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17Beta-Estradiol Triggers Postspawning in Spermatogenically Active Gilthead Seabream (*Sparus aurata* L.) Males[1]

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ABSTRACT

The testis is a tightly controlled dynamic tissue. In mammals, there is growing evidence that estrogen plays a role in the regulation of testicular functions. In teleosts, high levels of 17beta-estradiol (E_2) in serum correlate with the end of spermatogenesis, spawning, and the initiation of postspawning stages when spermatogonia are the main cell types in the testis. Moreover, E_2 modulates leukocyte functions in several teleost species. We hypothesized, therefore, that E_2 would induce the infiltration of acidophilic granulocytes and cause a resumption of testicular cell proliferation in spermatogenically active gilthead seabream males. Several studies of this species have reported that supraphysiological doses of E_2 are needed to induce histological and developmental changes in males. In fact, as gilthead seabream is a protandrous hermaphrodite teleost, long exposures (6–14 wk) to high doses of E_2 result in feminization of the males. Taking all this into account, we sharply increased E_2 levels during short times by i.p. injecting E_2 diluted in coconut oil as the vehicle and sampled the fish after 7, 13, and 18 days to assess the effects that E_2 had on spermatogenesis. It was observed that E_2 levels in plasma increased, while 11-ketotestosterone (11-KT) and testosterone (T) levels remained unaltered. However, 11-KT and T levels strongly increased in control fish 18 days postinjection. The most relevant result of our study was that E_2 accelerates the final events of spermatogenesis, inhibits the proliferation of spermatogonia in early stages, and induces some of the processes that usually occur during postspawning, such as the infiltration of acidophilic granulocytes and the apoptosis of primary spermatogonia. Strikingly, neither the shedding of spermatozoa nor an increase in the proliferative rate of spermatogonia stem cells was observed, probably because of the lack of other necessary stimuli, such as the increase in T levels that takes place during normal postspawning.

acidophilic granulocytes, estradiol, immunology, male sexual function, postspawning, seasonal reproduction, spermatogenesis, teleosts, testis

INTRODUCTION

Spermatogenesis is a complex process in which spermatogonia divide (renewal of spermatogonia stem cells and mitotic proliferation of spermatogonia), reduce their chromosome content by meiosis, and differentiate into spermatozoa. In fish, spermatogenesis occurs in a cystic structure in which all germ cells develop synchronously surrounded by a cohort of Sertoli cells, which also proliferate and nurse one germ cell type at a time [1, 2]. Therefore, the fish testis is an excellent model for studying spermatogenesis regulation. The gilthead seabream is a seasonally breeding, protandrous hermaphrodite teleost whose reproductive cycle, during the male phase, is divided into four stages: spermatogenesis, spawning, postspawning, and resting [1]. We have already demonstrated that the spawning stage in gilthead seabream is characterized by the shedding of spermatozoa and the lowest cell proliferative rates of the whole reproductive cycle, while during the postspawning stage, the proliferative rates are resumed concomitantly with primary spermatogonia apoptosis and a massive infiltration of acidophilic granulocytes, whose activities are heavily impaired [1, 3].

In mammals, there is growing evidence that estrogen plays a role in spermatogenesis. The infertility observed in estrogen receptor alpha (ERα) and aromatase knockout mice highlights the essential role of estrogen in spermatogenesis [4]. In fish, 17β-estradiol (E_2) promotes early spermatogonia renewal, although in some species, such as medaka, high concentrations (100 nM) of synthetic estrogen result in an inhibitory effect [5–7]. However, E_2 on its own is not able to induce all the stages of spermatogenesis [8]. Interestingly, in several species, including the gilthead seabream, increased levels of E_2 in serum correlate with the end of spermatogenesis, spawning, and postspawning stages, when spermatogonia are the main cell type in the testis [9, 10]. In addition, study of the reproductive hormone receptors in salmonid leukocytes has provided evidence that supports an immunoregulatory role for these steroids in fish [11]. In goldfish and carp, E_2 injected i.p. inhibited the immune response [12, 13], while both E_2 and testosterone (T) affected gilthead seabream leukocyte functions in vitro [14].

Taking all of the above into account, we hypothesized that E_2 induces a postspawning stage in spermatogenically active gilthead seabream males by promoting the infiltration of acidophilic granulocytes and the renewal of spermatogonia, as observed after the shedding of spermatozoa. In the gilthead seabream, supraphysiological doses of E_2 are needed to bring about observable histological and developmental changes in males [15]. Moreover, long exposures (6–14 wk) to supraphysiological doses of E_2 resulted in feminization of the males, whereas short-time exposures did not [15, 16]. Bearing this in mind, we exposed gilthead seabream males to supraphysiological doses of E_2 for 18 days to avoid feminization and analyzed

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its effect on testicular development and, more specifically, on testicular acidophilic granulocytes.

MATERIALS AND METHODS

Fish

Specimens of male gilthead seabream, with a body weight (bw) of 400 ± 20 g, were provided by Planta de Cultivos Marinos (Universidad de Cádiz, Puerto Real, Cádiz, Spain) and transferred to the laboratories at the Faculty of Marine Science (Puerto Real, Cádiz, Spain). Fish were acclimatized to seawater in 500-L aquaria in an open system. During the experiment (December 2004), fish were maintained under natural photoperiod and constant temperature (18°C) and fed daily with 1% bw of commercial dry pellets (Dibaq-Diprotg SA, Segovia, Spain). Fish were fasted for 24 h before sampling.

Experimental Design

Fish were caught by netting and then lightly anesthetized with 0.05% v/v 2-phenoxyethanol (Sigma). They were next weighed and injected i.p. with 5 μl/g bw of slow-release coconut oil (Sigma) as the vehicle (control) or the same containing 5 μg/g bw of E_2 (Sigma). Specimens (5–11 fish per group within each time period) were sampled at 7, 13, and 18 days postinjection (dpi). The dose of E_2 and the administration procedure were similar to those previously described for gilthead seabream [17, 18]. Specimens were weighed and injected i.p. with 50 mg/kg bw of 5-bromo-2′-deoxyuridine (BrdU; Sigma) 2 h before sampling. Fish were deeply anesthetized with 0.1% v/v 2-phenoxyethanol and weighed. Blood was obtained in ammonium-heparinized syringes from the caudal peduncle. Plasma samples were obtained by centrifugation of blood and immediately frozen and stored at −80°C until used. The gonads and head-kidneys (the bone marrow equivalent of fish) were removed and processed for light microscopy as described below. After removal, the gonads were weighed. The experiments described comply with the Guidelines of the European Union Council (86/609/EU) and the Bioethical Committee of the Universities of Cádiz (Spain) and Murcia (Spain) for the use of laboratory animals.

Light Microscopy

Gonads and head-kidneys were fixed in 4% paraformaldehyde solution, embedded in paraffin (Paraplast Plus; Themo Electron, Waltham, MA), and sectioned at 5 μm. After dewaxing and rehydration, some sections were stained with hematoxylin-eosin to determine the reproductive stage of each fish, or they were immunostained as described below.

Immunocytochemical Staining

After dewaxing and rehydration, some sections were incubated for 40 min in peroxidase-quenching solution (H_2O_2 in methanol, 1:9) to eliminate the endogenous peroxidase. The sections were then rinsed in Coon buffer (0.01 M sodium diethylbarbiturate and 0.1 M NaCl [pH 7.4]) and in Coon buffer containing 0.01% BSA and 0.2% Triton X-100 (CBT). After a 30-min incubation with skimmed milk powder in Coon buffer to block the nonspecific reaction, they were rinsed in CBT and incubated for 2 h at room temperature with a monoclonal antibody specific to gilthead seabream acidophilic granulocytes (G7) [19] at the optimal dilution of 1:10. After washing in CBT, the sections were exposed to anti-mouse immunoglobulin G (IgG) (whole-molecule) peroxidase conjugate (Sigma) diluted 1:100 for 1 h at room temperature. The sections were then washed in CBT and in 0.5 M Tris-HCl buffer (pH 7.6). The peroxidase activity for the immunoreactions was shown by incubation with 0.05% 3,3′-diaminobenzidine tetrahydrochloride (DAB) in Tris-HCl buffer (pH 7.6) containing 0.05% H_2O_2 for 15 min at room temperature. Some of the sections were lightly counterstained with hematoxylin. The specificity of the reactions was determined by omitting the first antiserum.

To determine cell proliferation, the sections were incubated in peroxidase-quenching solution for 40 min, in 1% periodic acid at 60°C for 30 min, and in 0.5% skimmed milk powder in PBS for 30 min. Afterward, they were incubated with a monoclonal antibody anti-BrdU (Caltag) at the optimal dilution of 1:500 in PBS for 2 h at room temperature. Subsequently, sections were washed in PBS, incubated with anti-mouse IgG (whole molecule) peroxidase conjugate (Sigma), and diluted 1:100 for 1 h at room temperature, and the reaction was revealed by incubation with DAB, as described previously.

In Situ Detection of DNA Fragmentation

The TUNEL assay was performed to identify apoptotic cells (in situ cell death detection kit; Roche). After hydration, gonad sections were permeabilized for 8 min with 0.1% sodium citrate and 0.1% Triton X-100 at room temperature, washed twice with PBS, and incubated for 1 h at 37°C in a humidified chamber with 50 μl of the TUNEL reaction mixture containing terminal deoxynucleotidyl transferase (TdT) and fluorescein-deoxyuridine triphosphate, following the supplier's guidelines. Negative controls were processed in an identical manner, except that the TdT enzyme was omitted. Positive controls were also performed treating the sections with DNase I (3–3000 U/ml; Sigma) in 50 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, and BSA (1 mg/ml) for 10 min at room temperature to induce DNA strand breaks before labeling. Slides were examined with an Axiolab (Zeiss) fluorescence microscope. Incorporated fluorescein was detected by anti-fluorescein antibody Fab fragments conjugated with peroxidase, and the reaction was shown by incubation with DAB, as described previously.

Analytical Techniques

Plasma levels of E_2, T, and 11-ketotestosterone (11-KT) were quantified by ELISA analysis following the method described by Rodríguez et al. [20]. Steroids were extracted from 3.5 to 8.5 μl of plasma in 1–1.3 ml of methanol (Panreac). E_2 and T standards were purchased from Sigma-Aldrich. The 11-KT standard, mouse anti-rabbit IgG monoclonal antibody, and specific anti-steroid antibodies and enzymatic tracers (steroid acetylcholinesterase conjugates) were obtained from Cayman Chemical. Microtiter plates (MaxiSorp) were purchased from Nunc. A standard curve from 6.1×10^{-4} to 2.5 ng/ml (0.03–125 pg/well) was established in all the assays. Standards and extracted plasma samples were run in duplicate. The lower limit of detection for all assays was 33 pg/ml. The interassay coefficients of variation at 50% binding were 3.4% for E_2 (n=2), 1.8% for T (n=2), and 4.5% for 11-KT (n=2). The intraassay coefficients of variation (calculated from the sample duplicates) were 4.7% ± 0.8% for E_2, 2.2% ± 0.4% for T, and 2.4% ± 0.3% for 11-KT assays. Details on cross-reactivity for specific antibodies

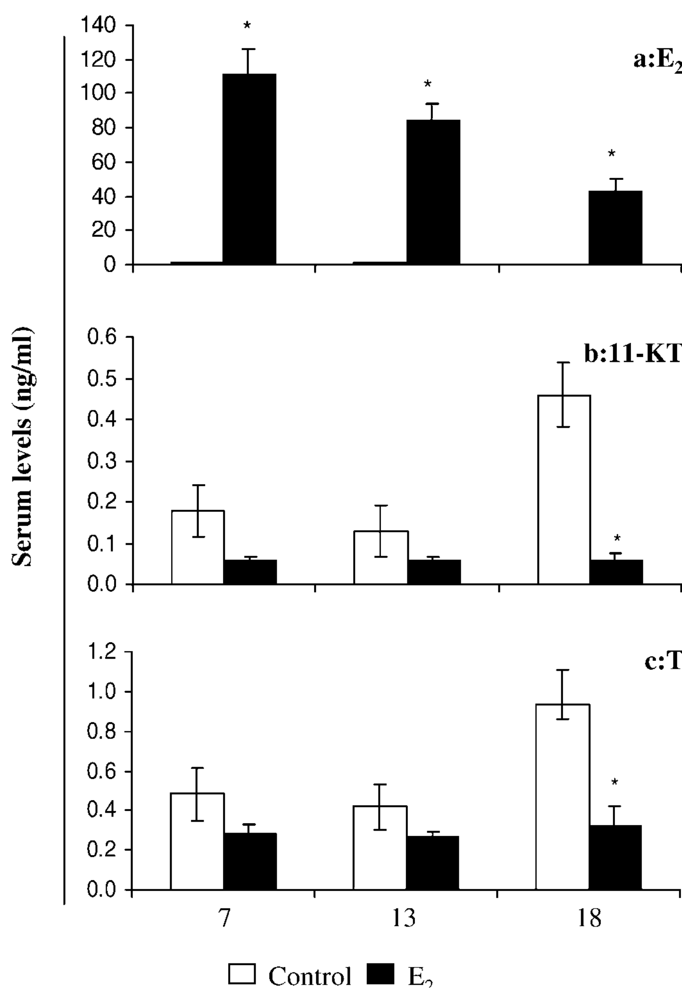

FIG. 1. Plasma levels of E_2 (**a**), 11-KT (**b**), and T (**c**) 7, 13, and 18 days after injection. Data represent the mean ± SEM of 5–10 fish/group. *$P \leq 0.05$ versus vehicle-injected fish.

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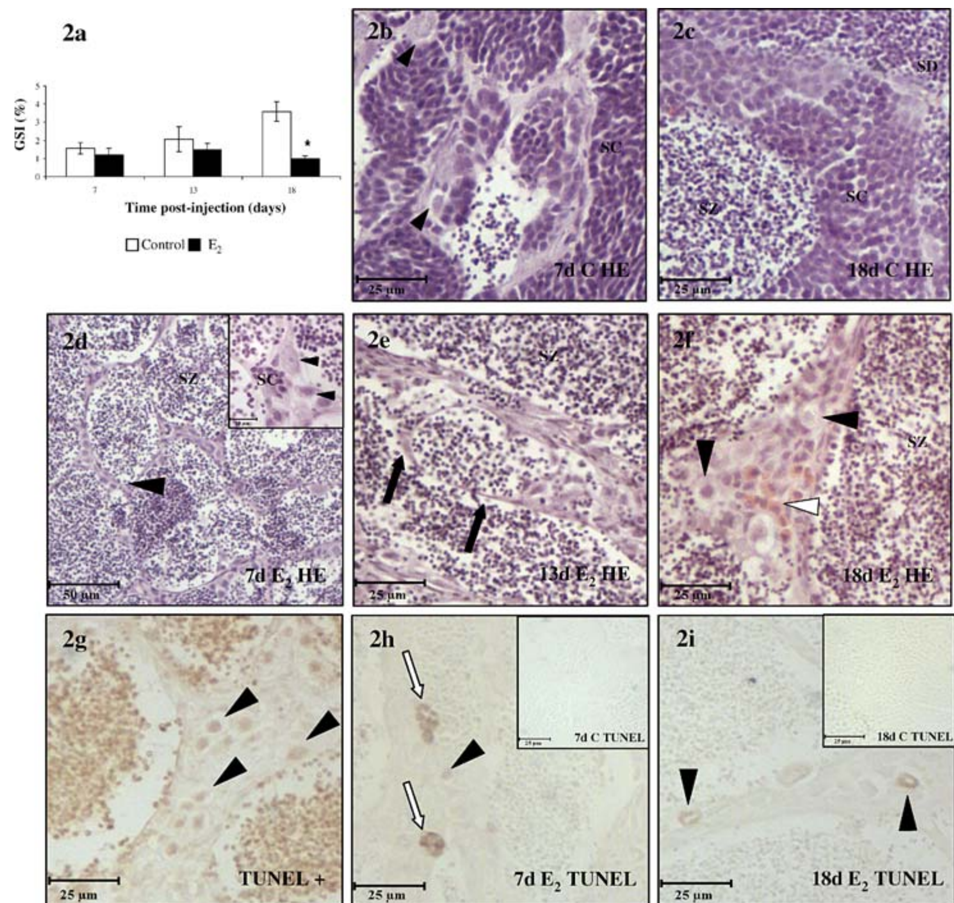

FIG. 2. Fish that were injected with E_2 show inhibited testicular growth, impaired spermatogenesis, and apoptotic primary spermatogonia compared with the control group. Fish were injected with a vehicle (coconut oil) alone (control) or with a vehicle containing 5 µg/g bw of E_2 (E_2). The GSI at 7, 13, and 18 dpi (**a**). Data represent the mean ± SEM of 5–10 fish/group. *$P \leq 0.05$ versus vehicle-injected fish. Sections of testis of gilthead seabream stained with hematoxylin-eosin (**b**–**f**) and labeled with TUNEL (**g**–**i**). Control fish at 7 (**b**, inset h) and 18 (**c**, inset i) dpi and E_2-injected fish at 7 (**d**, **h**, inset d), 13 (**e**), and 18 (**f**, **i**) dpi. TUNEL-positive control treated with DNase I before labeling (**g**). Scale bar = 50 µm (d), 25 µm (b, c, e–i, inset h, i), and 10 µm (inset d). SZ, Spermatozoa; SC, spermatocyte cysts; SD, spermatid cysts; Sertoli cells (black arrows); TUNEL-positive cysts (white arrows); spermatogonia (black arrowheads); and acidophilic granulocyte (white arrowhead).

were provided by the supplier (<0.01% of anti-11-KT reacts with T; 2.2% of anti-T reacts with 11-KT; and 0.1% of anti-E_2 reacts with T).

Calculations and Statistical Analysis

As an index of the reproductive stage, we calculated the gonadosomatic index (GSI) as 100·[GM/BM] (%), where GM is gonad mass (in grams), and BM is body mass (in grams). The proliferative rates were calculated as the mean number of proliferative cells (spermatogonia stem cells, Sertoli cells, and primary spermatogonia) and proliferative cysts in 50 optical fields at 400× magnification [1]. The areas measured were randomly distributed to cover the whole testis. Data were analyzed by a one-way ANOVA, the unpaired Student *t*-test, and a Waller-Duncan multiple range test ($P \leq 0.05$) to determine differences between groups.

RESULTS

Plasma Levels of Sex Steroids

Hormonal treatment induced supraphysiological levels of E_2 during the whole experimental period (Fig. 1a). The plasma levels of E_2 reached the highest level at 7 dpi (800-fold increase) and progressively decreased until Day 18 after injection, when E_2 levels were still 100-fold higher than in control fish (Fig. 1a). Interestingly, 11-KT (Fig. 1b) and T (Fig. 1c) plasma levels were not significantly modified at the time that maximal E_2 levels were observed in plasma (7 days), while at 18 dpi, the levels remained unchanged rather than showing the sharp increase seen in control fish.

The Germinal Compartment of the Testis Was Disturbed by E_2 Injection

The GSI gradually increased (from 1.6 ± 0.3 to 3.6 ± 0.5) in vehicle-injected fish as spermatogenesis developed. However, in E_2-injected fish, the GSI remained similar to that of control fish at 7 and 13 dpi but showed a 75% decrease at 18 dpi (Fig. 2a). Control fish were in mid-spermatogenesis at the beginning of the experiment when different, but always low, amounts of spermatozoa were present in the lumina of the tubules, which consisted of spermatogonia stem cells and all germ cell cysts: primary spermatogonia, spermatogonia A and B, spermatocytes, and spermatids (Fig. 2b). During the experiment, the amount of free spermatozoa and the spermatocyte and spermatid cysts slightly increased in control fish (Fig. 2c). Although no significant differences in the GSI were detected between vehicle and E_2-injected fish until 18 dpi, the spermatogenic process was already disrupted at 7 dpi (Fig. 2, d–f). Hence, the germinal epithelium was depleted of spermatid and spermatocyte cysts and was only formed of scarce spermatogonia, while the tubules were enlarged and full of free spermatozoa-spermatids (Fig. 2d). Sertoli cells appeared, forming empty cysts or hanging from the germinal epithelium in the lumen of the tubules (Fig. 2e). This morphology remained until 13 dpi. However, at 18 dpi, the germinal epithelium was again repopulated by spermatogonia (Fig. 2f). Interestingly, spawning was not induced, even though spermatozoa accumulated in increased amounts in E_2-injected fish. These results were consistently found in all individuals of each treatment group.

Apoptosis of Primary Spermatogonia Was Induced by E_2 Injection

Apoptosis is one of the most important mechanisms of cell death involved in different physiological processes. In the gilthead seabream testis, apoptotic cells were observed only

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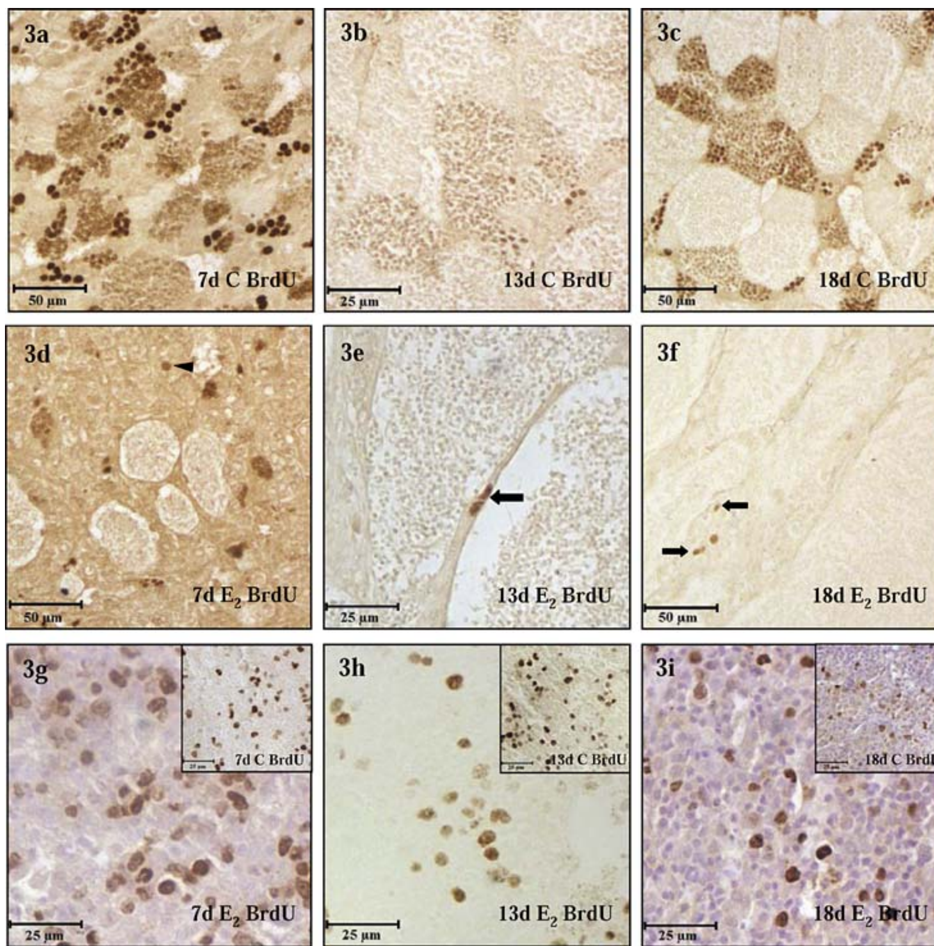

FIG. 3. Testis (**a–f**) and head-kidney (**g–i**) sections immunostained with the anti-BrdU monoclonal antibody. Control fish (**a–c**, inset **g–i**) and E_2-injected fish (**d–i**). Proliferative spermatogonia (arrowhead); proliferative Sertoli cells (arrows). Scale bar = 25 µm (**b**, **e**, **g–i**) and 50 µm (**a**, **c**, **d**, **f**).

during postspawning, when the remaining germ cells were being eliminated after the spermatogenic cycle, although some degenerative nuclei were observed during late spermatogenesis [1]. Nonapoptotic cells were observed in control fish, whereas apoptosis occurred in E_2-injected fish (Fig. 2, h and i, inset h and i). Interestingly, at Day 7 post-E_2-injection, some primary spermatogonia were seen to have undergone apoptosis, and some apoptotic bodies that resembled degenerative cysts were present (Fig. 2h). However, 13 and 18 days postspawning, only apoptotic primary spermatogonia were observed (Fig. 2i) (data not shown).

Proliferative Cell Rates Were Modified by E_2 Injection

Control fish showed the expected proliferative rates for the spermatogenesis stage, as we have already described [1]. As spermatogenesis progressed, the proliferative rates of spermatogonia stem cells, primary spermatogonia, Sertoli cells, spermatogonia A and B, and spermatocyte cysts gradually decreased (Figs. 3, a–c, and 4). However, in control fish, an unexpected decrease in the proliferation of spermatogonia A and B and spermatocyte cysts was observed at 13 dpi (Fig. 4b), although levels had risen again by 18 dpi (Fig. 4c).

In E_2-injected fish, the proliferative activity of the testis was affected at different rates, depending on the cell type and the postinjection time analyzed (Figs. 3, d–f, and 4). E_2 treatment resulted in 87% and 80% decreases in the proliferative rates of spermatogonia stem cells after 7 and 13 days of exposure (from 1.5 ± 0.2 to 0.2 ± 0.1 and from 0.5 ± 0.1 to 0.1 ± 0.04, respectively). Interestingly, at 18 dpi, the proliferative rate of spermatogonia stem cells was similar to the control. However, proliferative primary spermatogonia and the cysts of spermatogonia A and B and spermatocytes decreased after all the times assayed. The proliferative rates of primary spermatogonia were 90%, 80%, and 50% lower than in the controls at 7, 13, and 18 dpi, respectively. The number of proliferative cysts of A and B spermatogonia and spermatocytes had decreased 75% by 7 dpi, and no significant proliferation was found between 13 and 18 dpi. Interestingly, Sertoli cell proliferation was not affected at 7 dpi, had decreased by 13 dpi (from 4.8 ± 0.5 to 3 ± 0.3), and had increased (from 0.4 ± 0.1 to 2.6 ± 0.3) by 18 dpi (Fig. 4). Lastly, we observed no apparent effects of E_2 on the proliferative rate of head-kidney hematopoietic cells compared with their counterparts injected with vehicle alone at each time (Fig. 3, g–i).

Testicular and Head-Kidney Acidophilic Granulocytes Are Affected by E_2 Injection

Scattered testicular acidophilic granulocytes were present in the interstitial tissue of control fish throughout the experimental period (Fig. 5a) (data not shown). No differences between vehicle- and E_2-injected fish were observed at 7 dpi. From 13 dpi onward, however, clusters of acidophilic granulocytes were observed in the interstitial tissue, in the germinal epithelium between the spermatogonia, and in the lumen of the tubules of E_2-injected fish (Fig. 5, b and c). Interestingly, i.p. injection with E_2 resulted in a dramatic reduction of head-kidney acidophilic granulocytes (Fig. 5, d–f). The acidophilic granulocytes were abundant and occurred in clusters in the head-kidney of control fish (Fig. 5d), whereas they appeared isolated and scattered in the head-

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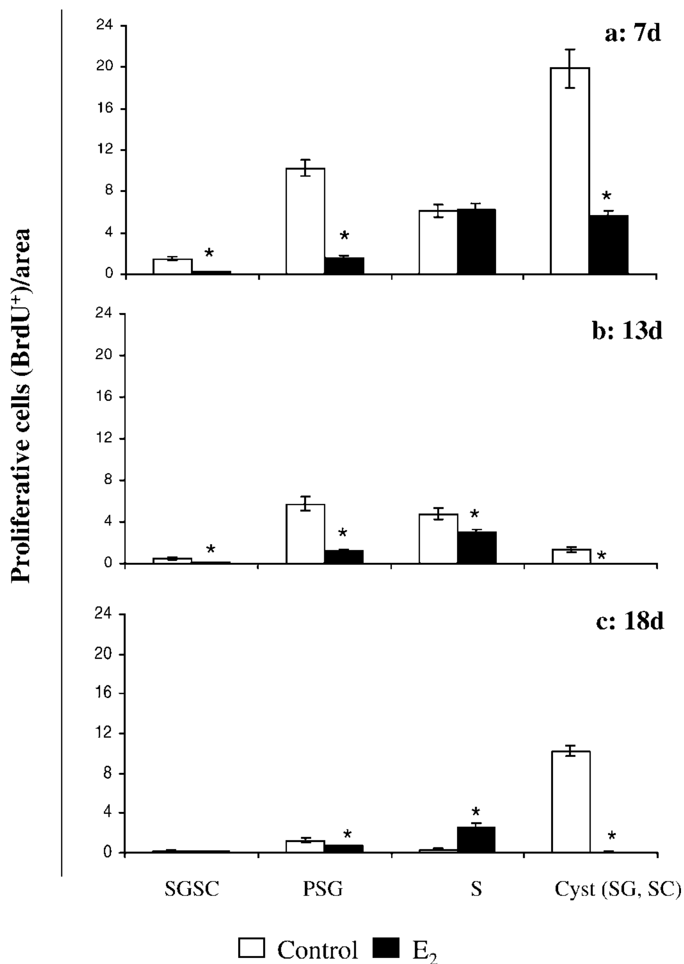

FIG. 4. The number of proliferative cells or cysts (BrdU) per area at 7 (**a**), 13 (**b**), and 18 (**c**) days after E_2 injection. SGSC, Spermatogonia stem cells; PSG, primary spermatogonia; S, Sertoli cells; SG, cyst of spermatogonia; SC, cyst of spermatocyte. Data represent the mean ± SEM for each (n = 4) fish/group. *$P \leq 0.05$ versus vehicle-injected fish.

kidney following E_2 injection (Fig. 5e). Eighteen days postinjection (Fig. 5f), the acidophilic granulocytes were more abundant and formed clusters, although they were still less abundant than in control fish.

DISCUSSION

Although it has been known for many years that estrogen administration has deleterious effects on male fertility, only recently has the use of mice lacking ERα or aromatase confirmed the important physiological role played by estrogen in spermatogenesis [4]. Studies involving estrogenic treatment have been performed in several fish species, including the gilthead seabream. In most of these studies, undifferentiated fish have been treated with E_2 to observe the plasticity of the gonad with regard to sex determination or sex reversal [6, 15, 19, 21, 22, 23]. In gilthead seabream, supraphysiological doses of E_2 inhibit testicular growth and male germ cell development after the spermatogonia stage. Furthermore, long exposures (at least 6 wk) to supraphysiological doses of E_2 are needed to bring about detectable changes in the ovarian area of the gonad [15]. Our data reveal that sexually mature (spermatogenically active) gilthead seabream treated with supraphysiological doses of E_2 during short periods of time (from 1 to 3 wk) show a decrease in the GSI at the same time as the testis becomes depleted of previously formed spermatocytes and spermatids and filled with free spermatozoa. Although some apoptosis of cysts was observed at 7 dpi, the accumulation of free spermatozoa in the lumen of the tubules, together with the inhibition of the early stages of spermatogonia proliferation, suggest that the i.p. injection of E_2 prevents the progression of spermatogonia stem cells and primary spermatogonia, while germ cells after this stage develop normally. Interestingly, E_2 also induced primary spermatogonia apoptosis, as occurs during the normal postspawning stage [1]. These data support the hypothesis that spermatogenesis in the gilthead seabream is a very efficient process, in which most of the premeiotic and postmeiotic cells are somehow programmed to finish the spermatogenesis process, although primary spermatogonia are liable to undergo cell death instead of proceeding to spermatogenesis. However, when spermatogenesis is induced, some deficient cells are produced, and some apoptotic cysts are also observed.

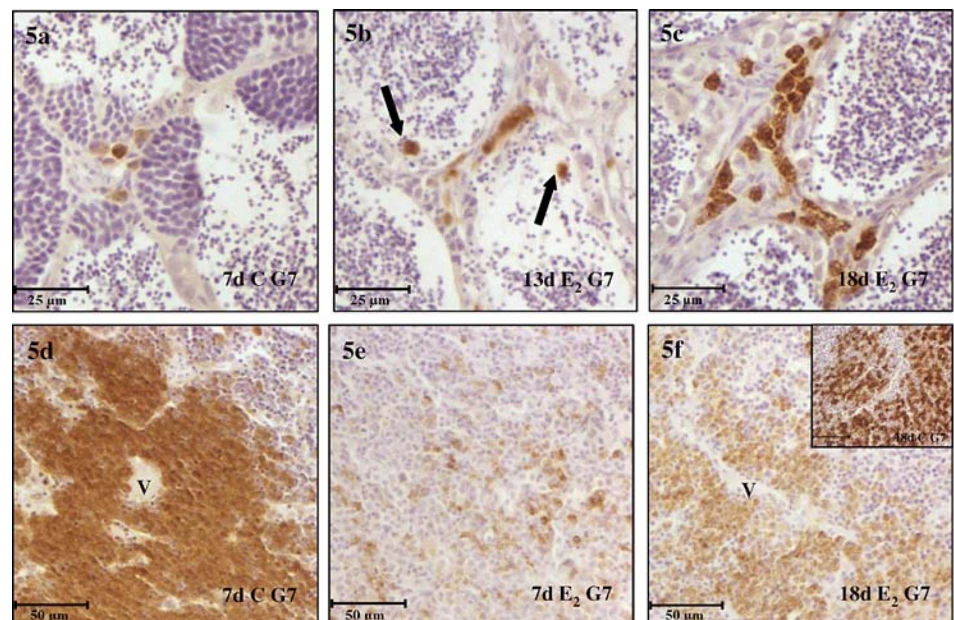

FIG. 5. Testis (**a–c**) and head-kidney (**d–f**) sections immunostained with G7 monoclonal antibody. Control fish (**a**, **d**, inset f) and E_2-injected fish after 7 (**e**), 13 (**b**), and 18 (**c**, **f**) dpi. Acidophilic granulocytes in the lumen of the tubules (arrows) and blood vessel (V). Scale bar = 25 µm (**a–c**) and 50 µm (**d–f**, inset **f**).

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Another interesting observation of this study was that E_2 did not induce the shedding of the spermatozoa. In most teleost species, spawning does not occur concomitantly with high levels of 11-KT, although in some of them, 11-KT increases slightly during the shedding of spermatozoa [24]. In the gilthead seabream, T peaks during the spawning and postspawning stages, while E_2 increases, and 11-KT remains low (unpublished results). Since E_2 treatment did not modify the plasma levels of T and 11-KT until 18 dpi, when a decrease in the levels of these two steroids was observed, the morphological changes observed at 7 and 13 dpi could be attributed to the direct effects of E_2 on the tissue. Together, these data suggest that although E_2 induces the regression of the testis, as occurs during postspawning, it is unable on its own to induce spawning, probably because high levels of T are also needed.

The in situ proliferation data showed a decrease in the proliferative rates of all germ cell types at 7 and 13 dpi, which resulted in the disruption of the germinal epithelium. Interestingly, at 18 dpi, the proliferative rate of spermatogonia stem cells was similar to that of control fish. These data suggest that E_2 was unable to completely block spermatogonia stem cell proliferation, resulting in a recovery of primary spermatogonia numbers in the germinal epithelium at 18 dpi. However, at this time, further development of primary spermatogonia was also blocked, since the proliferation of primary spermatogonia and cysts of spermatogonia A and B and spermatocytes remained inhibited. Although E_2 has been described as a proliferative factor for spermatogonia stem cells in immature fish [6], we observed no increase in spermatogonia stem cell proliferation triggered by a supraphysiological dose of E_2. It is probable that high levels of E_2 inhibit spermatogonia proliferation in mature gilthead seabream males, as occurs in the medaka testis when it is exposed to high doses of estrogenic compounds in vitro [7]. In addition, spermatogonia proliferation is not resumed in teleosts as long as spermatozoa are present in the lumen of the tubules, even after the application of a hormonal treatment that is supposed to be able to initiate spermatogenesis [1, 25, 26]. Therefore, as our E_2 treatment is not able to induce the shedding of spermatozoa, we cannot conclude whether E_2 is a spermatogonia proliferative factor during the normal postspawning stage in gilthead seabream.

An important contribution of this study was the observation that Sertoli cell proliferation may be induced by E_2. Thus, although Sertoli cell proliferation was inhibited at 13 dpi when E_2 levels were very high, it was induced at 18 dpi when E_2 levels were low, although still 100-fold higher than in the control fish. Moreover, at 18 dpi, low levels of T and 11-KT were observed in E_2-injected fish, suggesting that E_2 directly promotes the proliferation of Sertoli cells. These data suggest that the regulation of Sertoli cell proliferation is induced by regulatory pathways that are different from those occurring in germ cells. In agreement with these data, experiments with ERα −/− mice have established that E_2 is critical for somatic cell functions that, in turn, regulate the progression of spermatogenesis and sperm functioning [27, 28]. Furthermore, estrogens are also involved in prepubertal Sertoli cell proliferation in mammals [4].

We also studied whether E_2 was involved in the infiltration of acidophilic granulocytes in the testis during postspawning. In gilthead seabream, the head-kidney is the main source of acidophilic granulocytes [29]. We have previously reported that head-kidney acidophilic granulocyte functions are modulated in vitro by E_2 and 11-KT [14]. The present data unequivocally demonstrate that both head-kidney and testicular populations of acidophilic granulocytes are affected by E_2 injection i.p. Thus, the acidophilic granulocytes seemed to be mobilized from the head-kidney, since their numbers strongly decreased in the head-kidney at 7 dpi. In addition, the proliferative rate of head-kidney hematopoietic cells was similar to that seen in control fish, allowing the head-kidney acidophilic granulocyte population to recover by 18 dpi. Interestingly, acidophilic granulocytes are also mobilized from the head-kidney to the infection foci, although their numbers return to basal levels much more quickly [29, 30]. With regard to the testis, clusters of acidophilic granulocytes appeared at 13 dpi and were more abundant at 18 dpi. It is likely that high levels of E_2 in plasma trigger this behavior, which might be intensified by low levels of androgens, as occurs at 18 dpi. In addition, the changes observed in the testis would unleash the production of regulatory molecules, e.g., chemokines and cytokines that, in turn, would induce the infiltration of acidophilic granulocytes. Although our present data have not established which molecules are involved in this process, it is tempting to speculate on the involvement of interleukin 1β, a proinflammatory cytokine that is intracellularly accumulated by testicular acidophilic granulocytes during postspawning and whose production is regulated in vitro by E_2 and 11-KT [14].

All the above findings demonstrate that E_2 induces the final events of spermatogenesis and triggers some of the processes that occur during postspawning, such as acidophilic granulocyte infiltration. However, neither the shedding of spermatozoa nor an increase in the proliferative rates of spermatogonia stem cells occurred, probably because of the lack of other stimuli that are present during the postspawning stage, such as higher levels of androgens.

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REFERENCES

1. Chaves-Pozo E, Mulero V, Meseguer J, García-Ayala A. An overview of cell renewal in the testis throughout the reproductive cycle of a seasonal breeding teleost, the gilthead seabream (*Sparus aurata* L.). Biol Reprod 2005; 72:593–601.
2. Schulz RW, Menting S, Bogerd J, Franca LR, Vilela DA, Godinho HP. Sertoli cell proliferation in the adult testis—evidence from two fish species belonging to different orders. Biol Reprod 2005; 73:891–898.
3. Chaves-Pozo E, Mulero V, Meseguer J, García-Ayala A. Professional phagocytic granulocytes of the bony fish gilthead seabream display functional adaptation to testicular microenvironment. J Leukoc Biol 2005; 78:345–351.
4. O'Donnell L, Robertson KM, Jones ME, Simpson ER. Estrogen and spermatogenesis. Endocr Rev 2001; 22:289–318.
5. Miura T, Miura C. Japanese eel: a model for analysis of spermatogenesis. Zool Sci 2001; 18:1055–1063.
6. Miura T, Miura C, Ohta T, Nader MR, Todo T, Yamauchi K. Estradiol-17β stimulates the renewal of spermatogonial stem cells in males. Biochem Biophys Res Commun 1999; 264:230–234.
7. Song M, Gutzeit HO. Effect of 17-alpha-ethynylestradiol on germ cell proliferation in organ and primary culture of medaka (*Oryzias latipes*) testis. Dev Growth Differ 2003; 45:327–337.
8. Miura T, Yamauchi K, Takahashi H, Nagahama Y. Hormonal induction of all stages of spermatogenesis in vitro in the male Japanese eel (*Anguilla japonica*). Proc Natl Acad Sci U S A 1991; 88:5774–5778.
9. Kadmon G, Yaron Z, Gordon H. Sequence of gonadal events and estradiol levels in *Sparus auratus* (L.) under two photoperiod regimes. J Fish Biol 1985; 26:609–620.
10. Lone KP, Al-Ablani S, Al-Yaqout A. Steroid hormone profiles and correlative gonadal histological changes during natural sex reversal of sobaity kept in tanks and sea-cages. J Fish Biol 2001; 58:305–324.
11. Harris J, Bird DJ. Modulation of the fish immune system by hormones. Vet Immunol Immunopathol 2000; 77:163–176.

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12. Wang R, Belosevic M. Estradiol increases susceptibility of goldfish to *Trypanosoma danilewskyi*. Dev Comp Immunol 1994; 18:377–387.
13. Watanuki H, Yamaguchi T, Sakai M. Suppression in function of phagocytic cells in common carp *Cyprinus carpio* L. injected with estradiol, progesterone or 11-ketotestosterone. Comp Biochem Physiol C 2002; 132:407–413.
14. Chaves-Pozo E, Pelegrín P, Mulero V, Meseguer J, García Ayala A. A role for acidophilic granulocytes in the testis of the gilthead seabream (*Sparus aurata* L., Teleostei). J Endocrinol 2003; 179:165–174.
15. Condeça JB, Canario AVM. The effect of estrogen on the gonads and on *in vitro* conversion of androstenedione to testosterone, 11-ketotestosterone and estradiol-17β in *Sparus aurata* (Teleostei, Sparidae). Gen Comp Endocrinol 1999; 116:59–72.
16. Pinto P, Passos AL, Power DM, Canario AV. Sea bream (*Sparus auratus*) estrogen receptors: phylogeny and tissue distribution. Ann N Y Acad Sci 2005; 1040:436–438.
17. Guerreiro PM, Fuentes J, Canario AVM, Power DM. Calcium balance in sea bream (*Sparus aurata*). The effect of oestradiol-17β. J Endocrinol 2002; 173:377–385.
18. Sangiao-Alvarellos S, Polakof S, Arjona FJ, García-López A, Martí n del Río MP, Marínez-Rodríguez G, Miguel JM, Mancera JM, Soengas JL. Influence of testosterone administration on osmoregulation and energy metabolism of gilthead seabream *Sparus aurata*. Gen Comp Endocrinol 2006; 149:30–41.
19. Sepulcre MP, Pelegrín P, Mulero V, Meseguer J. Characterisation of gilthead seabream acidophilic granulocytes by a monoclonal antibody unequivocally points to their involvement in fish phagocytic response. Cell Tissue Res 2002; 308:97–102.
20. Rodríguez L, Begtashi I, Zanuy S, Carrillo M. Development and validation of an enzyme inmunoassay for testosterone: effects of photoperiod on plasma testosterone levels and gonadal development in male sea bass (*Dicentrarchus labrax* L.) at puberty. Fish Physiol Biochem 2000; 23: 141–150.
21. Lee YH, Wu GC, Du JL, Chang CF. Estradiol-17beta induced a reversible sex change in the fingerlings of protandrous black porgy, *Acanthopagrus schlegeli* Bleeker: the possible roles of luteinizing hormone in sex change. Biol Reprod 2004; 71:1270–1278.
22. Miura C, Takahashi N, Michino F, Miura T. The effect of para-nonylphenol on Japanese eel (*Anguilla japonica*) spermatogenesis in vitro. Aquat Toxicol 2005; 71:133–141.
23. Suzuki A, Nakamoto M, Kato Y, Shibata N. Effects of estradiol-17beta on germ cell proliferation and DMY expression during early sexual differentiation of the medaka *Oryzias latipes*. Zool Sci 2005; 22:791–796.
24. Borg B. Androgens in teleost fishes. Comp Biochem Physiol C 1994; 109: 219–245.
25. Scott AP, Sumpter JP. Seasonal variations in testicular germ cell stages and in plasma concentrations of sex steroids in male rainbow trout (*Salmo gairdneri*) maturing at 2 years old. Gen Comp Endocrinol 1989; 73:46–58.
26. Koldras M, Bieniarz K, Kime DE. Sperm production and steroidogenesis in testes of the common carp, *Cyprinus carpio* L., at different stages of maturation. J Fish Biol 1990; 37:635–645.
27. Mahato D, Goulding EH, Korach KS, Eddy EM. Estrogen receptor-alpha is required by the supporting somatic cells for spermatogenesis. Mol Cell Endocrinol 2001; 178:57–63.
28. Mahato D, Goulding EH, Korach KS, Eddy EM. Spermatogenic cells do not require estrogen receptor-alpha for development or function. Endocrinology 2000; 141:1273–1276.
29. Chaves-Pozo E, Pelegrín P, García-Castillo J, García-Ayala A, Mulero V, Meseguer J. Acidophilic granulocytes of the marine fish gilthead seabream (*Sparus aurata* L.) produce interleukin-1beta following infection with *Vibrio anguillarum*. Cell Tissue Res 2004; 316:189–195.
30. Chaves-Pozo E, Muñoz P, López-Muñoz A, Pelegrín P, García Ayala A, Mulero V, Meseguer J. Early innate immune response and redistribution of inflammatory cells in the bony fish gilthead seabream experimentally infected with *Vibrio anguillarum*. Cell Tissue Res 2005; 320:61–68.

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