

1 TRANSCRIPTION OF HISTONES H1 AND H2B IS REGULATED BY SEVERAL
2 IMMUNE STIMULI IN GILTHEAD SEABREAM AND EUROPEAN SEA BASS

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21 **Abstract**

22 Histones (H1 to H4) are the primary proteins which mediate the folding of DNA
23 into chromatin; however, and in addition to this function, histones have been also
24 related to antimicrobial peptides (AMPs) activity in vertebrates, in fact, mammalian H1
25 is mobilized as part as the anti-viral immune response. In fish, histones with AMP
26 activity have been isolated and characterized mainly from skin and gonads. One of most
27 threatening pathogens for wild and cultured fish species nowadays is nodavirus (NNV),
28 which target tissues are the brain and retina, but it is also able to colonize the gonad and
29 display vertical transmission. Taking all this into account we have identified the *h1* and
30 *h2b* coding sequences in European sea bass (*Dicentrarchus labrax*) and gilthead
31 seabream (*Sparus aurata*) fish species and studied their pattern of expression under
32 naïve conditions and NNV *in vivo* infection. The data obtained prompted us to study
33 their role on the immune response of gonad and head-kidney leucocytes upon viral
34 (NNV), bacteria (*Vibrio anguillarum* or *Photobacterium damsela*), pathogen-
35 associated molecular patterns (PAMPs) or mitogens stimulation. The *h1* and *h2b* genes
36 are expressed in a wide range of tissues and their expression is modify by infection or
37 other immune stimuli, but further studies will be needed to determine the significance of
38 these changes. These results suggest that *h1* expression is related to the immune
39 response against NNV in the brain, while *h2b* transcription seems to be more important
40 in the head-kidney. Moreover, the potential role of histones as anti-viral agents is
41 suggested and further characterization is in progress.

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43 **Keywords:** Histones; antimicrobial peptides (AMPs); nodavirus (NNV); head-kidney;
44 brain; gonad; European sea bass; gilthead seabream; teleost

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46 **1. Introduction**

47 Histones are usually classified as core (H2A, H2B, H3 and H4) and linker
48 histones (H1) due to their localization forming the basic units of the chromatin, the
49 nucleosome. Thus, the nucleosome is formed by 146 base pairs of DNA wrapped
50 around a protein octamer of two molecules of core histones. The linker H1 binds the
51 DNA at the union sites whether it enters or exits the core nucleosome [1]. Histones,
52 mainly core ones, are greatly conserved in eukaryotic organisms along evolution and
53 therefore their functions might be also conserved. In addition to this function, they are
54 also involved in other cellular functions and their implication in the epigenetic control
55 of gene expression is nowadays in fashion. However, they have been also linked to
56 immunity being their role as antimicrobial peptides (AMPs) the most described, which
57 were first characterized in mammals long time ago [2]. Thus, histones and histone-
58 derived fragments act as physiological barriers of cells exerting a variety of
59 antimicrobial actions and functions, including bacterial cell membrane permeabilization,
60 penetration into the membrane followed by binding to bacterial DNA and/or RNA,
61 binding to bacterial lipopolysaccharide (LPS) in the membrane, neutralizing the toxicity
62 of bacterial LPS, and entrapping pathogens as a component of neutrophil extracellular
63 traps (NETs) [3].

64 In fish, the connections between histones and immunity have been established.
65 First characterized was a catfish (*Ictalurus punctatus*) AMP isolated from the skin
66 closely related to the H2B [4]. Since then, proteins highly homologous to histones or
67 fragments derived by cleavage processes from histones (eg. Parasin I, hipposin) have
68 been defined as histone-like proteins (HLPs) and identified in some fish species [5-9].
69 Most studies in fish have focused on the antimicrobial function of HLP-1 and HLP-2
70 proteins homologous to H2B and H1, respectively; and usually isolated from skin or
71 gills [4, 10-14]. However, other AMPs have been widely distributed among several
72 tissues including immune-privileged tissues such as brain or gonads [15]. Recently, a
73 H1-like protein has been isolated from acidified testis extracts (fH1LP) of olive
74 flounder (*Paralichthys olivaceus*) and shown to be constitutively expressed in ovary and
75 testis and to have antibacterial (Gram+ and Gram-) and antifungal activity [16]. In
76 European sea bass (*Dicentrarchus labrax*), H2B and H1 coding genes were cloned and
77 their expression levels have been reported to be altered under stress conditions [14], and
78 also after *Vibrio anguillarum* infection [17].

79 Nodavirus (NNV) is a naked bipartite single stranded RNA virus which severely
80 affects European sea bass larvae and juveniles provoking high mortality rates [18, 19].
81 Nevertheless, other species such as the gilthead seabream (*Sparus aurata*) are infected
82 without showing disease symptoms, acting as a natural reservoir for most of the virus
83 strains [20]. NNV has demonstrated vertical transmission [21] and is able to colonize
84 and replicate in very low levels into the European sea bass and gilthead seabream testis
85 in order to not being detected by the immune response [22], altering the antimicrobial
86 activities and pattern of expression of several AMPs [23].

87 In this study, we identify the complete sequences of H1 and H2B coding genes
88 in European sea bass and gilthead seabream and study their pattern of expression in
89 immune, reproductive and other important tissues in naïve specimens and under NNV
90 infection. The results obtained, prompted us to analyse the modulation of both genes
91 upon *in vitro* viral, bacterial infection, pathogen-associated molecular patterns (PAMPs)
92 or mitogens stimulation of the immune response in gonad and/or head-kidney
93 leucocytes (HKLs) in order to determine whether these two genes might have a role in
94 the immune response of fish.

95 **2. Material and methods**

96 2.1. Animals

97 Healthy specimens of European sea bass (*Dicentrarchus labrax* L.) and gilthead
98 seabream (*Sparus aurata* L.) were bred and kept at the *Centro Oceanográfico de*
99 *Murcia* (IEO, Mazarrón, Murcia) in 14 m³ tanks with the water temperature ranging
100 from 14.6 to 17.8 °C, flow-through circuit, suitable aeration, filtration systems and
101 natural photoperiod. The environmental parameters, mortality and food intake, were
102 recorded daily. Juvenile specimens of both species with a mean body weight (bw) of
103 325 ± 37.5 g were used for the analysis of constitutive gene expression in naïve
104 conditions (see below). Adult specimens of both species with a bw of 774 ± 93 g were
105 used for *in vitro* treatments of the gonads (see below). Juvenile specimens of European
106 sea bass (n = 50) or gilthead seabream (n = 50) with a mean bw of 200 ± 15 g, were
107 transported to the University of Murcia (Spain) aquaria in order to perform *in vivo*
108 infections (see below). The experiments described comply with the Guidelines of the
109 European Union Council (2010/63/UE). The protocol was approved by the Committee
110 on the Ethics of Animal Experiments of the *Instituto Español de Oceanografía* (IEO)

111 (Permit Number: 2010/02) and of the University of Murcia (Permit Number:
112 A13150104).

113 2.2. Fish sampling

114 All specimens were anesthetized with 40 µl/l of clove oil before sampling, then
115 weighed, completely bled and immediately decapitated. Blood was obtained from the
116 caudal peduncle and the serum samples, obtained by centrifugation (10,000 xg, 1 min, 4
117 °C), were immediately frozen in liquid nitrogen and stored at -80 °C until use.

118 In order to analyse the constitutive expression in naïve conditions, brain, gill,
119 liver, skin, gonad, gut, head-kidney, spleen and thymus fragments from 6 independent
120 fish were removed and immediately frozen in TRIzol[®] Reagent (Life Technologies) at -
121 80 °C until used for RNA isolation. HKL suspensions were obtained as previously
122 described [24]. In brief, fragments of head-kidney tissue were transferred to 7 ml of
123 sRPMI [RPMI-1640 culture medium (Life Technologies) supplemented with 0.35 %
124 sodium chloride, 100 IU/ml penicillin (Life Technologies), 100 mg/ml streptomycin
125 (Life Technologies) and 5 % fetal bovine serum (FBS; Life Technologies)] under sterile
126 conditions. Cell suspensions were obtained by forcing fragments of the organ through a
127 100 µm nylon mesh, washed twice by centrifugation [400 xg, 10 min, room temperature
128 (RT)], counted and adjusted to 10⁷ cells/ml in sRPMI. In all cases, leucocyte viability
129 was determined by the trypan blue exclusion test and resulted higher than 98 %.

130 2.3. Viruses and bacteria

131 NNV (strain 411/96, genotype RGNNV) was propagated in the SSN-1 cell line
132 [19]. The SSN-1 cells were grown in Leibovitz's L-15 medium (Gibco) supplemented
133 with 10 % FBS, 2 mM L-glutamine (Life Technologies), 100 IU/ml penicillin, 100
134 µg/ml streptomycin and 50 µg/ml gentamicin (Gibco) at 25 °C using Falcon Primaria
135 cell culture flasks (Becton Dickinson). Inoculated cells were incubated at 25 °C until the
136 cytopathic effect (CPE) was extensive. Supernatants were harvested and centrifuged to
137 eliminate cell debris. Virus stock was titrated in 96-well plates and expressed as the
138 viral dilution infecting 50 % of the cell cultures (TCID₅₀), following a methodology
139 previously described [25].

140 Pathogenic bacteria *Vibrio anguillarum* (*Va*) R-82 and *Photobacterium*
141 *damselae* subsp. *piscicida* (*Pd*) were grown in sTSB [tryptic soy broth (Laboratorios

142 Conda) supplemented with 1.5 % NaCl] at 22 °C for 24 h. Absorbance at 600 nm was
143 measured and used to know the concentration based on growth curves. Both bacterial
144 cell cultures were washed in sterile 0.01 M phosphate-buffered saline (PBS, pH 7.4) by
145 centrifugation (6,000 xg, 15 min, 4 °C) and adjusted to 10¹⁰ bacteria/ml. For heat-
146 killing, cultures were washed with PBS, incubated at 60 °C for 30 min, washed and
147 adjusted to 10¹⁰ bacteria/ml with 0.01 M PBS.

148 2.4. In vivo infection

149 Once at the University of Murcia (Spain) facilities, juvenile specimens (n= 50)
150 of both species were randomly divided into two tanks, kept in 450–500 L running
151 seawater (28 ‰ salinity) aquaria at 25 °C and with a 12 h light: 12 h dark photoperiod
152 and acclimatised for 15 days prior to the infection. The infection was performed by
153 intramuscular injection of 100 µl containing 10⁶ TCID₅₀/fish of NNV in SSN-1 culture
154 medium, a mock-infected group was injected with 100 µl of SSN-1 culture medium
155 since this route of infection has been proven to be the most effective [26]. Fish (n = 5
156 fish/group and time) were sampled 1, 7 or 15 days upon infection and gonad and brain
157 were removed and immediately frozen in TRIzol[®] Reagent and stored at -80 °C for later
158 RNA isolation as described below.

159 2.5. In vitro treatments

160 Fragments of European sea bass ovaries (n = 6) or testis (n = 6) or gilthead
161 seabream gonads (n = 6) were removed, weighted and chopped into 1 mm² to culture
162 them in Leibovitz's L-15 medium supplemented with 2 mM L-glutamine, 100 IU./ml
163 penicillin, 100 µg/ml streptomycin, 2 µg/ml fungizone (Life Technologies) and 2 %
164 FBS. Six fragments of each tissue from independent fish specimens were incubated in
165 flat-bottomed 96-well microtiter plates (Nunc) with 200 µl of: culture medium alone
166 (control), NNV (10⁷ TCID₅₀/ml), *Va* (4 x 10⁷ bacteria/ml) or polyinosinic:polycytidic
167 acid (pI:C; 62,5 µg/ml; Sigma) at 25 °C during 24 h. Afterwards, the fragments of tissue
168 were washed in 0.01 M PBS and stored in TRIzol[®] Reagent at -80 °C for later isolation
169 of RNA as described below.

170 HKLs from healthy fish (n = 5) were isolated and maintained in Leibovitz's L-
171 15-medium supplemented with 10 % FBS, 2 mM L-glutamine, 100 IU/ml penicillin,
172 100 µg/ml streptomycin and 20 mM HEPES (Gibco). Aliquots of 10⁷ HKLs/ml were

173 incubated in flat-bottomed 48-well microtiter plates (Nunc) at 22 °C during 24 h with:
174 culture medium alone (control), 10⁶ TCID₅₀ NNV/ml, 10⁸ live bacteria/ml (*Va* or *Pd*),
175 50 µg/ml synthetic unmethylated cytosine-phosphodiester-guanosine
176 oligodeoxynucleotide 1668 (CpG ODN 1668; sequence 5'-
177 TCCATGACGTTTCCTGATGCT-3'; Eurogentec), 25 µg/ml pI:C, 5 µg/ml
178 lipopolysaccharide (LPS; Sigma), 10 µg/ml phytohemagglutinin (PHA; Sigma) or 5
179 µg/ml concanavalin A (ConA; Sigma). Afterwards, leucocytes were washed with 0.01M
180 PBS and stored in TRIzol[®] Reagent at -80 °C for later isolation of RNA as mentioned
181 below.

182 2.6. Gene sequences search and bioinformatics analysis

183 Complete sequences of European sea bass *h1* and *h2b* genes were obtained from
184 the European sea bass genome (<http://seabass.mpipz.mpg.de/>) and analysed for
185 similarity with known orthologue sequences using the BLAST program [27] within the
186 ExpASy Molecular Biology server (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>). This
187 program was also used to compare European sea bass sequences with the gilthead
188 seabream expressed sequence tags (ESTs) databases. Phylogenetic and molecular
189 evolutionary analyses were conducted using MEGA version 6 [28] to confirm that they
190 are *bona fide* gilthead seabream sequences.

191 The evolutionary history was inferred using the Neighbor-Joining method [29]
192 and the optimal tree was obtained. The percentage of replicate trees in which the
193 associated taxa clustered together in the bootstrap test (1,000 replicates) is shown next
194 to the branches [30]. The tree was drawn to scale, with branch lengths in the same units
195 as those of the evolutionary distances used to infer the phylogenetic tree. The
196 evolutionary distances were computed using the Poisson correction method [31] and are
197 in the units of the number of amino acid substitutions per site.

198 2.7. Analysis of gene expression by real-time PCR

199 Total RNA was isolated from TRIzol[®] Reagent frozen samples following the
200 manufacturer's instructions. One µg of total RNA was treated with DNase I (Promega)
201 to remove genomic DNA and the first strand of cDNA synthesized by reverse
202 transcription using the Superscript III (Life Technologies) with an oligo-dT12-18
203 primer (Life Technologies) followed by RNase H (Life Technologies) treatment. Real-

204 time PCR was performed with an ABI PRISM 7500 instrument (Applied Biosystems)
205 using SYBR Green PCR Core Reagents (Applied Biosystems). Reaction mixtures were
206 incubated at 95 °C for 10 min, followed by 40 cycles of 15 s at 95 °C, 1 min at 60 °C ,
207 and finally 15 s at 95 °C, 1 min at 60 °C and 15 s at 95 °C. For each mRNA, gene
208 expression was corrected by the elongation factor 1 alpha (*ef1a*) expression in each
209 sample and expressed as $2^{-\Delta Ct}$, where ΔCt is determined by subtracting the *ef1a* Ct value
210 from the target Ct. The primers used, specific for the histone forms studied herein and
211 described in section 2.6., were designed using the Oligo Perfect software tool (Thermo
212 Fisher Scientific) and are shown in Table 1. Before the experiments, the specificity of
213 each primer pair was studied using positive and negative samples. A melting curve
214 analysis of the amplified products validated the primer for specificity. All
215 amplifications were performed in duplicate cDNAs and repeated once to confirm the
216 results. Negative controls with no template were always included in the reactions.

217 2.8. Statistical analysis

218 Data were analysed by one-way ANOVA to denote statistical differences among
219 groups, followed by Tukey's post-hoc tests, except in the *in vivo* experiment in which a
220 t-Student test was used to determine statistical differences between infected and control
221 groups. A non-parametric Kruskal–Wallis test, followed by a multiple comparison test,
222 was used when data did not meet parametric assumptions. Statistical analyses were
223 conducted using SPSS 20 software. All data are presented as mean \pm standard error of
224 the mean (SEM). Minimum level of significance was fixed in 0.1.

225 **3. Results**

226 3.1 Identification of European sea bass and gilthead seabream *h1* and *h2b* gene 227 sequences

228 Complete cDNA sequences coding for European sea bass proteins H1 and H2B
229 were available at the European sea bass genome database
230 (<http://seabass.mpipz.mpg.de/>). We found one uncharacterized clone containing the
231 entire open reading frame (ORF) coding for each gilthead seabream proteins H1 and
232 H2B at the EST databases available at the NCBI GenBank database [GenBank
233 accession number *h1*: FM151953 (unpublished); *h2b*: AM953780 [32]]. The predicted

234 length, homology and e-values obtained from the gene sequences were compared with
235 their human orthologues (Table 2) resulting in *bona fide* sequences.

236 Phylogenetic tree showed two distinct clades for H1 and H2B proteins (Fig. 1).
237 The clustering provides evidences of high bootstrap support in the lineage of European
238 sea bass and gilthead seabream. Moreover, the teleost H1 proteins form an exclusive
239 clade opposed to human H1 sequences. Human H3 proteins were used as outgroup.

240 3.2. Expression of *h1* and *h2b* under naïve conditions

241 We found *h1* mRNA transcripts in brain, gills, liver, skin, gonad, gut, head-
242 kidney, spleen, thymus and blood tissues from both species (Fig. 2), although some
243 differences between species were observed. Thus, in European sea bass (Fig. 2a), the
244 tissues with the highest expression of *h1* gene were in thymus and blood followed by
245 brain and liver. Gills and gonad were the tissues with the lowest *h1* expression levels
246 (10,000 fold lower than thymus). However, in the gilthead seabream (Fig. 2b), the blood
247 showed the highest *h1* gene expression levels, followed by head-kidney and spleen
248 whereas liver, gut and gonad showed the lowest expression (100,000-fold lower than
249 blood).

250 Regarding the expression of H2B encoding gene (Fig. 3), no constitutive
251 expression were observed in brain, skin and spleen of European sea bass (Fig. 3a),
252 whilst the highest transcription levels were found in thymus. In contrast, in gilthead
253 seabream (Fig. 3b), all tissues constitutively expressed this gene. The highest level of
254 expression was found in gonad and blood while thymus has a medium level of
255 expression and the lowest expression was observed in liver (10,000-fold lower than in
256 blood). Overall, *h2b* transcription was lower than the expression of *h1* gene.

257 3.3. The expression of *h2b* but not of *h1* was increased in head-kidney upon 258 NNV infection in both species

259 Transcription of both *h1* and *h2b* genes was significantly regulated by NNV
260 infection (Fig. 4 and 5, supplementary table 1). The expression levels of *h1* were down-
261 regulated in brain but up-regulated in testis of European sea bass after 7 days of NNV
262 infection (Fig. 4a). In contrast, in gilthead seabream, the transcription levels of *h1* were
263 down- and up-regulated in brain after 7 and 15 days post-infection, respectively, and
264 down-regulated in gonad at day 15 post-infection (Fig. 4b).

265 In European sea bass (Fig. 5a), the *h2b* gene expression was down-regulated in
266 brain at day 1 and in testis at day 15 post-infection whilst it was down-regulated in
267 gilthead seabream gonad after 7 days (Fig. 5b). Interestingly, in both species the *h2b*
268 transcription was up-regulated in head-kidney at different time post-infection (7 days in
269 European sea bass or 15 days in gilthead seabream).

270 3.4. The expression of *h1* in European sea bass ovary and of *h2b* in the gilthead 271 seabream testis were inhibited after some *in vitro* treatments

272 When we analysed the pattern of expression of *h1* in the gonad of European sea
273 bass and gilthead seabream after 24 hours of *in vitro* treatment, we found that only
274 European sea bass ovaries showed down-regulated *h1* gene expression levels after NNV
275 infection, whilst in European sea bass testis and gilthead seabream gonad was
276 unchanged (Fig. 6a). However, the pattern of expression of *h2b* gene in European sea
277 bass gonads was unaltered by any treatment while was down-regulated after the
278 challenge with *Va* and pI:C in gilthead seabream gonad (Fig. 6b).

279 3.5. The expression of *h2b* gene was exclusively up-regulated in gilthead 280 seabream HKLs

281 Finally, we studied the pattern of expression of *h1* (Fig. 7) and *h2b* (Fig. 8)
282 genes in HKLs after 24 hours of treatment with known immune *stimuli* and our data
283 showed that *h1* gene expression was down-regulated after NNV, *Va* or *Pd* treatment in
284 European sea bass HKLs (Fig. 7a), and after NNV, *Va*, LPS, PHA or ConA treatment in
285 gilthead seabream HKLs (Fig. 7b).

286 Similarly, the *h2b* gene expression in HKLs of European sea bass was down-
287 regulated upon NNV, *Va*, *Pd*, pI:C or ConA treatments (Fig. 8a). In contrast to this and
288 what happened with *h1* gene expression, in gilthead seabream HKLs the *h2b* gene
289 expression was up-regulated after all immune *stimuli* assayed except with PHA (Fig.
290 8b).

291 **4. Discussion**

292 Histones, as chromatin structure proteins, were thought to be confined to the
293 nucleus. However, different studies have detected various histones and their fragments
294 in the cytoplasm of several cell types including leucocytes from mammals, bird, frogs,

295 fish and shrimps, showing those proteins a broad spectrum of antimicrobial activities
296 [33]. In fact, upon immune stimulation, leucocyte histones, mainly from macrophages
297 and neutrophils, are mobilized from the nucleus to the cytoplasm, the membrane and
298 even secreted to form the extracellular NETs [33] but no information exists at gene
299 level. In mammals, regarding to this, histones not only appear on the surface of
300 apoptotic cells but also on viable cell such as T-lymphocytes, macrophages or intestine
301 epithelial cells [33], whilst in fish they have been described on the cell surface of
302 macrophages, natural cytotoxic cells (NCC) [34, 35] and in the mucosa of gill and skin
303 tissues [6, 36]. In addition, histones are demonstrated to be innate immune effectors in a
304 wide range of tissues, being involved in the interaction with pathogens showing both
305 lytic activity and helping in their internalization through endocytic vesicles [33].
306 Although the mechanism of action of these histones is not completely known yet, a
307 specific conformation of histones and histone fragments is needed, suggesting that their
308 immune function is not only a consequence of their high amount of basic residues [37].

309 Both, core (H2A, H2B) and the linker (H1) histones showed antimicrobial
310 activity in several fish species [4, 6, 11, 13, 15, 33]. Concretely, in the European sea
311 bass, partial cDNA sequences coding for H1 and H2B proteins were isolated and their
312 pattern of expression analysed under stress conditions, resulted on a similar pattern of
313 expression in gills and epidermis than haemoglobin-like protein [14], a known
314 antimicrobial peptide [38]. In the present work we used the complete sequences coding
315 for these proteins for searching the gilthead seabream orthologue sequences. Thus, we
316 found two sequences annotated but not characterized in the GenBank database. Though
317 several histone forms are probably present in seabream and sea bass we only focused on
318 those previously documented [14, 32], which in addition showed good relation with
319 their zebrafish and human orthologues as evidenced by the phylogenetic tree,
320 suggesting that their function could be also conserved.

321 Since histones with antimicrobial activity were firstly identified in fish skin,
322 most studies in fish have avoided the study of the constitutive expression of histones in
323 other tissues apart from skin or liver [4, 8, 13], but other AMPs have been localized in a
324 wide range of tissues including immune-privileged tissues as brain or gonads [15, 23].
325 Our data showed that *h1* was constitutively expressed in all the tissues analysed in both,
326 European sea bass and gilthead seabream, as also occurred in the olive flounder [16].
327 Interestingly, in the olive flounder the highest expression of *h1* gene was found in gonad

328 [16], however, our data showed the highest *h1* gene expression in immune tissues of
329 both species. As far as we are concern, our study is the first analysing the pattern of
330 expression of *h2b* gene covering most of the tissues in fish. Therefore, we found that in
331 European sea bass, *h2b* gene transcription was not detected in brain, skin or spleen but,
332 was highly expressed in thymus. In contrast, in gilthead seabream *h2b* gene was highly
333 expressed in peripheral blood and gonad.

334 We next analysed the pattern of expression of *h1* and *h2b* upon NNV infection, a
335 virus which target tissues are the retina and brain [39] and colonizes the gonad to be
336 vertically transmitted [22]. Our data showed that upon *in vivo* infections with NNV, *h1*
337 is up-regulated in the gonad of European sea bass and in the brain of gilthead seabream,
338 which is greatly correlated to the increased immunity in seabream brain and sea bass
339 gonad as determined by the transcription levels of interferon, AMPs and leucocyte
340 markers [22, 23, 40], while *h2b* is up-regulated in the head-kidney of both species.
341 However, whether this is related to inflammation, immune response or tissue damage or
342 reparation merits further investigation. Interestingly, in Rohu (*Labeo rohita*), LHH1M
343 protein, that corresponds with the linker histone H1, is up-regulated in the brain of
344 specimens resistant to gram negative bacteria *Aeromonas hydrophila* [41] as occurred
345 with gilthead seabream, which is an asymptomatic carrier species of the NNV strain
346 used to perform the experiment [20]. On the other hand, European sea bass is very
347 susceptible to NNV [18] and our data showed that *h1* expression was down-regulated in
348 the virus target tissue, the brain, which is suffering great damage and no reparation is
349 performed. These data could suggest that histones are mobilized from the nucleus to
350 other cellular locations and this might lead to the down-regulation of histone genes.

351 Taking into account the high expression of *h2b* gene in the gonad of gilthead
352 seabream, the immune-privileged status of the gonad [42, 43] and the ability of NNV to
353 colonize the testis [22], we have analysed the expression of *h1* and *h2b* genes in mature
354 European sea bass male and females and gilthead seabream male gonads upon *in vitro*
355 treatment with alive NNV or *Va* or pI:C, and found slight down-regulations of *h1*
356 expression in the European sea bass ovary upon NNV infection and of *h2b* expression
357 in the gilthead seabream gonad upon *Va* and pI:C treatment. These data suggest that the
358 transcriptional changes observed on both genes upon *in vivo* infections were
359 orchestrated by the systemic immune response. However, it has been recently
360 demonstrated the presence of NNV in the testis of both species upon an infection [22].

361 Probably, the existence of other specific AMPs in the gonad together with the high
362 proliferative rates that this tissue showed during gametogenesis, avoids the use of H1
363 and H2B as antimicrobial proteins, whilst in other tissue such as HKLs or brain, this
364 function is enhanced and needed.

365 Histones are well known to be shed out of the cells in mammalian neutrophils
366 extracellular traps (NETs) and recently these NETs have been described to be produced
367 by some leucocytes of mainly cyprinid fish species [44-47]. In that sense and taking into
368 account the high expression of *h1* and of *h1* and *h2b* observed in European sea bass and
369 gilthead seabream blood, respectively, we next analysed the transcription levels of these
370 genes in HKLs stimulated with different immune stimuli. Thus, we observed that *h1*
371 gene expression was down-regulated in European sea bass upon challenge with live
372 virus and bacteria, while in gilthead seabream this down-regulation was also observed
373 upon LPS, PHA and ConA treatments. In human monocytes, and upon LPS stimulation,
374 H1 is able to bind LPS [48]. In contrast to what happened to *h1* expression, the
375 transcription of *h2b* gene was up-regulated in gilthead seabream HKLs and down-
376 regulated in European sea bass HKLs upon NNV and other immune *stimuli*. Although
377 further studies are needed, this study clearly suggests that the ability to use histones as
378 AMPs, either in traps or not, might be a clear difference in the susceptibility to
379 infections of each fish species.

380 **5. Conclusions**

381 In conclusion, this is the first study analysing the pattern of expression of H1
382 and H2B coding sequences in a broad spectrum of tissues of European sea bass and
383 gilthead seabream fish species. Moreover, both genes are regulated in different tissues
384 by pathogens, PAMPs and mitogens pointing to an important role in fish immunity.
385 Thus, our data suggest that H1 might have a role in the immune response against NNV
386 in the brain of both species, due to the fact that *h1* expression pattern is similar to that
387 found for other AMPs and several IFN pathway genes and correlated well with the
388 different susceptibility to infection of both species [23, 40]. In the other hand, *h2b*
389 expression seems to be more important in the head-kidney and HKLs immune response.
390 Nevertheless, further functional studies are needed to understand histones implication in
391 fish immunity, and concretely in antimicrobial responses upon NNV infection, and
392 several studies are in progress in our laboratory.

393 **Acknowledgements**

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 398 Pilar Fernández Somalo (*Laboratorio Central de Veterinaria de Algete, Ministerio de*
 399 *Medio Ambiente, Rural y Marino*).

400 Appendix A. Supplementary data

401 **Supplementary table 1:** Mean values of *h1* and *h2b* transcription in European sea bass
 402 and gilthead seabream brain, gonad and head-kidney tissues after 1, 7 and 15 days of
 403 infection with NNV. Data were corrected with *ef1a* gene expression levels and
 404 normalized with the mean of control group (mock-infected). Asterisks denote statistical
 405 differences (t Student test; *P<0.1, **P < 0.05) with control group.

<i>h1</i> gene expression			
	days	European sea bass	Gilthead seabream
Brain	1	0.55±0.19	0.63±0.24
	7	0.12±0.02*	0.39±0.10*
	15	0.95±0.34	2.75±0.64**
Gonad	1	1.10±0.34	0.84±0.40
	7	2.28±0.92*	0.26±0.10
	15	1.06±0.39	0.32±0.11*
Head-kidney	1	0.04±0.03	1.04±0.20
	7	0.38±0.16	0.43±0.10
	15	0.34±0.14	1.69±1.10

406

<i>h2b</i> gene expression			
	days	European sea bass	Gilthead seabream
Brain	1	0.14±0.03**	0.94±0.43
	7	0.30±0.07	0.83±0.29
	15	2.44±0.75	1.34±0.30
Gonad	1	1.27±0.67	0.95±0.34
	7	1.22±0.43	0.53±0.19 *
	15	0.21±0.11**	0.65±0.24
Head-kidney	1	0.16±0.07	1.15±0.27
	7	4.57±1.88**	0.72±0.27
	15	3.31±1.86	2.72±0.93*

407

408

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543

544

545 **Figure 1:** Phylogenetic analysis of the H1 and H2B proteins of European sea bass and
546 gilthead seabream with related sequences of fish and mammalian histone proteins. The
547 phylogenetic tree was drawn following the Neighbor-Joining method for the analysis of
548 evolutionary relationship. Genbank accession numbers are shown in parentheses.
549 Histones with “t” are thymus isolated histones.

550

551 **Figure 2:** Levels of expression of *h1* gene in European sea bass (a) and gilthead
552 seabream (b) tissues: brain (Br), gills (Gi), liver (Li), skin (Sk), gonad (Go), gut, head-
553 kidney (Hk), spleen (Sp), thymus (Th) and blood (Blo) studied by real-time PCR. Data
554 represent mean relative expression to the expression of endogenous control *ef1a* gene \pm
555 SEM (n = 6). Letters denote statistical differences among tissues according to ANOVA
556 and Tukey’s post-hoc test (P < 0.05).

557

558 **Figure 3:** Levels of expression of *h2b* gene in European sea bass (a) and gilthead
559 seabream (b) tissues: brain (Br), gills (Gi), liver (Li), skin (Sk), gonad (Go), gut, head-
560 kidney (Hk), spleen (Sp), thymus (Th) and blood (Blo) studied by real-time PCR. Data
561 represent mean relative expression to the expression of endogenous control *ef1a* gene \pm
562 SEM (n = 6). Letters denote statistical differences among tissues according to ANOVA
563 and Tukey’s post-hoc test (P < 0.05). ND, non detected.

564

565 **Figure 4:** Expression levels of *h1* gene in European sea bass (a) and gilthead seabream
566 (b) brain, gonad and head-kidney after 1, 7 and 15 days of *in vivo* NNV infection (10^6
567 TCID₅₀ per fish) studied by real-time PCR. Data are expressed as the mean \pm SEM (n =
568 5) of mRNA fold increase respect to control samples. Asterisks denote significant
569 differences with the controls at each sampling time (t Student test; *P<0.1, **P < 0.05).

570

571 **Figure 5:** Expression levels of *h2b* gene in European sea bass (a) and gilthead seabream
572 (b) brain, gonad and head-kidney after 1, 7 and 15 days of *in vivo* NNV infection (10^6
573 TCID₅₀ per fish) studied by real-time PCR. Data are expressed as the mean \pm SEM (n =
574 5) of mRNA fold increase respect to control samples. Asterisk denote significant

575 differences with controls at each sampling time (t Student test; *P<0.1, **P < 0.05).

576

577 **Figure 6:** Expression levels of *h1* (a) and *h2b* (b) genes in European sea bass testis and
578 ovaries and gilthead seabream gonad after 24 h of *in vitro* challenge with NNV (10^7
579 TCID₅₀/ml), *Va* (4×10^7 bacteria/ml) and poly I:C (pI:C 62,5 µg/ml) studied by real-
580 time PCR. Data are expressed as the mean ± SEM (n = 6) of mRNA transcripts relative
581 to *ef1a* gene expression. Letters denote statistical differences among tissues according to
582 ANOVA and Tukey's post-hoc test (P < 0.05).

583

584 **Figure 7:** The expression of *h1* gene in HKLs of European sea bass (a) and gilthead
585 seabream (b) after 24 h of *in vitro* challenge with culture medium alone (control), 10^6
586 TCID₅₀ NNV/ml, 10^8 live bacteria/ml (*Va* or *Pd*), 50 µg/ml CpG ODN 1668, 25 µg/ml
587 pI:C, 5 µg/ml LPS, 10 µg/ml PHA or 5 µg/ml ConA studied by real-time PCR. Data are
588 expressed as the mean ± SEM (n = 5) of mRNA transcripts relative to *ef1a* gene
589 expression.). Letters denote statistical differences among tissues according to ANOVA
590 and Tukey's post-hoc test (P < 0.05).

591

592 **Figure 8:** The expression of *h2b* gene in HKLs of European sea bass (a) or gilthead
593 seabream (b) after 24 h of *in vitro* challenge with culture medium alone (control), 10^6
594 TCID₅₀ NNV/ml, 10^8 live bacteria/ml (*Va* or *Pd*), 50 µg/ml CpG ODN 1668, 25 µg/ml
595 pI:C, 5 µg/ml LPS, 10 µg/ml PHA or 5 µg/ml ConA studied by real-time PCR. Data are
596 expressed as the mean ± SEM (n = 5) of mRNA transcripts relative to *ef1a* gene
597 expression.). Letters denote statistical differences among tissues according to ANOVA
598 and Tukey's post-hoc test (P < 0.05).

599

600 **Table 1:** Primers used for analysis of gene expression by real-time PCR.

601

Species	Molecule	Gene	Accession	Primer sequence
		Abbrev.	number	
European sea bass	Histone 1	<i>h1</i>	DLAgn00119260	AAGAAGACGGGTCCCTCAGT CTTGACCTTCTTCGCTTTGG
	Histone 2B	<i>h2b</i>	DLAgn00179560	GGAGAGCTACGCCATCTACG GCTCAAAGATGTCGCTCACA
	Elongation factor 1 alpha	<i>ef1a</i>	AJ866727	CGTTGGCTTCAACATCAAGA GAAGTTGTCTGCTCCCTTGG
Gilthead seabream	Histone 1	<i>h1</i>	FM151953	CGTGGTGAAGAACAGAGCAA TTGACCCTTTTCGTCTTTGG
	Histone 2B	<i>h2b</i>	AM953480	AGACGGTCAAAGCACCAAAG AGTTCATGATGCCCATAGCC
	Elongation factor 1 alpha	<i>ef1a</i>	AF184170	CTGTCAAGGAAATCCGTCGT TGACCTGAGCGTTGAAGTTG

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604

605 **Table 2:** Identity (in %; ^a) and e-value (^b) of the predicted proteins respect to the human
606 orthologues. Asterisk denotes the sequences with predicted full length.

607

Predicted protein	Fish species	Gene accession number	Protein length	Identity^a	e-value^b
H1	Sea bass	DLAgn_0011926	188*	71	1e-25
	Seabream	FM151953	192*	67	9e-23
	Zebrafish	XP_017209709	199*	63	1e-31
	Human	NP_005313	226*		
H2B	Sea bass	DLAgn_00179560	121*	92	6e-67
	Seabream	AM953480	134*	95	5e-67
	Zebrafish	NP_001013481	124*	98	2e-72
	Human	AAH98112	124*		

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Figure 1

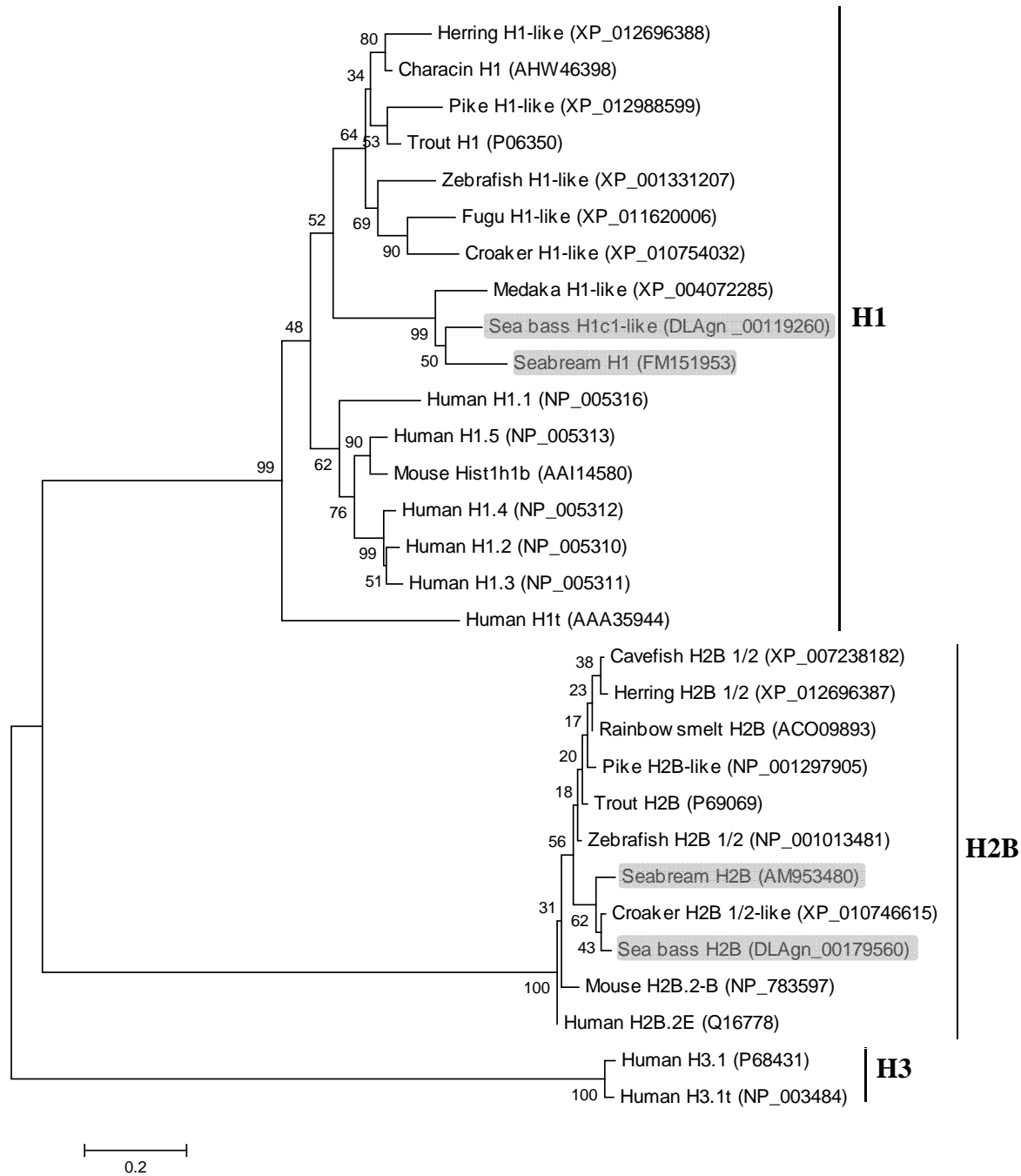


Figure 2

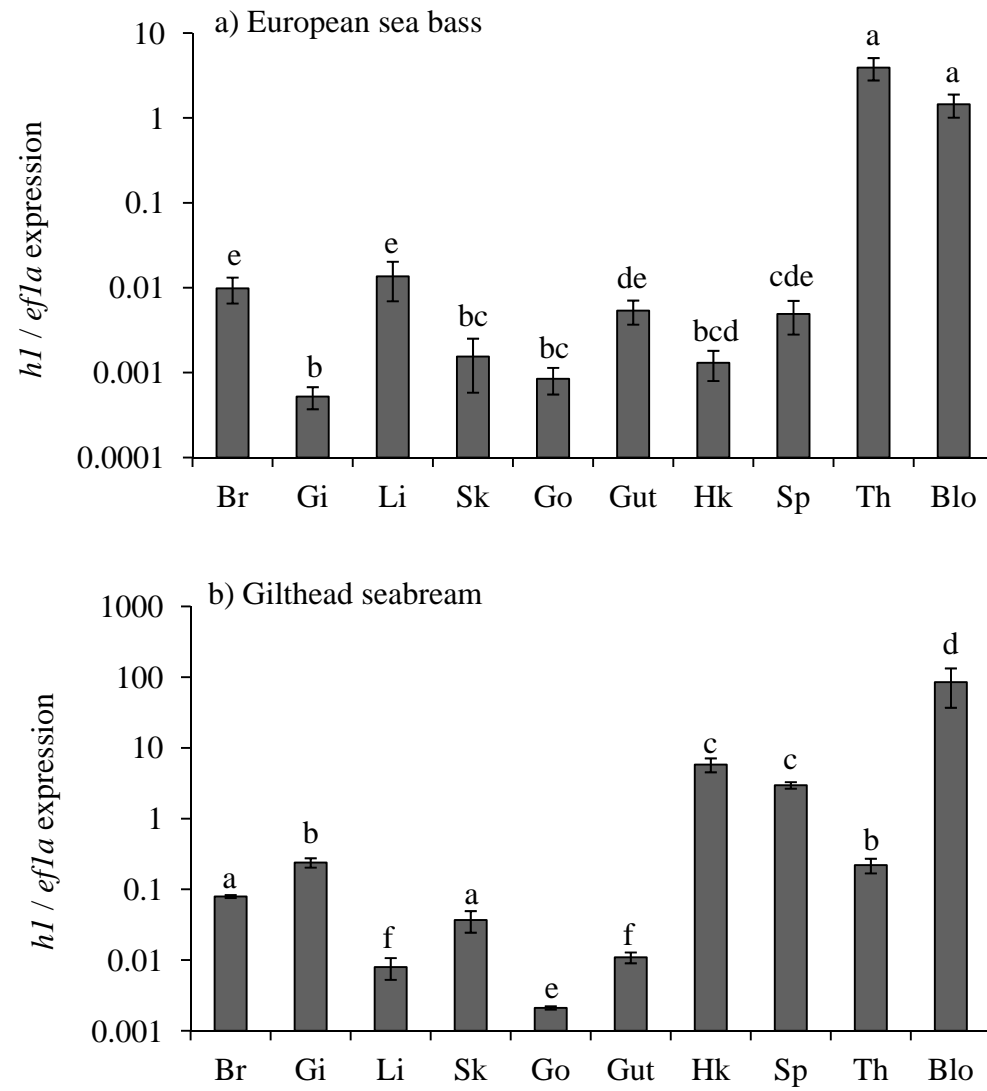


Figure 3

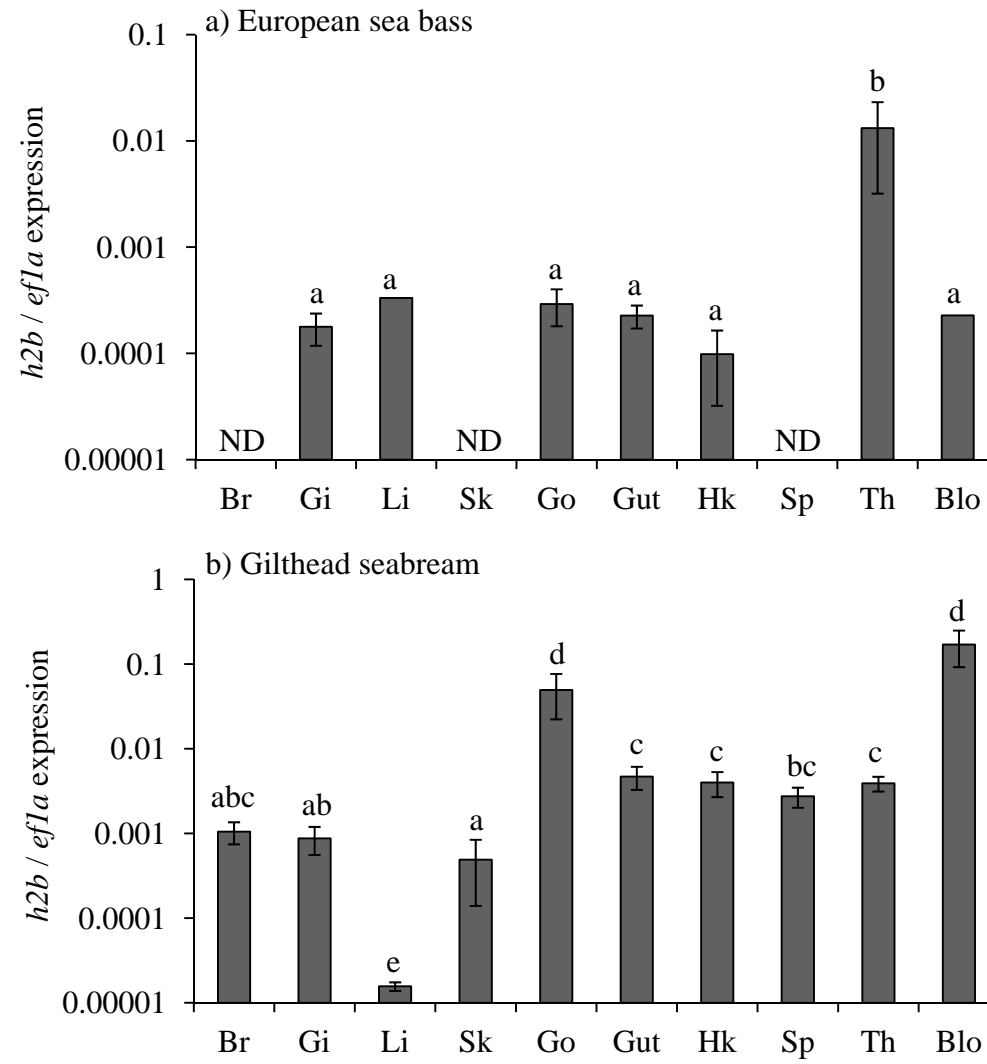


Figure 4

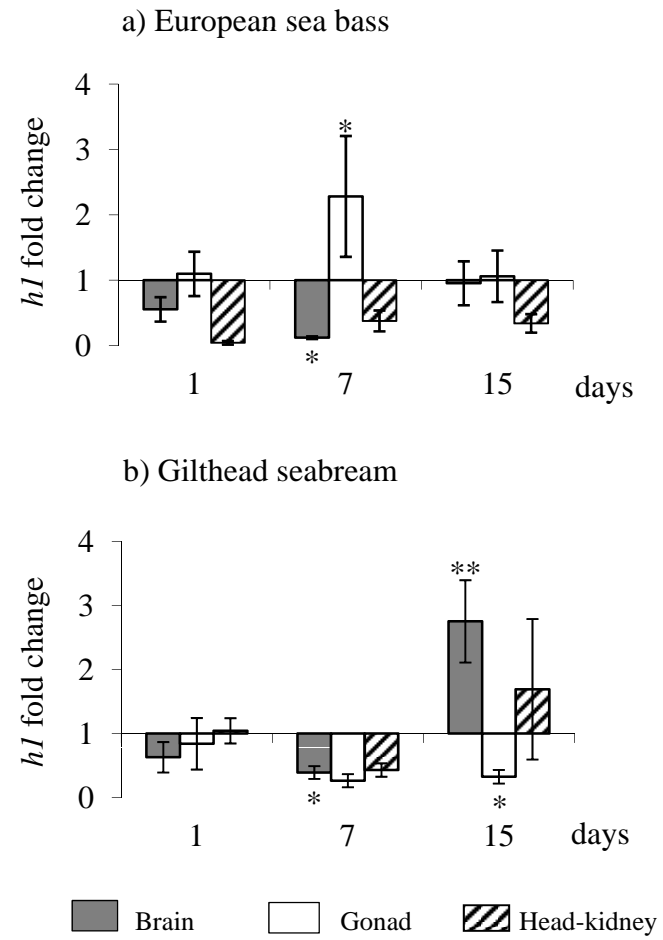


Figure 5

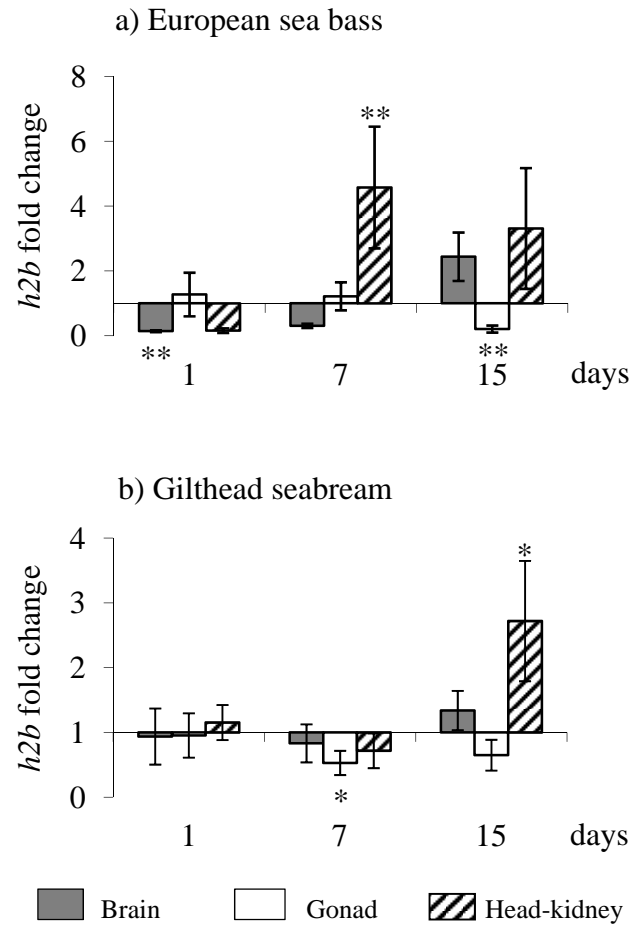


Figure 6

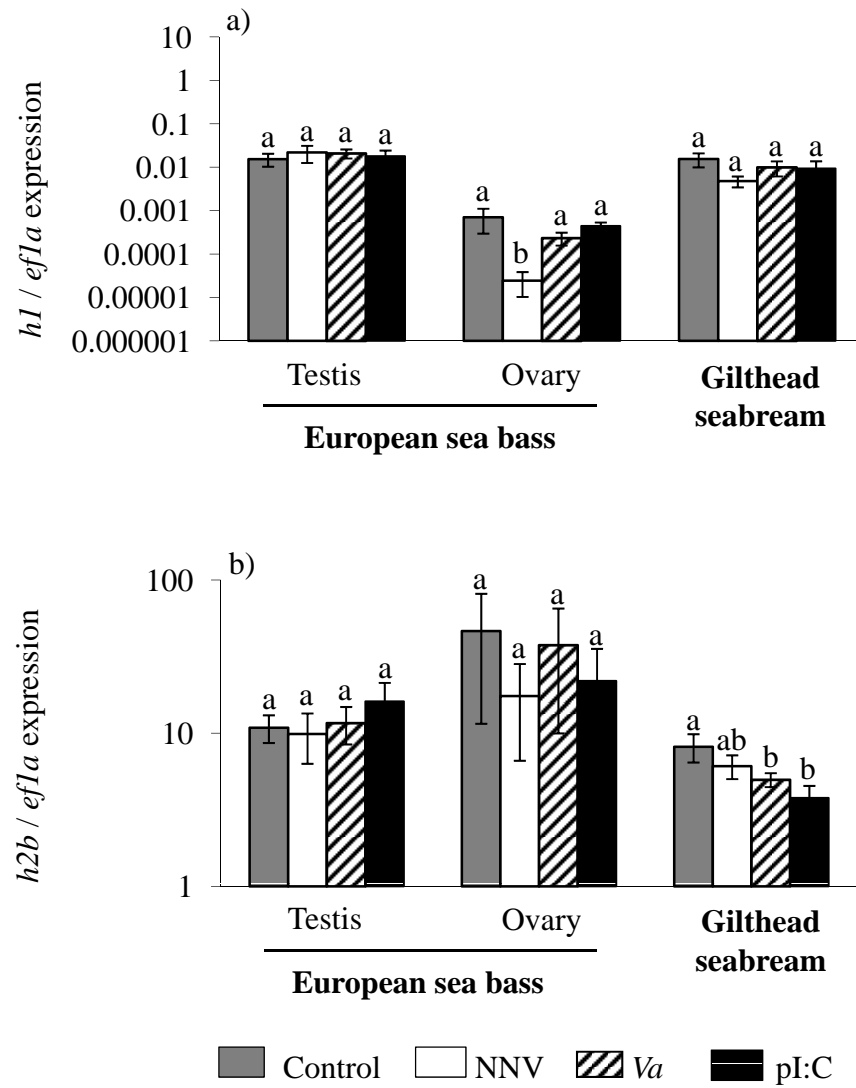


Figure 7

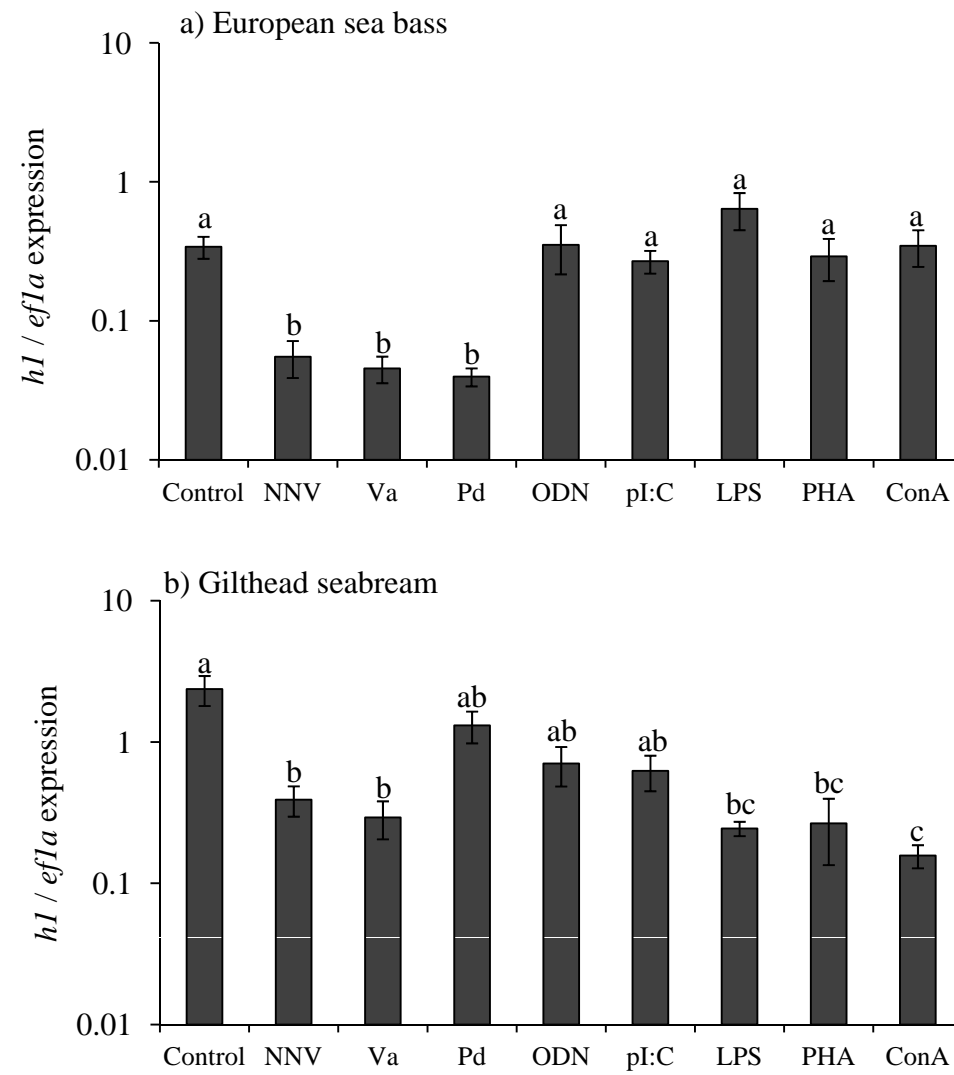
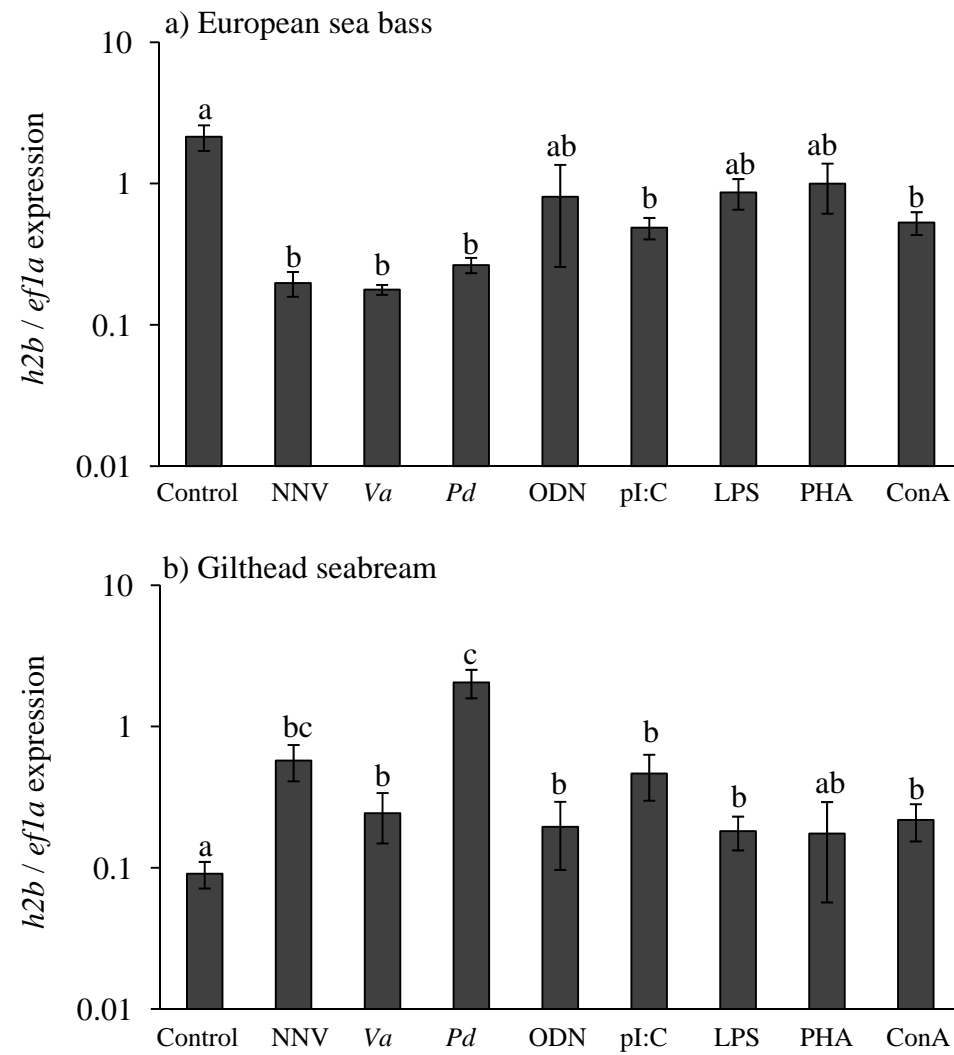


Figure 8



Highlights

- Histones H1 and H2b are characterized in European sea bass and gilthead seabream
- The transcription of *h1* gene may be related with immune response against NNV
- The transcription of *h2b* gene may be relevant in HKLs immune response against NNV
- The transcription of *h1* and *h2b* are differently regulated in HKLs