Antimicrobial response is increased in the testis of European sea bass, but not in gilthead seabream, upon nodavirus infection

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Footnote[1]: The genetic nomenclature used in this manuscript follows the guidelines of Zebrafish Nomenclature Committee (ZNC) for fish genes and proteins and the HUGO Gene Nomenclature committee for mammalian genes and proteins.

Abstract

Antimicrobial peptides (AMPs) have a crucial role in the fish innate immune response, being considered a fundamental component of the first line of defence against pathogens. Moreover, AMPs have not been studied in the fish gonad since this is used by some pathogens as a vehicle or a reservoir to be transmitted to the progeny, as occurs with nodavirus (VNNV), which shows vertical transmission through the gonad, and/or gonadal fluids but no study has looked into the gonad of infected fish. In this framework, we have characterized the antimicrobial response triggered by VNNV in the testis of European sea bass, a very susceptible species of the virus, and in the gilthead seabream, which acts as a reservoir, both *in vivo* and *in vitro*, and compared with that present in the serum and brain (target tissue of VNNV). First, our data show a great antiviral response in the brain of gilthead seabream and in the gonad of European sea bass. In addition, for the first time, our results demonstrate that the antimicrobial activities (complement, lysozyme and bactericidal) and the expression of AMP genes such as *complement factor 3* (*c3*), *lysozyme* (*lyz*), *hepcidin* (*hamp*), *dicentracin* (*dic*), *piscidin* (*pis*) or *β-defensin* (*bdef*) in the gonad of both species are very different, but generally activated in the European sea bass, probably related with the differences of susceptibility upon VNNV infection, and even differs to the brain response. Furthermore, the *in vitro* data suggest that some AMPs are locally regulated playing a local immune response in the gonad, while others are more dependent of the systemic immune system. Data are discussed in the light to ascertain their potential role in viral clearance by the gonad to avoid vertical transmission.

Keywords: fish; nodavirus; gonad; antimicrobial peptides; Sea bass

1. Introduction

The infection of the gonad by pathogens is the initial step to promote horizontal transmission through gonadal fluids and/or vertical transmission through infected gametes [1, 2]. In all vertebrates, the gonad is considered an immunologically-privileged site, as also occurs with the brain and retina. In those tissues, the immune response proceeds in a different manner in order to avoid cell damage [3, 4], which is used by some pathogens to be hiden and scape to the immunological control. In fish, the implications of this different regulation of the immune functions inside the reproductive organs and its implication on pathogen dissemination through the gonad have very recently been documented [5-7]. However, this immune response in the gonad from infected fish deserves deeper characterization as a mean to control this route of pathogens dissemination. In that sense, antimicrobial peptides (AMPs) are increasingly recognized as a critical first line of defence against many pathogens and have been extensively studied in invertebrates and vertebrate species, including fish [8]. Their specific characteristics of low molecular weight, polarity or amino acid composition confer them a broad-spectrum of antimicrobial activities against bacteria, virus, fungi, protozoa, and even tumour cells [9-11]. The AMPs expressed in the mammalian gonad are considered to assume an important part of a highly effective immune response against pathogens since the production of pro-inflammatory factors is strictly restrictive in this tissue in order to avoid germ cell damage [12, 13] as also described in gilthead seabream gonad [4]. In teleost fish, more than 60 AMPs have been described and determined its expression in several tissues, including gonad [14]. Unfortunately, to our knowledge, nothing is known about their regulation and immunological role in the fish gonad despite the immune peculiarities of this organ and the important roles attributed to AMPs.

Nodavirus (VNNV), a bipartite and positive single-stranded RNA virus, is a known vertical and horizontal transmitted pathogen [15-20] able to infect more than 50 marine fish species, some of them especially sensitive, as the European sea bass (*Dicentrarchus labrax*), and others only susceptible to some strains of VNNV, as occurs with gilthead seabream (*Sparus aurata*) [18, 21]. Interestingly, though the main target tissues of VNNV are the brain and the retina [18, 21], both immune-privileged tissues, as the gonad, the virus has also been detected in the European sea bass liver, spleen and caudal fin [22] and more recently we have also found it into the gonad [23]. Previous

studies have evaluated the role of several immune responses in the head-kidney or brain after VNNV infections such as the gene expression of interferon, pro-inflammatory cytokines, chemokines or leucocyte markers as well as leucocyte functions such as proliferation, respiratory burst or cell-mediated cytotoxic activity but never the role of AMPs [24-28]. Regarding the AMPs, it is unknown if they are triggered upon nodavirus infection but it has been well demonstrated that some isolated AMPs showed anti-VNNV activity *in vitro* [9]. However, no other study has evaluated the role of AMPs into the gonad from fish infected with nodavirus, nor any other immune response.

Therefore, with the knowledge that VNNV uses the fish gonad to be transmitted and that it is detected and isolated form European sea bass and gilthead seabream gonads [23], we aimed in the present study to assess the potential role of AMPs in the innate immune response triggered by VNNV in the gonad. Thus, we have evaluated the antimicrobial activities (complement, lysozyme and bactericidal activities) in serum and gonad extracts, as well as the expression profiles of several AMP coding genes (*c3*, *lyz*, *hamp*, *dic*, *pis* or *bdef*) in brain and gonad, upon *in vivo* infection, in two fish species with different susceptibility to VNNV, the European sea bass and the gilthead seabream. The local immune response triggered in the gonad by VNNV without the systemic influence by means of an *in vitro* challenge of the gonad with VNNV and poly I:C have also been determined. Moreover, the capability of VNNV to infect brain and gonad causing different effect on tissue functionality prompted us to dilucidate the possible correlations between the AMPs gene expression levels found in the brain and the gonad.

2. Material and methods

2.1 Animals

Healthy specimens of European sea bass (*Dicentrarchus labrax* L.) and gilthead seabream (*Sparus aurata* L.) were bred and kept at the *Centro Oceanográfico de Murcia* (IEO, Mazarrón, Murcia). The fish were kept in 14 m^3 tanks with the water temperature ranging from 14.6 to 17.8°C, a flow-through circuit, a suitable aeration and filtration system, natural photoperiod and fed daily with 1% of biomass of a commercial pellet diet (Skretting). Before sampling, all specimens were anesthetized with 40 µl/l of

clove oil, bled and immediately decapitated and weighed. All animal studies were carried out in accordance with the European Union regulations for animal experimentation and the Bioethical Committee of the *Instituto Español de Oceanografía* and of the University of Murcia.

2.2. Nodavirus stocks

Nodavirus (VNNV) (strain 411/96, genotype RGNNV) were propagated in the SSN-1 cell line (Frerichs et al., 1996). The SSN-1 cells were grown at 25°C in Leibovitz's L15-medium (Gibco) supplemented with 10 % fetal bovine serum (FBS; Gibco), 2 mM L-glutamine (Gibco), 100 i.u./ml penicillin (Gibco), 100 µg/ml streptomycin (Gibco) and 50 µg/ml gentamicin (Gibco) using Falcon Primaria cell culture flasks (Becton Dickinson). Cells were inoculated with VNNV and incubated at 25ºC until the cytopathic effect was extensive. Supernatants were harvested and centrifuged to eliminate cell debris. Virus stocks were titrated in 96-well plates as previously described [29].

2.3 In vivo infection

Specimens of European sea bass [n = 50; 125±25 g body weight (bw)] and gilthead seabream (n = 50; 305±77 g bw) were translated to the University of Murcia aquaria. Fish were randomly divided into two tanks, kept in 450-500 L running seawater (28 ‰ salinity) aquaria at 22-26ºC and with a 12 h light: 12 h dark photoperiod and acclimatised for 15 days prior to the experiments. Each group received a single intramuscular injection of 100 µl of SSN-1 culture medium (mock-infected) or culture medium containing 10^6 $TCID_{50}$/fish of VNNV since this route of infection has been proven as the most effective [30]. Fish (n = 6 fish/group and sampling time) were sampled 1, 7, or 15 days after viral infection and blood serum, gonad and brain were removed. The blood was obtained from the caudal peduncle and the serum samples were obtained by centrifugation at 10,000 g during 1 min at 4ºC, and immediately frozen and stored at -80ºC until used. Fragments of gonad and brain were immediately frozen in TRIzol Reagent (Invitrogen) and stored at -80ºC for later RNA isolation. Fragments of gonads were also immediately frozen in liquid nitrogen and stored at -80ºC for later analysis of antimicrobial activities.

2.4. In vitro treatments

Specimens of naïve European sea bass males (n = 6) or gilthead seabream males (n = 6) were bled and the gonad removed, weighed and chopped into 1 mm^2 fragments to culture them in flat-bottomed 96-well microtiter plates (Nunc) with sL-15 [Leibovitz's L15-medium supplemented with 2 mM glutamine, 100 u.i./ml penicillin, 100 µg/ml streptomycin, 2 µg/ml fungizone (Invitrogen), 2% FBS and 0.35% of NaCl] culture medium (control) or containing VNNV (10^7 $TCID_{50}$/ml) or poly I:C (62.5 µg/ml; Sigma) during 24 hours at 25ºC. Afterwards, fragments of tissue were washed with 0.01 M PBS and stored in TRIzol Reagent at -80ºC for later isolation of RNA.

2.5. Analysis of gene expression by real-time PCR

Total RNA was isolated from TRIzol Reagent (Invitrogen) frozen samples following the manufacturer's instructions. One µg of total RNA was treated with DNAse I (1 unit/µg RNA, Promega) to remove genomic DNA. The first strand of cDNA was synthesized by reverse transcription using the Superscript III (Invitrogen) with an oligo-dT12-18 primer (Promega) followed by RNAse H (Invitrogen) treatment, at 50ºC for 60 min.

The expression of the genes codifying for the interferon-induced GTP-binding protein Mx (*mx*), complement component 3 (*c3 1-2*), lysozyme (*lyz*), hepcidin (*hamp*), dicentracin (*dic*), piscidin (*pis)* or beta-defensin (*bdef*) was analysed by real-time PCR performed with an ABI PRISM 7500 instrument (Applied Biosystems) using SYBR Green PCR Core Reagents (Applied Biosystems) as previously described [28]. Reaction mixtures were incubated for 10 min at 95ºC, followed by 40 cycles of 15 s at 95ºC, 1 min at 60ºC, and finally 15 s at 95ºC, 1 min 60ºC and 15 s at 95ºC. For each mRNA, gene expression was corrected by the elongation factor 1 alpha (*ef1a*) content in each sample and expressed as $2^{-\Delta Ct}$, where ΔCt is determined by subtracting the *ef1a* Ct value from the target Ct. The specific primers used were designed using the Oligo Perfect software tool (Invitrogen) and are shown in Table 1. Before the experiments, the specificity of each primer pair was studied using positive and negative samples. A melting curve analysis of the amplified products validated the primers for specificity. Negative controls with no template were always included in the reactions.

2.6. Antimicrobial activities

Antimicrobial activities were determined in serum and homogenated gonad samples. Fragments of gonad were weighed and mechanically homogenized in 1 ml of 0.01 M PBS (9 mM sodium phosphate dibasic, 2 mM , sodium phosphate monobasic and 0.15 M NaCl), and centrifuged at 10,000 g during 10 min at 4 ºC to avoid cell debris. The supernatants of homogenated gonads, as well as the serum, were used for natural haemolytic complement, lysozyme and bactericidal activity assays.

2.6.1. Natural haemolytic complement activity

The activity of the alternative complement pathway was assayed using sheep red blood cells (SRBC, Biomedics) as targets [31]. Equal volumes of SRBC suspension (6 %) in phenol red-free Hank's buffer (HBSS) containing Mg^{+2} (Panreac) and EGTA (Sigma) were mixed with serially diluted serum or gonad homogenates (5.2 ± 0.2 or 4.9 ± 0.01 mg of protein/ml of sea bass or gilthead seabream, respectively) to give final serum concentrations ranging from 10 % to 0.078 % or gonad homogenates ranging from 0.5 to 0.004 mg of protein/ml. After incubation for 90 min at 22ºC, the samples were centrifuged at 400 g during 5 min at 4°C to avoid unlysed erythrocytes. The relative haemoglobin content of the supernatants was assessed by measuring their optical density at 550 nm in a plate reader (Nunc). The values of maximum (100 %) and minimum (spontaneous) haemolysis were obtained by adding 100 µl of distilled water or HBSS to 100 µl samples of SRBC, respectively. The degree of haemolysis (Y) was estimated and the lysis curve for each specimen was obtained by plotting Y/(1-Y) against the volume of serum or gonad homogenates added (ml) on a log-log scaled graph. The volume of serum or gonad homogenates producing 50 % haemolysis (ACH_{50}) was determined and the results were represented as ACH_{50} units/ml of serum or ACH_{50} units/g of gonad. Results were expressed as fold change of the infected group compared with the control group.

2.6.2. Lysozyme activity

The lysozyme activity of serum or gonad homogenates was measured according to a turbidimetric method modified from [32]. Briefly, 100 µl of serum or gonad homogenates diluted 1:2 with 0.01 M PBS at pH 6.2, were placed in flat-bottomed 96-well plates in triplicate. To each well, 100 µl of 0.3 mg/ml freeze-dried *Micrococcus lysodeikticus* (Sigma) in phosphate citrate buffer (0.13 M disodium phosphate, 0.11 M citrate and 0.015 M NaCl, pH 6.2) was added as lysozyme substrate. The reduction in

absorbance at 450 nm was measured immediately every 30 s during 15 min at 22ºC in a plate reader (Nunc). One unit of lysozyme activity was defined as a reduction in absorbance of 0.001/min. The units of lysozyme present in serum and gonads homogenates were obtained from a standard curve made with hen egg white lysozyme (HEWL, Sigma) and the results were expressed as units/ml of serum or units/mg of gonad. Results were expressed as fold change of the infected group compared with the control group.

2.6.3. Bactericidal activity

The pathogenic marine bacteria *Vibrio harveyi* (*Vh*) (strain Lg 16/100) was grown in agar plates at 25ºC in tryptic soy agar (TSA, Sigma). Then, fresh single colonies of 1-2 mm were diluted in 5 ml of tryptic soy broth (TSB; *Laboratorios Conda*), cultured for 16 h at 25°C on an orbital incubator at 200-250 revolutions per minute (rpm) and adjusted to 10^8 bacteria/ml TSB. The absorbance of bacteria cell cultures were measured at 600 nm and used to know the concentration based on growth curves.

The antibacterial activity of serum or gonad homogenates was determined by evaluating their effects on the bacterial growth of *Vh* curves using a method modified from [33]. Aliquots of 100 µl of the bacterial dilutions of *Vh* (1/10) were placed in flat-bottomed 96-well plates and cultured with 100 µl of European sea bass or gilthead seabream serum or gonad homogenates dilutions (1/10). The absorbance of the samples was measured at 620 nm every 30 min intervals during 36 h at 25ºC. Samples without bacteria were used as blanks (negative control). Samples without serum or gonad homogenates were used as positive controls (100 % growth or 0 % antibacterial activity). Bactericidal activity was expressed as % of bacterial growth inhibition per ml of serum or mg of gonad. Results were expressed as fold change of the infected group compared with the control group.

2.7. Statistical analysis

The data were analysed by a t-Student to determine differences between control and infected groups at each time point and specie (*P≤0.1; **P ≤ 0.05; ***P ≤ 0.01). In addition, non-parametric Pearson correlation tests were applied to test correlations among antibacterial activities and gene expression levels in the gonad or among the

gene expression levels in gonad or brain after *in vivo* infections with VNNV using Statgraphics 15.0 (StatPoint, Inc).

3. Results

3.1. VNNV infection induces the antimicrobial response in serum and gonad

The natural haemolytic complement, lysozyme and bactericidal activity in serum and gonad homogenates of European sea bass and gilthead seabream upon an *in vivo* infection with VNNV were analysed (Fig. 1). Haemolytic activity in the gonad of any specie was non-detectable for any group and at any assayed time. In serum, the haemolytic activity was inhibited at the beginning of the infection in both species (7 or 1 day post-infection in European sea bass or gilthead seabream, respectively), while only in the European sea bass the serum haemolytic activity increased and reached 2.6-fold after 15 days of infection (Fig. 1a, b). In contrast, lysozyme activity was unchanged in serum, but greatly increased in the gonad homogenates of both species upon VNNV infection (Fig. 1c, d). Thus, this activity increased 2.5- and 1.4-fold after 1 and 15 days of infection, respectively, in European sea bass gonad (Fig. 1c), and 3.1-fold increase after 7 days of infection in gilthead seabream gonad (Fig. 1d). Regarding the bactericidal activity, some differences were observed between species and tissues analysed (Fig. 1e, f). Thus, in the European sea bass, the bactericidal activity increased in serum, while decreased in the gonad after 7 and 15 days of infection (Fig. 1e). However, in the gilthead seabream, the bactericidal activity of serum decreased after 1 day and increased after 15 days of infection coinciding with an increase of the bactericidal activity in the gonad (Fig. 1f).

3.2. VNNV in vivo infection up-regulates the AMPs gene expression in sea bass gonad

Firstly, we evaluated the expression of *mx* gene upon *in vivo* challenge with VNNV as indicator of the antiviral response (Figs. 2a, 2b, 3a, 3b). The results showed a lower *mx* gene induction in the brain of European sea bass (Fig. 2b) than in the gilthead seabream (Fig. 3b), as previously documented [26, 28, 34]. Surprisingly, the

transcription of *mx* gene in the gonad was up-regulated at all time points in European sea bass (Fig. 2a), while kept unaltered in gilthead seabream (Fig. 3a).

Afterwards, we have studied the expression profiles in the brain and in the gonad of some known AMP genes upon *in vivo* challenge with VNNV and found a different pattern of expression between the both analyzed species (Figs. 2, 3). Thus, in the European sea bass (Fig. 2), the transcription of all AMPs analysed increased in at least one tissue, gonad or brain, while in the gilthead seabream (Fig. 3), those genes were down-regulated or kept steady. Thus, in the European sea bass gonad (Fig. 2c, e, g, i), VNNV infection increased the level of expression of *c3*, *hamp* and *dic* at days 1 and 7, 7 and 15 and 15 of infection, respectively, and decreased the levels of expression of *lyz* at day 7 of infection. However, in the brain (Fig. 2d, f, h, j), the VNNV infection decreased the expression level of *c3* gene throughout the trial and increased the expression levels of *lyz* and *dic* at days 1 and 15 of infection, and of *hamp* at all assayed times. In the gilthead seabream (Fig. 3), however, the VNNV infection decreased the expression levels of *c3* and *hamp* in the gonad (Fig. 3c, g) at days 7 and 15 and 7, respectively, and the expression levels of *c3* in the brain (Fig. 3d) at day 15, while no differences were observed in the expression levels of *lyz*, *bdef* and *pis* in any of the tissues and sampled times analysed (Fig. 3e, f, i, j, k, l).

3.3. Gonad AMPs gene expression and some antimicrobial activities are negatively correlated

There are previous available evidences about the interrelation of complement, lysozyme and bactericidal activities [35, 36], for this reason the correlation between these measured activities and the gene expression level of AMPs in the gonad upon *in vivo* infection with VNNV (Tables S1, S2) have also been studied. We found that, in both species, the expression of AMP genes negatively correlated with some antimicrobial activities. Thus, the gene expression of *lyz* negatively correlated with lysozyme activity in the gonad of European sea bass (Table S1), while in gilthead seabream gonad only the expression levels of *c3* gene negatively correlated with bactericidal activity (Table S2).

3.4. AMPs gene expression negatively correlated between gonad and brain upon VNNV infection.

When the correlation between the different gene expression in gonad and brain was studied, some differences between species were observed (Tables 2, 3). In the European sea bass brain, the expression of *mx* gene, up-regulated upon viral-infection, negatively correlated with *c3* or *hamp* gene expression (Table 2), while no correlations were observed in the gilthead seabream brain (Table 3). In the other hand, in the gilthead seabream gonad, positive correlations were observed between *mx* and either *c3*, *lyz*, *hamp* or *pis* gene expressions (Table 3), while no correlations were observed in the European sea bass gonad (Table 2). Moreover, in European sea bass gonad, the expression pattern of *c3* positively correlated with *hamp* gene expression (Table 2), while in the gilthead seabream gonad, strong correlations were observed between *c3* and either *lyz*, *hamp* or *pis* gene expression and between *hamp* and either *lyz* or *pis* gene expression (Table 3). Regarding the brain, the transcription levels of *hamp* and *lyz* as well as *hamp* and *pis* positively correlated in European sea bass and gilthead seabream, respectively (Tables 2, 3). Interestingly, in the European sea bass positive correlation was observed between the *mx* gene expression in brain and the *hamp* gene expression in gonad (Table 2). However, negative correlations were found between some AMPs gene expression in brain and gonad in both species. Thus, in European sea bass (Table 2), the transcription levels of *lyz* gene in brain was negatively correlated either with *c3* expression levels in gonad, while in the gilthead seabream (Table 3), the transcription levels of *bdef* in the brain were negatively correlated either with *c3* or *lyz* gene expression in the gonad.

3.5. In vitro exposure to VNNV differently alters the expression of AMP genes

Firstly, we found that both European sea bass and gilthead seabream gonad failed to mount an antiviral immune response after incubation with poly I:C or VNNV during 24 h (Fig. 4a, b), suggesting that these conditions might be suboptimal for this response. Regarding the AMPs, in the European sea bass (Fig. 4a), the expression of *lyz* gene was down-regulated upon VNNV infection, while *hamp* and *dic* gene expression was up-regulated upon VNNV and VNNV or poly I:C challenges, respectively. In the gilthead seabream (Fig. 4b), however, the *c3* gene expression was up-regulated upon

poly I:C challenge, while *pis* and *bdef* gene expression were down-regulated upon VNNV. Moreover, poly I:C completely blocked the transcription of *bdef* gene (Fig. 4b). Noteworthy, there was not transcription of *c3* gene in control gilthead seabream gonad unless stimulated with poly I:C (Fig. 4b).

4. Discussion

The role of AMPs as a part of innate immune response, as well as its regulation, has been studied in many vertebrates [37]. Interestingly, immune privilege is a term applied to eye, brain and reproductive organs where immune responses either do not act, or act in a different manner from other parts of the animal body. In this framework, the AMPs expressed in the reproductive system of vertebrates probably assume an important role in the innate immune response against pathogens [9, 38-40]. In fish, AMPs have been mainly studied in the immune organs [14], however, it is worth to study their role in the reproductive organs since the regulation of the immune response in those organs is different and it is also known that they allow to several virus colonize the gonad, persist and be transmitted [5, 6]. Between those pathogens, viruses and in particular VNNV can spread both horizontally and vertically from mother to offspring, producing persistent infections and giving raise to asymptomatic carriers in European sea bass and gilthead seabream specimens [25, 41, 42]. In fact, in addition to the nodavirus detection by PCR and ELISA techniques in asymptomatic brood fish and their embryos [16, 18, 20, 30], we have already detected and isolated infective VNNV particles from the gonads of infected European sea bass and gilthead seabream specimens [23]. Interestingly, in the brain, one of the target tissues of VNNV, the antiviral activity, determined as *mx* gene expression, was higher in gilthead seabream than in European sea bass, a fact that has been related with the resistance and susceptibility of these fish species to VNNV disease. Conversely, the European sea bass gonad from VNNV-infected fish showed an important up-regulation of the *mx* gene, which failed to do so in the gilthead seabream, indicating a strong interferon and antiviral response in this specie, which has never been observed. Therefore, we are trying to characterize the gonadal immune response under VNNV infection, focussing in this study on the AMP response.

C3, a major component of the complement system, is considered as an AMP because of their direct implication in the defence against pathogens [43, 44]. Thus, when the haemolytic activity of the complement in European sea bass and gilthead seabream serum were studied, small alterations were detected from its basal levels upon infection in contrast to the low increase in other trial [45]. However, non-detectable activity was observed in the gonads of any specie neither in control nor in infected specimens. Interestingly, a detectable transcription level of *c3* gene was observed in the gonad of control specimens of both species, which was greatly up-regulated in the European sea bass whilst down-regulated in gilthead seabream. Interestingly, upon *in vitro* challenge of the gonad, no variation on *c3* gene expression was observed in the European sea bass neither by poly I:C or VNNV. Curiously, in the gonad of gilthead seabream challenged *in vitro*, no detectable transcription of *c3* gene was observed, except after poly I:C stimulation, as also reported in a previous *in vivo* study [46]. However, in the *in vivo* experiment, we reported basal and regulated *c3* gene expression in the gonad. These differences could be due to different acute stress conditions, which could affect a gene expression during weeks [47]. Overall, these data suggest that C3 convertase is produced and this production is regulated upon infection in the testis of both species of teleost, however its activity could be inactivated by specific inhibitors produced locally, as occurs in humans [48]. All these data together suggest that *c3* gene expression might be regulated and influenced by multiple stimuli that could affect the local immune response of both gonad and brain.

Regarding lysozyme activity, previous data showed that this activity was decreased in European sea bass and gilthead seabream specimens exposed to VNNV [45]. We found that in the gonad, but not in serum, this activity was changed upon infection in both species. However, the lysozyme activity recorded in the gonad of European sea bass increased earlier (1 day upon VNNV infection) and lasted longer (15 days upon infection) than in gilthead seabream gonad (7 days upon VNNV infection). Interestingly, in the gonad, we found that lysozyme activity negatively correlated with the expression levels of *lyz* gene of European sea bass, suggesting that there are regulatory mechanisms of the protein activity involved in the up-regulation of lysozyme activity upon 1 day of infection and that this increased activity triggers a down-regulation of *lyz* gene transcription later on. In mammals, *lyz* gene is selectively expressed in the testis and its expression levels differ during its different developmental

stages [49-52] as also occurs during the reproductive cycle of European sea bass [23]. Furthermore, in both species, the *lyz* expression data obtained from the *in vivo* infection are in concordance with those observed in the gonad after 24 hours of *in vitro* challenge, suggesting that the *lyz* expression changes are triggered by a local immune response.

In this study, the bactericidal activity against *Vh* upon VNNV infection was clearly different in European sea bass and gilthead seabream. Thus, in European sea bass, this activity decreased in gonad and increased in serum from 7 days onwards; whether in gilthead seabream, after a slight down-regulation in serum at day 1, this activity was increased at day 15 post-infection in both gonad and serum. Evaluation of the direct lytic activity against pathogens is the most practical determination awaited for farmers whilst researchers also try to identify and characterize the molecules involved in this activity. Thus, determination of the bactericidal activity of the gonad might be more important than single AMP activities. In that sense, we have analysed the expression of several AMP coding genes known in both species, hepcidin and dicentracin in the European sea bass and hepcidin, piscidin and beta-defensin in the gilthead seabream; all of them cationic antimicrobial peptides [14]. Some of these peptides are suggested to be involved in the defence against virus, as happens with C3 and piscidin [53, 54]. Furthermore, some of them have demonstrated antiviral function, as occurred with lysozyme, hepcidin and beta-defensin [9, 14, 55]. Our data showed that in the European sea bass gonad, where the bactericidal activity is inhibited upon infection, the expression of most of the genes is up-regulated (*c3*, *hamp*, *dic*) with the exception of *lyz* mRNA. However, in the gilthead seabream gonad, where the activity is stimulated upon infection, the expression of most of the genes is down-regulated (*c3* and *hamp*) or unchanged (*lyz*, *bdef* and *pis*). Interestingly, the bactericidal activity was inversely correlated with *c3* gene expression in gilthead seabream gonad, while no correlations were found between the bactericidal activity and the expression of any of the AMP genes analysed in the European sea bass gonad. These data point to the complexity in the regulation of the processes analysed and to the need of further and deeper studies at molecular and functional levels.

When compared those AMPs gene expression in the gonad upon an *in vivo* infection with an *in vitro* challenge of the gonad with VNNV or poly I:C, we found that *lyz*, *hamp* and *dic* gene expression (but not *c3* gene expression) was similarly modified in both experimental situations in the European sea bass gonad, suggesting this that the

expression profile changes observed are triggered by immune local responses. However, our *in vitro* and *in vivo* data are difficult to compare since the *in vitro* stimulation of the gonad failed to change the antiviral *mx* gene expression in sharp contrast to what occurred in the *in vivo* infection. This fact would suggest that the antiviral and antimicrobial responses, at least those studied in this work, have different regulations and mediators. Furthermore, we also found that even when brain and gonad are immune-privileged organs, those tissues behave differently upon infection with VNNV at the gene expression level. Thus, in the European sea bass, the changes observed in the expression of *c3* and *lyz* genes were different in gonad and brain, while the *hamp* and *dic* expression levels were similarly modified upon infection in both tissues. Regarding gilthead seabream, all the genes analysed were similarly modified upon infection in both tissues, except *hamp* gene, which transcription was down-regulated in the gonad and unchanged in the brain. Interestingly, transcription of *mx* and some AMP genes were positively correlated in the gilthead seabream brain, adding more data to the idea that the high immune response in this tissue is the responsible for the viral clearance. Taking into account the brain-pituitary-gonadal axis, where both brain and gonad are closely linked by positive or negative feedback mechanisms [56] and that VNNV is capable to infect both, brain and gonad, and alter some reproductive functions as the steroid serum levels [23], we have analysed the relation between gonad and brain responses at the gene expression levels. Our analysis showed that in the European sea bass, *lyz* gene expression in brain negatively correlated with *c3* and *hamp* gene expressions in the gonad. Similarly, in the gilthead seabream, the *bdef* gene expression in brain negatively correlated with *c3* and *lyz* gene expressions in gonad. This data showed that the AMPs response, at the gene expression level, is inversely regulated in both tissues. This could partially explain the ability of VNNV to be transmitted through the gonad without severely affecting the reproductive functions of the specimens.

To conclude, the results obtained in this study demonstrate that the immune response based on AMPs in the European sea bass or gilthead seabream gonads are clearly different upon VNNV infection, at both expression and activity levels. These differences could be due to different susceptibility of the species to the infection and could determine the transmission rates of VNNV in each species. Moreover, the differences observed between the *in vivo* and *in vitro* experiment suggest that some AMPs are locally regulated by a local immune response in the gonad while others might

be more dependant of the systemic immune responses. In addition, our results determine clear differences in the immune responses triggered by VNNV in brain and gonad, explaining this, the differences observed in the affection of the functionality of both tissues upon VNNV infection.

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Figure legends

Figure 1: European sea bass and gilthead seabream antimicrobial activities in serum (black bars) and gonad homogenates (grey bars) upon *in vivo* VNNV infection. Haemolytic complement activity (a, b), lysozyme activity (c, d) and bactericidal activity (e, f). Data represent the mean ± standard error of the activity of VNNV-infected group respect to the control group (n = 6/group and time). Significance level (P) was fixed at 0.1 (P≤0.1*; P≤0.05**; P≤0.01***). ND, not detected.

Figure 2: Expression of *mx* (a, b) and antimicrobial peptide genes *c3* (c, d), *lyz* (e, f), *hamp* (g, h) and *dic* (i, j) in the gonad (a, c, e, g, i) or brain (b, d, f, h, j) from control (white bars) or VNNV-infected (grey bars) European sea bass pecimens. Data represent the mean ± standard error (n = 6/group and time). Significance level (P) was fixed at 0.1 (P≤0.1*; P≤0.05**; P≤0.01***).

Figure 3: Expression of *mx* (a, b) and antimicrobial peptide genes *c3* (c, d), *lyz* (e, f), *hamp* (g, h), *bdef* (i, j) and *pis* (k, l) in the gonad (a, c, e, g, i, k) or brain (b, d, f, h, j, l) from control (grey white bars) or VNNV-infected (grey bars) gilthead seabream specimens. Data represent the mean ± standard error (n = 6/group and time). Significance level (P) was fixed at 0.1 (P≤0.1*; P≤0.05**; P≤0.01***).

Figure 4: Expression of *mx* and antimicrobial peptide genes in the gonad of European sea bass (a) or gilthead seabream (b) upon *in vitro* treatment with medium (control group, white bars), VNNV (grey bars) or poly I:C (black bars) during 24 hours. Data represent the mean ± standard error (n = 6/group). Significance level (P) was fixed at 0.1 (P≤0.1*; P≤0.05**; P≤0.01***). ND, not detected.

Figure 1

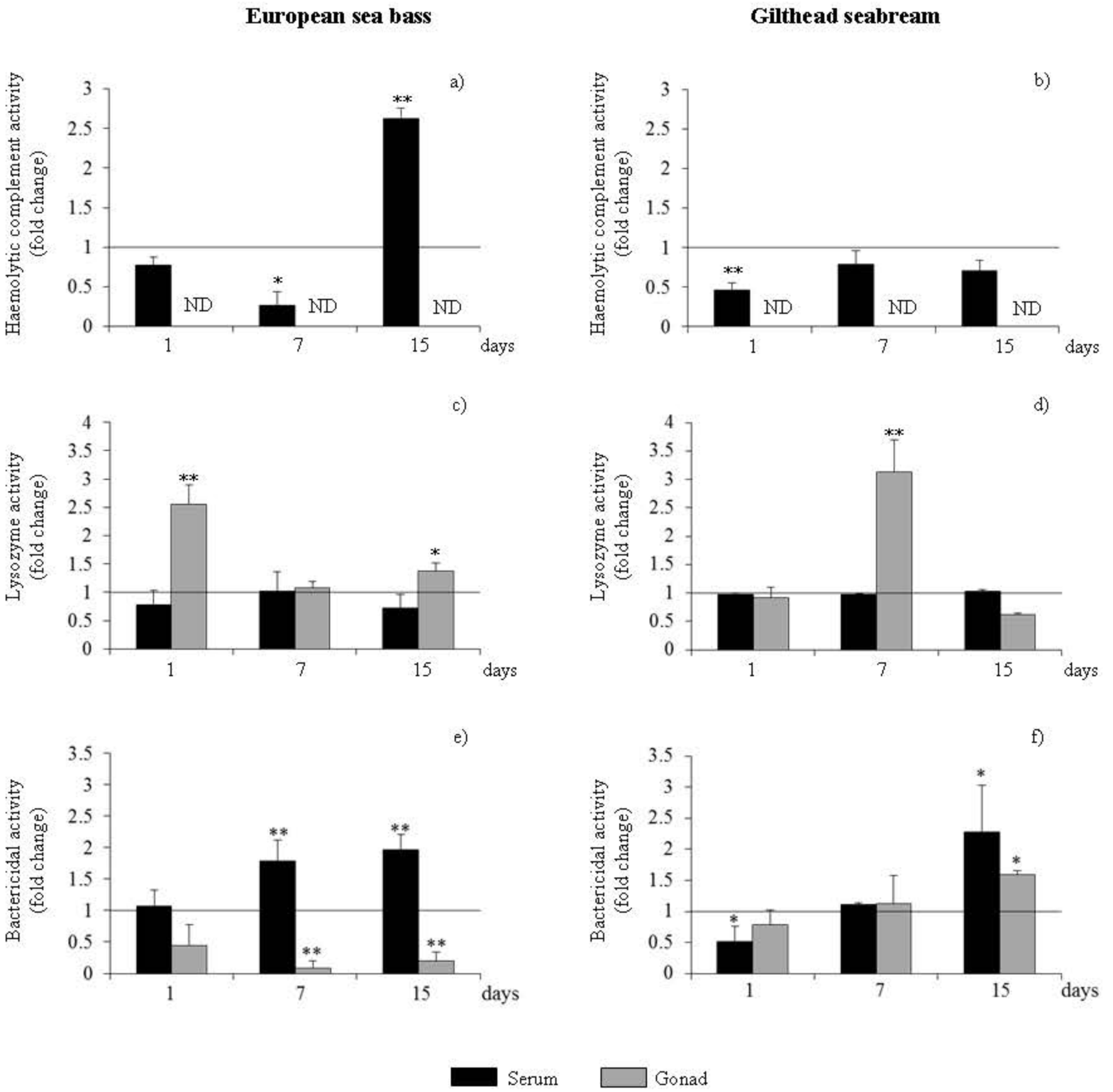
European sea bass
Gilthead seabream
a)
b)
c)
d)
e)
f)
Haemolytic complement activity (fold change)
Lysozyme activity (fold change)
Bactericidal activity (fold change)
ND
days
1
7
15
Serum
Gonad

Figure 2

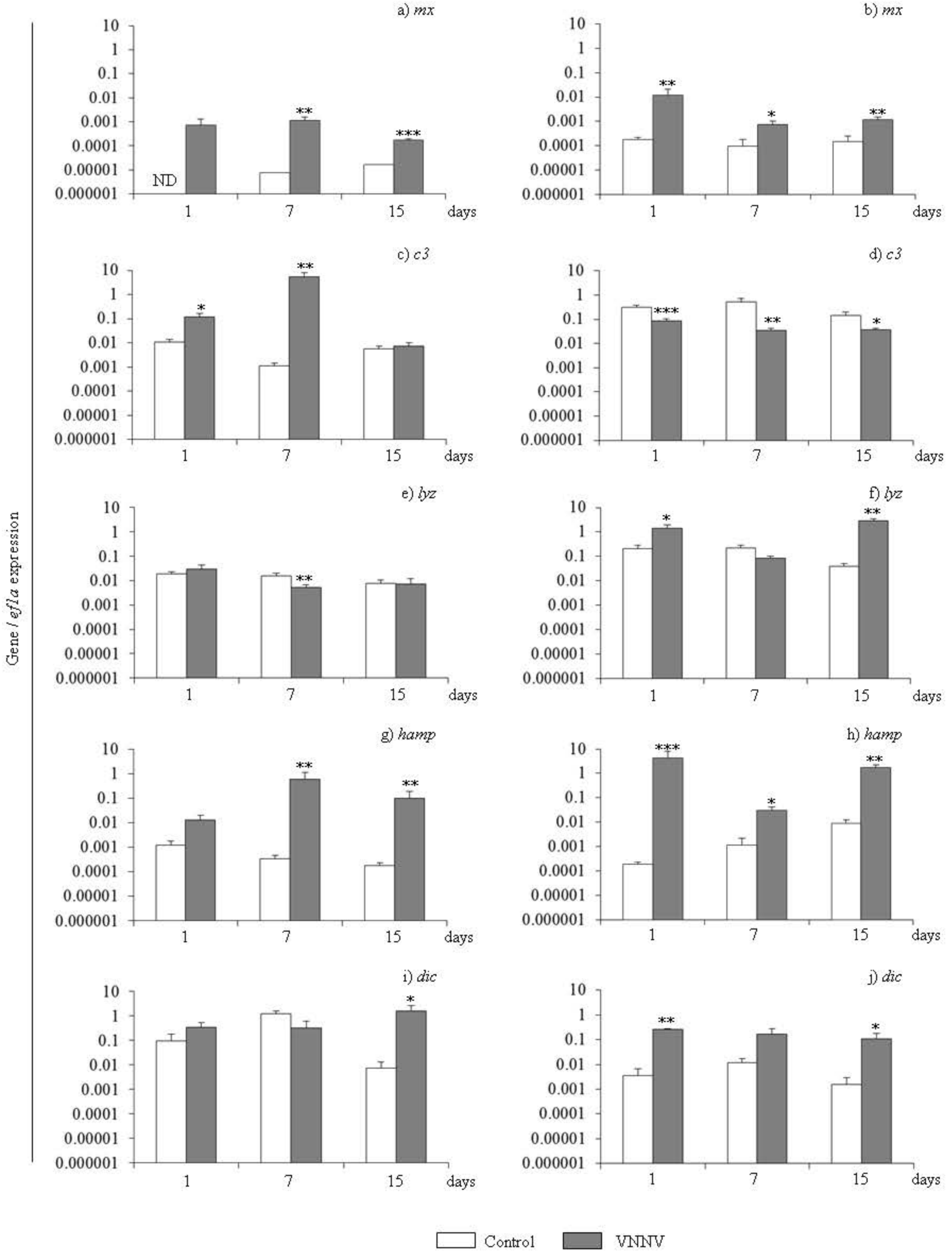
European sea bass
Gonad
Brain
a) mx
b) mx
c) c3
d) c3
e) lyz
f) lyz
g) hamp
h) hamp
i) dic
j) dic
Gene / efla expression
10
1
0.1
0.01
0.001
0.0001
0.00001
0.000001
ND
1
7
15
days
Control
VNNV

Figure 3

Gilthead seabream

Gonad | **Brain**

a) *mx* | b) *mx*

c) *c3* | d) *c3*

e) *lyz* | f) *lyz*

g) *hamp* | h) *hamp*

i) *bdef* | j) *bdef*

k) *pis* | l) *pis*

Figure 4

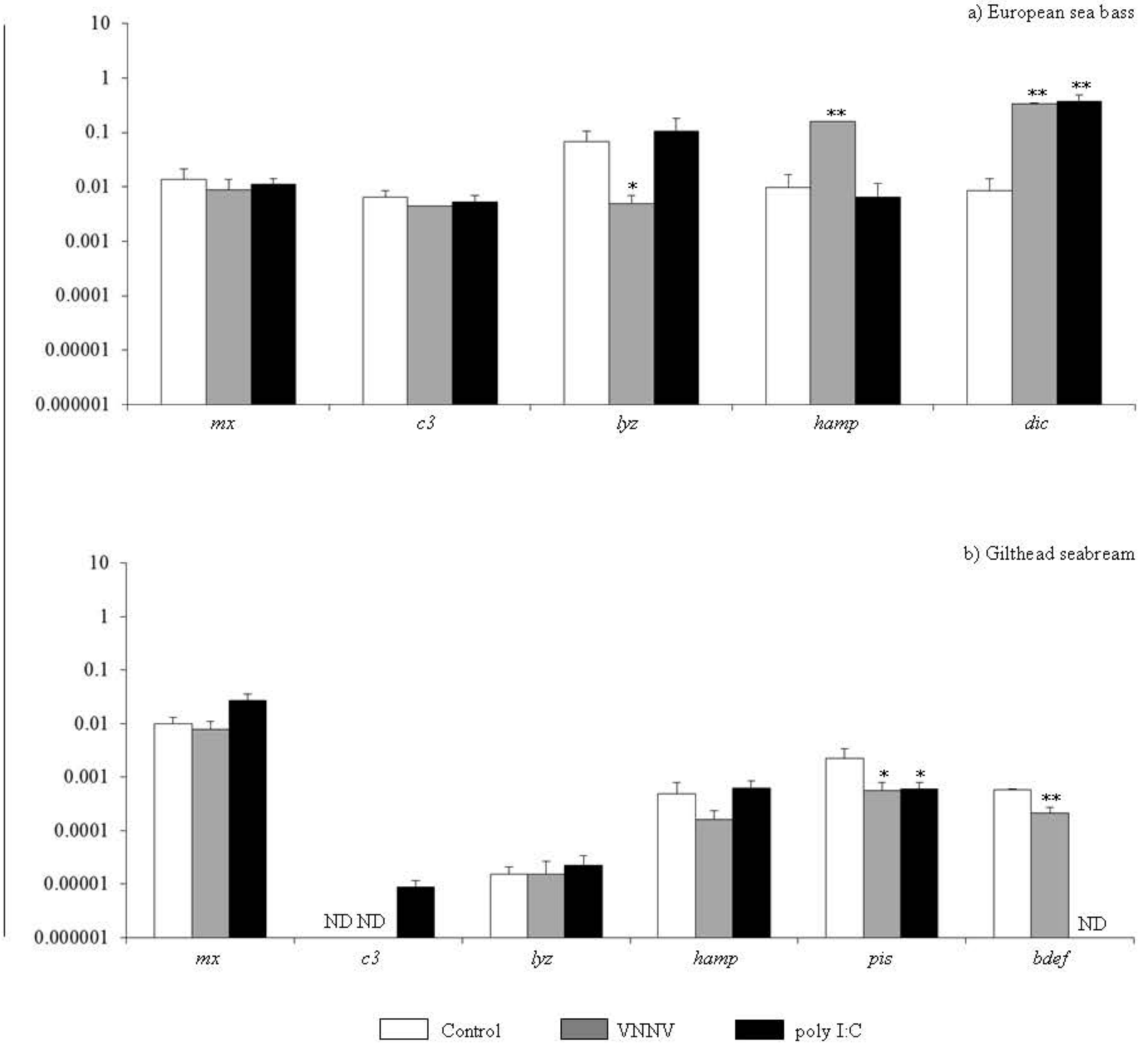
a) European sea bass
10
1
0.1
0.01
0.001
0.0001
0.00001
0.000001
*
**
**
**
mx
c3
lyz
hamp
dic
b) Gilthead seabream
ND ND
*
*
**
ND
mx
c3
lyz
hamp
pis
bdef
Gene / ef1a expression
Control
VNNV
poly I:C

Table 1: Gene accession numbers and primer sequences used for gene expression analysis.

Specie	Name	Abbreviation	Accession number	Sequence (5'-3')
European sea bass	Interferon-induced GTP-binding protein Mx	*mx*	*AM228977, HQ237501, AY424961*	GAAGAAGGGCTACATGATCGTC
				CCGTCATTGTAGAGAGTGTGGA
	complement component 3-1 and 3-2	*c3 1-2*	*HM563078, HM563079*	ACCAAAGAACTGGCAACCAC
				CTAGCAGTCGGTCAGGGAAC
	lysozyme	*lyz*	*FN667957*	ATTTCCTGGCTGGAACACAG
				GAGCTCTGGCAACAACATCA
	hepcidin	*hamp*	*DQ131605*	CCAGTCACTGAGGTGCAAGA
				GCTGTGACGCTTGTGTCTGT
	dicentracin	*dic*	*AY303940*	GGCAAGTCCATCCACAAACT
				ATATTGCTCCGCTTGCTGAT
	elongation factor 1 alpha	*ef1a*	*FM019753*	CGTTGGCTTCAACATCAAGA
				GAAGTTGTCTGCTCCCTTGG
Gilthead seabream	Interferon-induced GTP-binding protein Mx	*mx*	*FJ490556, FJ490555, FJ652200*	AAGAGGAGGACGAGGAGGAG
				CATCCCAGATCCTGGTCAGT
	complement component 3	*c3*	*CX734936*	ATAGACAAAGCGGTGGCCTA
				GTGGGACCTCTCTGTGGAAA
	lysozyme	*lyz*	*AM749959*	CCAGGGCTGGAAATCAACTA
				CCAACATCAACACCTGCAAC
	hepcidin	*hamp*	*CB184616*	GCCATCGTGCTCACCTTTAT
				CTGTTGCCATACCCCATCTT
	beta-defensin	*bdef*	*FM158209*	CCCCAGTCTGAGTGGAGTGT
				AATGAGACACGCAGCACAAG
	piscidin	*pis*	*FM158699*	CCTTGTGTTGTCCATGGTTG
				ACTGCTCCAGCTGCAAGTCT

	elongation factor 1 alpha	*ef1a*	*AF184170*	CTGTCAAGGAAATCCGTCGT
				TGACCTGAGCGTTGAAGTTG

Table 2: Correlation observed between *mx* and AMPs gene expression in brain and gonad of European sea bass after *in vivo* infection with VNNV. The first number corresponds to Pearson coefficient of correlation and the second to the significant difference P≤0.05. Written in bolds are the parameters correlated.

European sea bass		Gonad					Brain			
		mx	*c3*	*lyz*	*hamp*	*dic*	*mx*	*c3*	*lyz*	*hamp*
Gonad	*c3*	0.62 0.08								
	lyz	-0.30 0.39	0.54 0.14							
	hamp	0.40 0.28	**0.76** **0.02**	0.34 0.37						
	dic	-0.36 0.38	0.11 0.82	0.47 0.29	0.17 0.72					
Brain	*mx*	0.56 0.11	0.39 0.16	-0.19 0.49	**0.65** **0.02**	0.17 0.56				
	c3	-0.32 0.43	-0.09 0.78	0.47 0.34	-0.07 0.90	0.44 0.56	**-0.62** **0.03**			
	lyz	0.05 0.89	**-0.72** **0.03**	-0.61 0.08	-0.32 0.40	0.36 0.43	0.01 0.98	-0.16 0.76		
	hamp	0.42 0.24	-0.59 0.10	-0.16 0.68	-0.22 0.57	0.35 0.44	**-0.74** **0.01**	0.29 0.58	**0.81** **0.01**	
	dic	0.30 0.55	0.59 0.21	0.55 0.26	0.09 0.86	0.28 0.65	-0.20 0.55	0.21 0.79	-0.54 0.27	-0.38 0.45

Table 3: Correlation observed between *mx* and AMPs gene expression in brain and gonad of gilthead seabream after *in vivo* infection with VNNV. The first number corresponds to Pearson coefficient of correlation and the second to the significant difference P≤0.05. Written in bolds are the parameters correlated.

Gilthead seabream		Gonad						Brain				
		mx	*c3*	*lyz*	*hamp*	*bdef*	*pis*	*mx*	*c3*	*lyz*	*hamp*	*bdef*
Gonad	*c3*	**0.68** **0.00**										
	lyz	**0.79** **0.00**	**0.74** **0.00**									
	hamp	**0.79** **0.00**	**0.71** **0.00**	**0.77** **0.00**								
	bdef	0.30 0.16	0.01 0.98	-0.34 0.31	0.28 0.37							
	pis	**0.76** **0.00**	**0.67** **0.01**	0.48 0.09	**0.57** **0.03**	0.26 0.41						
Brain	*mx*	0.06 0.76	-0.22 0.29	-0.01 0.97	-0.03 0.89	-0.04 0.85	-0.13 0.51					
	c3	0.57 0.16	0.43 0.34	0.09 0.85	0.25 0.54	-0.03 0.95	-0.43 0.29	-0.18 0.66				
	lyz	-0.34 0.09	0.05 0.86	-0.06 0.85	-0.42 0.12	-0.54 0.07	0.12 0.67	-0.06 0.75	-0.61 0.14			
	hamp	-0.21 0.28	0.07 0.82	-0.07 0.83	-0.10 0.74	-0.05 0.87	0.32 0.26	-0.19 0.34	-0.61 0.14	0.50 0.06		
	bdef	-0.15 0.46	**-0.60** **0.02**	**-0.65** **0.02**	-0.35 0.19	0.41 0.18	-0.50 0.07	-0.11 0.56	-0.04 0.93	-0.24 0.40	-0.34 0.22	
	pis	0.02 0.93	0.32 0.26	0.03 0.92	0.20 0.47	0.05 0.88	0.44 0.12	0.15 0.45	-0.54 0.19	0.12 0.67	**0.77** **0.00**	-0.28 0.31

Table S1: Correlation observed between AMPs gene expression and antimicrobial activities in gonad of European sea bass after *in vivo* infection with VNNV. The first number corresponds to Pearson coefficient of correlation and the second to the significant difference P≤0.05. Written in bolds are the parameters correlated.

European sea bass		Gene expression				Antimicrobial activities
		c3	*lyz*	*hamp*	*dic*	lysozyme
Antimicrobial activities	lysozyme	-0.68	**-0.72**	-0.29	0.17	
		0.07	**0.04**	0.48	0.75	
	bactericidal	-0.31	0.34	-0.21	-0.06	0.19
		0.46	0.41	0.62	0.91	0.65

Table S2: Correlation observed between AMPs gene expression and antimicrobial activities in gonad of gilthead seabream after *in vivo* infection with VNNV. The first number corresponds to Pearson coefficient of correlation and the second to the significant difference P≤0.05. Written in bolds are the parameters correlated.

Gilthead seabream		Gene expression					Antimicrobial activities
		c3	*lyz*	*hamp*	*bdef*	*pis*	lysozyme
Antimicrobial activities	lysozyme	-0.50	-0.08	-0.17	-0.25	-0.52	
		0.08	0.80	0.55	0.47	0.07	
	bactericidal	**-0.80**	-0.48	-0.31	-0.23	-0.54	0.33
		0.00	0.11	0.28	0.50	0.06	0.25

Highlights

1. Great differences between brain and gonad immune responses are triggered by VNNV.
2. VNNV triggers a different AMPs response in European sea bass or gilthead seabream gonads.
3. Some AMPs are locally regulated in the gonad.