Molecular identification and characterization of haptoglobin in teleosts revealed an important role on fish viral infections

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Abstract

Haptoglobin (Hp) molecule has been cloned and characterized in two marine teleosts (gilthead seabream and European sea bass), obtaining putative proteins of 319 residues encoded by an ORF of 960 bp in both species. However, the matrix of similarity revealed low identities among bony fish species 78.9% (seabream-sea bass), 43% (seabream/seabass-zebrafish) and lower than 20% with sharks and human. The protein sequences showed a signal peptide from the position 1 to 23, a trypsin domain from 47 to 297, and several predicted disulfide bridges and glycosylation sites. The expression of *hp* transcript levels during ontogeny showed a progressive increase of expression in seabream whilst remained almost unaltered in sea bass. By tissues, this gene was found constitutively expressed with the highest levels on liver in both species. The main results on *hp* transcript levels showed the up-regulation in gilthead seabream suffering from naturally occurring lymphocystis disease; and the down-regulation and up-regulation after nodavirus infection in the resistant gilthead seabream and the susceptible European sea bass, respectively. These findings demonstrate for the first time an important role of haptoglobin against viral infections, operating differently in two of the most important marine farmed fish species.

Introduction

The animal reactions against infection, inflammation, or trauma are collectively known as the acute-phase response and comprise a wide range of pathophysiological responses to maintain the general homeostasis (Jain et al., 2011). Among these responses, liver produces a large number of plasma proteins which are known collectively as the acute-phase proteins (APP) being the most studied C-reactive protein (CRP) and serum amyloid P (SAP) and A (SAA). Acute phase response leads to increase some humoral components of the innate arm such as both complement and clotting systems, opsonins, antimicrobial peptides, lectins, lysozymes or metal-binding proteins among others. However, the contribution of APPs to fish immune response is not very well characterized (Bayne and Gerwick, 2001).

Haptoglobin (Hp) is the major hemoglobin-binding protein in the plasma of many vertebrates and all the mammals (Jayle et al., 1952; Wicher and Fries, 2006). Thus, this molecule binds to hemoglobin (Hb) forming Hp-Hb complexes and leading to the inactivation and clearance of oxidant radicals, and therefore it has a clear role in the oxidative stress (Alayash, 2011). Apart from being involved in the redox balance, Hp is considered a positive acute phase protein (APP), which has been reported to regulate the host immunity in mammals (Huntoon et al., 2008). In mammals, Hp has been reported to support the proliferation and functional differentiation of B and T lymphocytes (Huntoon et al., 2008) and affecting to the imbalance of T helper cell type 1/cell type 2 (Arredouani et al., 2003). Several APPs, including Hp as one of the most important, have been reported in different mammalian viral infections (Gómez-Laguna et al., 2010; Pomorska-Mól et al., 2012; Saco et al., 2016; Stenfeldt et al., 2011), and also in combination with bacterial infections (Pomorska-Mól et al., 2013), but most of them have only evaluated its levels in serum.

Among the most relevant virus of fish, lymphocystis disease virus (LCDV) is the causative agent of the main viral disease in cultured gilthead seabream, characterized by hypertrophy of fibroblastic cells in the connective tissue of skin (Borrego et al., 2015; Hick et al., 2016). On the other hand, nodavirus or nervous necrosis virus (NNV) produces viral encephalopathy and retinopathy altering the brain and retina structure and function (Breuil et al., 1991; Chaves-Pozo et al., 2012; Munday et al., 2002). NNV is nowadays considered one of the most serious viral diseases in marine aquaculture, which can be transmitted to the progeny through the gonad causing great mortality at early fish stages, especially in European sea (Breuil et al., 1991; Chaves-Pozo et al., 2012; Munday et al., 2002; Valero et al., 2015a).

In teleosts, some studies have evaluated the general role of APPs (including Hp) in different bacterial infections (Jayasinghe et al., 2015; Lin et al., 2007). However, little is known about the role of Hp in fish viral infections. Only one previous general study at protein level revealed the modulation of Hp levels in plasma by several agents such as yeast, bacteria and virus in rainbow trout (Gerwick et al., 2002) but nothing is known about its transcriptional regulation under naïve or infection conditions. To enlighten more about the fish *hp* gene and its role on antiviral immunity, the aim of this work was to clone and analyze its structure and evaluate its expression profile during the ontogeny, the tissue distribution and viral infections in gilthead seabream and European sea bass, two key farmed fish species in the marine aquaculture.

2. Material and methods

2.1 Animal maintenance and tissue

Adult specimens of the seawater teleost gilthead seabream (*Sparus aurata*) and European sea bass (*Dicentrarchus labrax*) (100- 200 g and 300-500 g body weight, respectively) were breed at the *Centro Oceanográfico de Murcia* (IEO, Mazarrón, Murcia) and transferred to the University of Murcia aquaria. Fish were kept in 450-500 L running seawater (28 ‰ salinity) aquaria at 24 ± 2ºC and with a 12 h light:12 h dark photoperiod. Animals were fed daily with 1 % body weight of a commercial pellet diet (Skretting) and acclimatized for 15 days prior to the experiments. These fishes were used for the *in vivo* infections as well as for tissue [brain, gill, gut, skin, liver, head-kidney (HK), spleen, thymus, gonad and blood] isolation by placing a piece of tissue in TRIzol reagent (Life Technologies) for later RNA isolation.

To obtain larvae, gilthead seabream and European sea bass healthy broodstocks were kept with natural seawater (38 ‰ salinity) and natural photoperiod at the *Centro Oceanográfico de Murcia* (IEO, Mazarrón, Murcia). Gilthead seabream and European sea bass larvae eggs were incubated at natural seawater (38 ‰ salinity) heated at 17 ± 1 ºC and filtered through mechanical and biological substrates. The temperature increased naturally and reached 26 ºC by the end of the developmental period studied. Gilthead seabream larvae were bred using phytoplankton with the "green water" technique (Papandroulakis et al., 2001) in a 5,000 L round tank with an initial density of about 60 eggs l^{-1}. During the experiment, the light intensity was 1,000 lux at the water surface, and the photoperiod was 16:8 (L:D). Water renewal was limited to 2 % daily during the first 20 days of culture and was achieved by the addition of 70 ml m^{-3} of a microalgae concentrated solution (Phytobloom, Necton) containing 80 % *Nanochloropsis oculata*. Subsequently, continuous water renewal (30 % h^{-1}) and light aeration were provided in the tank. Larvae were successively fed with enriched (Selco, Inve Animal Health) rotifers from 6 to 24 days post-hatching (dph), *Artemia* nauplii

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(Inve Animal Health) from 20 to 35 dph, enriched Instar II *Artemia* from 31 to 58 dph and a commercial dry pellet diet (Skretting) from 54 dph onward. The sea bass larvae were kept in dark during the first 40 days upon hatching. Afterwards, the light intensity was 1,000 lux at the water surface, and the photoperiod was 16:8 (L:D). The specimens were subsequently fed with enriched Instar II *Artemia* and a commercial dry pellet diet (Skretting) from 54 dph onwards. Eggs and larvae at different time points from hatching (0, 3, 10, 13, 17, 24, 31 and 59 dph) were sampled and stored at -80ºC in TRIzol reagent for later RNA isolation. The experiments described comply with the Guidelines of the European Union Council (2010/63/EU) and the Bioethical Committee of the University of Murcia (Spain) and the Bioethical Committee of IEO (reference REGA ES300261040017) for the use of laboratory animals.

2.2 SSN-1, SAF-1 and DLB-1 cell lines

Cell lines were cultured at 25ºC in 25 cm^2 plastic tissue culture flasks (Nunc) and maintained at exponential growth. The established striped snakehead SSN-1 (Frerichs et al., 1996), SAF-1 (Béjar et al., 2005) and DLB-1 (Morcillo et al., 2017) cell lines were cultured using Leibovitz's L-15-medium (Life Technologies) supplemented with 10% fetal bovine serum (FBS; Life Technologies), 2 mM L-glutamine (Life Technologies), 100 µg ml^{-1} streptomycin (Life Technologies), 100 U ml^{-1} penicillin (Life Technologies) and 10 mM HEPES (Life Technologies). The medium osmolarity of DLB-1 was adjusted according to the sea bass serum (353 ± 2 mOsm kg^{-1}).

2.3 Bacterial infection

The bacterial strain *Vibrio anguillarum* R-82 was grown in tryptic soy broth (TSB) culture medium overnight at 22ºC and continuous shaking. The culture was washed twice in phosphate-buffered saline (PBS), the optical density measured at 600 nm and bacterial

density adjusted to 10^8 bacteria ml^{-1} in PBS. They were then heat-killed at 60ºC for 30 min, washed and stored at -80ºC until used. Gilthead seabream specimens were divided into two groups, receiving an intraperitoneal (ip) injection of 1 ml of PBS alone (control group) or the same volume of PBS containing 10^8 bacteria ml^{-1} (infected group). Liver, HK and spleen tissues as well as peritoneal exudate leucocytes (PEL) were sampled after 4 h and 72 h of ip injection. Firstly, to obtain PELs 5 ml of culture medium were ip injected and the exudate collected by a slight peritoneal incision and a Pasteur pipette after 5 min of fish peritoneal massage. PELs were then washed by centrifugation and stored in TRIzol reagent at -80ºC for later RNA extraction. Tissues were obtained by dissection and immediately stored in TRIzol reagent.

2.4 Natural LCDV infection

Specimens of gilthead seabream (average weight 41.6 ± 3.6 g) with clear symptoms of LCDV (white nodules, individually or in clusters distributed by the body surface) or apparently healthy (control) were kept in re-circulating aquaria with a flow water of 900 L h^{-1}, 28 ‰ salinity, 20 ± 2ºC temperature and a photoperiod of 12h light: 12h dark. Control and diseased fish were sampled as described in a previous work (Cordero et al., 2016a). Skin and HK were dissected and immediately stored in TRIzol at -80ºC for later RNA isolation.

2.5 NVV infection

For the *in vitro* assays, duplicate cultures of SAF-1 and DLB-1 cells were incubated for 24 h with culture medium alone (controls) or containing 50 µg ml^{-1} polyinosinic acid (pI:C; Sigma-Aldrich) or 10^6 $TCID_{50}$ ml^{-1} NNV (strain 411/96, genotype RGNNV). After treatment, monolayers were carefully washed with PBS and stored in TRIzol reagent (Life Technologies) at -80ºC for later RNA isolation.

For the *in vivo* trial, specimens of gilthead seabream or European sea bass specimens received a single intramuscular injection of 100 µl of SSN-1 culture medium (mock-infected) or culture medium containing 10^6 NNV $TCID_{50}$ (Chaves-Pozo et al., 2012) since this route of infection has been proven as the most effective (Aranguren et al., 2002). Both species were sampled 1, 7, 15 days after the viral injection and brain, HK and gonad were stored for RNA isolation. Whilst seabream is reservoir and resistant (no mortality) to NNV infection, sea bass was susceptible with a mortality of 55% (Chaves-Pozo et al., 2012).

2.6 RNA extraction

Samples from the above experiments were homogenized with a pellet pestle and total RNA was extracted from each tissue with Trizol reagent (Life Technologies) according to the manufacturer's instructions. RNA present in samples was then quantified and the purity assessed by spectrophotometry; the 260:280 ratios were 1.8-2.0. The RNA was treated with DNase I (Promega) to remove genomic DNA contamination. To check the RNA quality, an agarose gel was also run. Complementary DNA (cDNA) was synthesized from 1-2 µg of total RNA using the SuperScript III reverse transcriptase (Life Technologies) with an oligo-dT18 primer.

2.7 Molecular cloning of *haptoglobin*

According to published sequences of *hp* orthologous we performed a BLAST analysis through NCBI database (http://www.ncbi.nlm.nih.gov/) against the gilthead seabream and European sea bass expressed sequenced tags (EST). When identified, mRNA sequence was obtained according to the 3' RACE and 5' RACE protocols (Life Technologies) for the 3'-UTR and 5'-UTR amplification.

Samples were run by nested PCR with the corresponding primers in each case (Table 1). Amplification was performed in 20 µl samples containing 2.5 µl of PCR reaction buffer

(10×, Life Technologies), 2 µl forward and reverse primers for *hp* in gilthead seabream and European sea bass (10 mM each), 0.5 µl dNTP mix (2.5 mM each), 1.25 µl MgCl$_2$ (50 mM), 0.25µl Taq DNA polymerase (5 U l^{-1}, Life Technologies), 15.25 µl DNase/RNase-free distilled water (Life Technologies) and 2 µl cDNA from the liver. PCR reactions were performed in a MasterCycler Gradient PCR: 94ºC for 5 min, 35 cycles of 94ºC for 45 s, 55ºC for 45 s, 72ºC for 45 s, and followed by 72ºC for 10 min. The PCR products were separated in a 1.2% agarose (Pronadisa) gel containing 0.01% (v/v) of Red Safe$^®$ (Life Technologies) and visualized under UV light. Bands were picked up and DNA extracted with GenJET Gel Extraction Kit (Thermo Scientific) according with the manufacturer's instructions. Ligation was performed with an incubation of 20 min containing 4µl of eluted DNA, 1µl of salt solution and 1µl of pcDNA 3.1/V5-HIS TOPO vector (Invitrogen). Transformation was performed using TOP10 chemically competent cells (Life Technologies) according to the manufacturer's instructions. Competent cells were spread in Luria Agar (Pronadisa) plates and incubated at 37ºC for 24h. Next day, a single colony was inoculated in Luria Broth (Pronadisa) with mixing at 250 rpm at 37ºC for 24h. Then, the plasmid was isolated with Gen Elute^{TM} Plasmid Mini Kit I (Sigma-Aldrich). The DNA was quantified by Nanodrop$^®$ as described elsewhere. Concentration was adjusted to 100 ng µl^{-1} to carry out the Sanger sequencing (Sanger et al., 1977).

2.8 Real-time PCR study

The expression of *hp* gene in both fish species was analyzed by real-time PCR (rtPCR), which was performed with an ABI PRISM 7500 instrument (Applied Biosystems) as described elsewhere (Cordero et al., 2015) and using the $2^{-\Delta Ct}$ method (Livak and Schmittgen, 2001). For each *hp* mRNA, gene expression was corrected by the elongation

factor 1α (*ef1a*) content in each sample. Information of primers is listed in Table 1. In all cases, each PCR was performed twice to confirm the results.

2.9 Bioinformatic analysis

After sequencing process, files were primarily analysed by Chromas software version 2.4.4. Next, we found the ORF and encoded protein with DNAstar Lasergene software version 7.1. The putative protein features were obtained with ProtParam tool (http://web.expasy.org/protparam/). A ClustalW was made with similar protein sequences after BLASTp analysis with different organisms in the evolution-scale, and then the Neighbor-joining tree was performed with MEGA software version 7.0 (Kumar et al., 2016). The alignments with the conserved domains were performed with BioEdit software version 7.2.5 (Hall, 1999). The signal peptide and the glycosylation sites were retrieved with SignalP 4.1 and NetNGlyc 1.0 servers, respectively.

2.10 Statistical analysis

The results are expressed as mean ± SEM. Data of challenges were statistically analyzed by T-student test to determine differences between groups. Data of expression from tissues and fish ontogenetic profile were statistically analyzed by one-way ANOVA and Tukey's post-hoc test. All the statistical analysis were conducted using Statistical Package for Social Science (SPSS for Windows; v19.0, USA) and differences were considered statistically significant when $p < 0.05$.

3. Results

3.1 Sequence of *hp* in gilthead seabream and European sea bass

The results of cloning and sequencing the haptoglobin in both species revealed mRNAs with 5'-UTR of 38 and 32 bp and 3'-UTR of 178 and 174 bp for gilthead seabream and

European sea bass, respectively; with both open reading frames (ORF) of 960 bp, which encode a polypeptide of 319 amino acids in both cases. The predicted molecular weight (MW) and the theoretical isoelectric point (pI) was 34.9 kDa and 7.6 for gilthead seabream and 35.1 kDa and 8.5 for European sea bass. Both sequences were submitted to NCBI databank with the following accession numbers KU940258 for gilthead seabream and KU940259 for European sea bass.

3.2 Structural and phylogenetic comparative analysis of haptoglobin

After BLAST analysis, the alignment of Hp sequences with other from important teleosts as well as from cartilaginous fish and mammals, including human, revealed a high variability of this molecule (Figure 1). We found no alignment matches with birds, reptilians or lamprey species. In addition, we only found three conserved domains between both species (positions 70 to 85, 158 to 174 and 242 to 256). Globally, there are only 27 conserved residues across all the analyzed species (Figure 1). Despite both sequences have 319 aa, the similarity of gilthead seabream and European sea bass was 78.9% whilst zebrafish showed similarity of only 42.3% with both studied species, human showed a similarity of 18.6% and 19.7% with gilthead seabream and European sea bass, respectively, and surprisingly the similarity was even lower (15-18%) with cartilaginous fish (Table 2).

The Neighbor-joining phylogenetic tree was also constructed and reflected three different clusters, one cluster with all the teleost fishes (including our two species), other cluster with mammals (including mousse and human) and the farthest cluster with cartilaginous fishes (Figure 2A). Regarding the putative protein structure, signal peptide of 23 aa and trypsin domains (positions 49 to 247) were found in both species (Figure 2B). About the conserved cysteine residues, we have found two disulfide bridges (between 214-233 and 244-273) in gilthead seabream and three disulfide bridges (49-297, 214-233 and 244-273) in European

sea bass, whilst potential N-glycosylation sites were found in the residues 2, 141 and 302 for gilthead seabream and 96, 123, 141 and 302 for European sea bass. Interestingly, SUSHI domain is missing in all the teleost fishes analyzed, including gilthead seabream and European sea bass, while two were found in cartilaginous fishes and human but only one SUSHI domain in mouse (Figure 2B).

3.3 Haptoglobin is constitutively expressed in both gilthead seabream and European sea bass

During the ontogeny, the transcript profile of *hp* revealed very low expression at all the analyzed stages from eggs to 73 dph in both species (Figures 3A and 3B). In gilthead seabream, the expression of *hp* was increasing constantly to 59 dph reaching a significant level compared to the rest of larval stages (Figure 3A); whilst in European sea bass high variability in the *hp* transcript profile was found from eggs to 13 dph achieving stability from 17 dph onward (Figure 3B). Interestingly, *hp* transcription was around ten-fold higher in seabream than in sea bass.

In naïve adults, the transcript profile of *hp* in tissues (Figure 3C and 3D) revealed its constitutive expression in all the assayed tissues as well as in the gilthead seabream cell line SAF-1, and in the European sea bass cell line DLB-1. In both species, liver showed the highest levels of *hp* transcript. Strikingly, and compared to the transcription during development, *hp* gene expression was around ten-fold lower in seabream compared to sea bass tissues.

3.4 Role of *hp* on fish bacterial infections

Based on the idea that Hp is an acute phase protein involved in bacterial infections we did a preliminary test to ensure this. Thus, our data showed the up-regulation of *hp* transcription

in gilthead seabream liver, HK, spleen and peritoneal exudate leucocytes after 4 h of ip injection of *Vibrio anguillarum* (Supplementary Figure 1).

3.5 LCDV natural outbreak up-regulated the *hp* transcript level

During a natural outbreak of the LCDV in gilthead seabream we evaluated the transcript levels of *hp* in the main lymphomyeloid organ, the HK, as well as in the target tissue of LCDV, the skin. The gene expression levels of *hp* after a natural outbreak of LCDV was significantly up-regulated in HK whilst it showed no differences in the skin, both compared to these tissues in the control or non-infected group of gilthead seabream (Figure 4).

3.6 NNV infection down-regulates *hp* transcription in seabream but up-regulates it in sea bass

We evaluated the transcription of *hp* after *in vitro* or *in vivo* NNV infections in gilthead seabream and European sea bass. First, poly I:C-treated and NNV-infected seabream SAF-1 cell line showed down-regulated transcription of *hp* (Figure 5A) whilst it was unaffected in the case of the sea bass DLB-1 cell line (Figure 5E).

In *in vivo* NNV infections, the expression of *hp* was evaluated in the target tissue of NNV, the brain, the primary lymphomyeloid organ, the HK, and the gonad, which is used to vertically transmit the virus. In the case of seabream (Figure 5B-D), a resistant species to the NNV strain used, *hp* transcription was significantly down-regulated in the brain (Figure 5B) 1 day post infection and in the HK (Figure 5C) after 7 days of infection whilst no changes were observed in the gonad (Figure 5D). In the case of European sea bass (Figure 5F-H), a very susceptible species, *hp* transcription was up-regulated in the brain (Figure 5F) after 15 days of infection but interestingly it was significantly up-regulated in HK (Figure 5G) and gonad (Figure 5H) tissues at all the assayed times, reaching up to 1,000-fold increments in the gonad after 7 days of NNV infection.

4. Discussion

In general, haptoglobin has been mainly studied in relation with its capacity to bind hemoglobin (Alayash, 2011; Dobryszycka, 1997; Polticelli et al., 2008; Wicher and Fries, 2006). In the last years, however, few studies have focused their interest on the role of haptoglobin in immunity (Huntoon et al., 2013, 2008).

From an evolutionary perspective, Hp appeared in fish but is not present in birds and amphibians (Wicher and Fries, 2006). Phylogenetic analysis with the available sequences of haptoglobin revealed strong differences among species. In general, the multiple alignments and the neighbor-joining tree matched for each species, but surprisingly, rainbow trout and Atlantic salmon showed very close genetic distance according to the neighbor-joining tree but quite different putative sequences according with the alignments of their sequences with 358 and 316 residues, respectively, which may indicate different evolution and/or function in these genetically similar species. On the other hand, in our two fish species, despite of showing lower similarity according with the neighbor-joining tree, the alignment of their sequences revealed that gilthead seabream and European sea bass are similar with 319 residues in both cases, suggesting a conserved function of this molecule in these two Mediterranean farmed fish species. Comparing the domains we found a very curious finding: cartilaginous fishes showed two SUSHI domains in a similar way than human, whilst mouse contains only one SUSHI domain, approaching the structure of human haptoglobin closer of cartilaginous fishes rather than mouse. The function of this protein deserves to be investigated as well as why these domains are not present in bony fish.

The acute phase proteins have been scarcely studied in fish (Bayne and Gerwick, 2001; Gerwick et al., 2002; Lin et al., 2007; Peatman et al., 2007). Among them, little attention has been paid to haptoglobin so far. At protein level, Hp has been only partially detected in plasma of rainbow trout (Gerwick et al., 2002), but not detected in 2D proteomic characterizations of fish serum (Brunt et al., 2008; Douxfils et al., 2011; Ghisaura et al., 2014; Isani et al., 2011; Russell et al., 2006) or mucus (Cordero et al., 2016c, 2015; Jurado et al., 2015; Patel and Brinchmann, 2017; Rajan et al., 2013, 2011; Sanahuja and Ibarz, 2015). More data are found at transcriptomic level where *hp* is easily found in skin (Valenzuela-Muñoz et al., 2017), liver (Asker et al., 2013), heart (Marques et al., 2008), larvae (Wang et al., 2014) and whole fish body (Meijer et al., 2005) of several fish species. In our study, we found that *hp* is constitutively expressed in all the analyzed tissues from gilthead seabream and European sea bass, with the highest transcript levels in liver. During the ontogenetic development, *hp* was expressed in all the larvae stages including eggs. However, the pattern of expression during ontogeny was very different in the two species; whilst the transcriptional levels of *hp* were concomitantly increasing over the time in gilthead seabream, we have observed a decrease of *hp* transcript levels around 13-17 dph in European sea bass. These transcriptional differences between gilthead seabream and European sea bass have been also observed in other molecules such as non-specific cytotoxic cell receptor protein (*nccrp1*) (Cordero et al., 2016b), suggesting an important role, and more remarkable in European sea bass, of *hp* in early larval stages.

Regarding the fish antibacterial activity, many papers have considered different molecules such as *g-lysozyme*, *leap-2* or *β-defensin* after bacterial infections (Buonocore et al., 2014; Cuesta et al., 2011; Li et al., 2015) but only one previous report focused on haptoglobin and showed an increase of Hp protein abundance after *Vibrio* infection in trout plasma

(Gerwick et al., 2002). These data are correlated with our transcriptional finding, in which the expression of *hp* was increased in liver, HK, spleen and peritoneal exudate leucocytes after *V. anguillarum* i.p. injection suggesting and important role against bacterial infections. All these data point to, and demonstrates, the importance of APPs, and concretely Hp, on the fish fight against bacteria.

However, the role of haptoglobin after viral infections has not been previously approached. At transcriptional level, a short communication reported serum amyloid A (*saa*) and *hp* modulation by poly I:C induction in liver of black rockfish (*Sebastes melanops*) (Jayasinghe et al., 2015). For this reason, we focused this study using two viral models: LCDV in gilthead seabream and NNV in seabream and European sea bass. In the case of LCDV, little is known about seabream immune response against this virus. Our previous study showed the up-regulation of *nccrp1* in HK but not in skin of naturally infected gilthead seabream (Cordero et al., 2016a), similarly to what happens in the present study for *hp* transcripts. In other fish species, after LCDV infection at larvae stages, molecules involved in cell division and apoptosis were down-regulated in fins as showed in a microarray assay in turbot (*Psetta maxima*) (Iwakiri et al., 2014). However, in agreement with our study, *grim19* was also up-regulated in the HK of infected turbot (Wang et al., 2014). All these data demonstrates an important induction of the immune response in LCDV-fish in the HK tissue but very low or inefficient induction in the target tissue, the skin, but anyhow the fish overcome the disease and recover. By contrast, antimicrobial peptides (AMPs) such as piscidin 3 are increased in the skin of naturally infected gilthead seabream (Dezfuli et al., 2012).

Regarding the infections with nodavirus, one of the most important marine virus, we found a great inhibition in the *hp* gene expression levels in the seabream SAF-1 cell line upon

infection and no effect on the European sea bass DLB-1 cell line, which is parallel to the effects observed for the poly I:C administration. These data could be due to the differential cell-type origin of the cell lines and probably different regulation. In *in vivo* infections, the study of the brain as the target tissue of NNV, the HK as the main hematopoietic organ, and the gonad that transmits the virus to the progeny, is basic to understand the viral disease. The expression levels of *hp* were much more inducible in European sea bass than in gilthead seabream after nodavirus infection. These data are in accordance with the transcript levels found in other immune-related molecules such as *tnfa*, *mx* or *nkef* after *in vivo* nodavirus infection in HK of European sea bass (Esteban et al., 2013; Poisa-Beiro et al., 2008; Valero et al., 2015b). Importantly, the greatest fold change in *hp* after infection was found in European sea bass gonad (more than 1,000-fold at 7 days), but in gilthead seabream *hp* was not induced or even down-regulated, which taken altogether with the inhibition of *hp* transcripts found in brain and HK of nodavirus-infected gilthead seabream may be in relation with the asymptomatic effects of nodavirus in this fish species. This hypothesis is supported by the absence of modulation of other molecules involved in inflammation such as *il1β* due to a short but effective response against the virus in the brain (López-Muñoz et al., 2012).

Overall, we have performed the molecular identification and characterization of haptoglobin, a very relevant but also unknown acute phase proteins. The protein comparison and phylogenetic tree revealed that the structure of haptoglobin is highly variable among species. In our study, this molecule is constitutively and widely expressed from eggs to adults of gilthead seabream and European sea bass. In addition, the role of haptoglobin in viral infections was unravelled for first time in fish, being inducible in gilthead seabream under LCDV, but not under NNV infections, in both cases virus that do

not produce fish death. However, in European sea bass, NNV, which is able to kill the fish, up-regulated the transcription levels of this molecule, demonstrating differential induction of this acute phase molecule depending on the species and pathogens and enlighten new strategies to understand the viral immunity at molecular level.

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Figure legends:

Figure 1. Multiple alignment of the predicted gilthead seabream and European sea bass haptoglobin amino acid sequences with other known haptoglobin molecules. The identical (*) and similar (. or :) residues are indicated. Accession numbers: gilthead seabream *Sparus aurata* (KU940258), European sea bass *Dicentrarchus labrax* (KU940259), rockfish *Sebastes schelegelii* (AKI07271), Nile tilapia *Oreochromis niloticus* (XP_003445796), Atlantic salmon *Salmo salar* (XP_014019196), rainbow trout *Oncorhynchus mykiss* (CDQ94979), channel catfish *Ictalurus punctatus* (AHH39487), pufferfish *Tetraodon nigroviridis* (CAF94447), zebrafish *Danio rerio* (XP_009301802), mouse *Mus musculus* (NP_059066), human *Homo sapiens* (AAI21126), nurse shark *Ginglymostoma cirratum* (AEB61473) and little skate *Leucoraja erinacea* (AFN85000).

Figure 2. A: Neighbor-joining tree of the available cartilaginous and bony fish, mouse and human propeptide haptoglobin sequences. 0.1 indicates the genetic distances, which were calculated based on protein differences (p-distance) with pairwise deletion of gaps. The number at each node indicates the percentage of bootstrapping after 1,000 replications. Accession numbers are specified in Fig. 1. B: Identification and organization of gilthead seabream and European sea bass putative haptoglobin protein domains with those of cartilaginous fish, mouse and human haptoglobin.

Figure 3. Transcript levels of *haptoglobin* during the ontogeny (from 0 to 60 days post-hatching) in gilthead seabream (A) and European sea bass (B). *Haptoglobin* expression profile in different adult naïve organs (brain, gill, gut, skin, liver, head-kidney, spleen, thymus, gonad, blood), and SAF-1 or DLB-1 cell lines from gilthead seabream (C) and

European sea bass (D), respectively. Results are expressed as the mean relative expression to the expression of endogenous control *ef1a* gene ± SEM (n=3). Different letters denote significant differences among ontogeny stages or among organs/cell lines when p≤0.05.

Figure 4. *Haptoglobin* gene expression profile in the head-kidney and skin of non-infected (white bars) and LCDV-infected (black bars) gilthead seabream during a natural outbreak. . Results are expressed as the mean relative expression to the expression of endogenous control *ef1a* gene ± SEM (n=5). Asterisks denote significant differences between control and infected groups when p≤0.05.

Figure 5. *Haptoglobin* transcription after nodavirus (NNV) infections (A-H). Relative gene expression of *haptoglobin* in SAF-1 (A) and DLB-1 (E) cell lines after poly I:C (grey bars) and NNV (black bars) treatments compared with controls (white bars). *Haptoglobin* profile after 1, 7 and 15 days of mock (white bars) or NNV infections (10^6 $TCID_{50}$ per fish; black bars) in the brain (B, F), head-kidney (C, G) and gonad (D, H) from gilthead seabream (A-D) and European sea bass (E-H), respectively. Results are expressed as the mean relative expression to the expression of endogenous control *ef1a* gene ± SEM (n=3).Asterisks denote significant differences between control and poly I:C or NVV groups when p≤0.05.

Table 1. Primers used for rtPCR study.

Gene name	Symbol	Species	Acc. number	Sequences (5' → 3')	Use
elongation *factor 1 alpha*	*ef1a*	Gilthead seabream	AF184170	F1: TGTCATCAAGGCTGTTGAGC	rtPCR
				R1: GCACACTTCTTGTTGCTGGA	
		European sea bass	FM019753	F1: CGTTGGCTTCAACATCAAGA	rtPCR
				R1: GAAGTTGTCTGCTCCCTTGG	
haptoglobin	*hp*	Gilthead seabream	KU940258	F1: TTCCTCTTACTTGCCCTGGA	rtPCR
				R1: CAGGGCCTGAAGCTCTACTG	rtPCR
				R2: AGAGCGTTTCATCCTTTCTT	5' RACE
				R3: CATAACCACAGCCTTTTTCA	5' RACE
		European sea bass	KU940258	F1: CTTTGACTGTGCTCCTGCTG	rtPCR
				R1: ACGGAGTGATGCTACCTTGG	rtPCR
				F2: TTGTTCTCCATCCAGGCTTC	5' RACE
				R2: GATGGGGGTCACTTCATCAC	5' RACE

Table 2. Identity matrix (%) with gilthead seabream and European sea bass haptoglobin.

Species	Accession number	Gilthead seabream	European sea bass
Gilthead seabream (*Sparus aurata*)	KU940258	100	78.9
European sea bass (*Dicentrarchus labrax*)	KU940259	78.9	100
Rockfish (*Sebastes schelegelii*)	AKI07271	71.4	74.2
Nile tilapia (*Oreochromis niloticus*)	XP_003445796	70.5	74.6
Atlantic salmon (*Salmo salar*)	XP_014019196	60.8	63.9
Rainbow trout (*Oncorhynchus mykiss*)	CDQ94979	53.9	58.3
Pufferfish (*Tetraodon nigroviridis*)	CAF94447	53.0	57.5
Channel catfish (*Ictalurus punctatus*)	AHH39487	49.0	51.2
Zebrafish (*Danio rerio*)	XP_009301802	42.3	42.3
Mouse (*Mus musculus*)	NP_059066	19.9	20.6
Human (*Homo sapiens*)	AAI21126	18.6	19.7
Nurse shark (*Ginglymostoma cirratum*)	AEB61473	17.6	18.7
Little skate (*Leucoraja erinacea*)	AFN85000	15.1	14.9

Figure 1

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Gilthead seabream   ------------------------------------------------------------------------   1
European sea bass   ------------------------------------------------------------------------   1
Rockfish            ------------------------------------------------------------------------   1
Nile tilapia        ------------------------------------------------------------------------   1
Atlantic salmon     ------------------------------------------------------------------------   1
Rainbow trout       ------------------------------------------------------------------------   1
Channel catfish     ------------------------------------------------------------------------   1
Pufferfish          -----------------------------------------------MRRKFILTNNPAVLTPEAAAGGR    23
Zebrafish           -----------------------------------------------MAKAKLTMNMTASVRFSSPQQRL    23
Mouse               MRALGAVVTLLLWGQLFAVELGNDAMDFEDDSCPKPPEIANGYVEHL-----------VRYRCRQ-FYRLRAEGDGVYTL   68
Human               MSALGAVIALLLWGQLFAVDSGNDVTDIADDGCPKPPEIAHGYVEHS-----------VRYQCKN-YYKLRTEGDGVYTL   68
Nurse shark         -MGSLSLQLVLLCTLLGCTLQDHVETDHSKVHCGVPVNITHGHGHYEYLTHHDEDTYLTVIRYVCDAPIYHLEHPEDATFVC   70
Little skate        -MWFLVLNVLALSSLV---LADH------HIDCPEPTNITHGHFHTITAEGHHAIGSVIKYECNSPRWVFEDEHDGIYVC    70


Gilthead seabream   ------------------------------------------MDITSKMWFSLTVLLLAT--WACL   22
European sea bass   ------------------------------------------MNITKRMWFSLTVLLLAA--WACL   22
Rockfish            ------------------------------------------MNIINNMWFSLTVLLLAA---LACL  22
Nile tilapia        ------------------------------------------MNMIHRMAFSLTVVVLAA--WACL   22
Atlantic salmon     ------------------------------------------MWSSLVVLLAASCVCLEQD         19
Rainbow trout       ------------------MGGQTGGEFSLQPWSYNFSRSTSSLQRRHSSTYKLINRYFHQNVVIPGSAPRGLLC  57
Channel catfish     ------------------------------------------MKWFSVAVFLVGL--MICL          17
Pufferfish          KEEKEKRRRKRHLGLEAGLVTSLAQALFLLQSFKSPYRYKYQSFVKLTLKEPKQPSALKDKMWFSLPLLLLAA---WSGL  100
Zebrafish           VQRVEVLVDWLQQCRPVVQPSFMPLTGTYKAEQFKSMRLVVLLSSGGNHNPVMQEQLFLVIIFRISVAVLLLGT---LTFL  100
Mouse               ND--------------------------------------------------------------EKQWVNT            77
Human               NDKKQWINKAVGDKLPECEADDGCPKPPEIAHGYVEHSVRYQCKNYY------------KLRTEGDGVYTLNNEKQWINK   136
Nurse shark         TDDNQWTNAKLKHKLPECHKVA-CGKPPAVDHGHFEYITTFGVDNYLSAVKYTCDDNYHERDFSDEGYVVCTIDGKWRNT   158
Little skate        ASNGHWKNKARAEVLPVCQLIH-CHAPHHIENATLEYLTSRHDFVYHSIIQYHCIDDHIEEKYSDEGIYVCAISGHWENK   149


Gilthead seabream   ADVAHSEERMKRSVSASRSASQRSRRMIGGNSA--PHVPWQAMVYLSEDVLDGGYAGGALISDRWVLTAGRN-LFVNKSR    99
European sea bass   ADVPHTEERMKRSVSASKVASLRSRRMIAGTIA--PHVPWQAMVYISDSVLDGGYAGGALISDRWILTAGRN-VFVRKSR    99
Rockfish            ADV--TEERVKRSVSASGLASLRSRRMVGGTIA--PHVPWQALVYISDNVLDGGHGGGALISDRWILTAGRN-VFVNKS-    96
Nile tilapia        ADVAHTEERMKGSVLASRTASLRSRRMVGGKIA--PHVPWQAMVYIAESILDGGYAGGALISDRWVLTAGRN-LFVKKSR    99
Atlantic salmon     KIKIKIKIKTKSIDEMSDNSDLRFRRMVGGTIA--PHVPWQAMVYLSKNVMNGGFAGGALISDRWVLTAGRN-LFVRKSR    96
Rainbow trout       KDKIKIKIKTKSIDEIPNNSDLRFRRMIRGTIA--PHVPWQAMVYLSKNVMNGGFAGGALISDRWVLTAGRN-LFVRKSR   134
Channel catfish     SESR-VVDGIKEHTEANTDAALRTRRMVGGLLTP-GHVPWQALVYISDSKLDGGIGGGALIAPQRILTAGRN-LFVRKTQ    94
Pufferfish          ADGVAGDDGIK--LSASRAAPLRSRRMIGGTIA--PLVPWQAMVYISDSVLTGGYAGGALISDRWVLTAGRN-LPLRKSR   175
Zebrafish           TDASPALERVGEHVSA-----LRSRRMVGGSLT--ASVPWQAMVYLSENILDGGFAGGALIAEVRWVLTAGRN-LFVGKSR   172
Mouse               VAGEKLPECE-AVCGKPKHPVDQVQRIIGSMDAGSFPWQAKMISRHGLTTG-----ATLISDQRVLTTAKN-LBLNHSE   151
Human               AVGDKLPECE-AVCGKPKNPANPVQRILGGHLDAKGSFPWQAKMVSHHNLTTG-----ATLINEQWVLTTAKN-LBLNHSE   210
Nurse shark         DLGYEFPTCEKVVCGRPIVPLEQHQRVGGHIVHNGATPWTVLMLGPSGTVVD-----GTLIDHHFVLTSAHALHGLNLSR   234
Little skate        DLGTTLPHCIVVCGHAVTHLDSVHETDGAQIVTKHATPWTALLKNASEDFHN------GVLISHQRILTSSH--IPTDHSP  223
                                                                 :.  :  .  . .* :  *   ::***::  *         15


Gilthead seabream   QDNCKGPVIPKVYLGITQKSDANPTNEVSVEKVVLHSG--FQNRSDWDNDLALICLKKAVVMSDKVTPIPLPERGQGTE   177
European sea bass   QDICKDPIIPKVYLGITHRSPADVSKEVAVEKVVLHPG--FQNCSDWDNDLALICLKEPVVMSDKVTPIPLPERGQDLE   177
Rockfish            QPAGKEGIVIPKVYLGITERSQANPSKEVAVDKVELHPH--FQNKSKWDNDLALICLKEVPVVITDKVTPIPLPERCQ---  171
Nile tilapia        EAICKEEVIIPKVYLGITKKDDANSSEVAVEKVVLHPG--FQNQSDWNNDLALICLKQPVVINDKVTPIPLPERGQDTA   177
Atlantic salmon     QDTCKEPIIPKVYLGITRYSQANDSKEVAVEKVVLHPG--FQSVSDWDNDLALICLKEPFTLSEAVTPIPLPERGEDIA   174
Rainbow trout       QDTCKEPIIPKVYLGITRQSQADDSKEVAVEKVVLHPG--FQNVSDWDNDLALICLKEPFTLSEAVMPIPLPERGQDTA   212
Channel catfish     KDTRCKEPIIPKVYLGIVRRAKADSASEVAVKKVELHPA--FQNASDVDNDLALICLKEPVVSYTDTIRPIPLPEKDDNLE  172
Pufferfish          QDTQGKNPLIPKVYLGISELABAKPSREVAVEFVVLHPG--FQNQSDWDNDLALICLKEPVVISDRVTPIPLPERGQDTA   253
Zebrafish           IQTRGQEPLIPKVYLGISKRADATASTEVAVEKVELHPG--FQNTSDWDNDLALIKLKRPVKFSKSIIPIPLPETGDNLE   250
Mouse               TASAKD----ITPTLTLY----VGKNQLVEIEKVVLHPN-------HSVVDIGLIKLKQRVLTERVTPICLPSKDYIA-   215
Human               NATAKD----IAPTLTLY----VGKKQLVEIEKVVLHPN-------YSQVDIGLIKLKQRVSVNERVTPICLPSKDYAE-   274
Nurse shark         EELKEK----LRVYVGIEDAREITAAHCVHVEDVHYHPR--MRDAYVYRNDIALVKLKEDVHFSNHIMPAGLPAHDYAE-   307
Little skate        EAIKKD----FVVYVGVEDLDDLHASHPHHVEKIFFEEIHDATNSSEYDNDIVLLKLDSVSYGDHIVTPICLPHEELVK-   298
                                                                *:  .  *::*:.  . . .  : :   * **          29


Gilthead seabream   NTMGQSGVITGWGWG--TFLTSATSLKHLVLELAGHSFCKAPYSRIP------------FTPDVDDNMFCTGPA-SFERN   242
European sea bass   GIVQGSGVITGWGWG--IYLTLATSLKHLILPLADHSICKAEYERIA------------LTPSVDDNMFCTGAT-KFGGN   242
Rockfish            DTMGGTGVITGWGWG--ILLTPSKSLKFLVLPLANHSVCKAETERNP------------FTPAVDDNMFCTGPT-KYDEN   236
Nile tilapia        KAVHGSGIITGWGWG--PLITPSPFLKHIVVPLANHSECRAEYESLA------------LTPTVDDDMICTAAT-KYQEN   242
Atlantic salmon     EAAQEKGIITGWGWG--VHFTPAESLKFLVLPVASHSFCKAPYNRGG--------------STPTIDDNMFCTGAS-KYCEN   239
Rainbow trout       EAAQEKGIITGWGWG--VFFTPAKSLKFLVLPVASHSFCKAPYNPGGQ--------VLSSTPTVDDNMFCTGAS-KYCEN   281
Channel catfish     ESEGQRGVIAGWGWG--PLLTFSESLKFLSLPVIPG--------------------------NVFDGKIWTIRT-EFCEN   223
Pufferfish          DGAQGSGVIAGWGWG--VYLNLASSLKFLLVELANHSACKAPYLEQSQ------------LAPSVDDSMFCTGSTGRFQEN   319
Zebrafish           ERDGERGIVAGWGWG--RLITPAPVLKFLSLPVLKSCKGNYQARVLES------------TPNIDDKQFCTGSG-RYLEN   314
Mouse               --PGRVGYVSGWGRN--ANFKFTDRLKYVMLPVADQDKCVVHYENSTVPEKKNLTSPVGVQPILNEHTFCAGLT-KYQED   290
Human               --VGRVGYVSGWGRN--ANFKFTDHLKYVMLPVADQDQCIRHYEGSTVPEKKTPKSPVGVQPILNEHTFCAGMS-KYQED   349
Nurse shark         --EGKTGHVAGWGVEGTGETSRANHIHWVSLAVANTTLCQAFEN--------EHHPGLFPADAPDCFCTQSL-SDGHN   374
Little skate        --GVEGAVTGWDLD---HAKGPHHLSYVLPVLPEEKARCVEHFS---------SHHHGLFPDDLNDBFCTHGL-EKHGQ   362
                     *  ::**..   .     * :.:  :.:          .    :  :            : :    :  : :          41


Gilthead seabream   VCFGDAGGALAVTDPETGDVYAAGILSYDKCCSRYNHGVYMKISSYLPWINRVIRGDTENSSALRTDAMSKIYSQQQ   319
European sea bass   VCFGDAGGALAVTDAKTGDIYAAGILSYDKSCTRYNYGVYMKISSYLPWIHRVIRGDAENSSTLRSDAMSKMYSRQP   319
Rockfish            VCFGDAGGALAVTDAETGDIYAAGILSFDKSCVSHKYGVYMKICSYLPWIHSVIRGDTDKSPALRADAMSKMYLWQQ   313
Nile tilapia        VCFGDAGGALAVTDPETGDIYAAGILSYDKSCTRYKHAVYMKLSSYLPWIHSIMRGDTDTSTAVRFKAMSSMYRRQE   319
Atlantic salmon     VCFGDAGGALAVQDPKDGRVYAAGILSFDKACAVRKYAVYMKLSAYMPWINSVLRGDSEKSASLRSSVMSEMFSQL    316
Rainbow trout       VCFGDAGGALAVQDPKDGRVYAAGILSFDKACAVERYAVYMKLSAYMPWINSVLRGDSETSASLRSSVMSEMYSRQL   358
Channel catfish     VCTGDAGGALALLNPVTKKVYAAGILSYDGPCTRNEEAVFMKISTYLPWIHSVMRADSDRFSSLRTSIMNDLLSK--   298
Pufferfish          VCFGDAGGALAVRDAQTGDVYAAGILSFDKPCSQHRYAVYMKLSSYLPWIHTVTRGDVPNSAAVRSSTMATMHSWQS   396
Zebrafish           VCFGDAGGAIAFLNTKTNAVYAAGILSFDKACSVEEHAVYTKISAHLPWIHSVMRGDSQDIASQRSSAIRHMFSQQL   391
Mouse               TCYGDAGSAFAIHDMEEDTWYAAGILSYDKSCAVAEYGVYVRATDLKDWVQETMAKN-------------------   347
Human               TCYGDAGSAFAVHDLEEDTWYATGILSFDKSCAVAEYGVYVKVTSIQDWVQKTIAEN-------------------   406
Nurse shark         VCFGDHGAALLVR--DGDDYAAGILSYDEGCAGEVIAVYTDVHHYLKWIDGIIHPQ-------------------   429
Little skate        NSERDRGAVFCVE--VGHKTYAVGCVLAYDAPEVGKGWAVYTDVYHHLDWINNVIEHN-------------------   417
                     .  *  *::. :    **.*:*:::*       .*:    *: :       *:.   :                     57
```

Figure 2

A

B

Figure 3

Figure 4

LCDV

Relative gene expression of haptoglobin

0.15
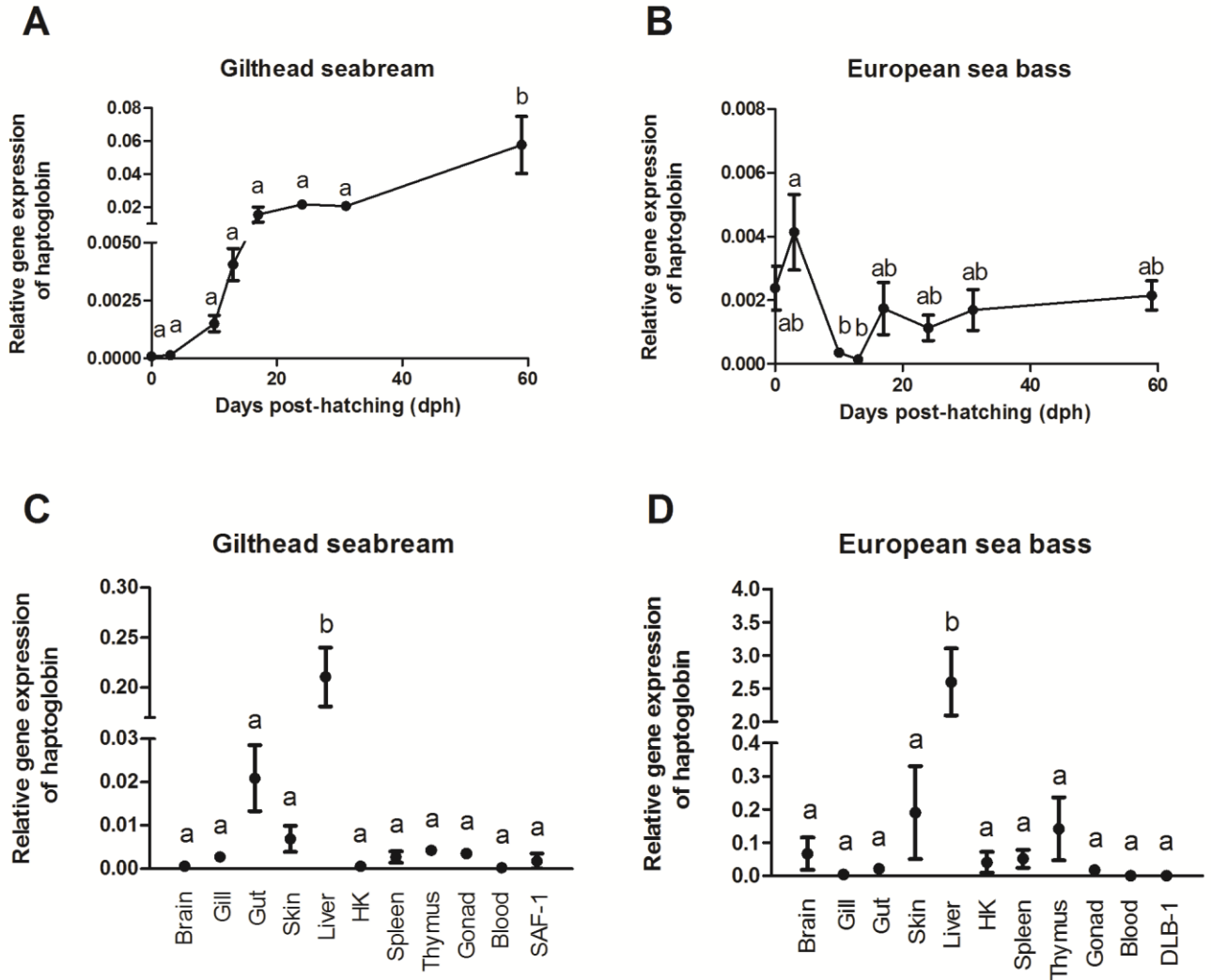0.10
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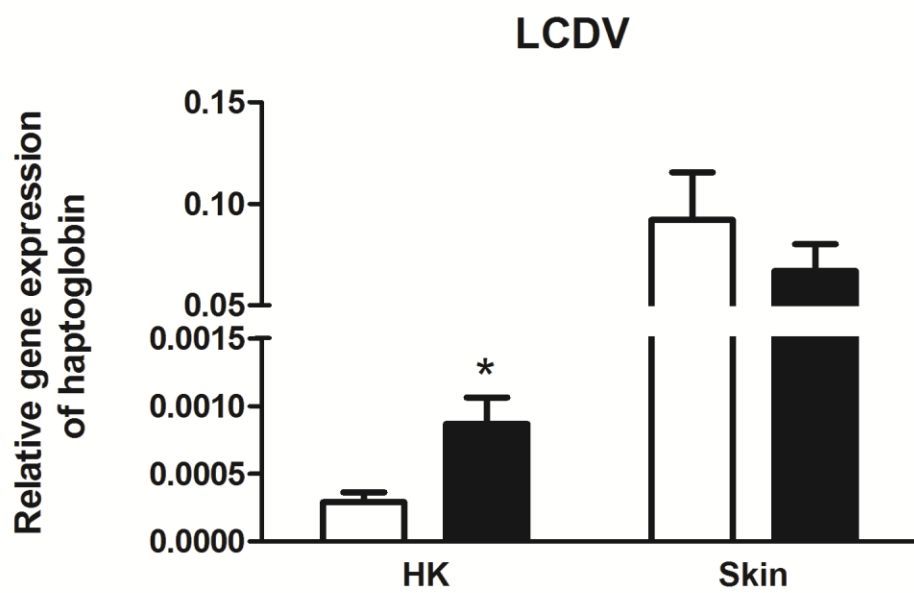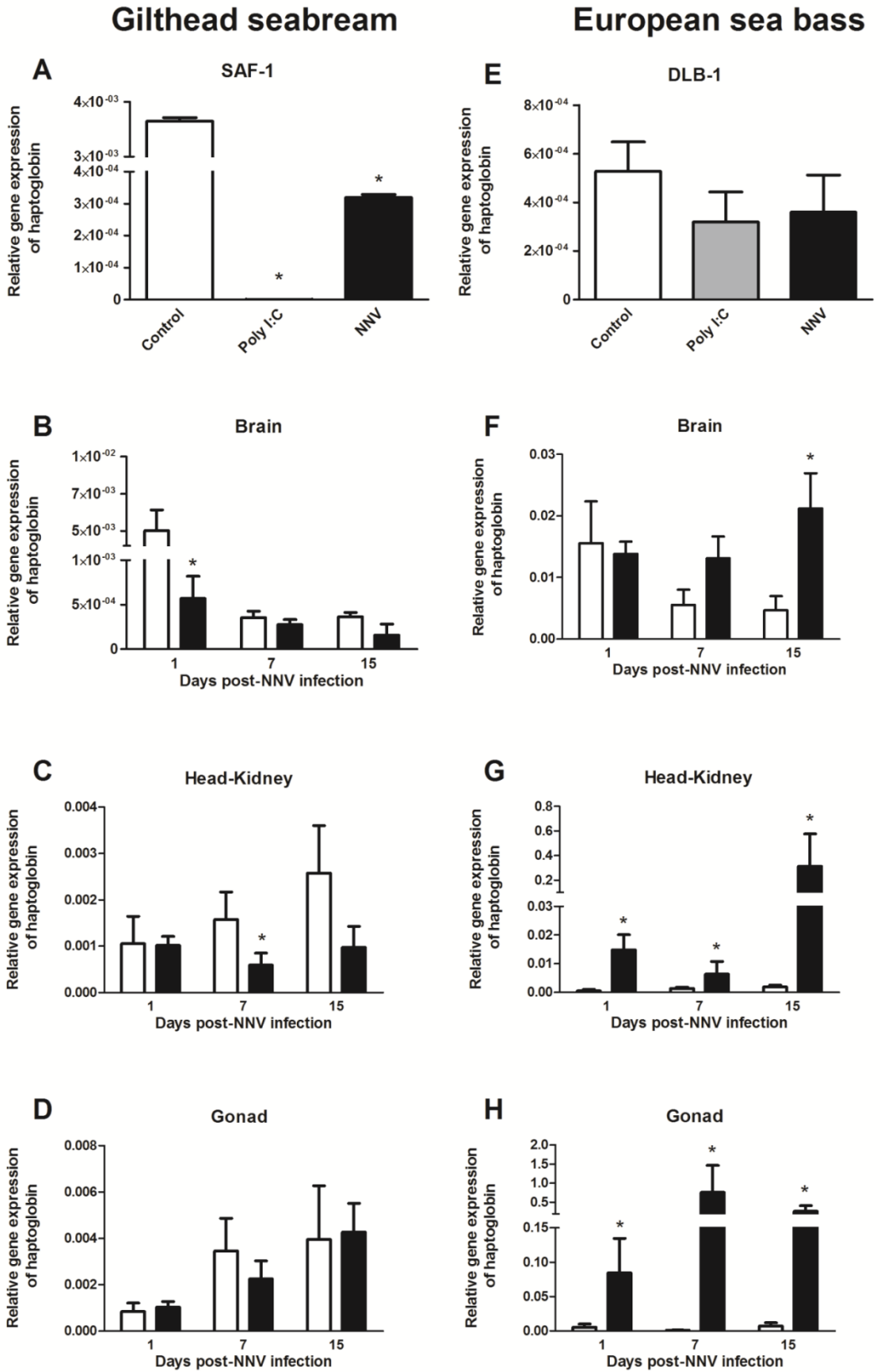0.0010
0.0005
0.0000

*

HK   Skin

Figure 5

Supplementary Figure 1. Haptoglobin gene expression in gilthead seabream after intraperitoneal (IP) injection of PBS (white bars) or *Vibrio anguillarum* (black bars) in different tissues. . Results are expressed as the mean relative expression to the expression of endogenous control ef1a gene ± SEM (n=2 pooled samples). Asterisks denote significant differences between control and infected groups when p≤0.05.