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Short communication

Effect of sex-steroid hormones, testosterone and estradiol, on humoral immune parameters of gilthead seabream

A. Cuesta [a], L. Vargas-Chacoff [b], A. García-López [c], F.J. Arjona [b], G. Martínez-Rodríguez [c], J. Meseguer [a], J.M. Mancera [b], M.A. Esteban [a,*]

[a] *Department of Cell Biology, Faculty of Biology, University of Murcia, 30100 Murcia, Spain*
[b] *Department of Biology, Faculty of Marine Sciences, University of Cadiz, 11510 Puerto Real, Cádiz, Spain*
[c] *Andalusian Institute for Marine Sciences, CSIC, 11510 Puerto Real, Cádiz, Spain*

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Abstract

The role of sex-steroid hormones, testosterone (T) and 17β-estradiol (E2), on the humoral immune parameters of the teleost gilthead seabream *Sparus aurata* was studied attempting to deepen on the knowledge of the immune–reproductive system interactions. Fish were injected intraperitoneally with coconut oil containing different dosages of T (0, 2, or 5 μg g^{-1} body weight [bw]) or E2 (0, 1, or 2 μg g^{-1} bw) and sampled 1, 3, and 7 days later. Hormonal levels and immune parameters (complement, peroxidase and antiprotease activities and IgM levels) were determined in plasma. Plasma hormone levels peaked at 1 day post-injection decreasing thereafter. Treatment with T significantly increased both complement and peroxidase activities after 3 days of injection but antiprotease activity and IgM levels remained unchanged. Treatment with E2 enhanced complement activity 1 day post-injection while decreased it after 3 and 7 days. However, peroxidase activity increased at 3 and 7 days post-injection while total IgM levels decreased. Implications of T and E2 in the immune-reproductive system interactions were discussed.

Keywords: Testosterone; Estradiol; Immune-reproductive system; Complement; IgM; Teleosts; Gilthead seabream

In addition to their role in fish reproduction, sex-steroids are known to be involved in fish growth [1], digestion and food utilization [2], gut transport [3], shifts in body composition [4], intermediary metabolism [5], osmoregulation [6,7] and immunity [8–15]. Focusing on the last one, sex-steroid hormones, such as 17β-estradiol (E_2) or testosterone (T), affect mammalian specific and non-specific defence responses [16] while several cytokines have been related to the growth and development of follicles, ovulation, luteal development, spermatogenesis and steroidogenesis [17]. Although some information exists confirming similar patterns in teleost fish less attention has been paid to this group of vertebrates. In rainbow trout, *Oncorhynchus mykiss*, the elevation of sex-steroids (E_2 and T in females, and T and 11-ketotestosterone [11-KT] in males during sexual maturation) was correlated with immunosuppression and increased disease susceptibility, likely due to the decreased antibody secreting cells and circulating levels [8–10],

* Corresponding author. Tel.: +34 968367665; fax: +34 968363963.
E-mail address: aesteban@um.es (M.A. Esteban).

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but not in cyprinids like goldfish and common carp [11,14]. In sparids, enhancement of gilthead seabream serum complement and agglutinating activities [18] coincided with the post-spawning period, when both E_2 and T peaks have been reported [19,20] while in goldlined seabream, *Rhabdosargus sarba*, disease status was correlated with high T plasma but low E_2 levels [15]. Direct administration of T or E_2 was shown to suppress certain fish immune parameters [8–10,21–27] or not [12,13,25] although *in vivo* studies are still scarce. In gilthead seabream, *in vitro* leucocyte incubation with 11-KT increased their respiratory burst activity and the accumulation of the cytokine IL-1β, while incubation with E_2 was inhibitory [28]. Moreover, injection of males with E_2 effected mobilization of acidophilic granulocytes from the head-kidney to the testis [29]. Discrepancies in the immunomodulatory effects of fish sex-steroid hormones and the scarcity of *in vivo* studies demand further evaluation to understand the neuroendocrine–immune system crosstalk in teleosts. Therefore, with this aim, we studied the effect of the sex-steroid hormones testosterone and 17β-estradiol on gilthead seabream immune parameters after injection.

Sexually immature male gilthead seabream (101 ± 4 g bw) were provided by Planta de Cultivos Marinos (CASEM, Universidad de Cádiz, Puerto Real, Cádiz, Spain) and transferred to the laboratories of the Faculty of Marine Sciences (Puerto Real, Cádiz). Immature specimens of approximately 1-year-old were used in order to eliminate the possible interference of endogenous sex hormones (the first sexual maturation in male gilthead seabream occurs when fish reach approximately 400 g bw [30]). Fish were acclimatised to seawater (38 p.p.t. salinity, 1103 mOsm/kg H_2O) in 500 L aquaria in an open system. During the experimental period (June–July 2005), fish were maintained under natural photoperiod (36°N, 6°W) and at constant temperature (21 °C), being fed on commercial dry pellets (Dibaq-Diproteg) at a daily rate of 1% body weight. Fish were fasted for 24 h before sampling. The experiments here described follow the European Union Council (86/609/EU) and the University of Cádiz (Spain) guidelines for the use of laboratory animals. Fish were caught by netting, lightly anaesthetized with 2-phenoxyethanol (0.05% v/v; Sigma), weighed and injected intraperitoneally (5 μl g^{-1} bw) with a slow-release coconut oil implant alone (control) or containing T (2 or 5 μg g^{-1} bw) (Experiment 1) or E_2 (1 or 2 μg g^{-1} bw) (Experiment 2). Thereafter, one group of untreated fish was sampled serving as uninjected control at time 0 days. Fish ($n = 10$ per group and time) were sampled after 1, 3, and 7 days post-implantation. For sampling, fish were deeply anaesthetized with 2-phenoxyethanol (0.1% v/v) and weighed. Blood was obtained from the caudal peduncle using ammonium-heparinized syringes and the plasma samples, obtained by centrifugation (10,000 g, 1 min, 4 °C), were immediately frozen in liquid nitrogen and stored at −80 °C until use. The dose of hormones and the administration procedure were similar to those previously described for gilthead seabream [6,7,29]. Analytical techniques included determination of plasma levels of E_2 and T and humoral immune parameters. Enzyme-linked immunosorbent assay (ELISA) of steroids [29,31] started with the extraction of 3.5–8 μl plasma in 1–1.5 ml methanol. Steroid standards were purchased from Sigma–Aldrich, mouse anti-rabbit IgG monoclonal antibody, and specific anti-steroid antibodies and enzymatic tracers (steroid acetylcholinesterase conjugate) were obtained from Cayman Chemical Company while microtiter plates (MaxiSorp™) were obtained from Nunc. Standards and extracted plasma samples were run in duplicate. The lower limits of detection (90% of binding, ED90) were 24 pg/ml plasma for E_2 and 19 pg/ml plasma for T. The inter- and intra-assay coefficients of variation were 4.9% and 2.9 ± 0.3% for E_2 ($n = 3$), and 11.5% and 4.0 ± 0.3% for T ($n = 3$), respectively. Main cross-reactivity (>1%; given by the supplier) was detected with estradiol-3-glucoronide (17%) and estrone (4%) for E_2 antibody and with 5α-dihydrotestosterone (27.4%), 5β-dihydrotestosterone (18.9%), androstenedione (3.7%), and 11-KT (2.2%) for T antibody.

Among the humoral immune parameters, we determined the alternative complement [32], peroxidase [33] and anti-protease [34] activities as well as the total immunoglobulin (Ig) M level [35] in the plasma. Firstly, the activity of the alternative complement pathway was assayed using sheep red blood cells (SRBC; Biomedics) as targets [32]. SRBC were washed in phenol red-free Hank's buffer (HBSS) containing Mg^{2+} and EGTA (ethylene glycol tetraacetic acid) and resuspended at 6% (v/v) in HBSS. Aliquots (100 μl) of test plasma as complement source, diluted in HBSS, were added to 100 μl of SRBC in a flat-bottomed 96-well plate to give final plasma concentrations ranging from 40 to 0.31%. After incubation for 90 min at 22 °C and the removal of unlysed erythrocytes, the optical density was read at 550 nm in a plate reader. 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) (percentage of haemolytic activity with respect to the maximum) was estimated and the lysis curve for each specimen was obtained by plotting Y/(1-Y) against the volume of plasma added (ml) on a $\log_{10}$–$\log_{10}$ scaled graph. The volume of plasma producing 50% haemolysis (ACH_{50}) was determined and the number of ACH_{50} units/ml was obtained for each specimen. To measure peroxidase activity [33], 5 μl of plasma was diluted with 50 μl of HBSS without Ca^{2+} or

Mg^{2+} in flat-bottomed 96-well plates. Then, 100 μl of a solution containing 80 μM 3,3′,5,5′-tetramethylbenzidine hydrochloride (TMB; Sigma) and 2.5 mM H_2O_2 were added. The colour-change reaction was stopped by adding 50 μl of 2 M sulphuric acid and the optical density was read at 450 nm in a plate reader. The wells without plasma were used as blanks. The peroxidase activity (units/ml plasma) was determined defining one unit of peroxidase as that which produces an absorbance change of 1 OD. The antiprotease activity [34] was measured from 20 μl of seabream plasma, mixed with 50 μl of a standard trypsin solution (2 mg/ml) and incubated for 10 min at 22 °C. Samples were then incubated with 100 μl of azocasein (1% in 0.1 M phosphate buffer, pH 7.2) followed by an incubation with 20% trichloroacetic acid. Following centrifugation, supernatants were diluted with an equal volume of 1 N NaOH and the absorbance measured at 450 nm and the results expressed as the percentage of trypsin inhibition compared with a plasma-free blank. Finally, plasma total IgM levels were measured by an indirect ELISA [35]. Briefly, flat-bottomed 96-well plates were coated overnight with seabream plasma (plasma diluted 1/500 in 50 mM carbonate–bicarbonate buffer, pH 9.6). Samples were blocked with bovine serum albumin and incubated for 1 h with the primary antibody (mouse anti-gilthead seabream IgM monoclonal antibody; Aquatic Diagnostics Ltd., 1/100 in blocking buffer). After incubation with the secondary antibody anti-mouse IgG-HRP (1/1000 in blocking buffer), samples were developed with TMB and H_2O_2. The plates were read at 450 nm in a plate reader. Negative controls consisted of samples with or without plasma or primary antibody, and these OD values were subtracted from each sample value. Immune parameters were represented as means + SE and analysed by one-way analysis of variance (ANOVA, $P \leq 0.05$) and a test of comparison of means.

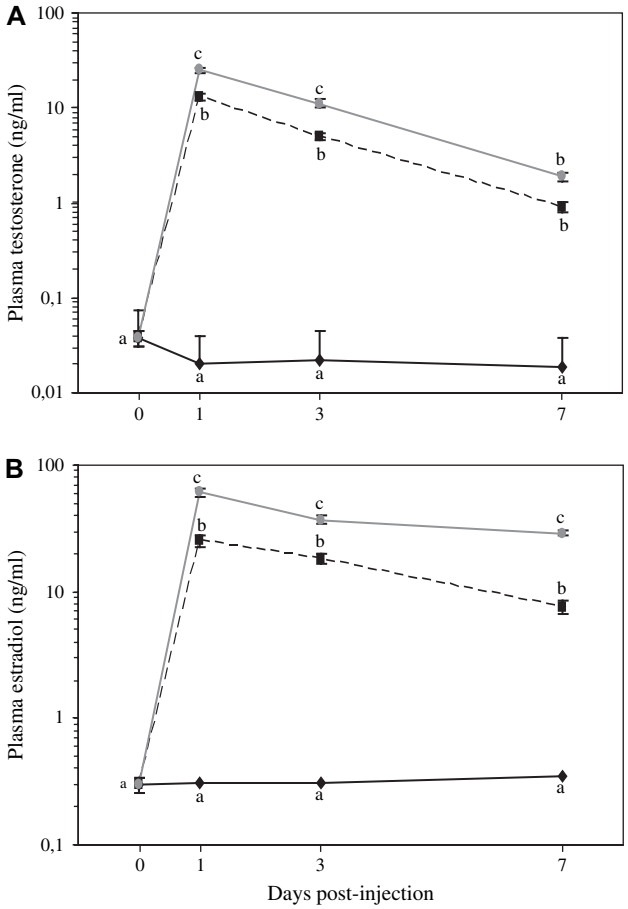

Fig. 1. Plasma levels of testosterone (A) and 17β-estradiol (B) in gilthead seabream after intraperitoneal injection. (A) Fish were injected with coconut oil with 0 (control; -◆-), 2 (-■-) or 5 (-●-) μg testosterone g^{-1} bw. (B) Gilthead seabream specimens were injected with coconut oil with 0 (control; ◆), 1 (■) or 2 (-●-) μg 17β-estradiol g^{-1} bw. Data represent mean + SE ($n = 10$). Different letters indicate statistically significant differences (ANOVA one-way, $P \leq 0.05$) between groups at the same sampling time.

The results show that hormonal levels and immune parameters of control fish were not different from those of uninjected fish (time 0; data not shown) indicating that the differences might be attributed to the hormone presence and not to handling or injection of the vehicle. Moreover, plasmatic levels of T and E_2 were significantly enhanced after hormonal treatment (Fig. 1), increasing in a dose- and time-dependent manner, peaking 1 day after injection and decreasing thereafter. As regards the immune parameters, single injection of T significantly increased humoral immune parameters 3 days after treatment (Fig. 2). Complement and peroxidase activities were significantly enhanced in fish treated with either 2 or 5 μg g^{-1} bw while the increase of total IgM with the latter dose was not significant. Little variations were detected at other sampling times. Interestingly, E_2 treatment differently affected gilthead seabream

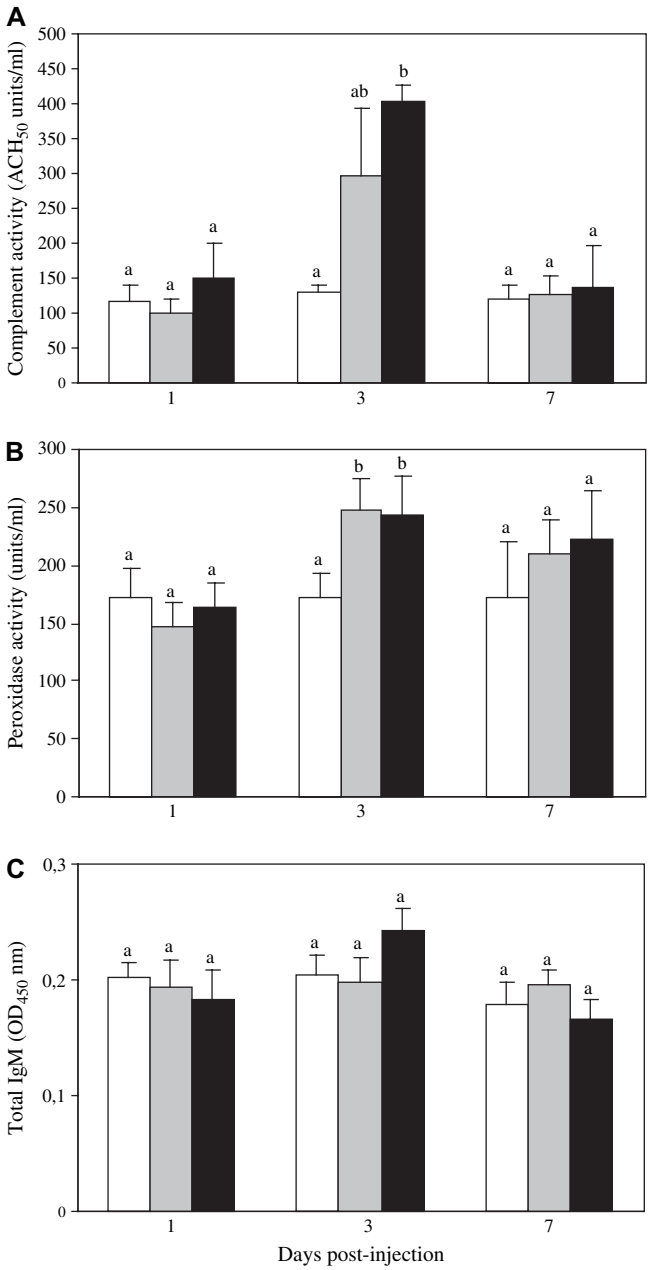

Fig. 2. Effect of testosterone on gilthead seabream humoral immune parameters. (A) Complement activity. (B) Peroxidase activity. (C) Total IgM level. Fish received an intraperitoneal injection of coconut oil alone (control; open bars) or with 2 (grey bars) or 5 (black bars) μg testosterone g^{-1} bw. Different letters indicate statistically significant differences (ANOVA one-way, $P \leq 0.05$) between groups at the same sampling time.

humoral immune parameters (Fig. 3). Complement activity was dose-dependently increased ($P < 0.05$) 1 day post-injection, while the peroxidase activity was augmented at 3 and 7 days. On the other hand, there was a concomitant inhibition of complement activity and IgM levels after 3 and 7 days of E_2 treatment. Antiprotease activity was unchanged by either T or E_2 (data not shown).

The bi-directional communication between reproductive and immune systems has been widely demonstrated and accepted in mammals but it is not completely understood in teleost fish. Overall, some studies have reported that

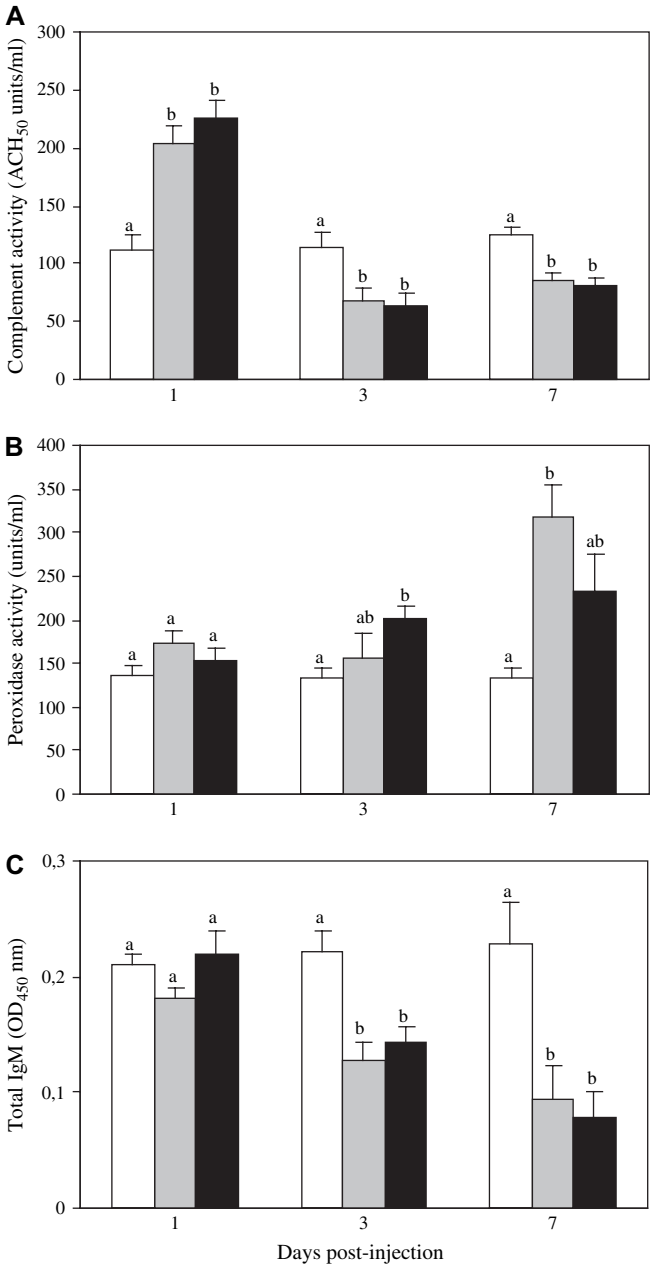

Fig. 3. Effect of 17β-estradiol on gilthead seabream humoral immune parameters. (A) Complement activity. (B) Peroxidase activity. (C) Total IgM level. Fish received an intraperitoneal injection of coconut oil alone (control; open bars) or with 1 (grey bars) or 2 (black bars) μg 17β-estradiol g^{-1} bw. Different letters indicate statistically significant differences (ANOVA one-way, $P \leq 0.05$) between groups at the same sampling time.

increments of sex-steroid levels during sexual maturation or hormonal-administration led to a strong inhibition of the fish immune response and disease resistance while other authors have failed to find this correlation [8–10,15,21–29]. Such contradictory results and the lack of knowledge in this field prompted us to design this study where the effects of treatment with T and E_2 on gilthead seabream immune response were determined. In this sense, the single injection of sex-steroid hormones, T and E_2, in immature male seabream induced a great increase in circulating plasma levels throughout the trials being maximum 1 day post-injection as observed earlier [6,7,29]. The administration route resulted in a much higher circulating hormone levels at days 1 and 3 than those found in a normal reproductive cycle while the levels at day 7 were close to those found in some stages of the cycle [6,7,20,29]. Therefore, some pharmacological effects might be observed.

As regards the immunomodulatory actions, T treatment significantly increased gilthead seabream serum complement and peroxidase activities while antiprotease activity and total IgM levels were unaffected. However, T and/or 11-KT inhibited antibody secreting cells and IgM production in several teleost fish species [9,10,13,21]. Moreover, T suppressed the production of superoxide anion, nitric oxide and phagocytosis of common carp leucocytes *in vitro* and *in vivo* [26,27]. On the contrary, goldlined seabream specimens diseased with vibriosis had elevated T plasma levels and leucocyte phagocytic activity [15]. Strikingly, T also increased respiratory burst activity and IL-1β production of gilthead seabream phagocytes *in vitro* [28] as occurs in rat macrophages [36]. Although some of the suppressive effects have been ascribed to the tendency of T to induce apoptosis and kill fish leucocytes [12,13,37], such effects seem to be related to the presence of androgen receptors in fish leucocytes [37]. More studies focusing on these aspects will throw light onto the mechanisms involved in the fish leucocyte regulation by testosterone.

E_2 treatment enhanced complement activity 1 day post-injection and peroxidase activity after 3 and 7 days. Concomitantly, E_2 treatment suppressed complement activity and production of IgM at the latest experimental time points. These results coincide with the suppression of IgM synthesis and inhibition of IgM producing cells in rainbow trout [9,10] but differ from the non-effect observed in common carp [13]. E_2 also decreased disease resistance and inhibited the leucocyte mitogen-induced proliferation, chemotaxis, superoxide anion and nitric oxide production and phagocytosis in cyprinids, such as goldfish and common carp, salmonids and hybrid tilapia *Oreochromis niloticus niloticus* × *O. aureus* [8–10,15,23–27]. Moreover, goldlined seabream diseased with vibriosis showed very low levels of circulating E_2 concomitantly with increased leucocyte phagocytosis [15]. Strikingly, and contrary to the role of T, E_2 did not induce apoptosis in common carp leucocytes [13]. Moreover, E_2 significantly reduced respiratory burst and IL-1β production in male gilthead seabream leucocytes [28] while increasing acidophil mobilization from the head-kidney to the testis after intraperitoneal injection [29]. The presence and expression of estrogen receptors (ERα and ERβ) have been found in gilthead seabream liver, gut, and kidney but not in organs involved in the immune response, such as the gills and head-kidney [38,39]. However, the immunosuppressive effect of E_2 seen in gilthead seabream head-kidney leucocytes [28] suggests the presence of estrogen receptors in such cells although there is no information confirming this hypothesis. Further characterization of the mechanisms whereby E_2 modulates the immune system cells will help to elucidate the reproductive-immune system interactions in fish.

In conclusion, both T and E_2 treatments differently affect complement and peroxidase activities and IgM levels and do not follow the proposed immunosuppressive patterns. These data clearly support the interaction between the reproductive and the fish immune systems, but further studies are needed in order to characterize the mechanisms of communication between both systems.

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