

1 **Tamoxifen disrupts the reproductive process in gilthead seabream males and**
2 **modulates the effects promoted by 17 α -ethynylestradiol**

3
4 **Running Title:** Effect of Tmx alone or combined with EE₂ on hermaphrodite fish

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1 **Abstract**

2 17 α -ethynylestradiol (EE₂), which is used in oral contraceptives and hormone
3 replacement therapy, is a well documented estrogenic endocrine disruptor and an
4 aquatic contaminant. In the present study, adult male specimens of the marine
5 hermaphrodite teleost gilthead (*Sparus aurata* L.) were fed a diet containing tamoxifen
6 (Tmx), an estrogen receptor ligand used in cancer therapy, alone or combined with EE₂,
7 for 25 days and then fed a commercial diet for a further 25 days (recovery period). The
8 effects of short (5 days) and long (25 days) treatments on several reproductive and
9 gonad immune parameters and the reversibility of the disruptive effects after the
10 recovery period were examined. Our data showed that Tmx acted as an estrogenic
11 endocrine disruptor as revealed by the increase in the hepatic transcription of the
12 *vitellogenin* gene in males, the serum levels of 17 β -estradiol and the gonad expression
13 levels of the estrogen receptor α and G protein-coupled estrogen receptor genes, and the
14 recruitment of leukocytes into the gonad, a well known estrogenic-dependent process in
15 gilthead seabream males. On the other hand, Tmx also increased sperm concentration
16 and motility as well as the serum levels of androgens and the expression levels of genes
17 that codify for androgenic enzymes, while decreasing the expression levels of the gene
18 that code for gonadal aromatase. When applied simultaneously, Tmx and EE₂ could act
19 in synergy or counteract, each other, depending on the parameter measured. The
20 disruptive effect of EE₂ and/or Tmx was not reversible after a 25 day recovery period.

21

22 **Keywords:** Tamoxifen, 17 α -ethynylestradiol, endocrine disruption reversibility,
23 spermatogenesis, steroidogenesis, gilthead seabream.

24

25 **Summary statement**

26 In gilthead seabream males, Tmx disrupts the reproductive process including the gonad
27 immune response and counteracts or enhances the effects of EE₂. A 25-day recovery
28 period did not reverse these effects in adult males.

1 **Introduction**

2 Endocrine disrupting chemicals (EDCs) exert their effects via agonistic/antagonistic
3 interactions with hormone receptors or by interfering with the normal synthesis,
4 transport, metabolism, and secretion of endogenous hormones (Segner et al., 2006).
5 Among EDCs, the most studied are the compounds that interfere with estrogen
6 receptors (ERs), which have hazardous and estrogenic effects on fish reproduction
7 (Folmar et al., 1996; Jobling et al., 1998, 2002; Hassanin et al., 2002; Penáz et al.,
8 2005). Some of these compounds are pharmaceutical products released in waste waters
9 which reach the aquatic environment through sewage treatment effluents (Mills and
10 Chichester, 2005).

11 In the group of estrogenic EDCs, 17 α -ethynylestradiol (EE₂), a major constituent
12 of contraceptive pills (Owen and Jobling, 2012), has a higher binding affinity to ERs
13 than natural 17 β -estradiol (E₂) (Blair et al., 2000) and is one of the most potent
14 compounds in the aquatic environment. Low concentrations of EE₂ (3-17 ng/L) are
15 sufficient to induce vitellogenin (Vtg) production in male fish (Holbech et al., 2001;
16 Rose et al., 2002; Andersen et al., 2003), to modify sexual behaviour (Coe et al., 2010;
17 Reyhanian et al., 2011; Filby et al., 2012) and to disrupt the reproductive capacities of
18 fish (Nash et al., 2004; Pawlowski et al., 2004; Fenske et al., 2005; Schäfers et al.,
19 2007). However, the ability of fish to recover from estrogen exposure has drawn little
20 attention, apart from some studies on sexual differentiation and reproductive capacity
21 (Hill and Janz, 2003; Nash et al., 2004; Schäfers et al., 2007; Larsen et al., 2009;
22 Baumann et al., 2014).

23 Other EDCs is tamoxifen (Tmx) which is widely used as a drug in cancer
24 therapy. Studies in humans have shown that approximately 65% of administered Tmx is
25 excreted with faeces, while its active metabolite OH-Tmx is excreted with bile and
26 urine. Tmx is a nuclear ER ligand which in mammals, acts as estrogen agonist on some
27 cell types but as an antagonist or partial agonist on others, which reflects the diversity of
28 the mechanisms that mediate ER actions in different tissues (Fitts et al., 2011). In
29 addition, Tmx acts as an agonist on the G protein-coupled estrogen receptor (GPER), a
30 transmembrane receptor that mediates rapid responses of estrogen and is widely
31 expressed in estrogen target tissues (Revankar et al., 2005), including fish testis and
32 ovary (Liu et al., 2009; Pang and Thomas, 2010). However, the effects of Tmx and its
33 action mechanisms in fish are just beginning to be understood, in part because of the
34 interest that binary mixtures of EDCs has attracted in the recent years (Sun et al., 2009,

1 2011a, 2011,b). Such studies have been performed in gonochoristic fish and showed
2 that the estrogenic or anti-estrogenic effects of Tmx depend on the gender,
3 concentration and tissue analyzed (Leaños-Castañeda et al., 2002; Chikae et al., 2004;
4 Sun et al., 2011a, 2011b). Tmx treatment leads to the masculinization of genetic female
5 fish (Kitano et al., 2007; Hulak et al., 2010; Liu et al., 2010). Moreover and although
6 the effects of Tmx mask or neutralize many signs of estrogen exposure, the impairment
7 of the fish reproductive process is not restored (Santos et al., 2006; Elias et al., 2007;
8 van der Ven et al., 2007; Sun et al., 2009). To the best of our knowledge, no such
9 studies have been studied in hermaphrodite fish such as gilthead seabream.

10 The gilthead seabream (*Sparus aurata* L.) is a marine, seasonally breeding,
11 protandrous teleost that develop a functional testicular area near by an immature
12 previtellogenic ovary during the first two reproductive cycles. We have recently
13 reported that EE₂ dietary intake increases the hepatic expression levels of *vgt*, disrupts
14 spermatogenesis and promotes leukocyte infiltration in the gonad (Cabas et al., 2011,
15 2013), a physiological process needed for gonad renewal after spawning (Chaves-Pozo
16 et al, 2005a, 2005b; Liarte et al., 2007). Moreover, most of these effects vary with the
17 reproductive stage of the specimens (Cabas et al., 2011, 2013). On the other hand, the
18 dietary intake of Tmx has been shown to be a suitable approach for studying its
19 potentially endocrine disruptive effects (Benninghoff and Williams, 2008; Singh et al.,
20 2014).

21 In the present study, we investigate the effect of the dietary intake of Tmx alone
22 or in combination with EE₂ on some reproductive events in gilthead seabream and the
23 possible reversibility of these effects after a recovery period of 25 days, during which
24 fish were again fed with a commercial diet. This approach, as a way to unbalance the
25 endocrine status of the fish, would improve our understanding of the complex network
26 acting on the regulation of the reproductive function in this species, which has a great
27 commercial interest in the Mediterranean area.

28 29 **Material and Methods**

30 Healthy specimens of gilthead seabream (Actinopterygii, Perciformes, Sparidae) were
31 bred and kept at the Centro Oceanográfico de Murcia (Instituto Español de
32 Oceanografía, Mazarrón, Murcia, Spain).

33 The experiment was performed using 80 male specimens of gilthead seabream,
34 all in the spermatogenesis stage, with a mean body weight of 215 ± 6.5 g. Fish were

1 kept in 2 m³ tanks with a flow-through circuit, suitable aeration and filtration system
2 and natural photoperiod. The water temperature ranged from 14.6 to 17.8°C.
3 Environmental parameters, mortality and food intake were recorded daily. The EE₂
4 (98% purity; Sigma) and Tmx (Sigma) were incorporated in the commercial feed (44%
5 protein, 22% lipids, Skretting, Spain) at doses of 0 (control), 5 µg EE₂/g food, 100 µg
6 Tmx/g food or 5 µg EE₂ + 100 µg Tmx/g food, using the ethanol evaporation method
7 (0.3 L ethanol/kg of food) as described elsewhere (Shved et al., 2007). The
8 concentration of EE₂ used in this study was previously assayed and shown to be the
9 lowest concentrations producing an effect on some reproductive events of gilthead
10 seabream (Cabas et al., 2011, 2013), while the concentration of Tmx used was twenty-
11 fold greater than the concentration of EE₂ in order to guarantee a Tmx-ER interaction,
12 considering that Tmx has a lower affinity than EE₂ to bind ER (Denny et al., 2005). In
13 any case, the Tmx concentration used in this study is similar to, or lower than, those
14 tested in previous investigations (Chikae et al., 2004; Hulak et al., 2010).

15 The specimens were fed with EE₂ and/or Tmx supplemented feed for 25 day,
16 after which they were fed with the commercial food for a further 25 days (recovery
17 period). The specimens were fed *ad libitum* three times a day and fasted for 24 h before
18 sampling, which was carried out after 5 and 25 days of the EE₂ and/or Tmx exposure
19 and after the recovery period (n=6 fish/group and time). Specimens were anesthetized
20 with 40 µL/L of clove oil and the urogenital pore was dried before collecting sperm as
21 described below. The specimens were then weighed, decapitated, and the gonads
22 removed and weighed. Fragments of liver and gonad were processed for gene analysis
23 and light microscopy, as described below. Serum samples from trunk blood were
24 obtained by centrifugation and immediately frozen and stored at -80°C until use.

25 The experiments comply with the Guidelines of the European Union Council
26 (2010/63/UE) and the Bioethical Committee of the University of Murcia (Spain) and
27 that of the “Instituto Español de Oceanografía” (Spain) for the use of laboratory
28 animals.

29 ***Analysis of gene expression***

30 Total RNA was extracted from liver and gonad fragments with TRIzol Reagent
31 (Invitrogen, Barcelona, Spain) following the manufacturer’s instructions, and quantified
32 with a spectrophotometer (NanoDrop, ND-1000). The RNA of five fish per group was
33 independently treated with DNase I (amplification grade, 1 unit/µg RNA, Invitrogen,
34 Barcelona, Spain) to remove genomic DNA traces that might interfere with the PCR

1 reactions, and the SuperScript III RNase H–Reverse Transcriptase (Invitrogen,
 2 Barcelona, Spain) was used to synthesize first strand cDNA with oligo-dT18 primer
 3 from 1 µg of total RNA, at 50°C for 50 min.

4 Real-time PCR was performed with an ABI PRISM 7500 (Applied Biosystems,
 5 Madrid, Spain) using SYBR Green PCR Core Reagents (Applied Biosystems, Madrid,
 6 Spain) and used to analyze the expression of the genes coding for (i) hepatic
 7 vitellogenin (*vtg*); (ii) steroidogenesis-related molecules: steroidogenic acute regulatory
 8 protein (*star*), cholesterol side chain cleavage cytochrome P450 (*cyp11a1*), steroid 11-
 9 beta-hydroxylase (*cyp11b1*), 11β-hydroxysteroid deshydrogenase (*hsd11b*), aromatase
 10 (*cyp19a1a*), 5α reductase (*srd5a*) and 3β-hydroxysteroid deshydrogenase (*hsd3b*); (iii)
 11 testicular specific protein, double sex-and mab3-related transcription factor 1 (*dmrt1*);
 12 (iv) hormone receptors: follicle stimulating hormone (FSH) receptor (*fshr*), luteinizing
 13 hormone (LH) receptor (*lhr*) and estrogen receptor α (*era*), G protein-coupled estrogen
 14 receptor (*gper*); (v) immune-relevant molecules: interleukin 1β (*illb*), tumor necrosis
 15 factor α (*tnfa*), transforming growth factor β1 (*tgfb1*), matrix metalloproteinase (*mmp*) 9
 16 and 13 (*mmp13*), major histocompatibility complex I α protein (*mhc1a*) and toll-like
 17 receptor 9 (*tlr9*). For each mRNA, gene expression was normalized to the ribosomal
 18 protein S18 gene (*rsp18*) content in each sample using the comparative Ct method
 19 ($2^{-\Delta\Delta Ct}$) (where Ct is a cycle threshold). The gilthead seabream specific primers used are
 20 shown in Table 1. In all cases, each PCR was performed in triplicate.

21 **Analytical techniques**

22 Serum levels of testosterone (T), 11-ketotestosterone (11KT) and E₂ were quantified by
 23 ELISA following the method described by Rodríguez et al. (2000) and previously used
 24 in gilthead seabream (Chaves-Pozo et al., 2008). Steroids were extracted from 20 µL of
 25 serum in 0.6 mL of methanol (Panreac). The methanol was then evaporated at 37°C and
 26 the steroids were resuspended in 400 µL of reaction buffer [0.1 M phosphate buffer with
 27 1 mM EDTA (Sigma), 0.4 M NaCl (Sigma), 1.5 mM NaN₃ (Sigma) and 0.1% albumin
 28 from bovine serum (Sigma)]. By using 50 µL in each well, 2.5 µL of serum was used in
 29 each well for all the assays. The T, 11KT and E₂ standards, mouse anti-rabbit IgG
 30 monoclonal antibody (mAb), and specific anti-steroid antibodies and enzymatic tracers
 31 (steroid acetylcholinesterase conjugates) were obtained from Cayman Chemical.
 32 Microtiter plates (MaxiSorp) were purchased from Nunc. A standard curve from $6.13 \times$
 33 10^{-4} to 2.5 ng/mL (0.03-125 pg/well) was established in all the assays. Standards and
 34 extracted serum samples were run in duplicate. The lower limit of detection for all the

1 assays was 12.21 pg/mL. The intra-assay coefficients of variation (calculated from
2 duplicate samples) were $8.26\% \pm 1.33\%$ for T, $8.80\% \pm 1.68\%$ for 11KT and $3.98\% \pm$
3 0.57% for E₂. Details on cross-reactivity for specific antibodies were provided by the
4 supplier (2.2% of anti-T reacts with 11KT; 0.01% of anti-11KT reacts with T; 0.1% of
5 anti-E₂ reacts with T).

6 ***Measurement of the sperm volume, concentration and motility***

7 Stripped sperm was obtained by gentle abdominal massage, the sperm being collected
8 and measured in the genital pore with a syringe as the semen flowed out (urine-
9 contaminated samples were discarded). The total semen from each fish (n=6 fish/group
10 and time) was used immediately to determine cell concentration and motility. To
11 determine the sperm concentration, semen was diluted in 1% formol (Panreac) and 5%
12 NaHCO₃ (Sigma) in water at a ratio of 1:400 and the spermatozoa were counted using a
13 Newbauer chamber. Motility was analyzed by activating 1 μL of sperm (diluted in
14 Ringer 200 mOsm solution at the optimal dilution of 1:5 with 20 μL of seawater
15 (Chereguini et al., 1997). The duration of sperm motility was determined by measuring
16 the time elapsing between the initiation of sperm motility and the cessation of cell
17 displacement using a light microscope at 400× magnification. The motility index was
18 expressed on a relative scale of 0 to 5 (Sánchez-Rodríguez, 1975).

19 ***Light microscopy and immunocytochemical staining***

20 The gonads were fixed in Bouin's solution, embedded in Paraplast Plus (Sherwood
21 Medical, Athy, Ireland), and sectioned at 5 μm. After dewaxing and rehydration, some
22 sections were stained with hematoxylin-eosin in order to determine the reproductive
23 stage and the degree of development of each specimen. Some sections were used to: i)
24 analyze cell proliferation with a commercial mammalian antibody specific to
25 proliferating cell nuclear antigen (PCNA, Sigma) or ii) localize acidophilic granulocytes
26 with a monoclonal antibody (mAb) specific to gilthead seabream acidophilic
27 granulocytes (G7) (Sepulcre et al., 2002) and B lymphocytes with a commercial mAb
28 specific to immunoglobulin M (IgM, Aquatic Diagnostic) (Sepulcre et al., 2011)
29 following an indirect immunocytochemical method previously described (Chaves-Pozo
30 et al., 2007). Rabbit polyclonal anti-PCNA cross-reacts with PCNA from all vertebrate
31 species investigated to date, including fish (Kilemade et al., 2002). The antibodies were
32 used at the optimal dilutions of 1:1000, 1:100 or 1:250, respectively. No
33 immunostaining was observed when the first antiserum was omitted.

34 ***Calculation and statistics***

1 All data were analyzed by one-way ANOVA and a post hoc test (Tukey Honestly
2 Significant Difference) to determine differences between groups ($P \leq 0.05$). Normality
3 of the data was previously assessed using a Shapiro–Wilk test and homogeneity of
4 variance was also verified using the Levene test. The stripped sperm volume, and the
5 sperm concentration and motility index data were analyzed by a Student t-test to
6 determine differences between untreated control and the treated group for each time
7 point. The critical value for statistical significance was taken as $P \leq 0.05$. The asterisks
8 mean: * $P < 0.05$; ** $P < 0.01$ and *** $P < 0.001$. All statistical analyses were carried
9 out using the GraphPad Prism 5 program.

11 Results

12 *Tmx up-regulates the hepatic expression of vitellogenin gene to a lesser extent than* 13 *EE₂*

14 The hepatic expression of *vtg* gene was up-regulated after the dietary intake of EE₂ and
15 Tmx. The Tmx-induced up-regulation was lower than that promoted by EE₂ (Fig. 1A).
16 After the recovering period, expression levels of *vtg* remained high, although the
17 differences that existed between treatments became less pronounced (Fig. 1A).
18 Interestingly, Tmx decreased the levels of *vtg* transcription triggered by EE₂ when they
19 were applied together at all time points analyzed (Fig. 1A).

20 *EE₂ and Tmx differently affect sex steroid serum levels and the expression levels of* 21 *some steroidogenic enzyme genes*

22 After 5 days of Tmx treatment, the serum levels of T (Fig. 1B), 11KT (Fig. 1C) and E₂
23 (Fig. 1D) were higher than those of control ones, while EE₂ exposure had no effect in
24 this respect (Fig. 1B-D). Interestingly, the administration of EE₂+Tmx prevented the
25 increases in serum steroid levels induced by Tmx (Fig. 1B-D). No differences in T,
26 11KT or E₂ serum levels were observed after 25 days of any treatment used (Fig. 1B-
27 D). By the end of the recovery period, T levels had fallen in the EE₂ treated fish (Fig.
28 1B) and E₂ serum levels had increased in Tmx treated fish (Fig. 1D); however, these
29 effects were annulled in the fish fed with EE₂+Tmx (Fig. 1D).

30 Although no effect was observed in the expression levels of *star* gene after 5
31 days of EE₂ or Tmx dietary intake, after 25 days, they were down-regulated in the
32 gonad of EE₂ treated fish and up-regulated in the gonad of Tmx treated fish (Fig. 2A).
33 However, in EE₂+Tmx treated fish the transcription levels of *star* decreased at day 5

1 and increased at day 25 of exposure (Fig. 2A). After the recovery period, they remained
2 similar to those of control in all treatment groups (Fig. 2A).

3 Regarding the transcription of the genes coding for the steroidogenic enzymes
4 studied (Fig. 2B-G), *cyp11a1*, *hsd11b*, *srd5a* and *hsd3b* expression levels were down-
5 regulated after 5 days of exposure to all the treatments (Fig. 2B,D,F,G). However, after
6 25 days, EE₂ down-regulated the expression of *cyp11b1*, *hsd11b*, *srd5a* and *hsd3b* genes
7 (Fig. 2C,D,F,G), Tmx up-regulated the *hsd11b*, *srd5a* and *hsd3b* genes (Fig. 2D,F,G)
8 and EE₂+Tmx up-regulated the expression of *cyp11a1*, *hsd11b*, *srd5a* and *hsd3b* genes
9 (Fig. 2B,D,F,G). Interestingly, the expression of *cyp19a1a* was down-regulated by Tmx
10 and up-regulated by EE₂ and EE₂+Tmx (Fig. 2E). At the end of the recovery period, the
11 expression levels of all these genes, with the exception of the *cyp11a1*, were up-
12 regulated in EE₂ treated fish, (Fig. 2B), while any effect depended on the gene in
13 question in Tmx and EE₂+Tmx treated fish (Fig. 2B-G). Thus, the transcription of
14 *cyp11a1*, *cyp11b1* and *cyp19a1a* (Fig. 2B,C,E) was down-regulated, while the
15 transcription of *hsd11b* and *hsd3b* (Fig. 2D,G) was up-regulated, in Tmx treated fish.
16 Expression levels of *cyp11a1* and *hsd3b* (Fig. 2B,G) were similar in Tmx and EE₂+Tmx
17 treated fish, while those of *cyp11b1* and *srd5a* were similar in the control and EE₂+Tmx
18 treated fish (Fig. 2C,F). On the other hand, the expression of *hsd11b* and *cyp19a1a* in
19 the EE₂+Tmx treated fish was higher than in the control and lower than in EE₂ treated
20 fish (Fig. 2D,E).

21 ***EE₂ reduces seminal fluid volume and sperm concentration, while Tmx increases***
22 ***sperm concentration and motility***

23 After 5 days of treatment, EE₂ decreased the volume of seminal fluid to such an extent
24 that it was not possible to measure sperm concentration or motility (Table 2). Tmx did
25 not affect the volume of seminal fluid, or the sperm concentration or motility and
26 EE₂+Tmx did not affect seminal fluid volume or sperm motility but decreased the sperm
27 concentration (Table 2). After 25 days of EE₂ or Tmx treatments, seminal fluid volume
28 was unaffected but respective decrease and increase in sperm concentration were
29 evident. However, fish treated with EE₂+Tmx showed a decreased seminal fluid volume
30 and sperm concentration. Sperm motility was increased only in Tmx treated fish (Table
31 2). Moreover, no detectable seminal fluid was observed after the recovery period in EE₂
32 or EE₂+Tmx treated fish, while in Tmx treated fish both the seminal fluid volume and
33 sperm concentration were higher than in control fish (Table 2).

34 ***EE₂ and Tmx affect spermatogenesis in a different way***

1 The control fish remained in spermatogenesis throughout the experimental period. The
2 testicular area of the gonad was formed by tubules with a germinal epithelium
3 consisting of spermatogonia stem cells and cysts of germ cells in all developmental
4 stages (spermatogonia, spermatocytes and spermatids); a varying amount of free
5 spermatozoa was also observed in the lumen of the tubules (Fig. 3A). Interestingly,
6 scattered degenerative cysts with very condensed genetic material were observed (Fig.
7 3A).

8 Although the general morphology of the testicular area did not change after 5
9 days of EE₂ (Fig. 3B) or EE₂+Tmx treatment, more degenerative cysts were evident
10 than in control fish. However, after 25 days, the germinal epithelium was made up of
11 spermatogonia stem cells and some cysts of primary spermatogonia and no meiotic
12 germ cells were observed (Fig. 3C). After the recovery period, the germinal epithelium
13 of these two groups of fish still consisted of spermatogonia and Sertoli cells and
14 abundant interstitial tissue (Fig. 3D,E). However, while most of the tubules had a
15 collapsed lumen in EE₂ treated fish (Fig. 3D), some tubules with a small amount of free
16 spermatozoa were observed in the EE₂+Tmx treated fish (Fig. 3E). Testis morphology
17 in fish fed with Tmx did not show any noticeable change compared with that of control
18 fish during the treatment period or after resuming the commercial diet (data not shown).

19 Cysts of proliferating spermatogonia and spermatocytes were observed in
20 control fish (Fig. 4A) and in Tmx exposed fish (Fig. 4B) at all the time points analyzed.
21 However, in fish treated with EE₂ (Fig. 4C) and EE₂+Tmx, only some Sertoli cells were
22 immunolabelled with anti-PCNA after 25 days of treatment. Interestingly, after the
23 recovery period, while a few spermatogonia and Sertoli cells were seen to divide in the
24 testis of EE₂ treated fish (Fig. 4D), numerous cysts of proliferating spermatogonia and
25 some proliferative Sertoli cells were observed in the testis of EE₂+Tmx treated fish (Fig.
26 4E).

27 Five days of EE₂, Tmx or EE₂+Tmx treatment promoted a decrease in *dmrt1*
28 gene expression, the EE₂ treated group showing the strongest effect (Fig. 4F). However,
29 after 25 days of treatment, the expression level of *dmrt1* gene remained down-regulated
30 in EE₂-treated fish, while it was up-regulated in Tmx- and EE₂+Tmx-treated fish (Fig.
31 4F). After the recovery period, the expression levels of *dmrt1* gene were down-regulated
32 in the fish that had been exposed to EE₂ or EE₂+Tmx (Fig. 4F).

33 The expression levels of *fshr* (Fig. 5A), *lhr* (Fig. 5B) and *gper* (Fig. 5D) genes
34 decreased after 5 days of Tmx or EE₂+Tmx dietary intake, while the expression of *era*

1 was not affected by any treatment (Fig. 5C). After 25 days of treatment, the expression
2 levels of *lhr* were similar to control ones (Fig. 5B), while those of *fshr* were up-
3 regulated by all the treatments (Fig. 5A). The expression levels of *era* (Fig. 5C) and *gper*
4 (Fig. 5D) were increased by Tmx and EE₂+Tmx, the up-regulation seen in *gper* gene
5 expression being more pronounced in the EE₂+Tmx-treated than in Tmx-treated fish
6 (Fig. 5A-D). However, after the recovery period, the expression levels of *fshr* were up-
7 regulated in the EE₂-treated group (Fig. 5A), and those of *lhr* were up-regulated in the
8 EE₂-treated group and down-regulated in the EE₂+Tmx treated group (Fig. 5B), while
9 expression levels of *era* and *gper* had returned to control levels.

10 ***EE₂ and Tmx differently affect the immune response in the gonad***

11 Acidophilic granulocytes and B lymphocytes (IgM⁺ cells) were revealed by
12 immunolabelling with G7 (Fig. 6) and anti-IgM (Fig. 7), respectively. In control fish,
13 scattered or, occasionally, small groups of acidophilic granulocytes were located in the
14 interstitial tissue of the testicular area (Fig. 6A) or in the connective tissue that limited
15 the ovarian and testicular areas of the gonad. Although the localization of these cells did
16 not change at any time during the experimental period in any treatment, they were more
17 numerous after 25 days of EE₂, Tmx and EE₂+Tmx dietary intake (Fig. 6B,C).
18 Interestingly, after the recovery period, this increase was not observed in fish fed with
19 Tmx (Fig. 6E) but was evident in fish that had been fed with EE₂+Tmx and, especially,
20 EE₂ (Fig. 6D,F). IgM⁺ cell (Fig. 7) recruitment was triggered only by EE₂ after 5 days
21 of exposure (Fig. 7B). However, after the recovery period, an increase in the amount of
22 IgM⁺ cells was observed in the fish that had been fed with diets containing EE₂ or
23 EE₂+Tmx (Fig. 7C,D).

24 As regards the pro-inflammatory cytokine genes, *illb* and *tnfa*, the former was
25 up-regulated by EE₂ and Tmx after 25 days of exposure (Fig. 8A), while the *tnfa* was
26 down-regulated by Tmx after 5 days (Fig. 8B). Both cytokine gene expression levels
27 were down-regulated after 5 days and up-regulated after 25 days of EE₂+Tmx dietary
28 intake. After the recovery period, the expression levels of *tnfa* had only increased in the
29 EE₂ treated fish (Fig. 8B). Regarding the expression of the anti-inflammatory cytokine
30 gene *tgfb1*, its transcription was inhibited after 5 days of exposure with all the
31 treatments (Fig. 8C). However, the *tgfb1* expression was up-regulated after 25 days of
32 Tmx and EE₂+Tmx dietary intake and after the recovery period in all treated groups.

33 When the expression pattern of two metalloproteinase genes, *mmp 9* (Fig. 8D)
34 and *mmp13* (Fig. 8E), was analyzed, they were seen to have an almost inverted pattern

1 of expression. Thus, after 5 and 25 days of EE₂ exposure, the transcription of *mmp9*
2 gene had increased, whilst the transcription of *mmp13* gene had decreased. Tmx dietary
3 intake for 5 days triggered a decrease and an increase of the transcription levels of
4 *mmp9* and *mmp13* genes, respectively, while after 25 days of treatment, only the
5 transcription levels of *mmp9* gene increased. Interestingly, the expression of *mmp13*
6 was down-regulated after 5 days of EE₂+Tmx dietary intake, while the expression of
7 both *mmp9* and *mmp13* was up-regulated after 25 days of this treatment. On the other
8 hand, after the recovery period, the expression levels of *mmp9* were higher in all the
9 treated groups compared with the control, while *mmp13* transcription was only
10 increased in the fish treated with EE₂ or EE₂+Tmx.

11 Regarding the expression of some antigen recognition genes, the expression
12 levels of *mhc1a* (Fig. 8F) or *tlr9* (Fig. 8G) genes decreased after 5 days of EE₂, Tmx or
13 EE₂+Tmx treatments and after 25 days of EE₂ dietary intake. Interestingly, the *mhc1a*
14 transcription levels increased after 25 days of Tmx or EE₂+Tmx dietary intake. After
15 the recovery period, both genes were up-regulated in the EE₂ treated group, while only
16 the *mhc1a* gene was up-regulated in the Tmx and EE₂+Tmx treated groups.

17

18 **Discussion**

19 The expression level of the hepatic *vtg* has been widely used as a marker of estrogenic
20 endocrine disruption in fish (Hiramatsu et al., 2005; Bugel et al., 2013; Genovese et al.,
21 2014; Hultman et al., 2015; Saunders et al., 2015). The present study shows that both
22 EE₂, as described previously (Cabas et al., 2011, 2013), and Tmx act as estrogenic
23 endocrine disruptors in gilthead seabream males, since they up-regulate the expression
24 of the hepatic *vtg* gene. Tmx also increased the *vtg* gene expression in some fish species
25 (Sun et al., 2007; Benninghoff and Williams, 2008) and the VTG concentration in fish
26 homogenates of zebrafish males (Baumann et al., 2014), although it did not have any
27 effect in other fish species (Leaños-Castañeda et al., 2002; Maradonna et al., 2009).
28 However, when Tmx was applied with EE₂, a reduction in the EE₂-induced *vtg* gene
29 expression was observed, as reported in other fish species upon exposure to Tmx and
30 other estrogenic compounds (Leaños-Castañeda et al., 2002; Benninghoff and Williams,
31 2008; Maradonna et al., 2009; Sun et al., 2011b). Our results indicate that Tmx activates
32 some ERs at the same time as it reduces their availability for binding to EE₂, which is
33 consistent with the formation of the relatively stable Tmx-ER complex described by
34 Jordan et al. (1977).

1 Although exposure to EE₂ does not affect plasmatic levels of T and E₂ in some
2 fish (Swapna and Senthilkimaran, 2009; Colli-Dula et al., 2014), in gilthead seabream
3 adult males serum levels of the main fish sex steroid vary according to the reproductive
4 stage of the specimens and the EE₂ concentrations and time point analyzed (Cabas et al.,
5 2011, 2013). In this sense, no effect of EE₂ was observed in our study during treatment,
6 although it promoted an increase in T levels after the recovery period. As regards Tmx,
7 an increase in androgen plasma levels was observed in our study as also occurs in carp
8 males (Bottero et al., 2005), while in addition to this effect, we observed an increase in
9 E₂ serum levels upon short term treatment. However, longer treatment led to the
10 restoration of control levels of the sex steroid, which indicates that fish adjusted to
11 sustained exposure. Interestingly, combined exposure to EE₂ and Tmx counteract their
12 individual effects on serum androgen levels, which suggests an antagonistic action of
13 EE₂ and Tmx beyond their direct competition to bind some ERs.

14 Despite the unaffected steroid plasma levels seen in EE₂-treated fish, our data on
15 steroidogenic enzyme gene expression levels reflect an alteration in the steroidogenesis
16 capability of the specimens, as occurs in other fish species upon EE₂ exposure (Sridevi
17 et al., 2013; Colli-Dula et al., 2014). Thus, the gene expression of most of the
18 steroidogenic enzymes analyzed in this study fell after short exposure to EE₂ and Tmx,
19 whether separately or in combination. However, after longer treatment, the effects of
20 Tmx became the opposite of those promoted by EE₂ but were reinforced when both
21 compounds were applied together. Only the *cyp19a1a* gene showed a different
22 expression pattern, being down-regulated by Tmx, as reported in other fish (Kitano et
23 al., 2007) and up-regulated by EE₂+Tmx. In medaka males, the transcription of *star*,
24 *cyp11a* and *cyp19a* genes was up-regulated by EE₂+Tmx, but not by EE₂ exposure,
25 which led to the suggestion of a compensatory feedback in response to the anti-
26 estrogenic property of Tmx (Sun et al., 2011b). Our results, however, underline the
27 complexity of the action mechanisms of Tmx, which may, as occurs in mammals, bind
28 to more than one ER (Fitts et al., 2011) and recruit tissue-specific co-regulators that
29 would determine different downstream effects, depending on the cellular context
30 (McDonnell and Wardell, 2010).

31 The depletion of *dmrt1* gene expression in mammals leads to the loss of mitotic
32 germ cells, which precociously enter meiosis (Don et al., 2011). In addition, we found
33 that EE₂ decreases the expression levels of *dmrt1* gene, concomitant with a depletion of
34 meiotic cells in testis (Cabas et al., 2011, 2013). Thus, the consumption of meiotic cells

1 observed in the testis of fish exposed to EE₂, as reported also in catfish upon EE₂
2 exposure (Swapna and Senthilkimaran, 2009), or EE₂+Tmx for 25 days was probably
3 triggered by the initial decrease in the expression levels of *dmrt1*. In addition, E₂ caused
4 the mitotic division of germ cells in testicular fragments of Japanese eel *in vitro* (Miura
5 et al., 1999). Hence, initial decreases in the expression levels of *dmrt1* in fish exposed to
6 Tmx did not lead to the subsequent exhaustion of meiotic cells, probably due to the high
7 serum levels of E₂ occurring in these fish. Accordingly, the renewal of mitotic cells after
8 the recovery period in gilthead seabream treated with EE₂+Tmx could have been
9 triggered by the increase in the expression levels of *dmrt1*, aided by the high expression
10 levels of *era* and *gper*, which would imply a certain estrogenic effect even though E₂ in
11 serum is at control levels, by the end of treatment period. All these data support that E₂
12 has a role in spermatogonia stem cell renewal in gilthead seabream as also suggested in
13 Japanese eel (Miura et al., 1999). Moreover, if 11KT is needed for meiosis to begin, as
14 established for Japanese eel (Miura et al., 1999), the high 11KT serum levels in Tmx-
15 treated fish could have prevented the accumulation of degenerative cysts in the rest of
16 the treated groups following short treatment, which would be explained by impaired
17 spermatogonia stem cell divisions promoted by the decrease in expression levels of
18 *dmrt1* and the initiation of meiosis. Moreover, our data demonstrate that Tmx partially
19 neutralizes the effects of EE₂ on spermatogenesis, accelerating the recovery of the
20 spermatogenic process upon the cessation of exposure, although it was not able to
21 prevent the increase in interstitial tissue caused by EE₂ treatment reported in fish (Elias
22 et al., 2007; Kaptaner and Ünal, 2010) and also observed in our study.

23 Although no differences between control and Tmx-treated fish were observed in
24 testis morphology after 25 days of treatment, the spermatogenic process was somehow
25 affected by Tmx, as shown by the increased sperm concentration and higher motility
26 index. In addition, in Tmx-treated fish the expression levels of *cyp19a1a* gene
27 decreased, while those of *dmrt1* gene increased, which have been described as being
28 necessary to induce testicular differentiation and for the maintenance of testicular
29 function in fish, respectively (Marchand et al., 2000; Liarte et al., 2007; Guiguen et al.,
30 2010). The same was recorded during female-to-male sex reversal caused by Tmx in the
31 Southern catfish (Liu et al., 2010). FSH regulates Sertoli cell proliferation in sea bass
32 (Mazón et al., 2014) and the expression of *fshr* gene has been related with this process
33 and with early spermatogenesis (Rahman et al., 2003; Rocha et al., 2009). In gilthead
34 seabream, the sharp increase in *fshr* expression levels after 25 days of EE₂+Tmx
35 treatment could have helped the restoration of mitotic activity in the testis of these fish

1 after the recovery period. Thus, our data point to the existence of a testicular network
2 that involves the *dmrt1*, *era*, *gper* and *fshr* genes, whose rates of expression could
3 determine the rates of mitotic proliferation and entrance of germ cells into
4 spermatogenesis, aided by relevant plasma levels of E₂ and 11KT.

5 EE₂ affects the recruitment of leukocytes and the regulation of the cytokine
6 network in fish gonads (Cabas et al., 2011, 2013; Seemann et al., 2013), up-regulating
7 the expression of genes coding for the molecules involved in gilthead seabream (Cabas
8 et al., 2011, 2013). In the present study, the expression patterns of pro- and anti-
9 inflammatory cytokines (*illb*, *tnfa* and *tgfb*) were stimulated or decreased according to
10 the time of exposure, as occurred in fish treated with Tmx. However, in all cases,
11 treatment with EE₂+Tmx enhanced the effect of Tmx alone, indicating a non-
12 competitive but synergic action of both compounds on the expression of these genes. As
13 regards the expression patterns of *mmp* genes, those of *mmp9* agree with the recruitment
14 of acidophilic granulocytes in the testicular tissue of fish treated with EE₂, Tmx and
15 EE₂+Tmx. On the other hand, it has been suggested that EE₂ stimulates the ability of the
16 gonad to recognize and respond to pathogens (Cabas et al., 2011). In the present study,
17 however, the expression of *tlr9* and *mch1a* genes was up-regulated by EE₂ only when
18 applied together with Tmx for 25 days or after the recovery period. These data suggest
19 that the effect of endocrine disrupter on this process might not only depend on the
20 compound itself but, on the physiological state of the individuals.

21 The long-term effects of Tmx, supplied alone or with EE₂, on most parameters
22 studied were contrary to those caused by short treatment. Changes in the effects of Tmx
23 over time have also been described during tumour treatment (McDonnell and Wardell,
24 2010). Interaction with different ERs at different times could be involved in these
25 striking responses, as various *er* genes are expressed in fish, including those coding for
26 nuclear ER α , ER β -I and ER β -II (Nelson and Habibi, 2013) and the membrane-
27 associated GPER (Liu et al., 2009; Pang and Thomas, 2010). However, the similar
28 response of genes coding for ER α and GPER to the treatments could indicate a
29 homeostatic response to sustained exposure, resulting in an up-regulation of the
30 expression of the genes involved. Also, a non-receptor mediatory mechanism, such as
31 an inhibitory effect of the chemicals on the activity of steroidogenic enzymes, as
32 suggested by Colli-Dula et al. (2014), could contribute to the imbalance in the
33 reproductive process.

1 In the present study, the effects promoted by Tmx and/or EE₂ were neutralized
2 after the recovery period in the case of *era* and *gper* genes. However, neither the
3 expression levels of *vtg* gene, the genes coding for the steroidogenic enzymes analyzed,
4 nor serum steroid levels were restored to control values after 25 days of recovery.
5 Studies on the ability to recover from EE₂ exposure, mostly carried out on zebrafish,
6 have focused on the sexual differentiation and reproductive capacity (Hill and Janz,
7 2003; Nash et al., 2004; Schafers et al., 2007; Baumann et al., 2014). Developing catfish
8 maintained high expression levels of genes coding for steroidogenic enzymes up to 300
9 days after a 50-day exposure to EE₂ (Sridevi et al., 2013). Further studies are needed to
10 clarify the action mechanisms of these disruptive compounds and the ability of fish,
11 particularly mature fish, to recover from their effects.

12 In conclusion, our data indicate that Tmx acts as an endocrine disruptor in
13 gilthead seabream males. It has estrogenic effects, such as the up-regulation of
14 expression levels of hepatic *vtg* and gonadal *era* and *gper* genes and the increase of E₂
15 serum levels. In addition, Tmx has some effects that do not fit with an estrogenic action,
16 such as an increase in serum levels of androgens, and the up- and down- regulation of
17 expression levels of *dmrt1* and *cyp19a1a* genes, respectively. However, when combined
18 with the estrogenic compound EE₂, Tmx may counteract (*vtg*, *dmrt1*) or enhance (*fsH*,
19 *era*, *gper*) its effects on gene expression levels. It was also found that the disruptive
20 effect of EE₂ and/or Tmx on the reproductive process is not reversible after a 25 day
21 recovery period, since expression levels of hepatic *vtg* and other parameters studied
22 were still disturbed after this time.

23

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33

1 **Author contributions**

2 Conceived and designed the experiments: MPGH, AIGA, ECP, AGA. Performed the
3 experiments: MPGH, MCR, IC, ECP. Analyzed the data: MPGH, MCR, IC, AIGA,
4 ECP, AGA. Contributed reagents/materials/analysis tools: AIGA, ECP, AGA.
5 Contributed to the discussion of the result and the writing of the manuscript: MPGH,
6 AIGA, ECP, AGA.

7

8 **Author competing interests**

9 The author(s) declare that they have no competing interests.

10

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27 99.

28
29

30 **Figure legends**

31 **Figure 1:** *EE₂, Tmx and EE₂+Tmx increase the transcription levels of vtg gene and*
32 *differently affect the serum levels of main steroid hormones in gilthead seabream males.*
33 *The transcription levels of vtg in the liver (A) and the serum levels of T (B), 11KT (C)*
34 *and E₂ (D) of gilthead seabream males treated with 0 (control), 5 µg EE₂/g food, 100 µg*
35 *Tmx/g food or 5 µg EE₂+100 µg Tmx/g food for 5 and 25 days and after 25 days of*

1 reverting the commercial diet (d25 recovery). The asterisks denote statistically
 2 significant differences between the groups according to Tukey's test. * P < 0.05, ** P <
 3 0.01 and *** P < 0.001.

4 **Figure 2:** *EE₂, Tmx and EE₂+Tmx modulate the expression of genes coding for*
 5 *steroidogenic-relevant molecules in the gonad of gilthead seabream males.* The
 6 specimens were treated with 0 (control), 5 µg EE₂/g food, 100 µg Tmx/g food or 5 µg
 7 EE₂+100 µg Tmx /g food for 5 and 25 days, and after 25 days of reverting to the
 8 commercial diet (d25 recovery). The mRNA levels of *star* (A), *cyp11a1* (B), *cyp11b1*
 9 (C), *hsd11b* (D), *cyp19a1a* (E), *srd5a* (F), and *hsd3b* (G) were determined in the gonad
 10 by real-time reverse transcription polymerase chain reaction (RT-PCR). Data represent
 11 means ± S.E.M. of the gene expression from 5 independent fish/group and time. The
 12 asterisks denote statistically significant differences between the groups according to
 13 Tukey's test. * P < 0.05, ** P < 0.01 and *** P < 0.001.

14 **Figure 3:** *Effects of EE₂, Tmx and EE₂+Tmx dietary intake on the testicular*
 15 *morphology of gilthead seabream males.* Paraplast embedded sections (A-E) of the
 16 gonad of gilthead seabream males treated with 0 (control, C) (A) or 5 µg EE₂/g food (B,
 17 D), or 5 µg EE₂ + 100 µg Tmx /g food (C, E) for 5 (A, B) and 25 (C) days and after 25
 18 days of reverting to the commercial diet (rp, D, E) stained with hematoxylin-eosin (HE).
 19 White arrowheads: primary spermatogonia; white arrows: degenerative cysts; asterisk:
 20 spermatogonia cysts; Sc: spermatocytes, Sz: spermatozoa. Scale bar = 35 µm.

21 **Figure 4:** *Effect of EE₂, Tmx and EE₂+Tmx on cell testicular proliferation and on the*
 22 *dmrt1 gene expression.* Paraplast embedded sections immunostained with anti-
 23 proliferating cell nuclear antigen (anti-PCNA) serum (A-E) and the transcription levels
 24 of *dmrt1* gene (F) of the gonad of gilthead seabream males treated with 0 (control, C)
 25 (A), 5 µg EE₂/g food (C, D), 100 µg Tmx/g food (B) or 5 µg EE₂+100 µg Tmx /g food
 26 (E) for 5 (A, B) and 25 (C) days and after 25 days of reverting to commercial diet (rp,
 27 D, E). (A-E) white arrowheads: spermatogonia stem cells; white arrows: proliferative
 28 primary spermatogonia; asterisks: cyst of proliferative spermatogonia; black arrows:
 29 proliferative Sertoli cells. Scale bar = 35 µm. The mRNA levels of *dmrt1* (F) were
 30 determined in the gonad by real-time reverse transcription polymerase chain reaction
 31 (RT-PCR). Data represent means ± S.E.M. of the gene expression from 5 independent
 32 fish/group and time. The asterisks denote statistically significant differences between
 33 the groups according to Tukey's test. * P < 0.05, ** P < 0.01 and *** P < 0.001.

34 **Figure 5:** *EE₂, Tmx and EE₂+Tmx modulate the expression of genes coding for*
 35 *hormone receptors in the gonad of gilthead seabream males.* The specimens were

1 treated with 0 (control), 5 μg EE_2/g food, 100 μg Tmx/g food or 5 μg EE_2+100 μg
 2 Tmx/g food for 5 and 25 days and after 25 days of reverting to the commercial diet (d25
 3 recovery). The mRNA levels of *fshr* (A), *lhr* (B), *era* (C) and *gper* (D) were determined
 4 in the gonad by real-time reverse transcription polymerase chain reaction (RT-PCR).
 5 Data represent means \pm S.E.M. of the gene expression from 5 independent fish/group
 6 and time. The asterisks denote statistically significant differences between the groups
 7 according to Tukey's test. ND, not detected; * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$.

8 **Figure 6:** *EE₂, Tmx and EE₂+Tmx recruited acidophilic granulocytes into the testis of*
 9 *gilthead seabream.* Paraplast embedded sections of the testis of gilthead seabream
 10 males treated with 0 (control, C) (A), 5 μg EE_2/g food (D), 100 μg Tmx/g food (B, E) or
 11 5 μg EE_2+100 μg Tmx/g food (C, F) for 5 (A) and 25 (B, C) days and after 25 days of
 12 reverting to the commercial diet (rp, D-F) immunostained with the serum against
 13 gilthead seabream acidophilic granulocytes (G7, black arrows). Scale bar = 35 μm .

14 **Figure 7:** *EE₂, and EE₂+Tmx, but not Tmx, recruited B-lymphocytes into the testis of*
 15 *gilthead seabream.* Paraplast embedded sections of the testis of gilthead seabream
 16 males treated with 0 (control, C) (A), 5 μg EE_2/g food (B, C) or 5 μg $\text{EE}_2 + 100$ μg Tmx
 17 /g food (D) for 5 days (A, B) and after 25 days of reverting to the commercial diet (rp,
 18 C, D) immunostained with the serum against gilthead seabream IgM. Black arrows: B-
 19 lymphocytes stained with the anti-gilthead seabream IgM serum. Scale bar = 35 μm .

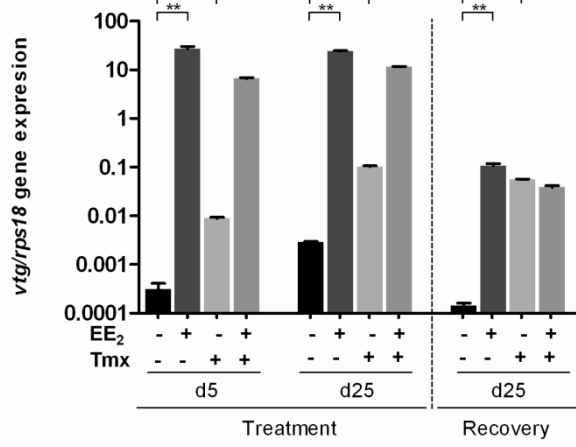
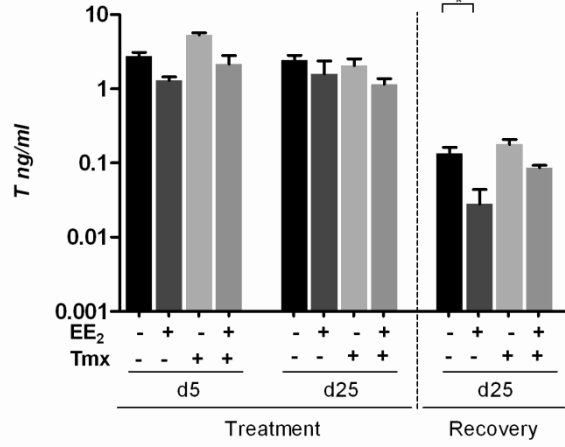
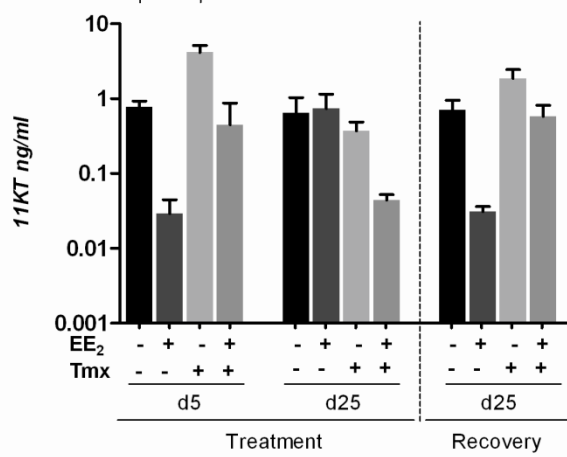
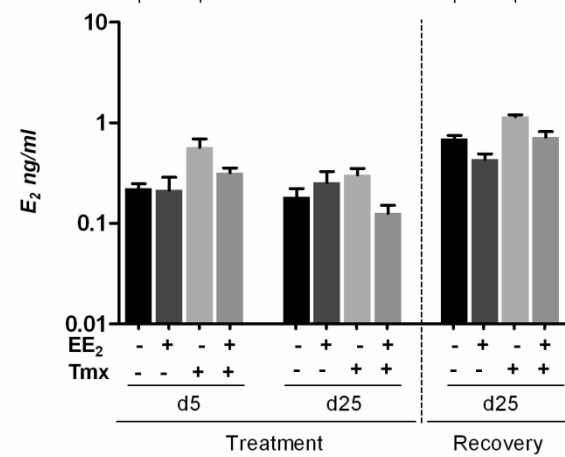
20 **Figure 8:** *EE₂, Tmx and EE₂+Tmx modulate the expression of genes coding for some*
 21 *immune-relevant molecules in the gonad of gilthead seabream males.* The specimens
 22 were treated with 0 (control), 5 μg EE_2/g food, 100 μg Tmx/g food or 5 μg EE_2+100 μg
 23 Tmx/g food for 5 and 25 days and after 25 days of reverting to the commercial diet (d25
 24 recovery). The mRNA levels of *il1b* (A), *tnfa* (B), *tgfb1* (C), *mmp9* (D), *mmp13* (E),
 25 *mhc1a* (F), and *tlr9* (G) were determined in the gonad by real-time reverse transcription
 26 polymerase chain reaction (RT-PCR). Data represent means \pm S.E.M. of the gene
 27 expression from 5 independent fish/group and time. The asterisks denote statistically
 28 significant differences between the groups according to Tukey's test. * $P < 0.05$, ** $P <$
 29 0.01 and *** $P < 0.001$.

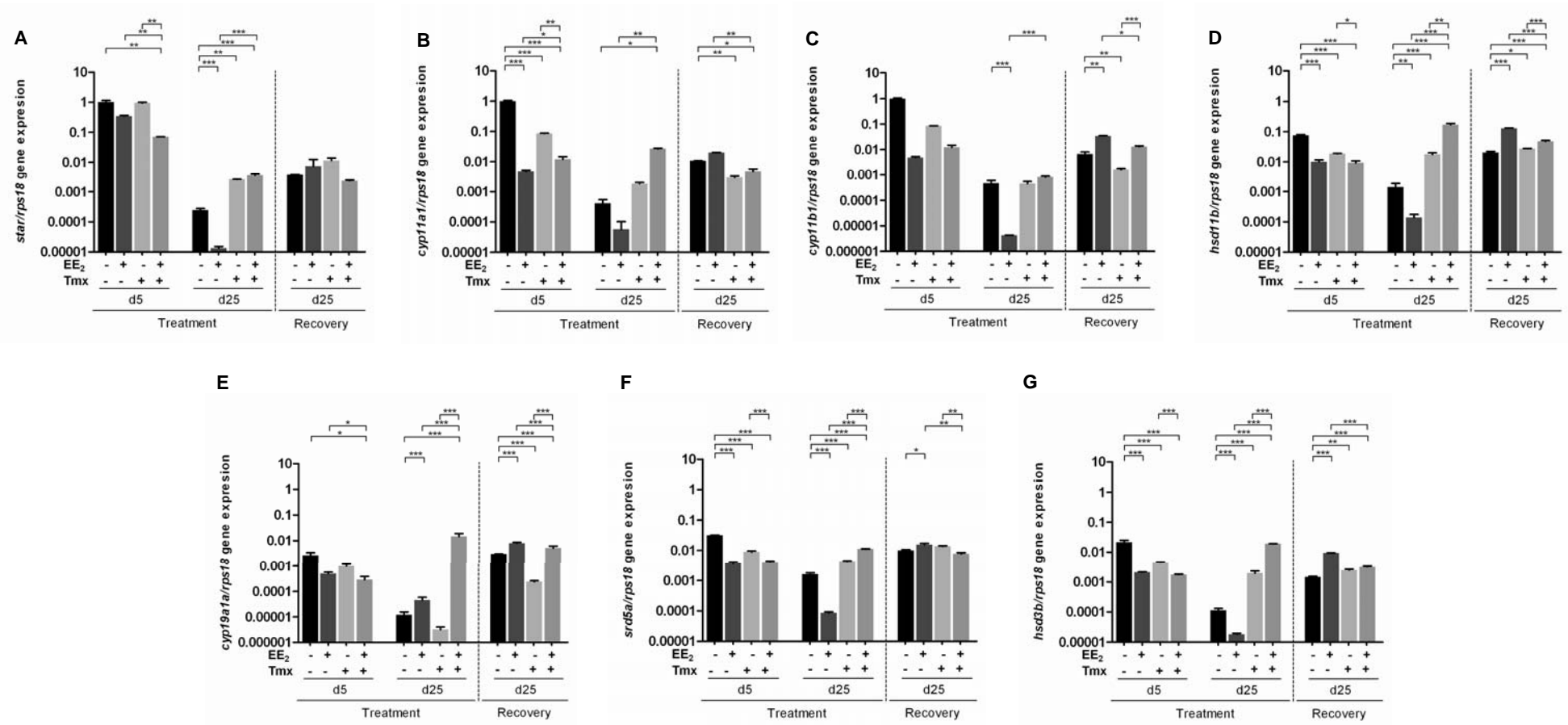
1 **Table 1:** Gene accession numbers and primer sequences used for gene expression
 2 analysis by real time PCR.
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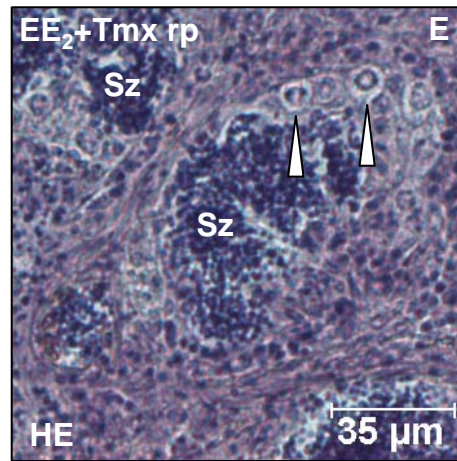
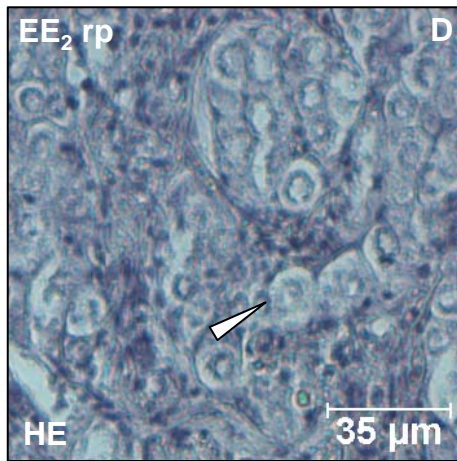
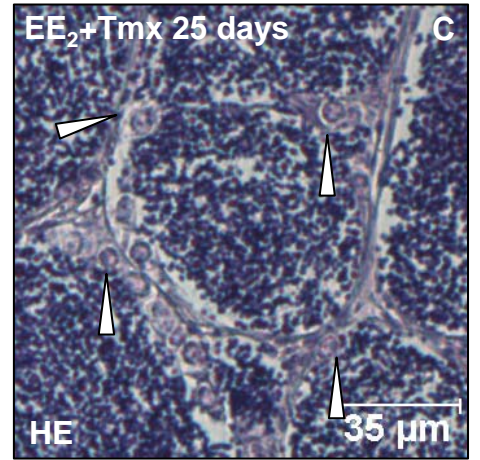
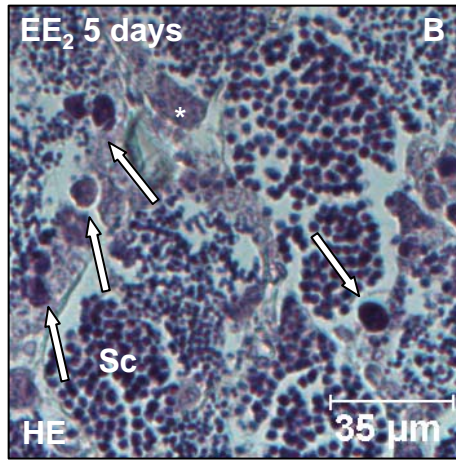
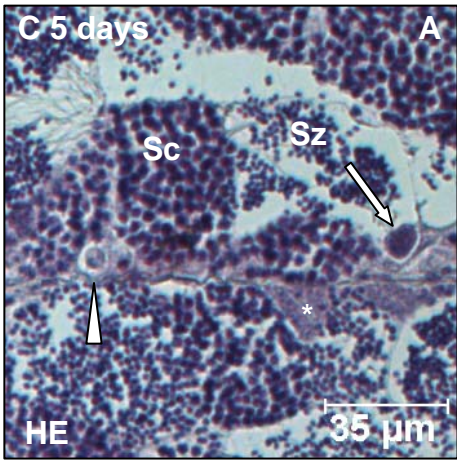
Gene	Accession number	Name	Sequence (5'-3')
<i>vtg</i>	AF210428	F1	CTGCTGAAGAGGGACCAGAC
		R1	TTGCCTGCAGGATGATGATA
<i>star</i>	AM905934	F1	ACATCGGGAAGGTGTTCAAG
		R1	TCTCTGCAGACACCTCATGG
<i>cyp11a1</i>	FM159974.1	F	CGCTGCTGTGGACATTGTAT
		R	CATCATGTCTCCCTGGCTTT
<i>cyp11b1</i>	FP332145	F	GCTATCTTTGGACCCCATCA
		R	CTTGACTGTGCCTTTCAGCA
<i>hsd11b</i>	AM973598	F	AGACATGGGCAACGAGTCAG
		R	TCCACATCTCCCTCCCACAT
<i>cyp19a1a</i>	AF399824	F2	CAATGGAGAGGAAACCCTCA
		R2	ATGCAGCTGAGTCCCTGTCT
<i>srd5a</i>	AM958800	F	TGCACTTTCGTGACTCTGCT
		R	TTTCGCACAAGACGTCCAGA
<i>hsd3b</i>	HS985587	F	GGAGGACAAACTGGTGGAGG
		R	ACATTCTCCGTTCCGGTGAC
<i>dmrt1</i>	AM493678	F	GATGGACAATCCCTGACACC
		R	GGGTAGCGTGAAGGTTGGTA
<i>fshr</i>	AY587262	F2	TCCCACTACGGATCCTCATC
		R2	AACGGGAACAGTCAGTTTG
<i>lhr</i>	AY587261	F2	ATACACGACCACGCATTCAA
		R2	CGCCGGTAACTTCTTGAGAG
<i>era</i>	AF136979	F	GCTTGCCGTCTTAGGAAGTG
		R	TGCTGCTGATGTGTTTCCTC
<i>gper</i>	HG004163	F1	GGCTGCCAGAGAATGTCTTC
		R1	GTGGCCTGTGAGTGGGTAGT
<i>il1b</i>	AJ277166	F2	GGGCTGAACAACAGCACTCTC
		R3	TTAACACTCTCCACCCTCCA
<i>tnfa</i>	AJ413189	FE1	TCG TTCAGAGTCTCCTGCAG
		RE3	CATGGACTCTGAGTAGCGCGA
<i>tgfb1</i>	AF424703	F	AGAGACGGGCAGTAAAGAA
		R	GCCTGAGGAGACTCTGTTGG
<i>mmp9</i>	AM905938	F1	GGGGTACCCTCTGTTCGATTT
		R1	CCTCCCCAGCAATATTCAGA
<i>mmp13</i>	AM905935	F	CGGTGATTCTTACCATTG
		R	TGAGCGGAAAGTGAAGGTCT
<i>mhc1a</i>	AY292461	F	CCAGAGCTTCCCTCAGTGTC
		R	CATCTGGAAGGTTCCATCGT
<i>tlr9</i>	AY751798	F2	GGAGGAGAGGGACTGGAT
		R2	GATCACACCGTCACTGTCTC
<i>rps18</i>	AM490061	F	AGGGTGTGGCAGACGTTAC
		R	CTTCTGCCTGTTGAGGAACC

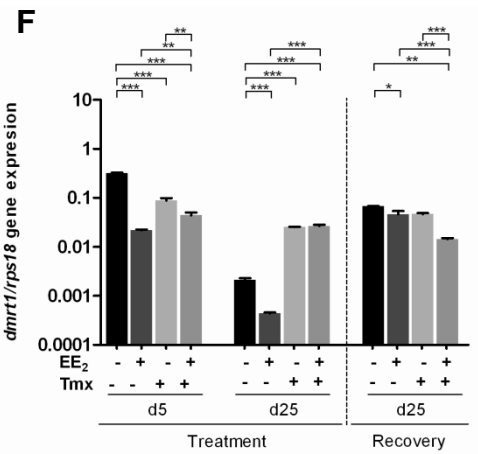
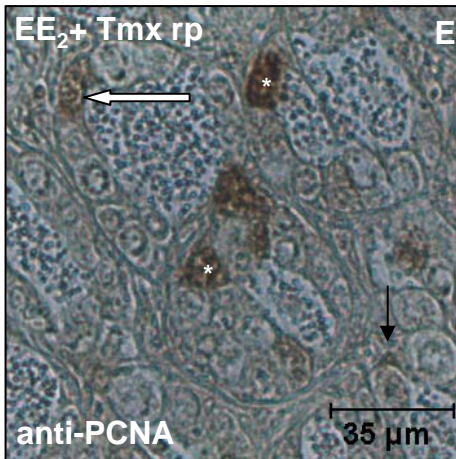
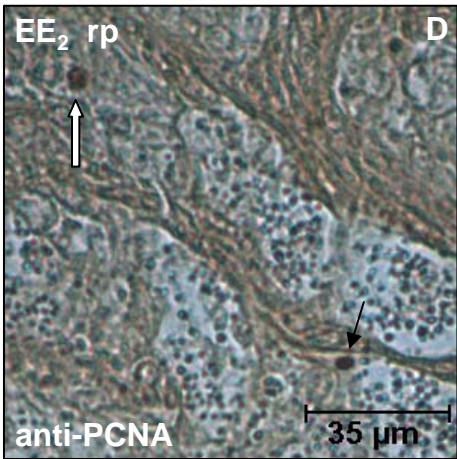
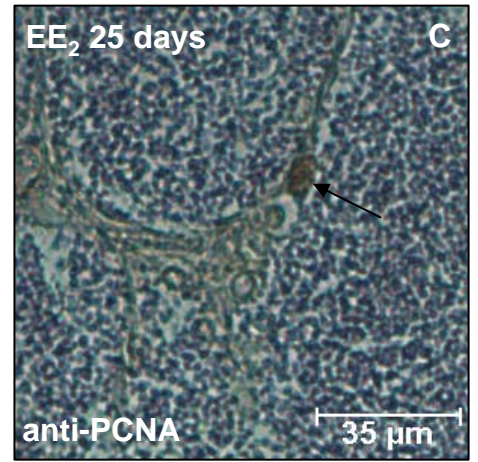
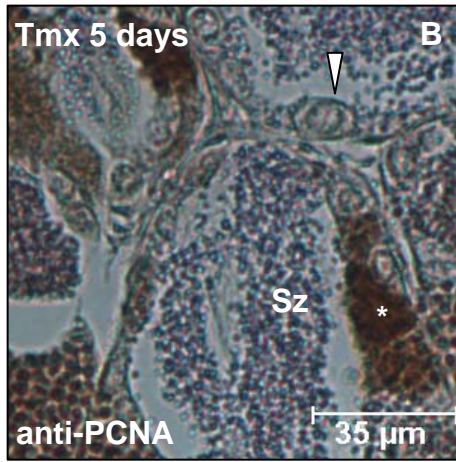
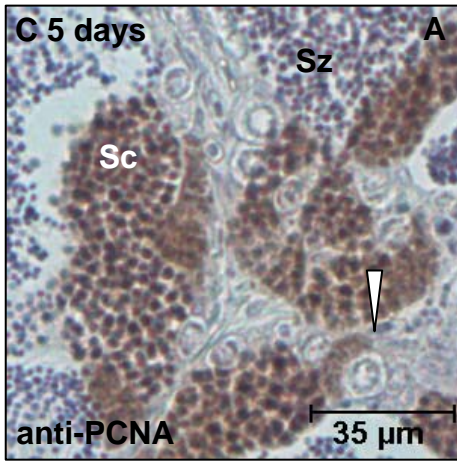
Table 2. Effects of the dietary intake of 5 μg 17 α -ethynylestradiol (EE₂)/g food, 100 μg tamoxifen (Tmx)/g food or 5 μg EE₂ +100 μg Tmx/g food during 5 and 25 days (Treatment) and after resuming normal diet (Recovery) during 25 days on volume of seminal fluid (mL), sperm concentration (cell/mL) and motility index. Data represent means \pm SEM of six independent fish per group. Asterisks denote statistically significant differences between treatment and control groups according to a Student t test (*P \leq 0.1; **P \leq 0.05). ND: not detected

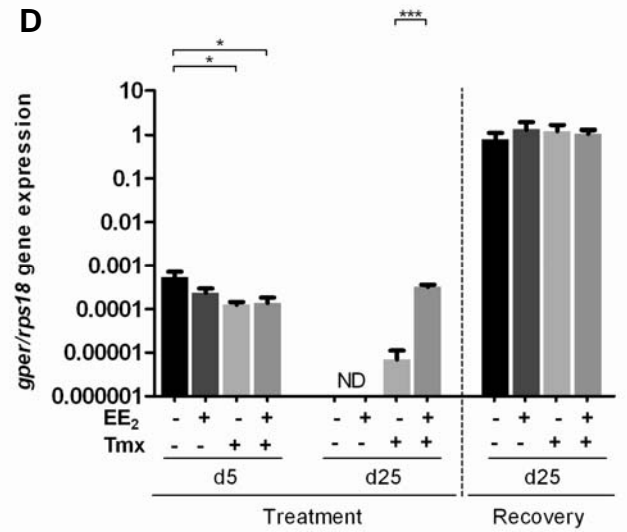
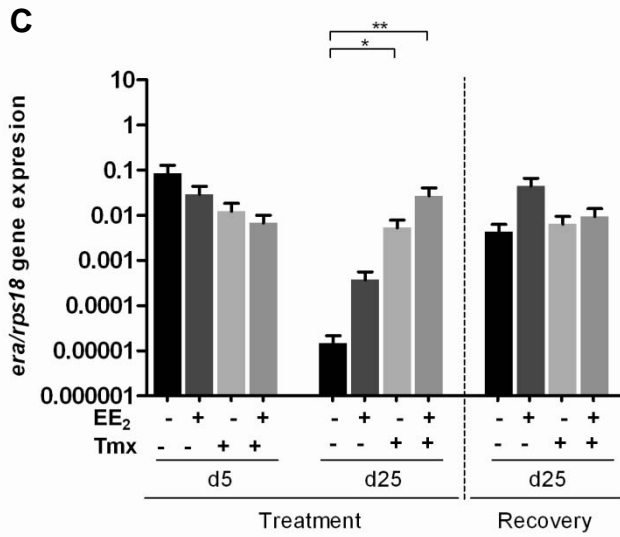
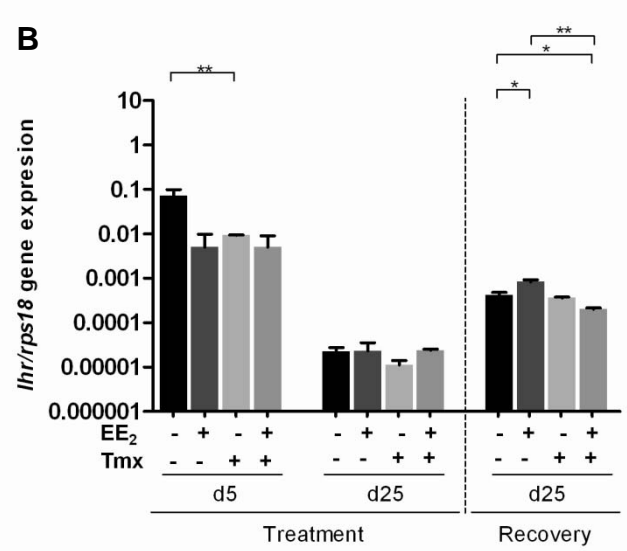
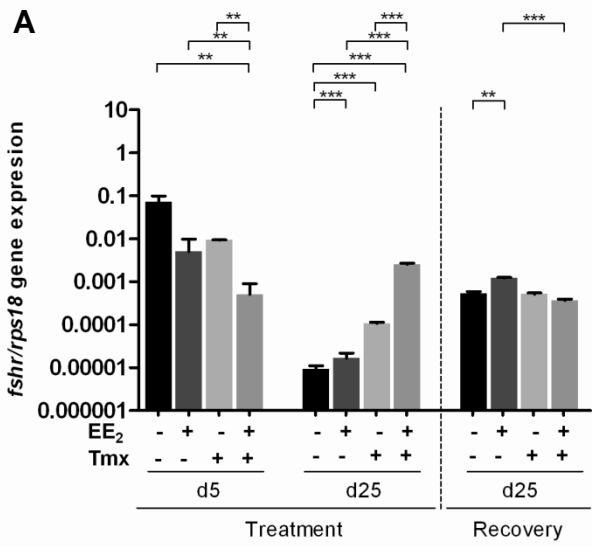
Treatment	Volume of seminal fluid (mL)			Sperm concentration (cell/mL)			Sperm motility index		
	Treatment		Recovery	Treatment		Recovery	Treatment		Recovery
	5 days	25 days	25 days	5 days	25 days	25 days	5 days	25 days	25 days
Control	0.83 \pm 0.14	1.03 \pm 0.31	0.9 \pm 0.24	16.37 \pm 3.0	(9.32 \pm 0.60) $\times 10^3$	(7.66 \pm 0.44) $\times 10^3$	0.83 \pm 0.31	0.33 \pm 0.25	2.08 \pm 0.27
EE ₂	0.02 \pm 0.02**	0.68 \pm 0.45	ND	ND	(4.54 \pm 2.96) $\times 10^3$ **	ND	ND	0.67 \pm .033	ND
Tmx	0.73 \pm 0.18	2.67 \pm 1.19	2.65 \pm 0.88*	9.17 \pm 2.29	(13.33 \pm 0.75) $\times 10^3$ **	(9.81 \pm 0.60) $\times 10^3$ **	1.38 \pm 0.33	1.42 \pm 0.20*	1.58 \pm 0.30
EE ₂ +Tmx	0.73 \pm 0.23	0.08 \pm 0.07**	ND	8.5 \pm 1.95**	(3.31 \pm 2.88) $\times 10^3$ **	ND	1.0 \pm 0.37	0.58 \pm 0.37	ND

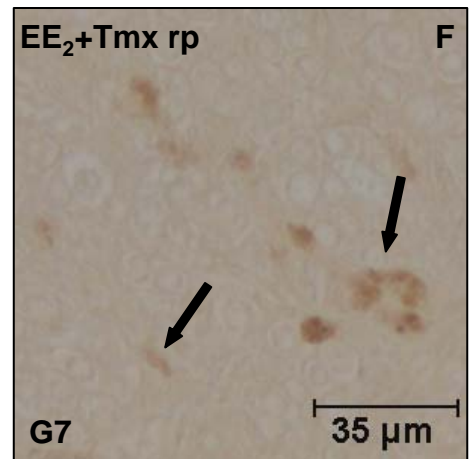
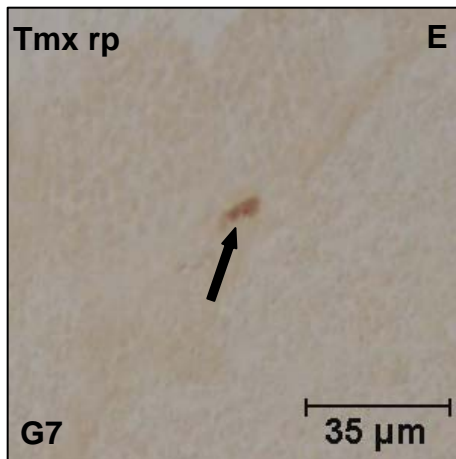
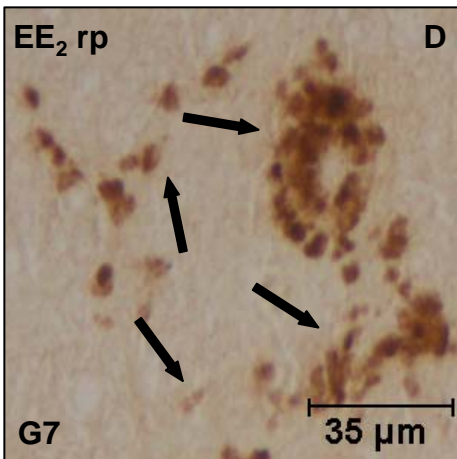
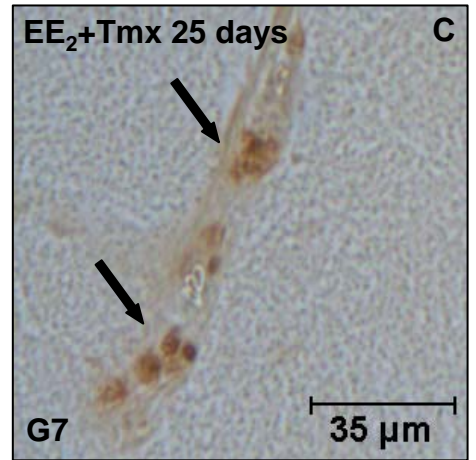
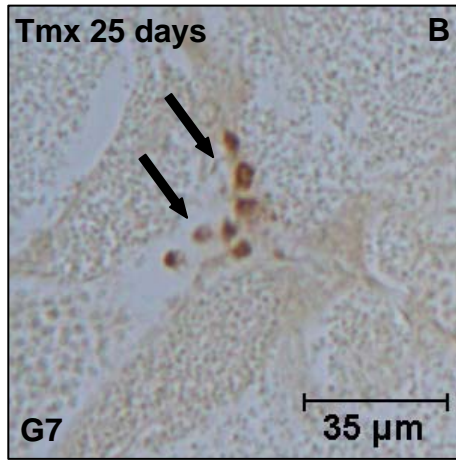
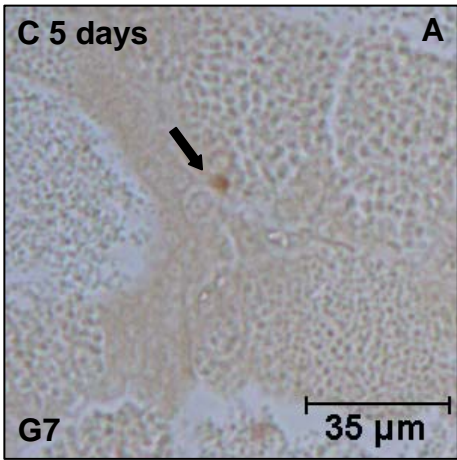
A**B****C****D**

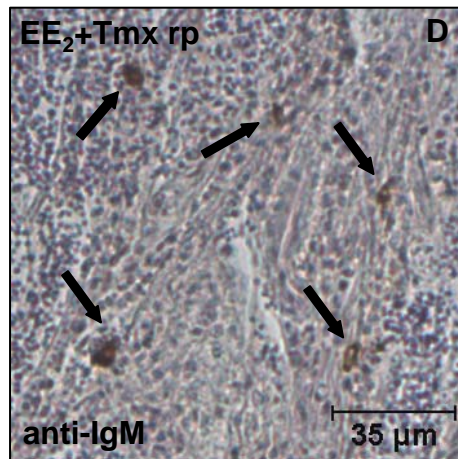
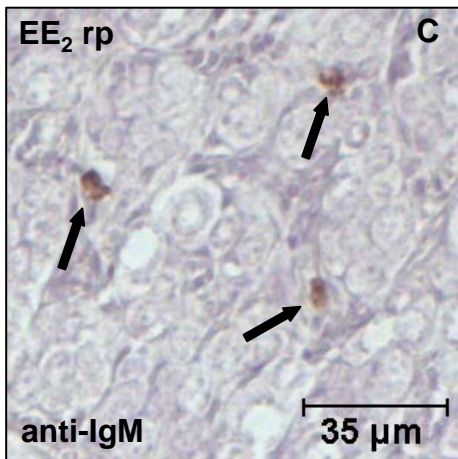
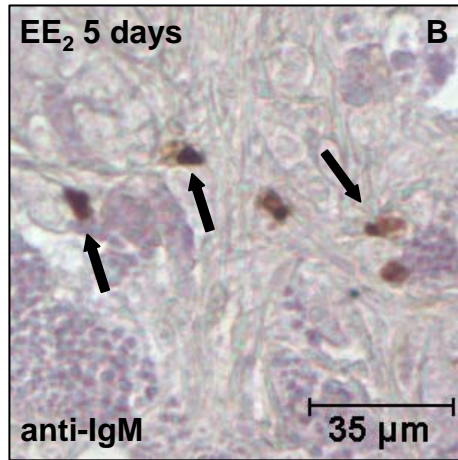
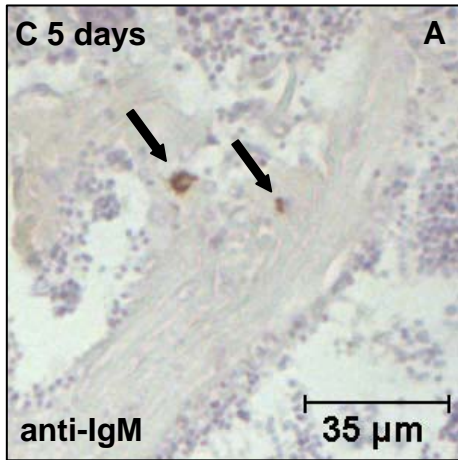


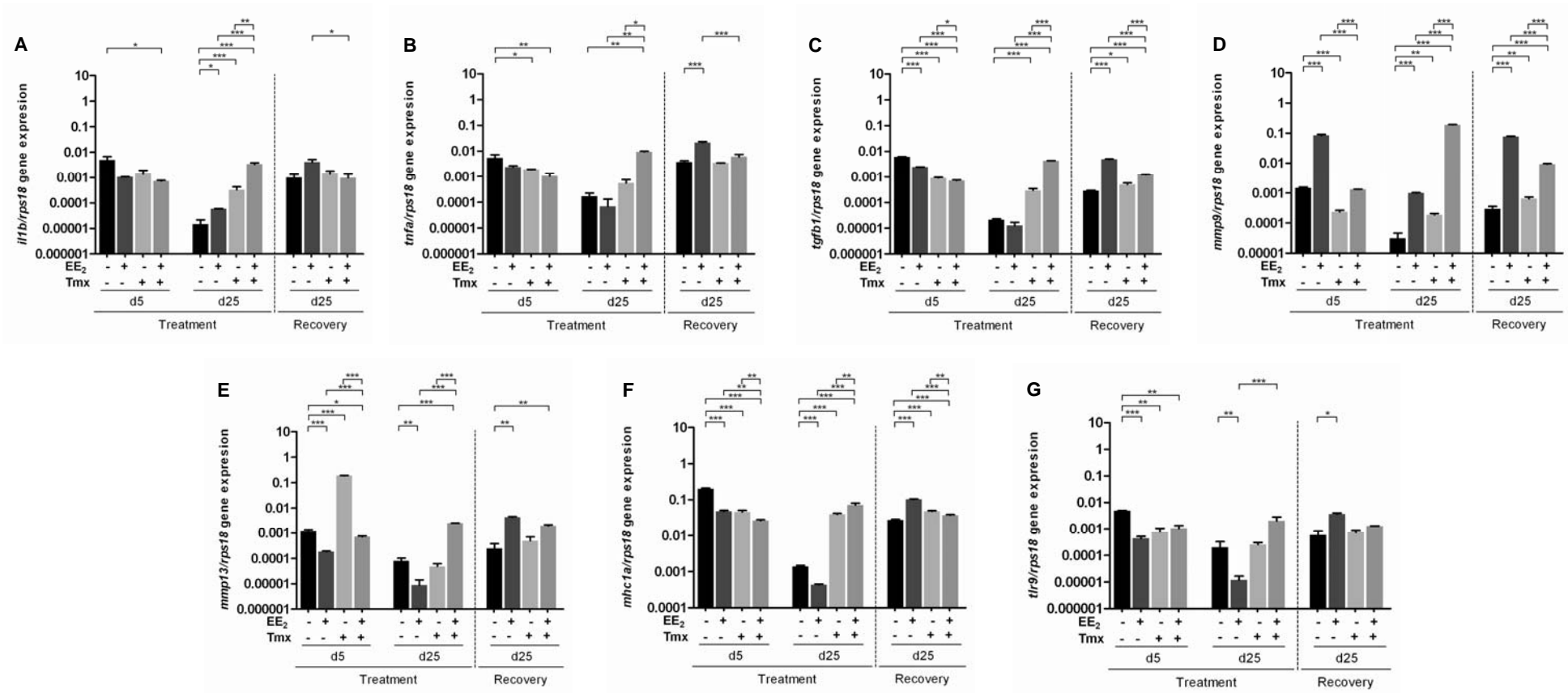












Highlights

Tmx disrupts the gilthead seabream reproductive process including the gonad immune response

Tmx alters the expression profile of hepatic vitellogenin gene in males of a protandrous fish

Tmx can counteract or enhance the effects of EE₂ on reproductive several parameters

The disruptive effects of Tmx and/or EE₂ on reproduction are not reversed after a 25-day recovery period