1　**Nodavirus increases the expression of Mx and inflammatory**
2　**cytokines in fish brain.**

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20

Abstract

21

22   Nodavirus has become a serious pathogen for a wide range of cultured marine fish

23   species. In the present work, the expression of genes related to immune and inflammatory

24   responses of sea bream (*Sparus aurata* L.), considered as non susceptible species, was

25   studied both *in vitro* and *in vivo*. No replication of the virus was observed in head kidney

26   macrophages and blood leukocytes. Moreover, the enhancement of expression of several

27   immune genes (tumor necrosis factor alpha (TNF-α), interleukin-1-beta (IL-1β), interferon-

28   induced Mx protein) was not detected in both head kidney macrophages and blood

29   leucocytes in response to an *in vitro* infection with nodavirus. However*, in vivo,* nodavirus

30   was detected 1 day post-infection (p.i.) by a reverse transcription-polymerase chain reaction

31   (RT-PCR) in blood, liver, head kidney and brain of experimentally infected sea bream,

32   while its presence clearly decreased in blood after 3 days p.i. Also, a transitory increment of

33   the expression of TNFα and IL-1β was detected in the brain of intramuscular (i.m.)

34   infected sea bream 3 days p.i. In head kidney, the over expression of TNFα was only

35   observed 1 day p.i. The expression of Mx, an interferon induced gene, was increased in

36   brain and head kidney of infected sea bream, reaching values of 1300 fold compared to

37   controls in brain three days post infection.

38   For comparative purposes, we analyzed the expression of the same genes on a

39   susceptible species, such as sea bass (*Dicentrarchus labrax*) and, although the same pattern

40   of expression was observed both in brain and kidney, the magnitude was different mainly in

41   the case of brain, the key organ of the infection, where higher expression of TNFα and

42   lower expression of Mx compared with control was observed.

43

44   *Keywords*: Nodavirus, sea bream (*Sparus aurata*), sea bass (*Dicentrarchus labrax*),

45   immune system, cytokines, TNFα, IL-1β and Mx

1. Introduction

Viral encephalopathy and retinopathy (VER), also referred to as viral nervous necrosis (VNN) is an emerging disease caused by several *Betanodaviruses*, members of the family *Nodaviridae* inducing high mortalities in infected marine fish. The disease caused by these viruses is identified by abnormal swimming behaviour and neurological lesions, which are characterized by cellular vacuolization and neuronal degeneration mostly found in the brain, retina, spinal cord and ganglia of the affected fish. Since its first description in larvae and juvenile sea bass (*Dicentrarchus labrax*) reared in Martinique (Bellance and Gallet de Saint-Aurin, 1988), the disease has spread to many other marine species worldwide (Nakai et al., 1994; Munday and Nakai, 1997; Curtis et al., 2001; Barke et al., 2002) and recently in freshwater species (Hegde et al., 2003; Athanassopoulou et al., 2004).

Sea bream and sea bass are species of a high economic value cultured in the Mediterranean Sea. Sea bream has been initially reported as an asymptomatic carrier of the disease (Castric et al., 2001). However, we have previously shown that sea bream can be experimentally susceptible to nodavirus, depending upon the temperature and route of infection (Aranguren et al., 2002). Also, sea bream is often cultured in the Mediterranean in the vicinity of sea bass and other susceptible species, raising the possibility of cross infection.

So far, little is known about the interactions between nodavirus and the fish immune system. Antibodies to nodavirus were detected by ELISA (Enzyme-Linked Immuno Sorbent Assay) in the serum of adults of striped jack (Mushiake et al., 1992), sea bass (Breuil et al., 2000), barfin flounder (Watanabe et al., 2000) and barramundi (Huang et al., 2001), regardless of the sex or origin (wild or cultivated) of the fish examined. Vaccines have been experimentally tested in fish with preliminary positive results (Husgaro et al., 2001; Sommerset et al., 2003; 2005) and the effect of nodavirus-neutralizing antibodies on virus clearance or survival has been reported (Tanaka et al., 2001).

73   The aim of this work was to study if the experimental infection of sea bream and sea

74   bass with nodavirus could affect the expression of inflammatory cytokines, such as tumor

75   necrosis factor alpha (TNF-α), interleukin-1 beta (IL-1β), and an interferon-induced Mx

76   protein, both *in vitro* and *in vivo*. Moreover, we have compared the viral replication and the

77   gene expression between the two fish species with the aim to find possible explanations of

78   the differential susceptibility to the disease.

79

80   **2. Materials and methods**

81

82   *2.1. Fish*

83

84   Adult sea bream and sea bass of approximately 200 g were obtained from a

85   commercial fish farm. Fish were then acclimatized to laboratory conditions for 2 weeks,

86   maintained at 20 ºC and fed daily with a commercial diet (Trouw, Spain).

87

88   *2.2. Virus*

89

90   The nodavirus strain, 475-9/99, was provided by The Istitute Zooprofilattico delle

91   Venize (Italy) after isolation from diseased sea bass. The virus was propagated in the SSN-

92   1 cell line (Frerichs et al., 1996) and then titrated in 96-well plates (Falcon). $TCID_{50}$ ml^{-1}

93   (tissue culture infectious dose infecting 50 % of inoculated cultures) was calculated

94   according to Reed and Müench (1938).

95

96   *2.3. Isolation of head kidney macrophages and blood leukocytes*

97

98   Head kidney macrophages and blood leukocytes were isolated following the method

99   previously described by Chung and Secombes (1988). The viable cell concentration was

100   determined by Trypan blue exclusion.

101

102   *2.4. Replication of nodavirus in sea bream and sea bass leukocytes and kidney*

103   *macrophages*

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105   Primary cultures of total blood leukocytes and kidney macrophages from sea bass

106   and sea bream were infected with nodavirus (1×10^4 TCID$_{50}$ ml^{-1}). After 1 h of incubation

107   with the virus at 25 ºC, cells were washed twice with L-15 medium and incubated at 25 ºC

108   with L-15 + 5 % fetal calf serum (FCS). After 1, 3, 5 and 7 days, supernatants and cells

109   were collected by scraping the bottom of the wells and separated by centrifuging at 12000 x

110   g for 10 minutes at 4 ºC. Cells were then suspended in the same medium previously used

111   for the culture. Supernatants and cells were frozen until use and, in the case of cells,

112   another freezing cycle was conducted in order to lyse them. Titration of supernatants and

113   cells was made in SSN-1 96-well plates and the TCID$_{50}$ calculated.

114

115   *2.5. Cytokines induction after a nodavirus infection*

116

117   The level of expression of TNFα, IL-1β and Mx was tested after infection both *in*

118   *vitro* and *in vivo* using quantitative Real Time PCR (qPCR).

119   The *in vitro* induction of these genes was tested after infecting head kidney

120   macrophages and blood leukocytes (5×10^6 cells ml^{-1}) with nodavirus at a final

121   concentration of 7.8×10^5 TCID$_{50}$ ml^{-1}. After 6 hours of incubation at 25 ºC, supernatants

122   were removed by centrifuging 5 min at 12000xg and RNA was extracted from the cells

123   using Trizol (Gibco). RNA was then used to obtain cDNA by Superscript Preamplification

124   System (Gibco), which was stored at −20 ºC.

125   The *in vivo* induction was tested by intramuscular injection of sea bream and sea

126   bass. Eighteen fish from each species were challenged with 50 µl of nodavirus (3 x 10^5

127    $TCID_{50}$ ml^{-1}/fish) and eighteen fish were injected with 50 µl of cell culture medium as

128   control. Fish were sacrificed by MS-222 overdose 1, 3 and 7 days post challenge (three

129   pools of two fish each one) and brain and head kidney were removed aseptically and frozen

130   for RNA isolation and cDNA transcription, as previously described.

131   Quantitative PCR assays were performed using the 7300 Real Time PCR System

132   (Applied Biosystems). cDNA amplification was performed using specific primers designed

133   by Primer 3 software (Rozen and Skaletsky, 2000). 0.5 µl of each primer (10 µM) was

134   mixed with 12.5 µl of SYBR green PCR master mix (Applied Biosystems) in a final

135   volume of 25 µl. The standard cycling conditions were 95 º for 10 min, followed by 40

136   cycles of 95 º 15 s and 60 º for 1 min. The comparative CT method (2-ΔΔCT method) was

137   used to determine the expression level of analyzed genes (Livak and Schmittgen, 2001).

138   The expression of the candidate genes was normalized using β–actin as a housekeeping

139   gene. Fold units were calculated dividing the normalized expression values of infected

140   tissues by the normalized expression values of the controls. Primer sequences are shown in

141   Table 1.

142

143   *2.6. Nodavirus detection by RT-PCR*

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145   In order to determine whether nodavirus was present in the different organs of sea

146   bream in which the expression of cytokines was studied in a similar way than it happens in

147   sea bass, viral detection was performed using an RT-PCR based on the amplification of a

148   highly conserved region of the coat protein gene as previously described by Dalla Valle et

149   al. (2000). Products of the amplification reaction were visualized on a 2 % agarose gel.

150

151   *2.7. Statistics*

153   Data were compared using Student's *t* test. Results are expressed as mean $\pm$
154 standard deviation and differences were considered statistically significant at $p < 0.05$.

155

3. Results

157

3.1. Replication of nodavirus in head kidney macrophages and blood leukocytes

159

160   The viral titer did not increase with time in sea bream and sea bass kidney
161 macrophages or in blood leukocytes (Figure 1), neither in the cells nor in the supernatants,
162 indicating that these cell populations do not support viral replication in any of the two
163 studied species. No cytopathic effect was ever observed in head kidney macrophage or
164 blood leukocyte cultures during the nodavirus infection.

165

3.2. Nodavirus detection by RT-PCR

167

168   In order to confirm that the lack of susceptibility of sea bream to nodavirus infection
169 was due to a problem in the accessibility to the key organ, the presence of nodavirus was
170 assessed in infected sea bream at days 1 and 3 post-infection in blood, liver, kidney and
171 brain. Nodavirus, as in the case of sea bass (data not shown), was strongly detected in blood
172 1 day post-infection but the amount of virus detected highly decreased 3 days after
173 infection (Figure 2a). However, nodavirus presence was confirmed 1 and 3 days p.i. in the
174 remaining tissues, especially in brain as the target organ of the disease (Figure 2b, 2c and
175 2d). Nodavirus was never detected in control sea bream tissues (Figure 2a, 2b, 2c and 2d).

176

3.3. Cytokines expression analysis in sea bream and sea bass

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179   The expression of TNF-α, IL-1β and Mx both in sea bream and sea bass

180   macrophages and blood leukocytes was not enhanced after exposure to nodavirus *in vitro* in

181   this study (data not shown).

182   However, with regard to the *in vivo* infection of sea bream, a significant but

183   transitory up-regulation of the expression of TNFα and IL-1β was detected in the brain of

184   infected sea bream 3 days p.i. (Figure 3a and 3c, respectively). In head kidney, the over

185   expression of TNF-α was only observed 1 day p.i. (Figure 4a), and a down-regulation was

186   detected 3 days p.i. in the case of IL-1β (Figure 4c). The expression of Mx protein was

187   increased both in brain and head kidney (Figures 3e and 4e, respectively), reaching values

188   of 1300 fold compared to controls in brain three days post infection (Figure 3e).

189   The pattern of expression described above for sea bream was similar to the one

190   observed both in brain and kidney of infected sea bass. Nevertheless, the magnitude was

191   different mainly in the case of brain, the target organ of the infection, where higher

192   expression of TNF-α and lower expression of Mx compared with control was observed

193   (Figure 3b and 3f).

194

195   **4. Discussion**

196

197   Nodavirus is an increasingly important pathogen for several marine fish species,

198   causing mortalities mainly in larvae and juveniles due to a degenerative process in brain,

199   retina and spinal cord. Despite of many species are affected by this disease including sea

200   bass and sea bream, the pathogenesis and immune response of nodaviriosis is not well

201   known so far. Innate immunity is the first line of defense in fish and other invertebrates and

202   therefore has a relevant role after body injury or infection. Pro-inflammatory cytokines

203   such as interleukins and tumor necrosis factors that participate in the Acute Phase Response

204   (APR) or the effectors molecules involved in the antiviral interferon (IFN) pathway such as

205   Mx proteins are one of the most studied.

206   In the present study, in contrast to what occurs with other fish viruses (Chilmonczyk

207   et al., 1995; Tafalla et al., 1998), the *in vitro* experiments suggested that nodavirus

208   replication in sea bream and sea bass immune system cells (head kidney macrophages and

209   blood leukocytes) was limited or non existent. Even when viral replication is not supported

210   by cells of the immune system, viruses often cause an alteration of their immune functions

211   (Stolhman et al., 1982). However, this seems not to be the case in sea bream and sea bass

212   macrophages and leukocytes infected *in vitro*, as at least in the genes analyzed (TNF-α, IL-

213   1β and Mx), no modulation of expression was detected (data not shown). *In vivo* studies

214   seem to support the *in vitro* results since, although nodavirus was present in the blood and

215   several organs 1 day post-infection, after three days, the analysis of RT-PCR products

216   indicated low nodavirus concentration in the blood of infected sea bream but a high

217   concentration in liver, head kidney and above all in brain. This confirmed the spread of

218   nodaviruses through the circulating system towards the target organ for replication and

219   development of the disease. This result indicates that the virus behaves in a similar way in

220   both fish species reaching the brain where the viral pathogenesis is evident. The lack of

221   susceptibility of sea bream cannot then be explained by a different ability to reach and

222   replicate in the brain, indicating that a stronger response should be present in sea bream

223   which confers resistance against the disease.

224   TNF-α is an important mediator in resistance against parasitic, bacterial and viral

225   infections among other therapeutic roles (Aggarwal and Vilcek, 1991; Vilcek and Lee,

226   1991; Czarniecki, 1993; Wride and Sanders, 1995; Goldfeld and Tsai, 1996; Steinshamn et

227   al., 1996; Krueger et al., 1998; Secombes et al., 2001). IL-1β on the other hand plays a

228   pivotal role in the inflammatory response as initiates and/or increases a wide variety of non-

229   structural function associated genes that are characteristically expressed during

230   inflammation, particularly other cytokines (Dinarello, 1994; Bird et al., 2002). In this study,

231   we observed a strong up-regulation of TNF-α expression in head kidney 1 day post-

232   infection both in sea bream and sea bass infected with nodavirus (Figure 4a and 4b,

233   respectively), this up-regulation was no longer obvious 3 and 7 days after infection. This

234   result could be explained as head kidney is the main immune organ in fish and therefore

235   responds in the APR to fight against the infection (Dinarello, 1996; Bayne et al., 2001). In

236   the case of IL-1β, a down-regulation was detected 3 days p.i. in both species (Figure 4c and

237   4d) which was not longer observed 7 days post infection. The regulation of these two

238   cytokines in kidney in the first stages of the disease in both species could be then

239   considered as a generalized response against nodavirus.

240   In the case of brain, the key organ of the disease, TNF-α and IL β over-expression

241   in sea bream was mainly observed 3 days post-infection (Figure 3a and 3c). The fact that

242   TNF-α was modulated in brain 3 days after infection unlike to what happened in kidney (1

243   day p.i.), may suggest that immune system seems to be activated in brain when nodaviruses

244   reach their target organ and start replication, as we previously reported (Dios et al., 2007).

245   This pattern of expression was similar to the one observed in the brain of infected sea bass.

246   Nevertheless, the expression values for TNF-α were much higher in sea bass (more than 30

247   times) than in sea bream (Figure 3b). We suggest that the strong up-regulation of this pro-

248   inflammatory cytokine in the brain of a susceptible species like sea bass, may be

249   responsible of the vacuolization and the neuroinflammatory process associated to this

250   disease in brain, retina and spinal cord. In fact, inflammation has been described as an

251   important factor causing irreparable brain damage in the pathogenesis of neurodegenerative

252   diseases and microbial infections of the nervous system (Brabers and Nottet, 2006; Kim

253   and Joh, 2006; Lafon et al., 2006; Sutton et al., 2006; Wei et al., 2006; Ghoshal et al., 2007;

254   Konsman et al., 2007).

255   The interferon system is one of the most important mechanisms for antiviral defense

256   and the Mx proteins one of its effectors molecules best known (Meier et al., 1990; Staeheli

257   et al., 1993; Arnheiter et al., 1996; Robertsen et al., 1997; Trobridge et al., 1997; Haller et

258   al., 1998; Jensen and Robertsen, 2000; Haller and Kochs, 2002; Ko et al., 2002; Caipang et

259   al., 2003; Plant and Thune, 2003; Larsen et al., 2004; Chen et al., 2006; Wu and Chi, 2006).

260   In the present work, an over-expression of Mx was not observed *in vitro* however, a

261   significant up-regulation was detected in general both in brain and head kidney of sea

262   bream and sea bass infected with nodavirus in all sampling points (Figures 3e, 3f, 4e, and

263   4f). These results corroborated the unequivocal participation of Mx proteins in the antiviral

264   responses in sea bream and sea bass. Noteworthy, just like we previously described for

265   TNF-α in brain, even when the Mx expression pattern is similar in both species, the

266   magnitude of expression in terms of fold change values is higher in sea bream in this case

267   (more than 1300 times) (Figure 3e). This strong up-regulation of Mx protein in the brain of

268   sea bream with respect to the one observed in sea bass could be related to the effectiveness

269   in solving the infection and could explain why sea bream is an asymptomatic carrier of the

270   disease. Also, all the results taking together seem to support recent findings, in which was

271   suggested that human neurons, although are not located in an immune organ, have the

272   intrinsic machinery to mount robust inflammatory, chemoattractive, and antiviral responses

273   (Lafon et al., 2006). To our knowledge, this is the first time this response in fish brain

274   against a viral infection is described.

275   In summary, the results presented here for sea bream and sea bass pointed out the

276   early activation of TNF-α and Il-1β in head kidney as a generalized response against

277   nodavirus infection. Their expression increased 3 days after infection in brain, where the

278   immune responses seem to be activated when nodaviruses reach the target organ and start

279   replication. Also, TNF-α was highly over-expressed in the brain of infected sea bass, which

280   seems to be related to the vacuolization and neurodegenerative symptoms of the

281   disease. Mx protein was also up-regulated as an antiviral mechanism in both species but the

282   expression level (in fold change units) in brain was higher in sea bream than in sea bass,

283   suggesting an explanation why sea bass is a susceptible species and sea bream is an

284   asymptomatic carrier. Moreover, these results support the fact that fish brain, in the same

285   way that human neurons, is able of triggering a strong inflammatory response characterized

286   by the expression of inflammatory cytokines, chemokines, and antiviral molecules.

287   Further studies will be conducted to elucidate another genes involved in the immune

288   response of sea bream and sea bass against a nodavirus infection.

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524 **Figure legends**

525

526 **Figure 1**. Nodavirus titers in supernatants and cells from sea bream and sea bass at

527 different times after *in vitro* infection. (A) Sea bream kidney macrophages; (B) sea bream

528 blood leukocytes; (C) sea bass kidney macrophages and (D) sea bass blood leukocytes.

529 Data are expressed as mean Log TCID$_{50}$ mL^{-1} $\pm$ SD for 3 replicates.

530

531 **Figure 2**. RT-PCR nodavirus detection in control and experimentally infected sea bream

532 tissues at different times post-infection. (A) Blood; (B) liver; (C) kidney and (D) brain.

533 Lanes 1 and 2: control samples; lanes 3-6: infected.

534

535 **Figure 3**. Real time PCR results for TNFα, IL-1β and Mx expression level in the brain of

536 intramuscular infected sea bream and sea bass. Fold units were calculated dividing the

537 expression values of infected tissues by the expression values of the controls once

538 normalized regarding to the β–actin expression. (A, C and E) sea bream; (B, D and F) sea

539 bass.

540

541 **Figure 4**. Real time PCR results for TNFα, IL-1β and Mx expression level in the head

542 kidney of intramuscular infected sea bream and sea bass. Fold units were calculated

543 dividing the expression values of infected tissues by the expression values of the controls

544 once normalized regarding to the β–actin expression. (A, C and E) sea bream; (B, D and F)

545 sea bass.

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Figure 1

Figure 1

Figure 2

Figure 2

a   BLOOD

b   LIVER

c   KIDNEY

d   BRAIN
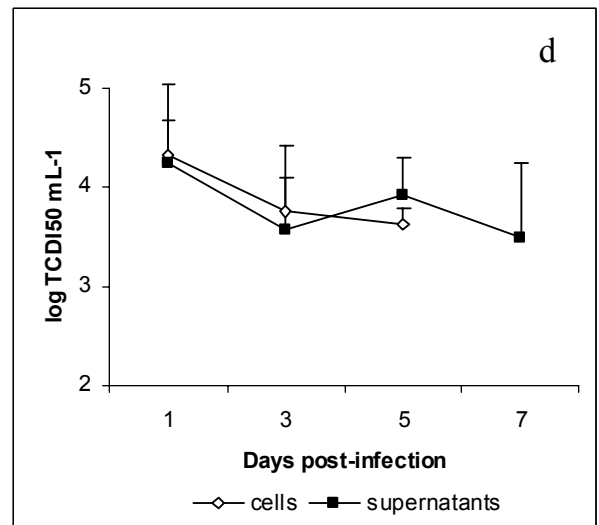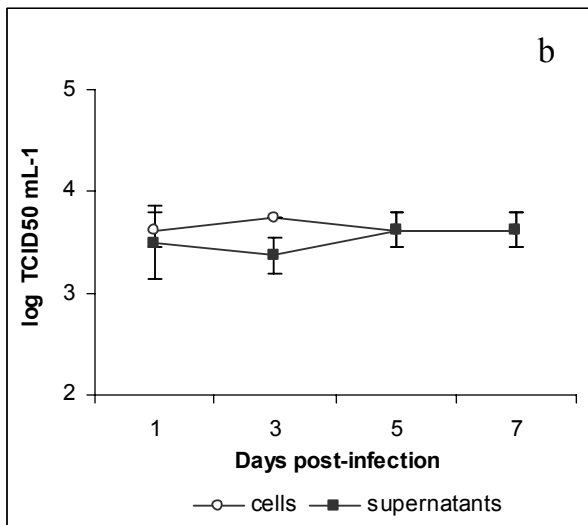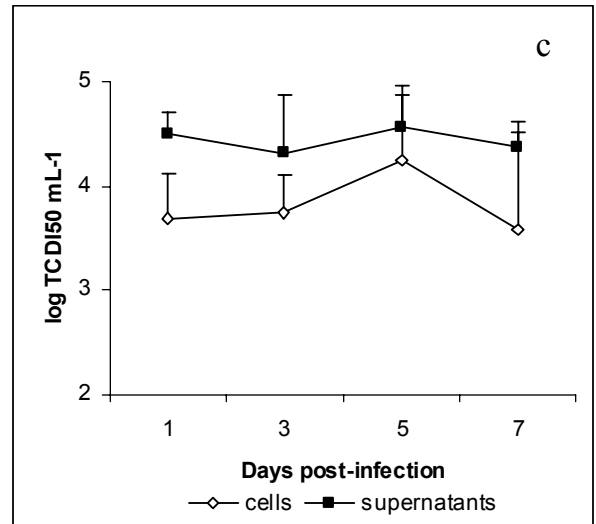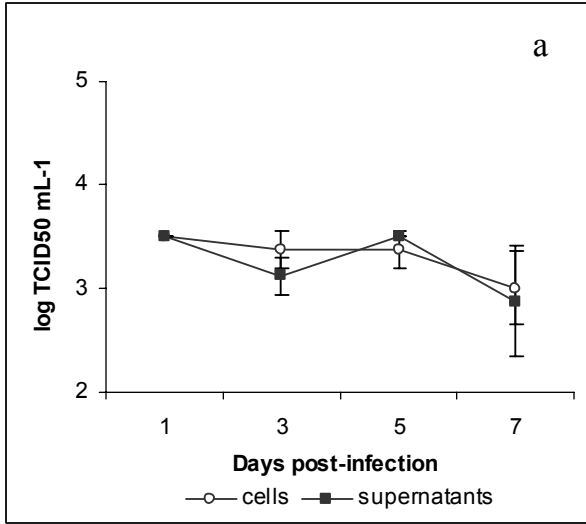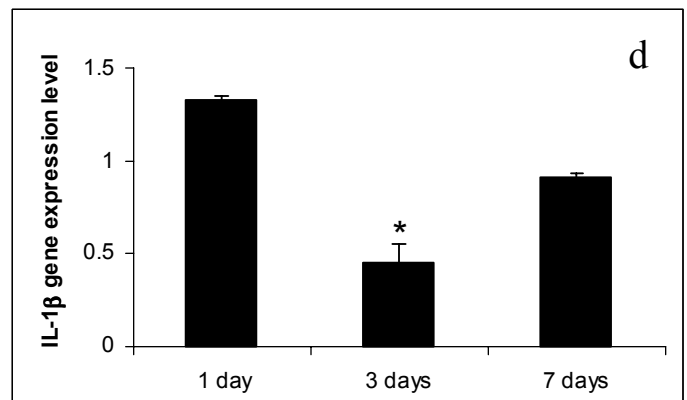
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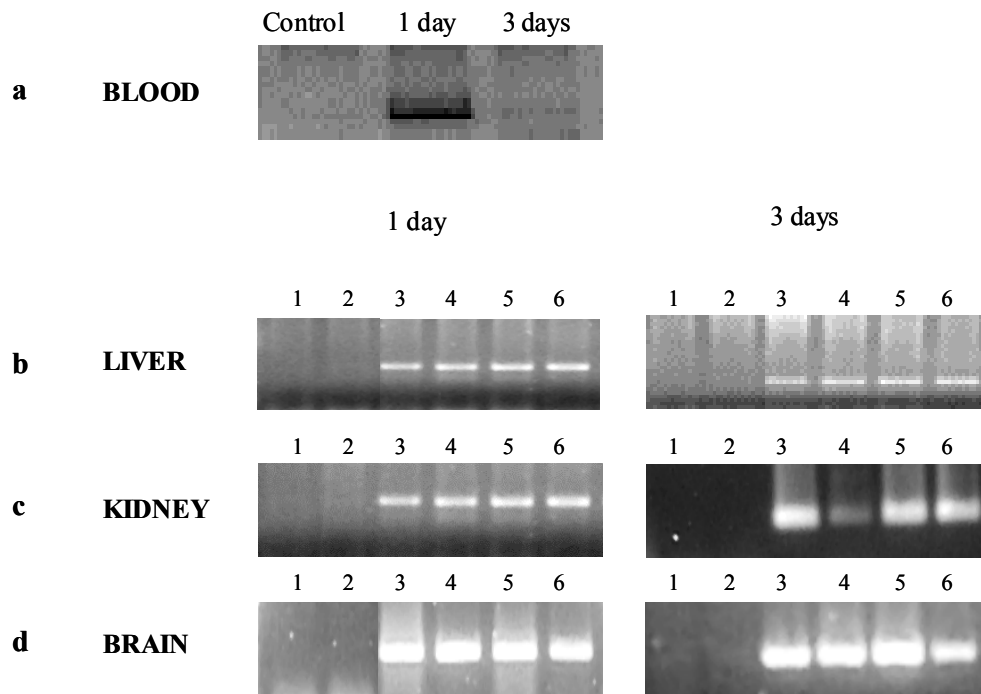
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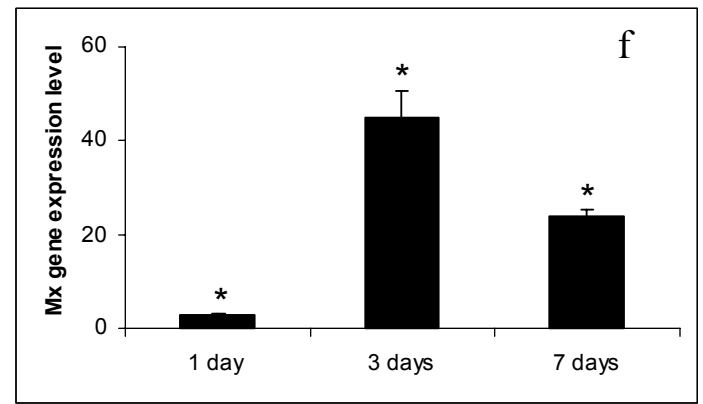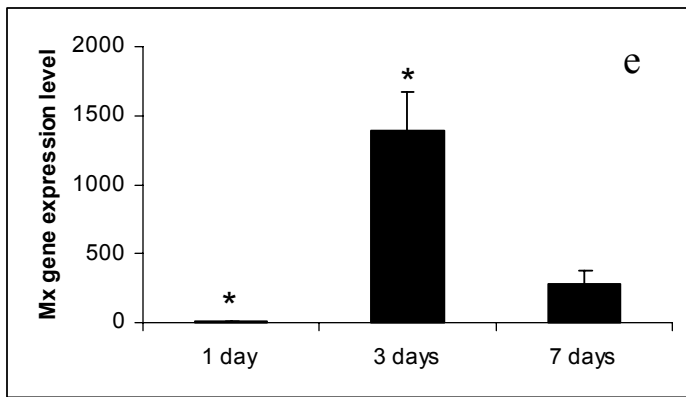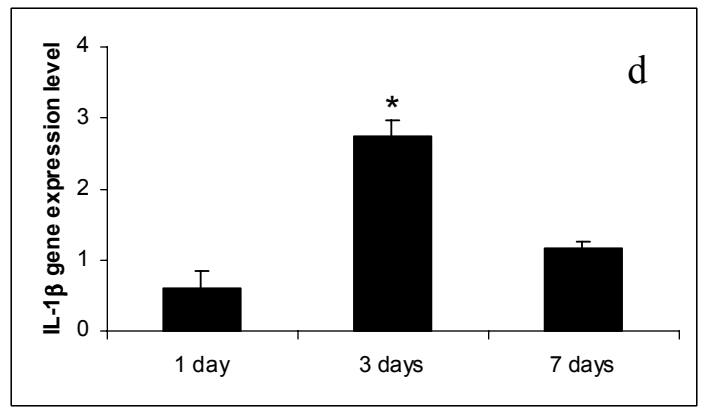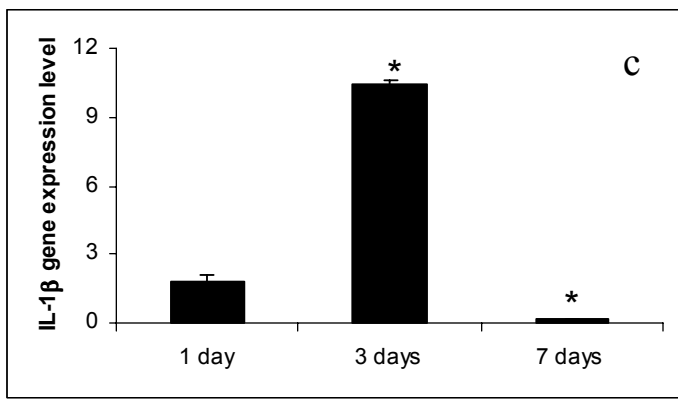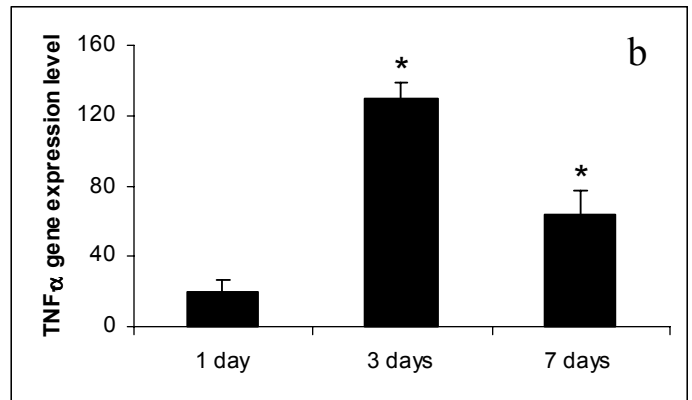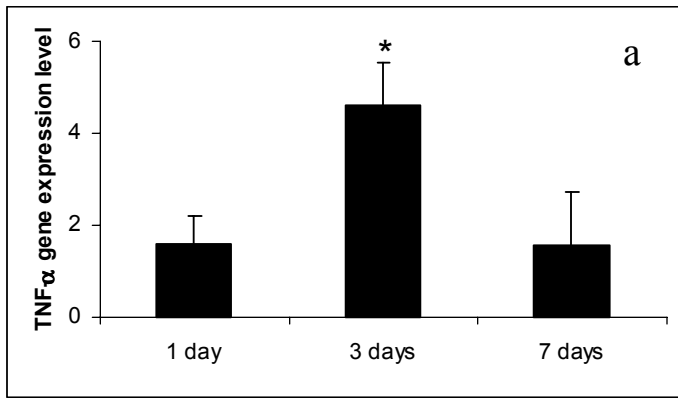
Figure 4

Figure 4
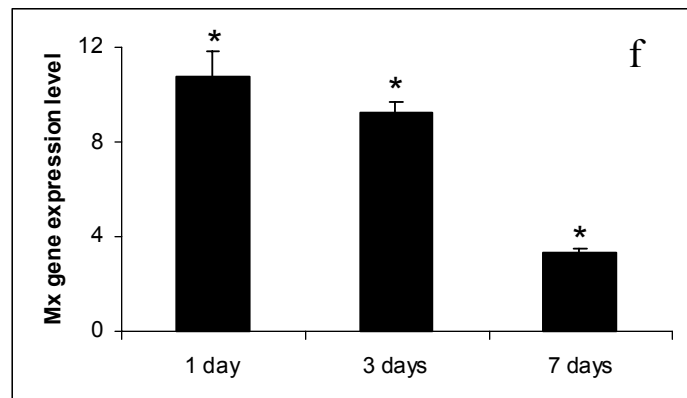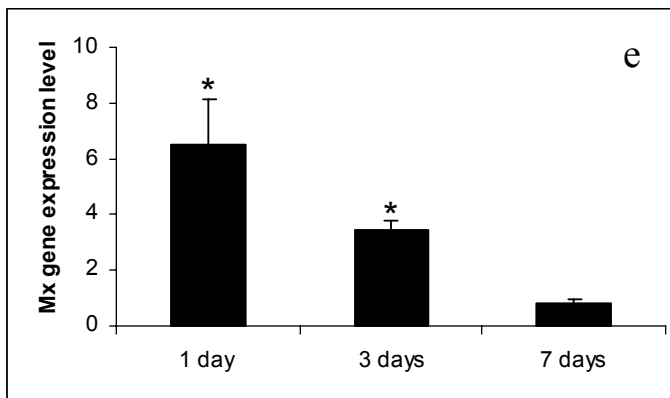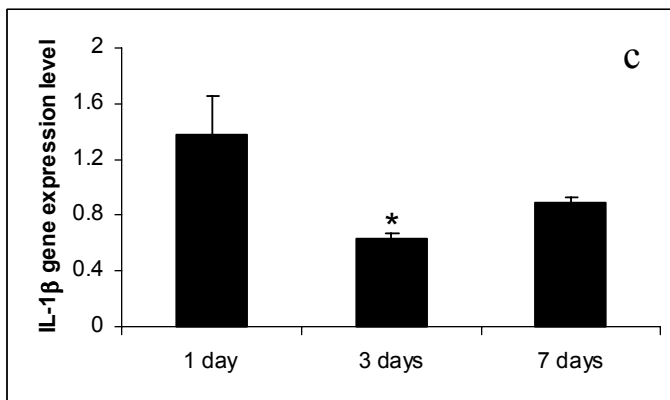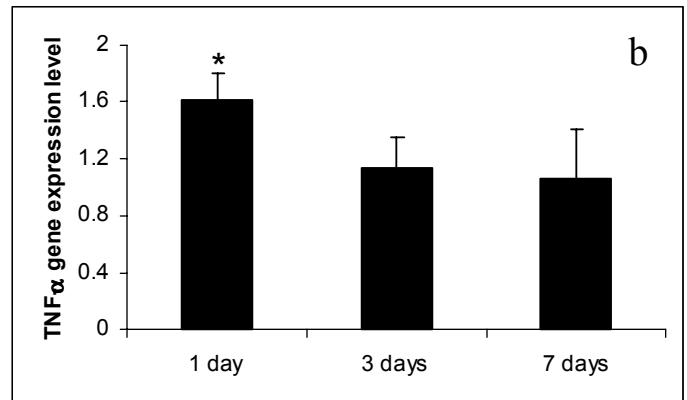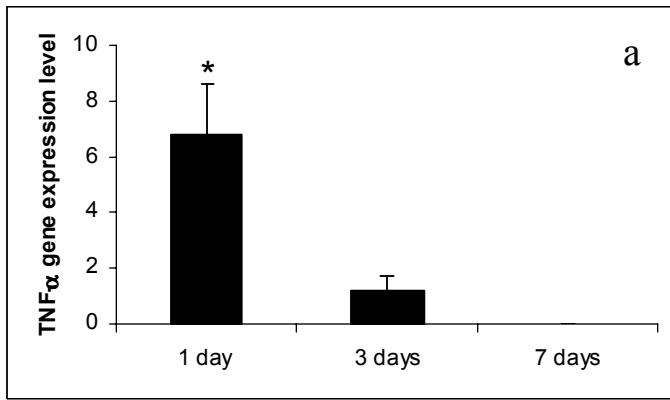
Table 1

Table 1. Primer sequences of the genes analyzed.

	F / R	SEA BREAM	SEA BASS
β-actin	Forward	TCGGTCGCCCCAGGCATC	GTGCGTGACATCAAGGAGAA
β-actin	Reverse	CTCCTTAATGTCACGATTT	GCTGGAAGGTGGACAGAGAG
Tumor necrosis factor α	Forward	CAAGCCGGAAATTCTGGTAA	CGAGGGCAAGACTTTCTTTG
Tumor necrosis factor α	Reverse	TTTCTCAGCGTGGTCCTTCT	GCACTGCCTGTTCAGCTACA
Interleukin-1β	Forward	ATGCCCGAGGGGCTGGGC	CAGGACTCCGGTTTGAACAT
Interleukin-1β	Reverse	CAGTTGCTGAAGGGAACAGAC	GTCCATTCAAAAGGGGACAA
Mx protein	Forward	CTCTGCTGAGGACCCAGTTC	GGGGTCAGAAGGAGATCACA
Mx protein	Reverse	GTGCAGCATCAACTCCTTCA	ATGATGCACCAGCTCAAGTG