17α-Ethynylestradiol alters the peritoneal immune response of gilthead seabream

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Abstract

17α-Ethynylestradiol (EE_2), a synthetic estrogen used in most oral contraceptives pills and hormone replacement therapies, is found in many water bodies, where it can modulate the fish immune response. EE_2 acts as an endocrine disruptor in gilthead seabream, *Sparus aurata* L., a marine teleost fish of great economic value in Mediterranean aquaculture, as it induces hepatic vitellogenin gene (*vtg*) expression. Moreover, EE_2 also alters the capacity of gilthead seabream to appropriately respond to infection although it does not behave as an immunosuppressor. Nevertheless, these previous studies have mainly focused on the head kidney leukocytes and no information exists on peritoneal leukocytes, including mast cells. In the present work, juvenile gilthead seabream fish were fed a pellet diet supplemented with EE_2 for 76 days and intraperitoneally injected with hemocyanin plus imject alum adjuvant at the end of EE_2 treatment and 92 days later, and the peritoneal immune response was analyzed. EE_2 supplementation induced *vtg e*xpression but returned to basal levels by 3 months post-treatment. Interestingly, gilthead seabream peritoneal leukocytes express the genes encoding for the nuclear estrogen receptor α and the G protein-coupled estrogen receptor 1 and the dietary intake of EE_2 induced these expression. Moreover, EE_2 induced an inflammatory response in the peritoneal cavity in unvaccinated fish, which was largely maintained for several months after the cessation of the treatment. However, the impact of EE_2 in vaccinated fish was rather minor and transient. Taken together, the study provides fresh information about endocrine immune disruption, focusing on peritoneal leukocytes.

1. Introduction

It is well known that estrogens are key modulators of the immune system (Straub, 2007), including that of fish, due to the presence of nuclear estrogen receptors (ERs) in piscine leukocytes (Cabas et al., 2013a; Liu et al., 2009; Lynn et al., 2008; Shved et al., 2009; Slater et al., 1995; Todo et al., 1999). Moreover, the identification of a G protein-coupled estrogen receptor 1 (GPER1), a membrane estrogen receptor that binds 17β-estradiol (E_2) (Revankar et al., 2005; Thomas et al., 2005) and other estrogens (Prossnitz and Hathaway, 2015), and its identification in immune cells of both mammals and fish (Blasko et al., 2009; Brunsing et al., 2013; Cabas et al., 2013a), has opened up the possibility of exploring additional estrogen-mediated effects.

Anthropic activity produces the release of a wide variety of chemical compounds into the environment, most of which affect in some way the species living there. Among these compounds, endocrine disruptor chemicals (EDCs) are a group of substances able to mimic and/or antagonize the effects of endogenous endocrine hormones, and disrupt the synthesis and metabolism of endogenous hormones and the synthesis of hormone receptors (Aris et al., 2014). EDCs alter fish reproduction, but also growth, osmoregulation and immunity and induce stress (Filby et al., 2007).

17α-Ethinylestradiol (EE_2), considered a xeno-estrogen EDC, is a synthetic estrogen and the main active component of contraceptive pills, which are also used in hormonal therapy. EE_2 can be found not only in sewage treatment works (Baronti et al., 2000; Desbrow et al., 1998; Quinn et al., 2004; Vethaak et al., 2005). Due to its capacities to be bio-accumulated and to alter endocrine homeostasis, much research effort has focused on its effects on animals living in aquatic environments (Aris et al., 2014). It has been found that EE_2 can modify the expression of ERs and the expression of the genes classically regulated by these receptors (Milla et al., 2011), such as the vitellogenin (vtg) gene (Kausch et al., 2008), which is widely accepted to be a biomarker of endocrine disruption by xeno-estrogens (Sumpter and Jobling, 1995).

The gilthead seabream (*Sparus aurata* L.), a marine protandrous teleost fish of great commercial value in the Mediterranean Sea and in aquaculture, has been widely used to study the effect of EE_2 on the reproductive (Cabas et al., 2011) and immune systems at different times of its reproductive cycle (Cabas et al., 2013b; Cabas et al., 2012; Rodenas et al., 2015; Rodenas et al., 2016). Thus, EE_2 provokes an estrogenic response, inducing

hepatic *vtg* gene expression (Cabas et al., 2013b; Cabas et al., 2012; Rodenas et al., 2015; Rodenas et al., 2016), and alters the expression of the gene encoding for the ERα of head kidney leukocytes (Cabas et al., 2012). EE_2 might alter the capacity of fish to appropriately respond to infection although it does not behave as an immunosuppressor, as assayed *in vivo* and *in vitro* using head kidney leukocytes (Cabas et al., 2012; Rodenas et al., 2015). Interestingly, the estrogenic response of EE_2 disappears when exposure to it ceases, although the adaptive immune response remains altered to some extent (Rodenas et al., 2015; Rodenas et al., 2016). To increase our knowledge of the effect of EE_2 on the immune response of gilthead seabream, we focus our study on its effects on peritoneal cells. The peritoneal exudate of healthy gilthead seabream, as occurs in sea bass, consists of granulocytes, macrophages, lymphocytes and mast cells (Gómez-González et al., 2014; Meseguer et al., 1993) and its cellular composition may vary depending on the extent of tissue damage or injury. Although some studies have revealed the effect of estrogens, both natural and synthetic, on peritoneal leukocytes, there is a noticeable lack of information about the effects of xenoestrogens on piscine peritoneal leukocytes, and, to the best of our knowledge, no information exists concerning how they may be modulated by EE_2.

The aim of this work was, therefore, to evaluate the impact of dietary administration of EE_2 in leukocyte activation (respiratory burst and cytokine gene expression), and mast cell and acidophilic granulocyte abundance in the peritoneal exudate of fish upon intraperitoneal (i.p.) vaccination

2. Materials and Methods

2.1. Animals and experimental design

Healthy specimens of gilthead seabream *Sparus aurata* L. (Actinopterygii, Perciformes, Sparidae) were reared at the Centro Oceanográfico de Murcia (Instituto Español de Oceanografía, Mazarrón, Murcia, Spain), where they were kept in running seawater aquaria (dissolved oxygen 6 ppm, flow rate 20% aquarium vol/h) with a natural temperature and photoperiod, and fed three times per day with a commercial pellet diet (44% protein, 22% lipids; Skretting) at a feeding rate of 1.5% of fish biomass. Environmental parameters, mortality and food intake, as well as behaviour, were recorded daily. The experiments described were approved by the Consejería de Agua, Agricultura y Medio Ambiente of the Región de Murcia (approval number A13160507).

I*n vivo* EE_2 treatment was carried out with juvenile gilthead seabream specimens (n = 400) with a body weight of 26.6 ± 4.2 g in 170 L aquaria. Briefly, EE_2 (5 µg/g food, 98% purity; Sigma) was incorporated in the commercial food using the ethanol evaporation method (0.3 L ethanol/kg of food), as described previously (Shved et al., 2007). The specimens were fed three times a day *ad libitum* with the pellet diet supplemented with EE_2 (treated fish) or the pellet diet prepared with ethanol but without EE_2 (untreated fish) for 76 days (days of treatment, dot) after which they were fed with a commercial feed for a further 142 days. In order to evaluate the effect of EE_2 on an induced immune response, the specimens were i.p. injected with hemocyanin (45 µg/fish; Sigma-Aldrich) plus alum adjuvant (3.6 µg/fish; Thermo Scientific) (vaccinated fish) or phosphate buffered saline (PBS) (unvaccinated fish) 1 day after treatment (dat) (priming) and 92 dat (booster) (Supplementary Fig. 1).

Samples of liver and peritoneal exudate leukocytes were collected 1, 93 and 142 dat (1 day post-priming, dpp and 1 and 50 days post-booster, dpb, respectively) (Supplementary Fig. 1). Specimens (n=6 fish/treatment/time of sampling) were fasted for 24 h before each sampling. They were tranquilized by 8 µl/l of clove oil and immediately anesthetized using 40 µl/l of clove oil and weighed. Two-three mL of sRPMI culture medium [RPMI culture medium (Sigma-Aldrich) with 2 mM glutamine (Sigma-Aldrich), 100 i.u./ml penicillin and 100 µg/ml streptomycin (Sigma-Aldrich) adjusted with 0.35% NaCl to seabream serum osmolarity (353.33 mOs)] were i.p. injected at 1 dpp, 1 dpb and 50 dpb. Fish abdomens were massaged for 10 min to dislodge tissue-attached cells into the sRPMI culture medium. Then, incisions were made below of the lateral fin to access the peritoneum and the peritoneal exudates were aspirated and collected into 15 mL Falcon tubes, after which the specimens were decapitated and the liver samples were keep on RNAlater (Sigma-Aldrich) and on ice until analysis.

2.2. Peritoneal exudate cell suspensions

Peritoneal exudates (approximately 2-3 mL) were passed through 70 µm cell strainers in order to remove large clumps of cells. Then, the cells were centrifuged at 600 x *g* for 5 min and resuspended in 5 mL of sRPMI. The total number of cells in peritoneal exudate was counted using an automatic cell counter (Bio-Rad).

2.3. Determination of the percentage of acidophilic granulocytes and mast cells in peritoneal exudate cell suspensions

The percentage of acidophilic granulocytes and mast cells was analyzed using 0.1 x 10^6 peritoneal exudate cell suspensions. Cells were incubated on ice with a specific monoclonal antibody (mAb) against gilthead seabream acidophilic granulocytes (G7, 1:100) (Sepulcre et al., 2002), to measure the percentage of acidophilic granulocytes, or with a mAb against seabream mast cells (GB10, 1/1) (Gómez-González et al., 2016), to determine the percentage of mast cells in 100 µL of FACS buffer (PBS, 0.35% NaCl, 2% fetal bovine serum (FBS, Sigma-Aldrich) and 0.05% sodium azide (Sigma-Aldrich). After 30 min, cells were centrifuged at 600 x *g* for 5 min, the supernatant was removed by aspiration and the cells were washed twice with FACS buffer. Then, cells were labelled with the polyclonal antibody Alexa Fluor 488 F(ab')2 fragment (1:500) of goat anti-mouse IgG (H+L) (ThermoFisher Scientific) for 30 min at 4 °C. The supernatant was removed and cells were washed, as previously described. Fluorescence intensity was measured by flow cytometry using a FACSCalibur flow cytometer (BD Biosciences) and data were analyzed with FlowJo v10.0.4 software.

2.4. ROS production assay

ROS production was measured using luminol-dependent chemiluminescence produced by 0.5 x 10^6 peritoneal exudate cell suspensions. Cells were incubated at 21 °C and 5% CO_2 during 20 min. Then, the ROS production was determined by adding 100 µM luminol (Sigma-Aldrich) and 1 µg/mL phorbolmyristate acetate (PMA) (Sigma-Aldrich) in Hanks' Balanced Salt Solution (Sigma-Aldrich) to the cell culture. Chemiluminescence was recorded every 127 s for 1 h using a FLUOstartluminometer (BGM, LabTechnologies). Three experimental replicates were performed for all samples (6). The values reported are the average of the replicate samples, expressed as maximum of the reaction curve from 127 to 1016 s, from which the background (only culture medium) was subtracted.

2.5. Gene expression analysis

Liver samples were defrosted and RNAlater removed. Total RNA was extracted from liver and peritoneal exudate cell suspensions of 1, 93 and 142 dat (1 dpp, 1dpb and 50 dpb, respectively) with TRIzol Reagent (ThermoFisher Scientific) as indicated by the manufacturer's instructions, and the RNA concentration was quantified by

spectrophotometry (NanoDrop, ND-1000). RNA was treated with DNase I (amplification grade, 1 unit/µg RNA, Invitrogen) to remove genomic DNA traces that could interfere with the PCR reactions. SuperScript III RNase H- Reverse Transcriptase (Invitrogen) was used to synthesize first strand cDNA with oligo $(dT)_{18}$ primer (Invitrogen) from 1 µg of total RNA, at 50 °C for 50 min. The quality of cDNA was analyzed by performing a semi-quantitave PCR of ß-actin (*actb*) housekeeping gene using an Eppendrof Mastercycle Gradient Instrument (Eppendorf). The reaction mixture was incubated for 2 min at 94 °C, followed by 30 cycles of 45 s at 94 °C, 45 s at 55 °C (the specific annealing temperature), and 45 s at 72 °C and finally holding at 72 °C for 10 min. Real-time PCRs were performed with an ABI PRISM 7500 (Applied Biosystems) using SYBR Green PCR Core Reagents (Applied Biosystems) to analyze the gene expression of: i) the hepatic *vtg*, ii) *erα* and *gper1*, iii) gene markers of macrophages (colony stimulating factor 1 receptor, *csf1r*), IgM^+ B lymphocytes (immunoglobulin M heavy chain, *ighm*) and IgT^+ B lymphocytes (immunoglobulin T heavy chain, *ight*), iv) the pro-inflammatory cytokine interleukin 1β, *il1b*, and v) the anti-inflammatory cytokine *il10*. Reaction mixtures were incubated for 10 min at 95 °C, followed by 40 cycles of 15 s at 95 °C, 1 min at 60 °C, and finally 15 s at 95 °C. For each mRNA, gene expression was corrected by the ribosomal protein S18 gene (*rps18*) content in each sample. The gilthead seabream specific primers used are shown in Supplementary Table 1. In all cases, each PCR was performed with triplicate samples. Less than 3% variation in the *rps18* gene expression was observed among samples.

2.6. Statistical analysis

Normal distribution of the data was analyzed with the Statgraphics Centurion XVI v16.1.15 software. Statistical differences were analyzed by Student's t tests (when only two groups were compared) or two-way ANOVA and a *post hoc* Holm-Sidak's multiple comparison test (when more than two groups were compared) using the GraphPad Prism v5.01 software.

3. Results

3.1. EE_2 modulates the expression of hepatic vitellogenin

The expression of the hepatic *vtg* gene was analyzed 76 dot and 93 dat to assess the endocrine disruption activity of dietary intake of EE_2. Hepatic *vtg* mRNA levels of EE_2-treated fish were much increased 76 dot compared with the untreated fish (Supplementary

Fig. 2A). However, no statistical differences were found at 93 dat between EE_2-treated and untreated fish (Supplementary Fig. 2B).

3.2. EE_2 modulates estrogen receptor expression in peritoneal leukocytes

Total peritoneal leukocytes were found to express *erα* and *gper1* by *RT-qPCR* (1 dat, 1dpp) (Supplementary Fig. 3). Moreover, we observed that vaccination decreased the *erα* and *gper1* transcript levels (Supplementary Fig. 3A), while EE_2 treatment increased the *era* and *gper1* mRNA levels in both unvaccinated and vaccinated fish (Supplementary Fig. 3A, B).

3.3. EE_2 modulates leukocyte recruitment to the peritoneal cavity and peritoneal leukocyte populations

Changes in the number of peritoneal exudate leukocytes and leukocyte populations produced by EE_2-treatment and vaccination were studied 1 dat (1 dpp) (Fig. 1A-F). As expected, vaccination increased the number of peritoneal leukocytes (Fig. 1A).

Surprisingly, dietary intake of EE_2 increased leukocyte recruitment in both vaccinated and unvaccinated fish (Fig. 1A). In addition, changes in leukocyte populations were also observed in peritoneal exudate 1 dpp (Fig. 1B-F). Firstly, the percentage of mast cells was substantially lower in vaccinated fish (Fig. 1B), while dietary EE_2 further reduced it in vaccinated fish (Fig. 1B). Secondly, vaccination promoted an increase in the percentage of acidophilic granulocytes while dietary EE_2 did not modify it (Fig. 1C). Thirdly, dietary EE_2 increased the transcript levels of genes encoding specific markers for macrophages and IgM^+ and IgT^+ B lymphocytes (*csf1r*, *ighm* and *ight*, respectively) in both unvaccinated and vaccinated fish (Fig. 1D-4F). Finally, vaccination induced a reduction of the transcript levels of *ight* (Fig. 1F).

Ninety-three days after suspending the EE_2 treatment, which coincided with 1 dpb, the picture was rather similar to 1 dat (1 dpp) (Fig. 1G-1L). The most noticeable differences were that EE_2 increased the expression of *csf1r* only in unvaccinated fish (Fig. 1J), while the effect of dietary EE_2 in the transcript levels of *ighm* (Fig. 1K) and and *ight* (Fig. 1L) were no longer observed.

Finally, the total leukocyte numbers were found be reduced in vaccinated and unvaccinated and EE_2-treated fish at 142 days after EE_2 treatment, which coincided with 50 dpb (data not shown).

3.4. EE_2 alters the respiratory burst of peritoneal exudate leukocytes

The respiratory burst was analyzed 1, 93 and 142 dat (1 dpp, 1 dpb and 50 dpb, respectively) in peritoneal exudate leukocytes (Fig. 2A-C). ROS production robustly increased 1 dat (1 dpp) and 142 dat (50 dpb) in vaccinated fish, while was slightly inhibited at 93 dat (1 dpb) (Fig. 2A-5C). In addition, dietary intake of EE_2 significantly induced ROS production 1 dat in unvaccinated fish (Fig. 2A), while had no effect at 93 and 142 dat (Fig. 2B and 2C).

3.5. EE_2 induces peritoneal *il1b* gene expression

The mRNA levels of the genes encoding the pro-inflammatory IL-1β and the anti-inflammatory IL-10 were analyzed by RT-qPCR in total peritoneal exudate leukocytes 1, 93 and 142 dat (1 dpp, 1 dpb and 50 dpb, respectively) (Fig. 2D-I). At 1 dpp (Fig. 2D) *il1b* expression was found to be higher in vaccinated fish. Strikingly, dietary intake of EE_2 strongly increased *il1b* transcript levels 1 (Fig. 2D) and 142 dat (Fig. 2E) in unvaccinated fish, while had no effect in vaccinated fish at any of the time analyzed (Fig. 2D-F). On the other hand, *il10* expression was not altered at any of the time analyzed (Fig. 2G-I).

4. Discussion

Xeno-estrogens EDCs are able to mimic or antagonize the action of natural estrogens. That, as well as other sex steroids, have demonstrated effects beyond the reproductive context, with drastic effects along molecular to organism levels, particularly on aquatic animals (Castillo-Briceno and Kodjabachian, 2014). Despite, most fish studies on these compounds have focused on the alterations they produced on the reproductive system, it is also known that they can also alter other aspects of fish biology, including immunity (Filby et al., 2007). In this study, we analyze the effect that EE_2 has on the immune response of a marine hermaphrodite species, the gilthead seabream, focusing on peritoneal exudate leukocytes.

First of all, EE_2 promotes a significant increase in hepatic *vtg* gene expression in gilthead seabream, which is considered as a maker of endocrine disruption for estrogenic exposure in male fish (Sumpter and Jobling, 1995), as previously demonstrated in gilthead seabream, both in adults and juveniles, and in other fish species (Cabas et al., 2012; Kausch et al., 2008; Rodenas et al., 2016). Notably, this increase disappeared when

the treatment ceased, as we also previously described (Rodenas et al., 2015; Rodenas et al., 2016). Moreover, we have observed that ERs, both nuclear ERs and membrane-anchored GPER1, are expressed in peritoneal exudate leukocytes as described in mammal and in fish immune cells from head kidney (Blasko et al., 2009; Cabas et al., 2013a; Liarte et al., 2011; Straub, 2007), and that EE_2 increased the expression of both receptors. The present results extend these earlier observations by demonstrating that (i) EE_2 would exert its effect on gilthead seabream peritoneal leukocyte biology not only through the classical nuclear receptor pathway but also through the novel GPER1 and (ii) EE_2 would increase estrogen signaling in these cells by inducing ER. In addition, it is also interesting that vaccination decrease the expression of gene encoding ERα and GPER1 in peritoneal exudate leukocytes, as increases the expression of both genes in the head kidney of this species (Cabas et al., 2012; Cabas et al., 2013a). Such differences may be due to the alterations of different leukocyte populations after an immune challenge (Chaves-Pozo et al., 2005; Chaves-Pozo et al., 2004; García-Castillo et al., 2002).

One of the most interesting observations of this study is that dietary intake of EE_2 was able to promote leukocyte recruitment to the peritoneal cavity and the expression of genes encoding the *csf1r*, *ighm* and *ight*, what strongly suggests that macrophages and B lymphocytes were recruited to the peritoneal cavity of unvaccinated fish, as occurred in the gonad of gilthead seabream exposed to dietary EE_2 (Cabas et al., 2011) and in agreement with the changes observed in leukocyte populations of Japanese sea bass blood after E_2 treatment (Thilagam et al., 2009). Nevertheless, once treatment had ceased, *cfsr1* gene expression remained at high levels for 3 months in EE_2-unvaccinated fish, despite the number of total peritoneal exudate leukocyte returned to basal levels. Moreover, EE_2 increased the respiratory burst and the expression of the gene encoding IL-1β of peritoneal exudate leukocytes in unvaccinated fish, even 3 months after ceasing the treatment. These data are in agreement with the induction of ROS production in blood leukocytes of Japanese sea bass promoted by E_2 (Thilagam et al., 2009) and the induction of IL-1β expression levels in the head kidney of gilthead seabream exposed to dietary EE_2 (Cabas et al., 2012). Similarly, other xeno-estrogens, such as bisphenol A, nonylphenol and different phthalates, were found to increase the ROS production by common carp phagocytic cells (Gushiken et al., 2002; Watanuki et al., 2003). The biological consequences of the long lasting effects of dietary intake of EE_2 shown here for the first time deserve further investigations. It is tempting to speculate that epigenetic mechanisms

294 are involved (Bhandari et al., 2015), since the endocrine disruption effect of EE_2 is over
295 at this time.

296 The effect of dietary intake of EE_2 in vaccinated fish was also interesting, although
297 weaker than the observed in their unvaccinated counterparts. EE_2 exerted its greatest
298 effects in the total number of leukocytes and in the transcript levels of *ight* 1 dpp, as
299 occurred in unvaccinated fish, but further decreased the percentage of mast cells.
300 Therefore, EE_2 showed a synergistic effect with vaccination increasing the total number
301 of leukocytes and reducing mast cell abundance. In contrast, other EDCs, such as PCB
302 126, have been shown to increase the abundance of mast cells in gills and intestine of
303 gilthead seabream (Lauriano et al., 2012).

304 To conclude, our data point to a very complex role of estrogens in fish immunity, as
305 it is widely accepted in mammals (Straub, 2007).We found that dietary intake of EE_2
306 promotes a long lasting inflammatory response in the peritoneal cavity of unvaccinated
307 gilthead seabream juveniles after ceasing the treatment and even though its endocrine
308 disruption effect was over. However, the impact of dietary EE_2 in vaccinated fish was
309 rather minor and transient. These results, therefore, paves the way to futures studies aimed
310 at understanding the molecular mechanisms involved in the long lasting effect of EDCs
311 in fish immunity.

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319

320 **Conflict of interest**

321 The authors declare no conflict of interest.

322

323 **Footnotes**

[1]Abbreviations: ERs: estrogen receptors; GPER1: G protein-coupled estrogen receptor 1; E_2: 17β-estradiol; EDCs: endocrine disruptor chemicals; EE_2: 17α-ethynylestradiol; i.p.: intraperitoneal; PBS: phosphate buffered saline; dot: days of treatment; dat: days after treatment; dpp: days post-priming; dpb: days post-booster; mAb: monoclonal antibody; PMA: phorbolmyristate acetate; ROS: reactive oxygen species.

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Figure legends

Figure 1. Dietary intake of EE_2 promotes a peritoneal leukocyte recruitment and modulates the peritoneal populations. The total leukocyte numbers (A and G), the percentage of mast cells (MC, $GB10^+$ cells) (B and H), the percentage of acidophilic granulocytes (AG, G7+ cells) (C and I),the *csf1r* mRNA levels (D and J), the *ighm* mRNA levels (E and K) and the *ight* mRNA levels (F and L) at 1 and 93 dat (1 dpp and 1 dpb, respectively) in the peritoneal exudate leukocytes of fish i.p. injected with PBS (unvaccinated) or with hemocyanin plus imject alum adjuvant (vaccinated) previously exposed to dietary EE_2 (EE_2, solid bars) or not (C, open bars). dat: days after treatment; dpp: days post-priming; dpb: days post-booster. Data are mean ± SEM of 6 individuals. The asterisks denote statistically significant differences between groups according to two-way ANOVA and Holm-Sidak's multiple comparison tests. ns, not significant; *p < 0.05; **p<0.01and ****p<0.0001.

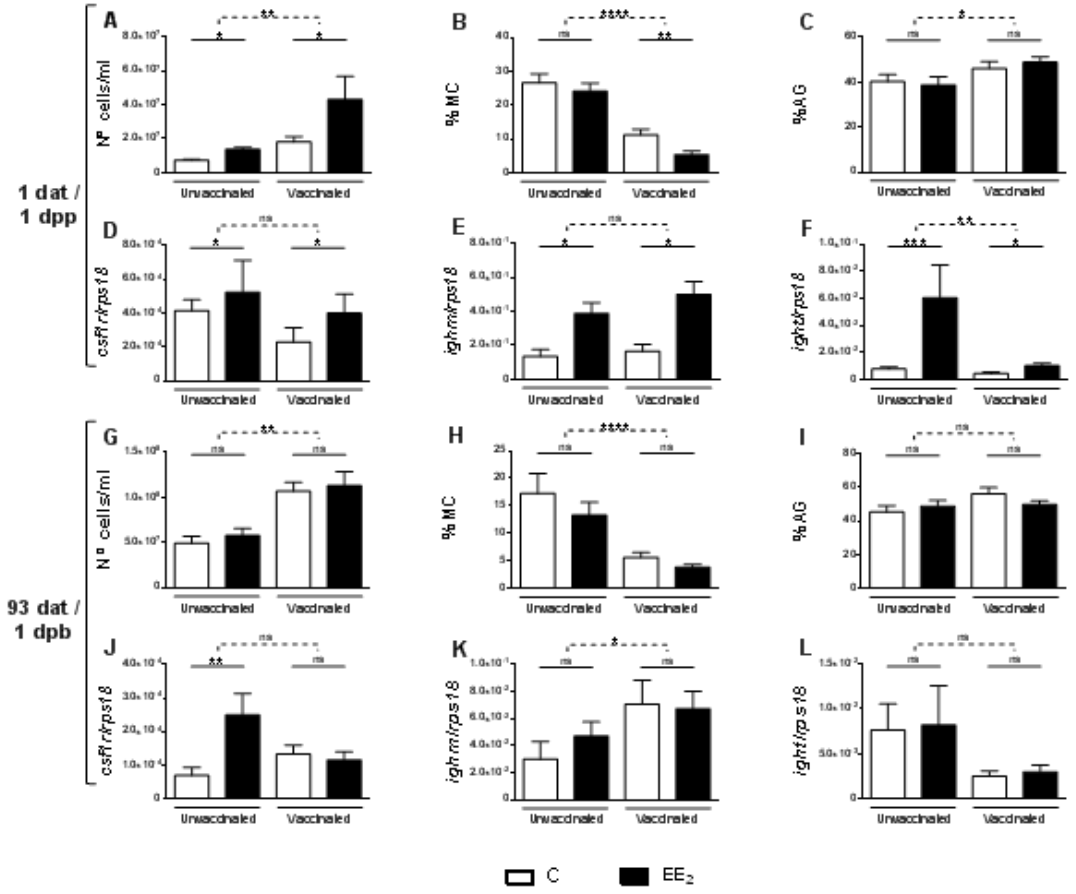
Figure 2. Dietary intake of EE_2 modulates the respiratory burst activity and induces *il1b* gene expression of peritoneal exudate leukocytes. ROS production (A-C) triggered by PMA was measured as maximum values of luminol- dependent luminescence at 1 (A), 93 (B) and 142 (C) dat (1 dpp, 1 dpb and 50 dpb, respectively) generated by total peritoneal exudate leukocytes of fish i.p. injected with PBS (unvaccinated) or with hemocyanin plus imject alum adjuvant (vaccinated) previously exposed to dietary EE_2 (EE_2, solid bars) or not (C, open bars). The mRNA levels of the genes encoding IL-1β and IL-10 (D-I) was analyzed by RT-qPCR at 1 (A and D), 93 (B and E) and 142 (C and F) dat (1 dpp, 1 dpb and 50 dpb, respectively) in total peritoneal exudate leukocytes of fish i.p. injected with PBS (unvaccinated) or with hemocyanin plus imject alum adjuvant (vaccinated) previously exposed to dietary EE_2 (EE_2, solid bars) or not (C, open bars).dat: days after treatment; dpp: days post-priming; dpb: days post-booster. Data are mean ± SEM of 6 individuals. The asterisks denote statistically significant differences between groups according to two-way ANOVA and Holm-Sidak's multiple comparison tests. ns, not significant; *p < 0.05; **p<0.01and ****p<0.0001.

Supplementary Figure 1. Schematic drawing of the experimental design. Animals were exposed to 0 (untreated fish) and 5 (treated fish) μg EE_2/g food for 76 days (days of treatment, dot), after which they were fed with commercial food for a further 142 days. Fish were i.p. injected with PBS (unvaccinated fish) or with hemocyanin plus imject alum adjuvant (vaccinated fish) at 76 dot (priming) and 92 days after treatment (dat) (booster). Samples were collected 1, 93 and 142 dat (1 day post-priming, dpp, 1 day post-booster, dpb, and 50 dpb, respectively)**.**

Supplementary Figure 2. Dietary intake of EE_2 increases the expression of the gene encoding vitellogenin (*vtg*) in gilthead seabream liver. RT-qPCR analysis of hepatic *vtg* mRNA levels in untreated (C, open bars) and dietary EE_2-treated fish (EE_2, solid bars) at 76 days of treatment (dot) (A) and 93 days after treatment (dat) (B). Data are mean ± SEM of 6 individuals. The asterisks denote statistically significant differences between groups according to Student's t-test. ns, not significant and ***p<0.001.

Supplementary Figure 3. Dietary intake of EE_2 alters the mRNA expression levels of the estrogen receptors *erα* and *gper1* in peritoneal exudate. RT-qPCR analysis of *erα* (A) and *gper1* (B) mRNA levels at 1 dat (1dpp) in the peritoneal exudate leukocytes of fish i.p. injected with PBS (unvaccinated) or hemocyanin plus imject alum adjuvant (vaccinated) previously exposed (EE_2, solid bars) or not (C, open bars) to dietary EE_2. dat: days after treatment; dpp: days post-priming; dpb: days post-booster. Data are mean ± SEM of 6 individuals. The asterisks denote statistically significant differences between groups according to two-way ANOVA and Holm-Sidak's multiple comparison tests. *p < 0.05; **p<0.01and ***p<0.001.

Figure 1


1 dat / 1 dpp
93 dat / 1 dpb
A
B
C
D
E
F
G
H
I
J
K
L
Nº cells/ml
% MC
%AG
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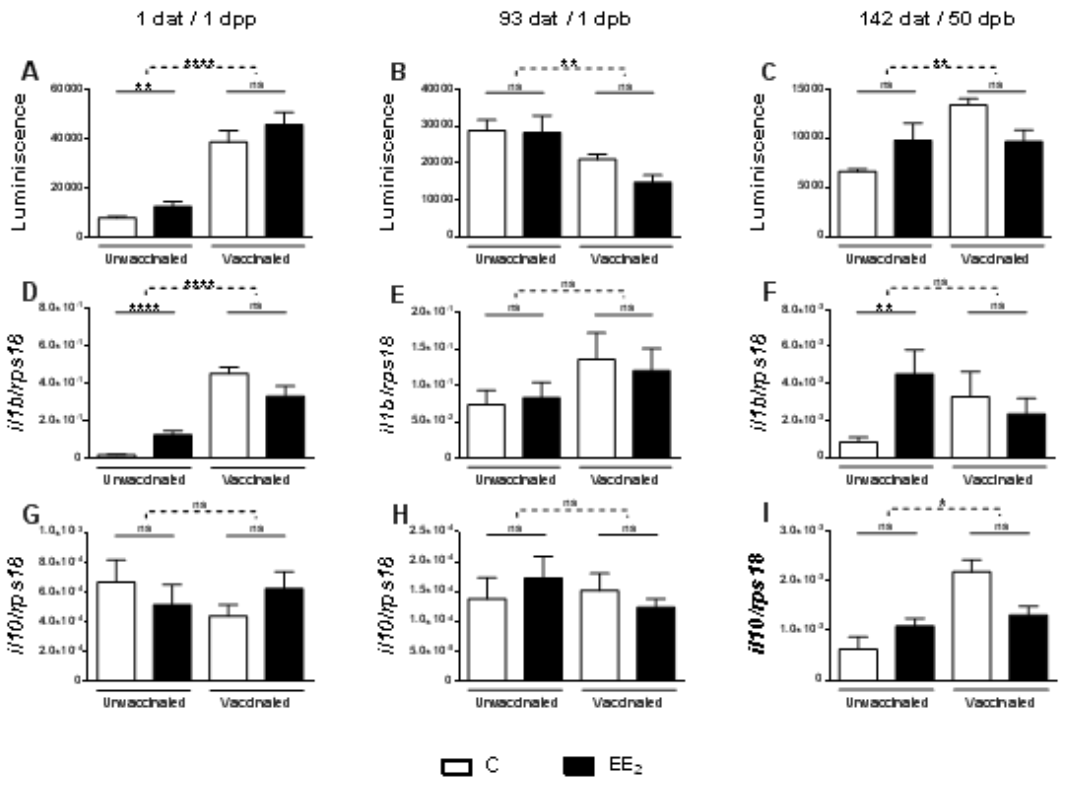
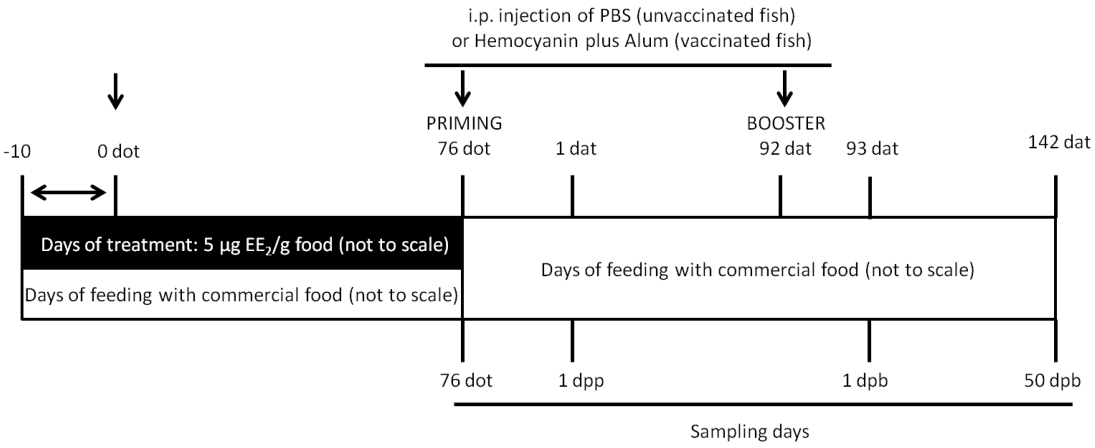
ightlrps18
Unvaccinated
Vaccinated
C
EE2

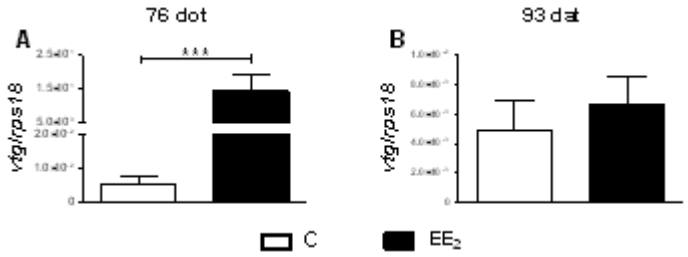
Figure 2



Supplementary Figure 4



Supplementary Figure 2



Supplementary Figure 3