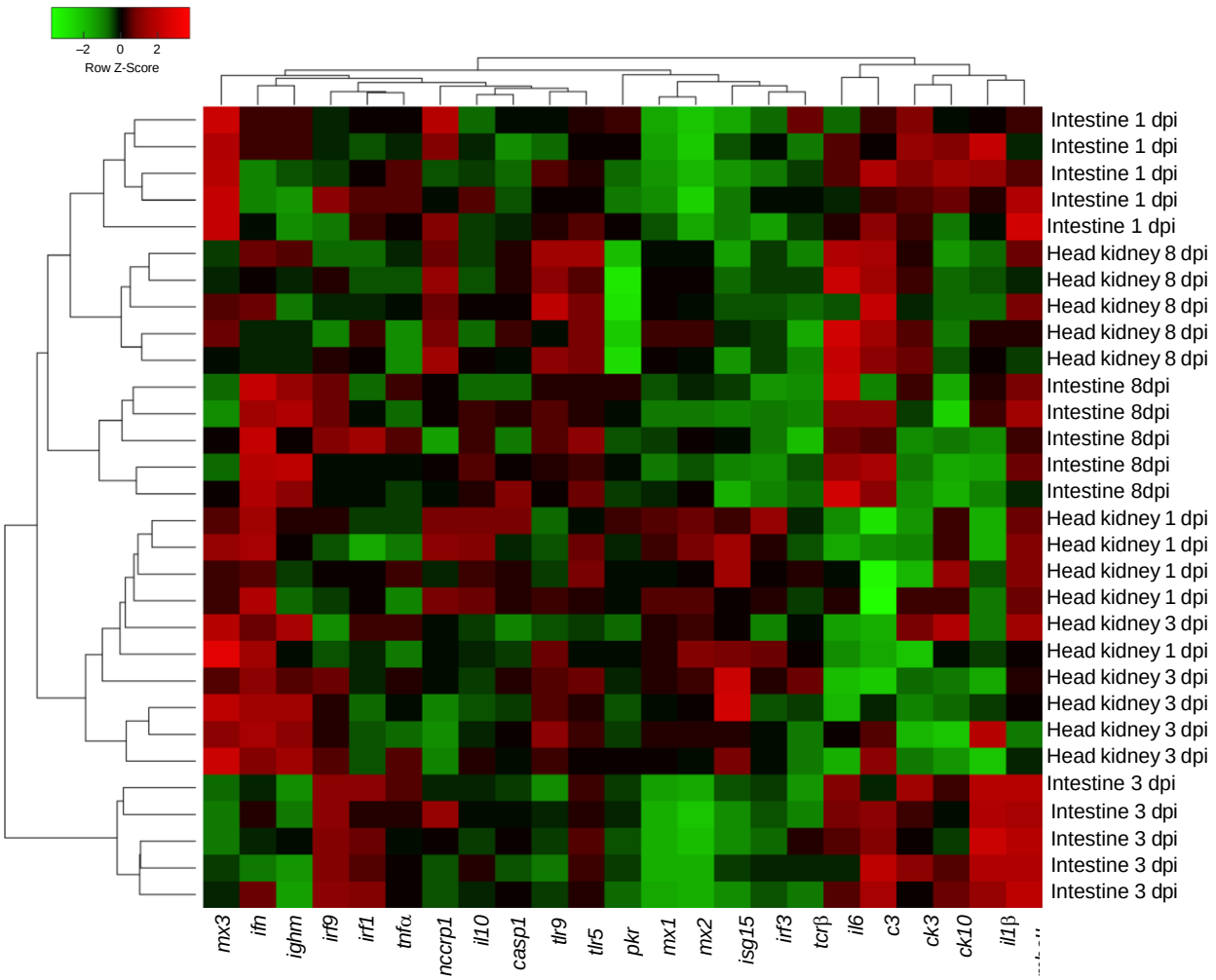

Figure 2 Hierarchical clustering analysis of the head kidney and intestine samples from LCDV-Sa infected gilthead seabreams based on 23 immune-related genes. Data are expressed as $\log_2$ fold change. Green and red colours indicate low and high expression values according to the scale shown. [Colour figure can be viewed at wileyonlinelibrary.com]

abundance not significantly different from the controls (or down-expressed in the case of *isg15*) at 8 dpi. On the other hand, the expression of the type I interferon (IFN)-stimulated genes was downregulated in the intestine samples at 1–3 dpi, except for the *mx3* gene that appeared upregulated at 1 dpi (3·8-fold), reversing its regulation at 3 dpi (Fig. 4).

The expression profiles of the DEGs coding cytokines are shown in Fig. 5. An early upregulation of *il10* and *ck10* was observed in HK samples (1·6- and 1·7-fold respectively), that was reversed at 8 dpi, while only *ck10* showed a downregulation in the intestine at 8 dpi. The expression of *il6* at 3 dpi was upregulated in the intestine (2·4-fold), maintaining the expression level at 8 dpi, and downregulated in the HK, whereas the *il1b* was differentially downregulated in HK at 1 dpi and in both organs at 8 dpi, no significant differences in the fold change were recorded among samples. The complement fraction *c3* transcription was initially downregulated in the HK at 1 dpi and upregulated at 3 dpi in the intestine with a similar transcription level at 8 dpi in the HK. Finally, an overexpression of *nccrp1* was observed in the HK at 1 and 8 dpi (mean transcription level of 1·7-fold), and for *ighm* at 3 and 8 dpi in HK and intestine samples, respectively (2·2- and 4·2-fold), whereas the expression of *tcrb* was downregulated in both organs at 8 dpi. The differences in the fold change among organs and time points for receptor markers genes were not statistically significant (Fig. 5).

Discussion

LCD is the main viral disease reported in gilthead seabream, a fish species with high economic value in the

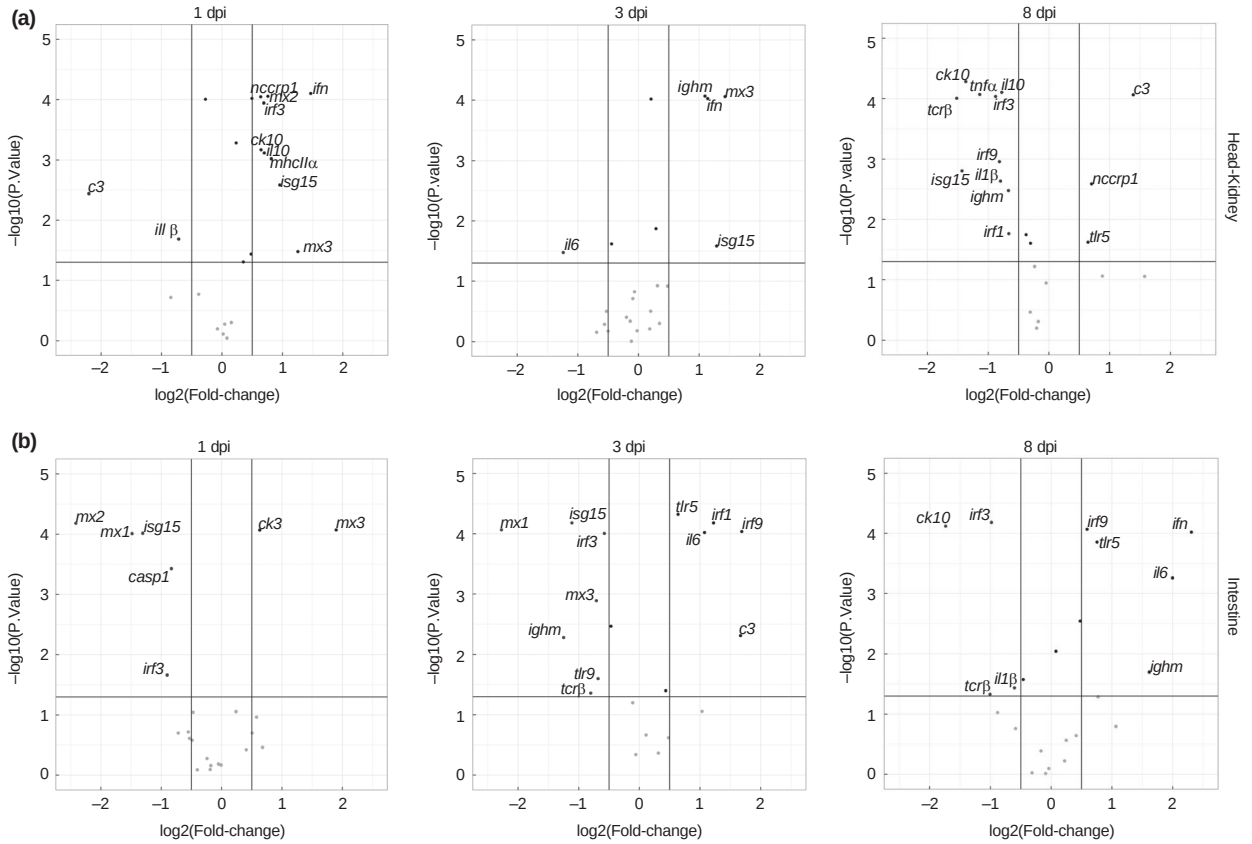

Figure 3 Volcano plots displaying differentially expressed genes (DEGs) in head kidney (a) and intestine (b) of gilthead seabreams infected with LCDV-Sa at different times post-inoculation. The vertical axis corresponds to $-\log_{10}(P\text{-value})$ and the horizontal axis shows the $\log_2$ fold change.

Mediterranean aquaculture (Borrego *et al.* 2001). A few studies have been focused on host–pathogen interactions in LCDV infections, but in the case of gilthead seabream all of them have analysed natural infections (Cano *et al.* 2009; Dezfuli *et al.* 2012; Cano *et al.* 2013; Cordero *et al.* 2016, 2017; Valverde *et al.* 2017a). This is the first study focused on the immune response of gilthead seabream infected with LCDV-Sa under experimental conditions.

In the present study, gilthead seabream specimens were intramuscularly inoculated with an isolate of LCDV-Sa and the course of the infection was investigated analysing viral load and *mcp* expression. Viral DNA and transcripts were detected in caudal fin, HK and intestine from 1 dpi, indicating that the virus spreads quickly from the injection site to the skin and internal organs, establishing a systemic infection as it has also been demonstrated in larvae feeding in LCDV-contaminated rotifers (Cano *et al.* 2013). The viral load in caudal fin remained low through the experiment, with values similar to those reported in fish sampled from populations suffering subclinical infections (Valverde *et al.* 2016, 2017a, 2017b). In HK and intestine, viral loads were significantly lower at the end of the experimental period, which could indicate a decrease in viral replication. Nevertheless, the overall *mcp* transcription levels were higher in HK and intestine than in caudal fin, suggesting that viral infection was more efficient in those organs, at least in terms of MCP expression. The MCP has been identified as a highly immunogenic protein in several iridoviruses including LCDV-C (Jang *et al.* 2011; Liu *et al.* 2015), which could explain the immune gene expression response triggered in both HK and intestine. The Japanese flounder can also be infected with LCDV-C by intramuscular injection, but, in contrary to our results, the viral load in all the organs analysed increased with time, reaching the higher value ($2{\cdot}4\ 9\ 10^7$ copies per μg total DNA) in skin samples at the end of the experiment (4 weeks pi), although the fish remained asymptomatic (Wu *et al.* 2015). In the Japanese flounder experimental model, LCD signs appeared 4–6 weeks pi at 20°C (Hossain *et al.* 2009; Iwakiri *et al.* 2014).

The immune response of gilthead seabream against LCDV-Sa was evaluated analysing the expression levels of 23 immune-related genes in two organs, the HK and the intestine. These genes included those coding two Toll-like receptors (*tlr5* and *tlr9*), members of the type I IFN

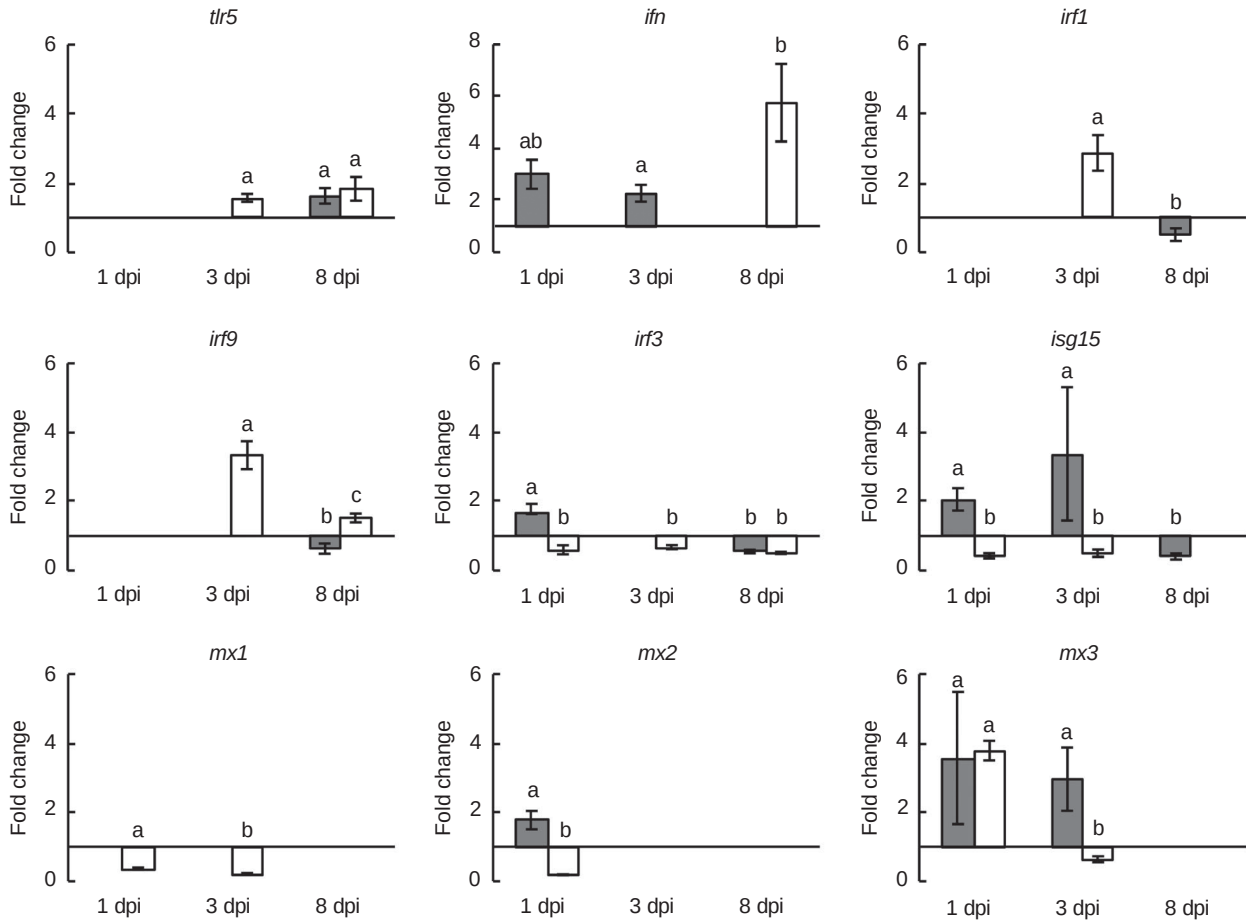

Figure 4 Relative expression levels (2^{-DDCt}) of *tlr5* and type I interferon-related genes differentially expressed in head kidney (grey bars) and intestine (white bars) at different times post-inoculation. Data are expressed as mean SEM (n = 5). Different letters denote significant differences between organs and time points analysed ($P < 0{\cdot}05$).

pathway (*ifn*, *irf1*, *irf3*, *irf9*, *pkr*, *mx1*, *mx2*, *mx3*, *isg15*), which plays a critical role in the innate immune response against viruses (Robertsen 2006; Collet 2014), several cytokines related to the inflammation process (*tnfa*, *il1b*, *il6*, *il10*) (Roca *et al.* 2008; Collet 2014), the pro-inflammatory caspase 1 (*casp1*), chemokines (*ck3* and *ck10*), the complement component C3 (*c3*) and several receptor markers characteristic of nonspecific cytotoxic cells (*nccr-p1*), T and B lymphocytes (*tcrb* and *ighm* respectively) and antigen-presenting cells (*mhcIIa*) (Cuesta *et al.* 2006; Castro *et al.* 2011; Chaves-Pozo *et al.* 2012; Rauta *et al.* 2012).

Human TLR9 recognizes foreign DNA molecules from bacteria and/or viruses that typically contain short sequences of unmethylated CpG dinucleotides in higher proportion than eukaryotic DNA (Pan *et al.* 2012). In the case of fish, *tlr9* expression is induced after bacterial challenge in different species (Pietretti and Wiegertjes 2014), but also after viral infection as has been recently demonstrated in rock bream (*Oplegnathus fasciatus*) infected by rock bream iridovirus (RBIV) (Jung and Jung 2017). On the other hand, human TLR5, but also zebrafish TLR5, recognizes flagellin, the principal component of the bacterial flagella, and the expression of *tlr5* is upregulated in fish species upon infection with motile bacteria (Pietretti and Wiegertjes 2014; He *et al.* 2019). In the present study, *tlr9* was not differentially expressed in the HK in LCDV-infected fish, whereas *tlr5* expression was upregulated in both organs at 8 dpi. In RBIV-infected rock bream, the expression of *tlr9* was dependent on the course of the infection; in fish maintained at 26°C, with a recorded mortality of 100% at 15 dpi, an overexpression in the HK was observed at 8 dpi, while no differential expression appeared in subclinically infected fish maintained at 17°C (Jung and Jung 2017). The *tlr5* is one of the DEGs found in a recent transcriptomic analysis of Japanese flounder infected with viral haemorrhagic septicaemia virus (Hwang *et al.* 2018), and its expression

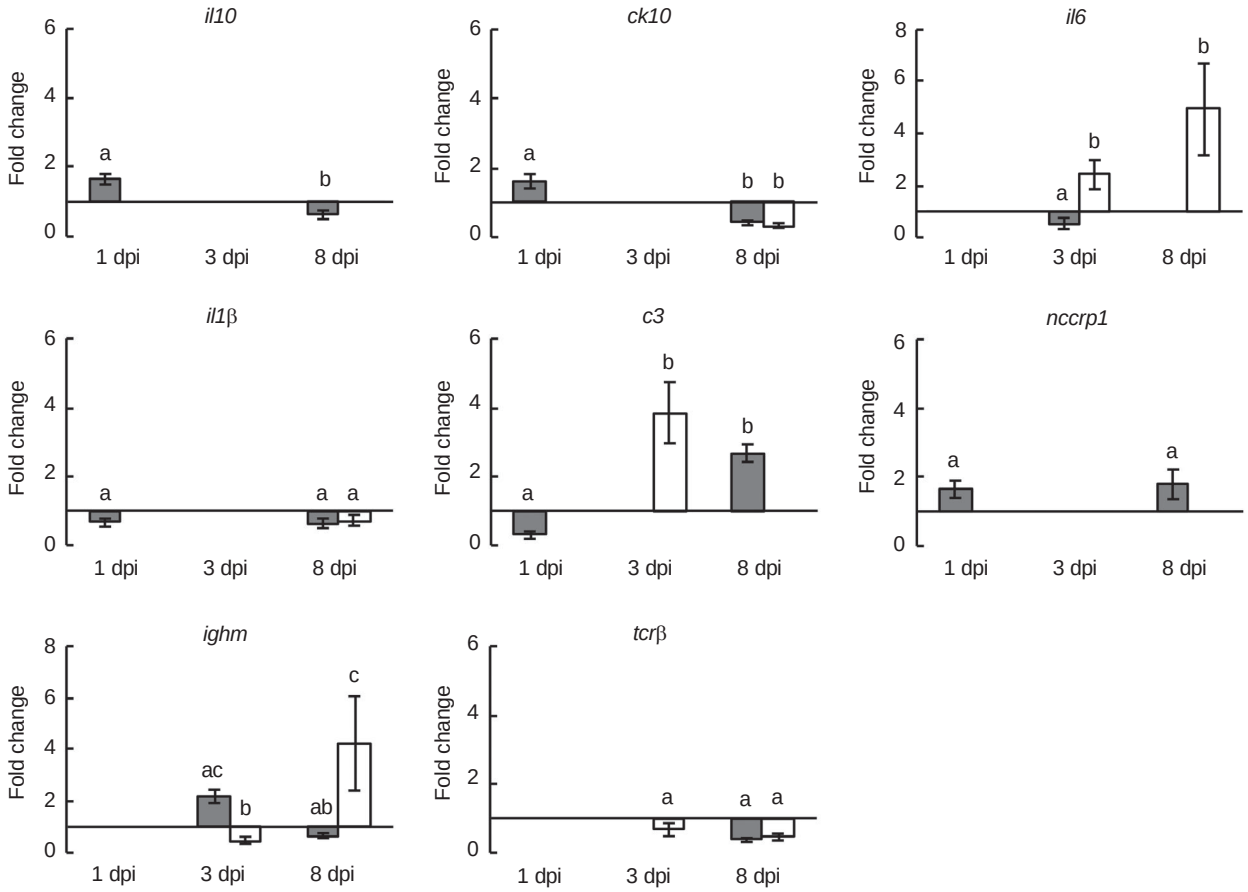

Figure 5 Relative expression levels (2^{-DDCt}) of differentially expressed genes coding cytokines, the complement component C3 and receptor markers of immune cells in the head kidney (grey bars) and intestine (white bars) at different times post-inoculation. Data are expressed as mean SEM ($n = 5$). Different letters denote significant differences between organs and time points analysed ($P < 0{\cdot}05$).

is also upregulated in Senegalese sole infected by LCDV-Sa (Carballo *et al.* 2017). Although the mechanism of regulation of *tlr5* expression in fish viral infections remains unclear, these results suggest that fish TLR5 sense other ligands besides flagellin.

Induction of type I IFN is crucial for innate antiviral immunity and is primarily controlled at the gene transcriptional level, the IRFs playing central roles. The IRF1, IRF3 and IRF7 act as positive regulators of type I IFN gene transcription through different regulatory pathways, while the IRF9 participates in the IFN signalling pathway to trigger the transcription of hundreds of genes through STAT-dependent mechanisms (Honda *et al.* 2006). In humans, more than 50 of these IFN-stimulated genes (ISGs) codify antiviral proteins (Schmeisser *et al.* 2010; Waddell *et al.* 2010). A small number of antiviral ISGs has been characterized in fish species, the *pkr*, *mx* and *isg15* among them (Verrier *et al.* 2011; Collet 2014). In gilthead seabream infected by LCDV-Sa, an upregulation of *ifn*, *irf3*, *mx2*, *mx3* and *isg15* expression was observed early in infection, whereas the expression of *irf1*, *irf9*, *pkr* and *mx1* remained un-affected. At 8 dpi all these genes lowered their transcription to levels not significantly different to control fish or were slightly downregulated. Fish Mx proteins present antiviral activity against a wide range of viruses, although the antiviral specificity varies among fish species and Mx isoforms (Garc´ıa-Rosado *et al.* 2010; Verrier *et al.* 2011). Mx1 and Mx2 from gilthead seabream, but not Mx3, showed *in vitro* activity against LCDV-Sa (Fern´andez-Trujillo *et al.* 2013). In addition, the expression of the three Mx coding genes was induced in SAF-1 cells infected with LCDV-Sa, reaching values up to fivefold higher than those observed after induction with poly I:C (Valverde *et al.* 2012). In LCDV-infected gilthead seabreams, *mx3* is the ISGs gene with the highest overexpression level, with *mx2* only slightly upregulated in HK at 1 dpi. The expression of *ifn*, *irf3* and *mx* was also upregulated at 1–4 dpi in Japanese flounder injected with LCDV-C (Hu *et al.* 2011), while *mx* and *isg15* were downregulated 1 week after infection (Wu *et al.* 2018).

These results suggest that even though an antiviral innate response related to type I IFN is produced, both in gilthead seabream and Japanese flounder, it is not sufficient to eliminate the virus.

Upon LCDV-Sa challenge, both by intramuscular or intraperitoneal injection, juvenile gilthead seabream developed an asymptomatic infection, with low viral loads detected in caudal fin samples for at least 2 months (unpublished results). On the contrary, in Japanese flounder infected by LCDV-C, a highly productive infection is established leading to the appearance of the disease (Wu *et al.* 2015). The transcriptomic analysis carried out in LCDV-infected Japanese flounder showed an upregulation of numerous genes related to inflammation and apoptosis, among others process, whereas the expression of some anti-inflammatory cytokines was downregulated. Nevertheless, the virus multiplication was not affected in spite of the host response observed at 7 dpi (Wu *et al.* 2018). In Senegalese sole infected with LCDV-Sa, a high activation of IRF-encoding transcripts (including *irf1*, *irf3* and *irf9*) and *mx* expression was observed, both in HK and intestine samples, and furthermore, the expression of pro-inflammatory cytokines (*tnfa*, *il1b*, *il6*, *if8* and *il12*), chemokines, and complement components was also activated. In these animals, the viral load decreased from 1 to 7 dpi, indicating that the robust immune response limits virus replication (Carballo *et al.* 2017). These results highlight the importance of the inflammation as an early response to LCDV infection, and point out the presence of potential immune evasion mechanisms in LCDV-C that need to be addressed.

The inflammatory reaction is characterized by the systemic release of specific cytokines and chemokines and by the chemotactic migration of leucocytes (neutrophils and macrophages) to the site of inflammation (Collet 2014). This process is critical to an efficient innate and subsequent adaptive immune response to viral infections (Hussell and Goulding 2010). The expression of pro-inflammatory cytokines, *casp1*, and chemokines were not induced in the HK of LCDV-infected gilthead seabreams, with the exception of a slight upregulation of *ck10* at 1 dpi. Moreover, *il10* was upregulated at 1 dpi. The presence of the anti-inflammatory cytokine IL-10 early in the course of infection is a factor in the development of persistence in different viruses infecting mammals (Wilson and Brooks 2011). In addition, low levels of activation of pro-inflammatory cytokines, accompanied by induction of an ineffective type I IFN response and upregulation of *il10*, have been related to the establishment of persistent IPNV infections in Atlantic salmon (Reyes-Cerpa *et al.* 2012). The results obtained in the present study suggest that a similar mechanism could be implicated in the presence of long-term asymptomatic LCDV infections in gilthead seabream. These kind of infections are frequently detected in gilthead seabream farms, with prevalence values up to 100% (Valverde *et al.* 2016).

The expression profile of immune-related genes in the intestine of LCDV-infected gilthead seabream was different than observed in HK. The transcription of *irf1* and *irf9* was upregulated at 3 dpi, which could be related to the delayed overexpression of *ifn*, while the *irf3* showed downregulation through the experimental period. Regarding ISGs, *mx3* was upregulated at 1 dpi, but the other four genes analysed were downregulated at 1 and 3 dpi. At 8 dpi, both *ifn* and *il6* were strongly upregulated, and the expression level of *il1b* was also very high, although not statistically different from control non-infected fish. This expression profile could be related with a scenario of local inflammation in the intestine which could explain the decrease in viral load observed in this organ at the end of the experimental period.

The complement component C3 plays a central role in all the activation pathways of the complement system, and is involved in both innate and adaptive immune responses participating in biological processes like lysis of micro-organisms, promotion of phagocytosis, triggering inflammation and immune clearance (Dunkelberger and Song 2010). In the present study, an induction of the *c3* transcription was observed in the intestine and HK at 3 and 8 dpi respectively. An upregulation of *c3* has also been described in Japanese flounder gills in response to LCDV infection at 7 dpi (Wu *et al.* 2018), indicating that complement activation could be a common host defensive strategy triggered by LCDV infection.

The transcription of *nccrp1* was induced in HK early in the infection, and it was maintained at 8 dpi, which indicates that nonspecific cytotoxic cells (NCCs) can be important in the innate cell-mediated cytotoxicity against LCDV-infected cells. A positive regulation of *nccrp1* was also described in the HK of lymphocystis (LC)-diseased gilthead seabream, collected from a natural LCD outbreak, suggesting that this gene plays a major role in LCDV infection (Cordero *et al.* 2016). In addition, *mhcIIa* was also overexpressed in HK at 1 dpi, whereas *tcrb* expression was downregulated at 8 dpi in both organs analysed, and *ighm* expression was upregulated at 3 and 8 dpi in HK and intestine samples respectively. These results could indicate that LCDV infection triggers an efficient humoral adaptive immune response in gilthead seabream, in which antigen-presenting cells and B lymphocytes are stimulated, but an impaired T-specific cell immunity. In LC-diseased fish, the expression of all these three genes was strongly downregulated (Cordero *et al.* 2016). The downregulation of *tcrb* is of special relevance, since T lymphocytes plays a major role in the

control of virus infections (Rauta *et al.* 2012; Fischer *et al.* 2013).

In summary, the immune response triggered by LCDV-Sa in gilthead seabream seems to be characterized by a slightly and transitory activation of type I IFN system and a lack of systemic inflammatory response. Although NCC activity could be implicated in virus clearance, an impairment of the cellular adaptive immune response would allow to an unsuccessfully immune-mediated elimination of infected cells. This type of response could lead to the establishment of an asymptomatic persistent infection and it may be the result of immune evasion mechanisms present in LCDV-Sa which warrants further investigation.

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Conflict of Interest

No conflict of interest declared.

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