Role of 5α-dihydrotestosterone in testicular development of gilthead seabream following finasteride administration

Running title: Testicular regulation on gilthead seabream males

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

Footnote[2]: 5α-reductase gene (*srd5a*), 11β-hydroxytestosterone (OHT), 11β-hydroxysteroid dehydrogenase gene (*hsd11b*), 11-ketotestosterone (11KT), 17β-estradiol (E_2), aromatase gene (*cyp19a1a*), body mass (bm), cholesterol side chain cleavage cytochrome P450 gene (*cyp11a1*), 5α-dihydrotestosterone (DHT), dimethyl sulfoxide (DMSO), finasteride (FIN), intraperitoneally (ip), phosphate buffer saline (PBS), reproductive cycles (RCs), ribosomal protein S18 gene (*rps18*), steroid 11-β-hydroxylase gene (*cyp11b1*), testosterone (T).

Abstract

In teleosts, spermatogenesis is regulated by pituitary gonadotropins and sex steroids. 5α-dihydrotestosterone (DHT), derived from testosterone (T) through the action of 5α-reductase, has recently been suggested to play a physiologically important role in some fish species. In this study, gilthead seabream, *Sparus aurata* L., males received an implant of 1 µg T/g body mass (bm) or vehicle alone and, 7 days later, 1 mg finasteride (FIN, an inhibitor of 5α-reductase)/kg bm or vehicle. Serum levels of T, 11-ketotestosterone (11KT), DHT and 17β-estradiol (E_2), and the mRNA levels of the main enzymes involved in their synthesis, were analysed. T promoted a transient increase in the serum levels of T, 11KT and E_2 but a decrease in those of DHT at day 15 following T injection, in accordance with the up-regulation of mRNA levels of the enzymes involved in T transformation to 11KT (coding genes: *cyp11b1* and *hsd11b*) and the down-regulation of mRNA levels of the enzyme responsible for T transformation to DHT (coding gene: *srd5a*). Interestingly, a similar effect was observed when FIN was injected. However, when fish were injected with T and FIN successively (T+FIN), control levels were not recovered at the end of the experimental period (28 days). DHT seems to regulate E_2 serum levels via the down-regulation of mRNA levels of aromatase (coding gene: *cyp19a1a*), which is needed for the transformation of T into E_2. The testis histology, together with the proliferative rates recorded upon T, FIN or T+FIN treatment, suggests that DHT is involved in the onset of the meiotic phase of spermatogenesis.

Keywords: 5α-reductase, sex steroids, steroidogenic enzyme inhibitors, *Sparus aurata*.

1. Introduction

Androgens play an important role in controlling sexual differentiation and spermatogenesis in males. Testosterone (T) serves as precursor of other sex steroids - both androgens [1,2] and estrogens [3]. Although 5α-dihydrotestosterone (DHT) has been considered the most potent androgen in mammals, birds, reptiles and amphibians [4–6], T and 11-ketotestosterone (11KT) are generally considered to be the major androgens in fish [1]. However, significant serum levels of DHT have recently been detected in some fish species and its androgenic effect has been reported following its inclusion in the diet or in flow-through systems [2,7–9]. The main organ that synthesizes biologically active steroids is the gonad, in a process whereby the regulation of the amounts of the specific mRNAs coding for steroidogenic enzymes controls the activity levels of these enzymes [10,11]. The first step in steroid synthesis is catalyzed by the cholesterol side chain cleavage cytochrome p450 (coding gene: *cyp11a1*) [10]. This reaction is the first rate-limiting and hormonally regulated step in the synthesis of all steroids [12]. Subsequently, T is transformed into 11KT in a two-step process: the hydroxylation of T to 11β-hydroxytestosterone (OHT) by the enzyme 11β-hydroxylase (coding gene: *cyp11b1*), followed by its conversion into 11KT via 11β-hydroxysteroid dehydrogenase (coding gene: *hsd11b*) [13–15], or into DHT by 5α-reductase activity (coding gene: *srd5a*) [13]. Moreover, T is transformed into 17β-estradiol (E_2) by aromatase activity (coding gene: *cyp19a1a*) [10]. Few studies have focused on the disruptive effects of inhibitors of the 5α-reductase activity used in human pharmacological treatments, such as dutasteride [16] and finasteride (FIN) [17], on the reproduction of fish. The results obtained indicate that these treatments affect the reproductive process. In fact, the changes observed suggest that 5α-reductase could be involved in steroid biosynthesis and play a role in androgen signaling via DHT in fish [16,17]. On the other hand, it has been reported that FIN produces feminization in rats [18] and may lead to the development of abnormalities in rhesus monkeys [6]. As regards amphibians, FIN produced severe disruption of spermatogenesis and reduced proliferation activity in *Xenopus laevis* [19], and dutasteride accelerated ovarian differentiation in female tadpoles of *Bufo bufo* and *Rana dalmatina* [20,21].

The gilthead seabream (*Sparus aurata* L.) is a protandrous teleost fish that has a bisexual gonad with two coexisting areas: the testicular area which develops during the first two reproductive cycles (RCs) and the ovarian area which is not functional during this time. As regard the development of the testicular area, four stages have been

defined in the first RC: spermatogenesis, spawning, post-spawning and resting [22,23]. In this species, exogenous T treatment was seen not to affect the first step of steroid synthesis but enhanced the transcript levels coding for the enzymes involved in the transformation of T into DHT and E_2 [24].

These observations prompted us to experimentally inhibit the 5α-reductase activity using FIN in gilthead seabream males in order to elucidate the importance of DHT in the reproductive physiology of this hermaphrodite fish. The relationship between the different sex steroid levels and between them and the transcript levels coding for the steroidogenic enzymes was also analysed. A morphological study was made of the testicular area, including an assessment of proliferating cells with an anti-PCNA serum and the calculation of proliferative rates in the different experimental groups established.

2. Material and methods

2.1. Animals and experimental design

Gilthead seabream (*Sparus aurata* L.) (Actinopterygii, Perciformes, Sparidae) males of 169.15 ± 17.72 g mean body mass (bm) were bred and kept at the Centro Oceanográfico de Murcia (IEO, Mazarrón, Murcia). The fish were kept in 2 m^3 tanks with the water temperature ranging from 23.2 to 25.8 ºC, a flow-through circuit, an aeration and filtration system and a natural photoperiod. The environmental parameters, mortality and food intake were recorded daily. The experiment was performed in July, when the fish were in the resting stage of the first RC [22]. The fish were fed three times a day *ad libitum* and were ip injected with 5 µl slow-release coconut oil g^{-1} bm as vehicle or the same containing 1 µg T g^{-1} bm (n = 30 fish/group), a dose previously shown to promote supraphysiological T serum levels [24,25]. After 7 days, both groups were divided again and 15 fish were ip injected with 1 ml of phosphate buffer saline (PBS) with 0.1 % of dimethyl sulfoxide (DMSO) as vehicle and 15 fish were ip injected with PBS-DMSO containing 1 µg FIN g^{-1} bm, a dose that we estimated would inhibit 5α-reductase activity. Thus, four experimental groups were established: i) a control group consisting of fish injected with coconut oil and PBS, ii) T treated group consisting of fish injected with a T-implant and PBS, iii) FIN treated group consisting of fish injected with coconut oil and FIN, and iv) T+FIN treated group consisting of fish injected with a T-implant and FIN (Fig. 1). The fish were fasted for 24 h before sampling 7, 15 and 21 days after FIN injection (14, 21 and 28 days after T injection,

respectively). At each sampling time four fish per group were tranquilized by 20 µl L^{-1} of clove oil and, immediately, anesthetized with 40 µl L^{-1} of clove oil. Blood was obtained from the caudal peduncle and the serum samples were obtained by centrifugation (10,000 xg, 1 min, 4 ºC) and immediately frozen in liquid nitrogen and stored at - 80 ºC until use. Then, fish were weighed, decapitated and the gonads were removed and processed for mRNA analysis and light microscopy and immunocytochemistry as described below.

The experiments described comply with the Guidelines of the European Union Council (2010/63/EU) and Spanish directive RD53/2013. All specimens studied were handled in accordance with the Guidelines of the Bioethical Committee of the IEO (reference REGA ES300261040017) and the Bioethical Committee of the University of Murcia (reference REGA ES300305440012).

2.2. Analytical techniques

Serum (n=4 fish/sampling time and group) levels of T, 11KT and E_2 were quantified by ELISA following the method previously described [26]. Steroids were extracted from 10 µl of serum in 1.3 ml of methanol (Panreac). Then, the methanol was evaporated at 37 ºC and the steroids were resuspended in 400 µl of reaction buffer [0.1 M phosphate buffer with 1 mM EDTA (Sigma), 0.4 M NaCl (Sigma), 1.5 mM NaN_3 (Sigma) and 0.1 % albumin from bovine serum (Sigma)]. Aliquots of these solutions (50 µl) were dispensed into microtiter plate wells (MaxiSorp; Nunc) so that 1.25 µl of serum were used in each well for all the assays. T, 11KT and E_2 standards, mouse anti-rabbit IgG monoclonal antibody, specific anti-steroid antibodies and enzymatic tracers (steroid acetylcholinesterase conjugates) were obtained from Cayman Chemical. A standard curve from 6.13 x 10^{-4} to 5 ng ml^{-1} (0.03-250 pg $well^{-1}$), a blank and a non-specific binding control (negative control) were established for all the assays. Standards and extracted serum samples were run in duplicate and all the measurements were corrected with the blank and negative control. The lower limit of detection for all the assays was 12.21 pg ml^{-1}. The intra-assay coefficients of variation (calculated from sample duplicates) were 9.24 ± 1.45 % for T, 7.36 ± 1.15 % for 11KT and 15.74 ± 2.19 % for E_2 assays. The inter-assay coefficients of variation were 4.08 ± 0.09 % for T, 7.18 ± 0.32 % for 11KT and 3.02 ± 0.07 % for E_2 assays. Details on cross-reactivity for specific antibodies were provided by the supplier (0.01 % of anti-11KT reacts with T; 2.2 % of anti-T reacts with 11KT; and 0.1 % of anti-E_2 reacts with T). Serum (n=4

fish/sampling time and group) levels of DHT were quantified using the 5α-dihydrotestosterone ELISA kit (DRG diagnostics) following the manufacturer's instructions. Details on cross-reactivity for the specific antibody were provided by the supplier (8.7 % of anti-DHT reacts with T).

2.3. Analysis of mRNA levels

Total RNA was extracted from the gonad (n=4 fish/sampling time and group) with TRIzol reagent (Cayman) following the manufacturer's instructions. RNA was treated with RQ1 RNAse-free DNAse, amplification grade (1 unit μg^{-1} RNA, Promega). BioScript Reverse Transcriptase (Bioline) was used to synthesize first strand cDNA with oligo-dT18 primer from 1 µg of total RNA, at 42 ºC for 60 min.

Transcript levels of the genes coding for steroidogenesis-related molecules - the cholesterol side chain cleavage cytochrome P450 (*cyp11a1*), steroid 11-β-hydroxylase (*cyp11b1*), 11β-hydroxysteroid dehydrogenase (*hsd11b*), 5α-reductase (*srd5a*) and aromatase (*cyp19a1a*) - were analysed by real-time PCR performed with an ABI PRISM 7500 instrument (Applied Biosystems) using SYBR Green PCR Core Reagents (Applied Biosystems) as previously described [27]. For each mRNA, gene expression was corrected by the *ribosomal protein S18* gene expression (*rsp18*) content in each sample. The gilthead seabream specific primers used are shown in Table 1. Less than 2 % variation in *rsp18* gene expression was observed between samples.

2.4. Light microcopy and immunocytochemistry

Gonad fragments (n = 4 fish/group and time) were fixed in Bouin's solution for 16 h at 4 °C, embedded in paraffin (Paraplast Plus; Sherwood Medical) and sectioned at 5 µm. After dewaxing and rehydration, some sections were stained with haematoxylin–eosin in order to determine the degree of development of each specimen. Other sections were subjected to an indirect immunocytochemical method using a monoclonal antibody specific to proliferating cellular nuclear antigen (PCNA, Sigma) at the optimal dilution of 1:1000 as previously described [28]. The specificity of the reactions was determined by omitting the first antiserum. Slides were examined with an Axiolab (Zeiss) light microscope.

2.5. Calculations and statistics

All data are presented as mean ± standard error of the mean (SEM). The significance level (P) was fixed at 0.05. In order to determine differences between the

control and T, FIN, or T+FIN fish groups, respectively, and between T (or FIN) and T+FIN fish groups, Student's t tests were performed. In addition, Pearson correlation tests were applied to test correlations among steroid levels and between steroid and mRNA expression levels for each experimental condition. Statistical analyses were conducted using SPSS 12.0 (SPSS, Chicago, IL, USA).

PCNA immunostaining was calculated as the mean value ± SEM of the stained area/total area of 16 randomly distributed optical areas at X 200 magnification. The stained areas were measured by image analysis using a Nikon eclipse E600 light microscope, an Olympus SC30 digital camera (Olympus soft imaging solutions GMBH), and ImageJ software [29]. Proliferation rates in treated groups were expressed as fold change of control values and analyzed by the t-Student`s test ($P \leq 0.05$).

3. Results

3.1. T implants transiently alter testosterone-derived steroid serum levels and transcript levels of the enzymes involved in their production

Fifteen days after T treatment, increases in the T (Fig. 2A), 11KT (Fig. 2B) and E_2 (Fig. 2D) and a decrease in the DHT (Fig. 2C) serum levels were recorded compared with the serum levels of the control fish, while 21 days after T treatment, only a decrease in the T serum levels was observed (Fig. 2A). In control fish, T serum levels positively correlated with the levels of 11KT (Fig. 2E) and E_2 (Fig. 2F) and negatively correlated with those of DHT (Fig. 2G), while DHT serum levels negatively correlated with E_2 serum levels (Fig. 2H). However, these correlations were not observed in T treated fish (data not shown).

Then, in the gonad, we analysed how the T implants modified the transcript levels of the enzyme involved in the first reaction of cholesterol transformation into steroids (*cyp11a1*) and of the enzymes involved in T transformation into 11KT (*cyp11b1* and *hsd11b*), into DHT (*srd5a*) or E_2 (*cyp19a1a*) (Fig. 3). Fifteen days after T injection, the *cyp11b1* (Fig. 3A) and *hsd11b* (Fig. 3B) mRNA levels were seen to have increased, while the *srd5a* transcript levels had fallen (Fig. 3C) compared with those of the control fish. A decrease in the transcript levels of *cyp11b1* (Fig. 3A) and *hsd11b* (Fig. 3B) was also observed 21days after T treatment. In contrast, no differences were observed in the transcript levels of *cyp11a1* or *cyp19a1a* between control and T-implanted fish (data not shown).

Furthermore, in control fish, the *hsd11b* transcript levels positively correlated with T serum levels (Fig. 3D), while *cyp11a1* transcript levels negatively correlated with 11KT serum levels (Fig. 3E). However, in T-treated fish no such correlations were observed (data not shown).

3.2. Finasteride affects sex steroid serum levels and transcript levels of the enzymes involved in their production

Seven days after FIN injection, an increase was observed in T (Fig 4A), 11KT (Fig. 4B) and E_2 (Fig. 4D) and a decrease in the DHT (Fig. 4C) serum levels compared to those of control fish. Moreover, in FIN treated fish, T serum levels positively correlated with those of 11KT (Fig. 4E) and E_2 (Fig. 4F) and negatively correlated with DHT serum levels (Fig. 4G).

In T+FIN treated fish a decrease in T (Fig. 4A), DHT (Fig. 4C) and E_2 (Fig. 4D) serum levels at day 7 and an increase of T, at days 15 and 21, and of DHT, at day 21 were observed compared to the serum levels of T treated fish. However, compared to the levels measured in control fish, T serum levels had increased at days 7 and 15, and 11KT serum levels at day 7, while DHT serum levels had decreased at day 7 and increased at day 21 (Fig. 4A,B,C) and serum levels of E_2 were not affected (Fig. 4D). Moreover at day 21 the serum levels of DHT were higher in T+FIN than in FIN fish (Fig. 4C). However, 11KT serum levels were not modified at any time in the T+FIN treated fish compared to T treated fish (Fig. 4B).

Regarding the transcript levels of the genes coding for steroidogenic enzymes, *cyp11b1* at day 7 (Fig. 5A) and of *cyp19a1a* at day 15 (Fig. 5C) were up-regulated after FIN injection compared to control fish, while those of *hsd11b* were down-regulated at day 7 in T+FIN treated fish compared to the T treated fish (Fig. 5B). However, neither FIN nor FIN+T treatments modified the transcript levels of *cyp11a1* and *srd5a* (data not shown).

In FIN treated fish, no correlations were observed between the serum levels of sex steroids and the levels of mRNA coding for the enzymes involved in steroidogenesis (data not shown). However, in T+FIN treated fish, there was a positive correlation between T serum levels and the levels of mRNA coding for *cyp11a1* (Fig. 5D), *hsd11b* (Fig. 5E) and *srd5* (Fig. 5F).

3.3. Testosterone and finasteride modify the testicular proliferation rates

In the testis of control fish, which were at the resting stage, proliferative Sertoli cells, spermatogonia stem cells and a few cysts of primary spermatogonia were

observed using anti-PCNA serum (Fig. 6B). Implants of T did not produce any effect on the proliferative rate of the testes compared to that seen in control fish (Fig. 6A). However, FIN caused an increase in the proliferative rate of the testis compared with the control fish, the magnitude of which depended on the previous treatment (vehicle alone or vehicle plus T). Thus, in FIN treated fish, an increase in the proliferation rate was observed after 7 days, which decreased as the experiment progressed, while in T+FIN treated fish the proliferation rate increased gradually from day 15 after FIN injection until the end of the experiment (Fig. 6A).

Interestingly T, FIN or T+FIN treatments affected the cell type that proliferated in the testis differently (Fig. 6). Compared to the testis of control fish, T-implants enhanced Sertoli cells but not spermatogonia proliferation (Fig. 6B, C), while FIN injection promoted an increase in the number of all proliferative cell types (Fig. 6D) at all sampling times. However, the testes of T+FIN treated fish showed a similar aspect to those of control fish 7 days after FIN injection, but displayed a progressive increase in the number of proliferative spermatogonia and cysts of spermatogonia, and also contained cysts of spermatocytes 21 days after FIN injection (Fig. 6E-G).

4. Discussion

In teleosts, as in mammals, the balance that exists between androgens and estrogens is essential for the reproductive process [30,31], T being the source of other steroids [1–3]. Thus, E_2 is derived from T through local aromatase activity in several tissues, including the testis [3,10,27], and the dominant androgen in male fish, 11KT, is synthesized from T by the sequential action of 11β-hydroxylase and 11β-hydroxysteroid dehydrogenase [1]. In recent years, there has been growing evidence that teleost fish produce DHT, the most potent androgen among other vertebrate groups [4], from T through 5α-reductase activity [1,2], although its exact role is still unclear. DHT has androgenic effects and seems to be involved in regulating spermatogenesis in a number of teleost species, as illustrated when fish are exposed to this compound [2,32]. In addition, it has recently been demonstrated that DHT serum levels positively correlate with T serum levels in pubertal European sea bass males [33]. To increase our understanding of the role of DHT in the reproductive physiology of hermaphrodite male fish, FIN was used to inhibit the 5α-reductase activity needed to transform T into DHT. Since the synthesis of 11KT, DHT and E_2 depends on the availability of their precursor, T [1,24], changes in the serum levels of these sex steroids were expected. Indeed, the increased serum levels of T observed 15 days after T implantation were accompanied by

an increase in the levels of 11KT and E_2. However, serum levels of DHT had decreased by this time, which is consistent with the decrease in the gene expression levels of *srd5a,* which codes for the 5α-reductase, and with the negative correlation between T and DHT levels found in control fish. Interestingly, control levels of T-derived steroids in serum recovered before T serum levels were normalized after T-implants. The rapid up- or down regulation of transcripts that code for the enzymes needed to produce 11KT (*cyp11b1* and *hsd11b*) coincided with the respective increase or decrease in T serum levels. These data suggest that T serum levels regulate the kinetics of T transformation into 11KT, as further supported by the positive correlation between the T serum levels and *hsd11b* mRNA levels found in control fish. However, the transcript levels of *cyp11a1* in the first and hormonally regulated step of steroid synthesis [34] were not affected by exogenous T, reflecting the findings of previous studies [24]. Furthermore, the high serum levels of 11KT promoted by exogenous T could contribute to the restoration of T control levels, since a negative correlation was found between serum levels of 11KT and *cyp11a1* mRNA levels in control fish.

FIN administration inhibited 5α-reductase activity, as shown in the sharp decrease in DHT serum levels 7 days after its application. In addition, although the pharmacology potency of T and FIN might be different and some of the FIN actions might be masked by high levels of T, the dose of FIN used in this study was able to inhibit the 5α-reductase activity also in T-implanted fish. Interestingly, the inhibition of 5α-reductase activity had an effect similar to that caused by T treatment - an increase in T, 11KT and E_2 serum levels - which could compensate the failing conversion of T into DHT in control fish. By contrast, in T-implanted fish which exhibited increased serum levels of these steroids, FIN did not affect 11KT while induced a decrease in E_2 serum levels, possibly to avoid excessive levels that could harm the fish physiology. Serum levels of all steroids were restored at the end of the experiment in FIN treated fish; whilst only serum levels of 11KT and E_2 were recovered in T+FIN treated fish at this time.

DHT seems to regulate E_2 levels, since inhibition of its synthesis in FIN treated fish up-regulated *cyp19a1a* expression and increased E_2 serum levels, as occurs in amphibians [19,35]. E_2 has been seen to mediate spermatogonia renewal in fish [36,37], which could explain the initial increase in proliferative spermatogonia in FIN treated fish, as they showed a considerable increase in E_2 serum levels compared to the other experimental groups. However spermatogenesis did not progress in FIN treated fish, possibly because the subsequent restoration of control levels of all the steroids studied.

Interestingly, the proliferative rates and cell types observed in the testis of T+FIN treated fish were consistent with the onset of spermatogenesis 15 days after FIN injection, when cysts of spermatogonia increased in size and numbers, and with the progression of this process, as shown by the appearance of spermatocyte cysts 21 days after FIN injection. Of note is the fact that these fish showed higher DHT levels than those of the other experimental groups. These data suggests that DHT serum levels are not involved in triggering the mitotic or spermatogonial phase of spermatogenesis, as already seen in catfish [38] but in the passage from the mitotic phase to the meiotic phase of spermatogenesis when primary spermatocytes are produced.

In conclusion our data show that T serum levels regulate DHT serum levels by down-regulating the transcript levels of *srd5a*, which codes for the enzyme responsible for its synthesis. Furthermore, the inhibition of T transformation into DHT revealed that DHT might have a role in the passage from the mitotic to the meiotic phase of spermatogenesis and be involved in the regulation of E_2 serum levels in gilthead seabream males.

5. Competing interest: No competing interests declared.

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7. Figure legends

Figure 1. *Schema of the experimental design*. bm, body mass; DMSO, dimethylsulfoxide; FIN, finasteride; PBS, phosphate buffer saline; T, testosterone.

Figure 2. *Sex steroid serum levels and significant correlations after T administration.* **(A-D)** The serum levels of T (A), 11KT (B), DHT (C) and E_2 (D) in control and T-implanted gilthead seabream fish at 15, 21 and 28 days after T-implant injection. Data were obtained from 4 fish/group and represent the means ± S.E.M. of duplicate samples. Asterisks indicate a significant difference between groups accordingly to a t-student test ($P \leq 0.05$). **(E-H)** Significant correlations between T and 11KT (E), E_2 (F) or DHT (G) and between DHT and E_2 (H) serum levels were observed in the control group. The Pearson rank number is indicated. Asterisks indicate a significant correlation ($P \leq 0.05$). T, testosterone; 11KT, 11-ketotestosterone; DHT, 5α-dihydrotestosterone; E_2, 17β-estradiol.

Figure 3. *Transcript levels of some relevant steroidogenic enzyme coding genes and significant correlations with sex steroid serum levels after T administration*. **(A-C)** Transcript levels of *cyp11b1* (A), *hsd11b* (B) and *srd5a* (C) in the gonad of control and T-implanted gilthead seabream fish at 15, 21 and 28 days post-T-implant injection. All the mRNA levels were studied by real-time RT-PCR. Data represent means ± S.E.M. for 4 fish/group. Asterisks indicate a significant difference between groups accordingly to a t-student test ($P \leq 0.05$). **(D-E)** Significant correlations between T and *hsd11b* (D) and between 11KT and *cyp11a1* (E) were observed in the control group. The Pearson rank number is indicated. Asterisks indicate a significant correlation ($P \leq 0.05$). T, testosterone; 11KT, 11-ketotestosterone.

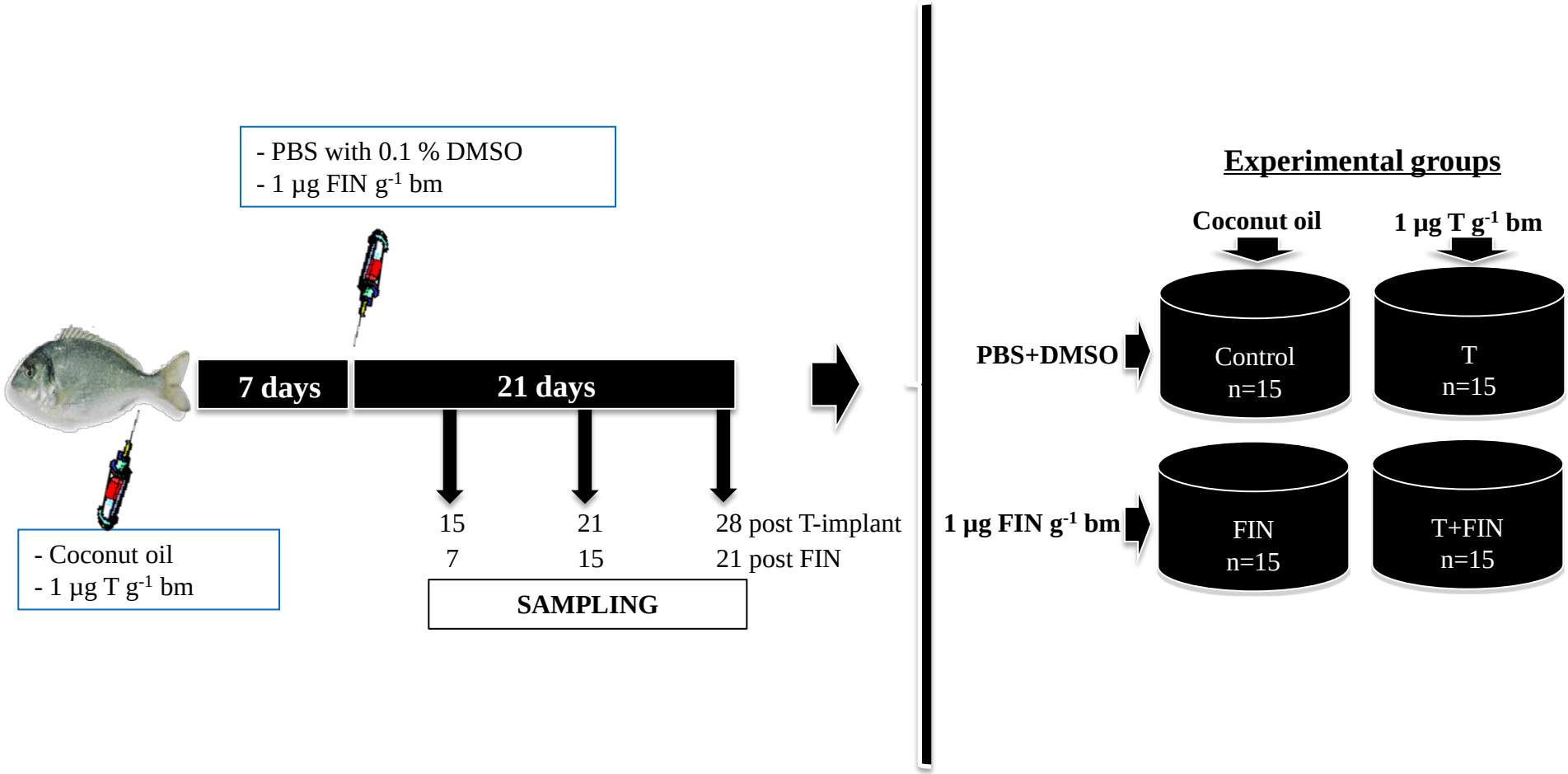
Figure 4. *Sex steroid serum levels and significant correlations after FIN administration.* **(A-D)** Serum levels of T (A), 11KT (B), DHT (C) and E_2 (D) in control, finasteride (FIN), T or T+FIN treated gilthead seabream fish 7, 15 and 21 days after

FIN injection. Data were obtained from 4 fish/group and represent the means ± S.E.M. of duplicate samples. Asterisks denote statistically differences between groups accordingly to a t-student test ($P \leq 0.05$). **(E-G)** Significant correlations between T and 11KT (E), DHT (F) or E_2 (G) serum levels observed in the FIN treated group. The Pearson rank number is indicated. Asterisks indicate a significant correlation ($P \leq 0.05$). T, testosterone; 11KT, 11-ketotestosterone; DHT, 5α-dihydrotestosterone; E_2, 17β-estradiol.

Figure 5. *Transcript levels of relevant steroidogenic enzyme coding genes and significant correlations with sex steroid serum levels after FIN administration*. **(A-C)** Transcript levels of *cyp11b1* (A), *hsd11b* (B) and *cyp19a1a* (C) in the gonad of control, finasteride (FIN), testosterone (T) or T+FIN treated gilthead seabream fish 7, 15 and 21 days after FIN injection. The mRNA levels of all genes were studied by real-time RT-PCR. Data represent means ± S.E.M. from 4 fish/group. Asterisks denote statistically differences between groups accordingly to a t-student test ($P \leq 0.05$). **(D-F)** Significant correlations between T serum levels and transcript levels of *cyp11a1, hsd11b* or *srd5a* were observed in the T+FIN treated group. The Pearson rank number is indicated. Asterisks indicate a significant correlation (*$P \leq 0.05$, **$P \leq 0.01$, ***$P \leq 0.001$).

Figure 6. *Testicular proliferative activity after T and/or FIN administration.* **(A)** Proliferative rate expressed as fold change of control values and measured as the mean of the staining area with anti-proliferative cell nuclear antigen (anti-PCNA) per optical area at 7, 15 and 21 days post-finasteride (FIN) administration Asterisks denote statistically differences between groups accordingly to a t-student test ($P \leq 0.05$). **(B-G)** Paraffin embedded section of control (B), testosterone (T) (C), FIN (D) and T+FIN (E-G) treated fish staining with anti-PCNA serum and counterstained with hematoxylin. Scale bar=15 µm. Asterisk denote proliferative primary spermatogonia, black arrows denote proliferative Sertoli cells, SG denote cyst of proliferative spermatogonia and SC denote cysts of proliferative spermatocytes.

Figure 1


- PBS with 0.1 % DMSO
- 1 µg FIN g-1 bm
7 days
21 days
- Coconut oil
- 1 µg T g-1 bm
15
7
21
15
28 post T-implant
21 post FIN
SAMPLING
Experimental groups
Coconut oil
1 µg T g-1 bm
PBS+DMSO
1 µg FIN g-1 bm
Control
n=15
T
n=15
FIN
n=15
T+FIN
n=15

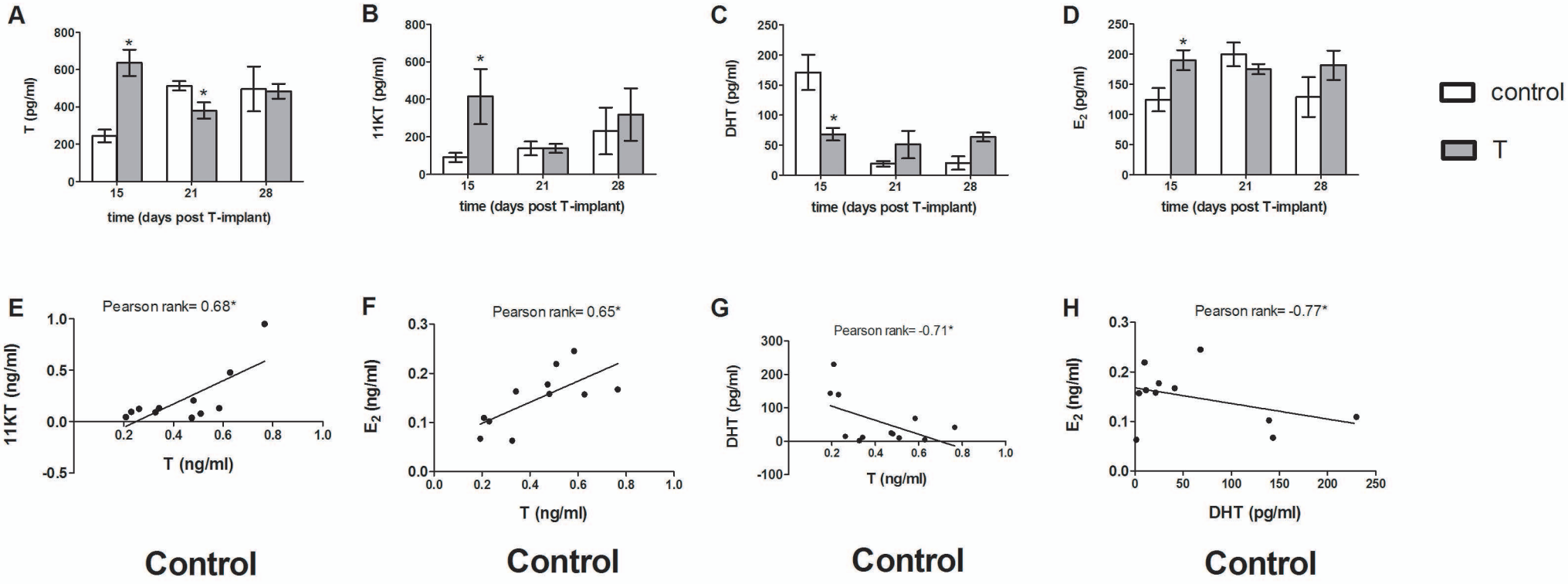

A
T (pg/ml)
time (days post T-implant)
B
11KT (pg/ml)
time (days post T-implant)
C
DHT (pg/ml)
time (days post T-implant)
D
E2 (pg/ml)
time (days post T-implant)
control
T
E
Pearson rank= 0.68*
11KT (ng/ml)
T (ng/ml)
Control
F
Pearson rank= 0.65*
E2 (ng/ml)
T (ng/ml)
Control
G
Pearson rank= -0.71*
DHT (pg/ml)
T (ng/ml)
Control
H
Pearson rank= -0.77*
E2 (ng/ml)
DHT (pg/ml)
Control

Figure 2

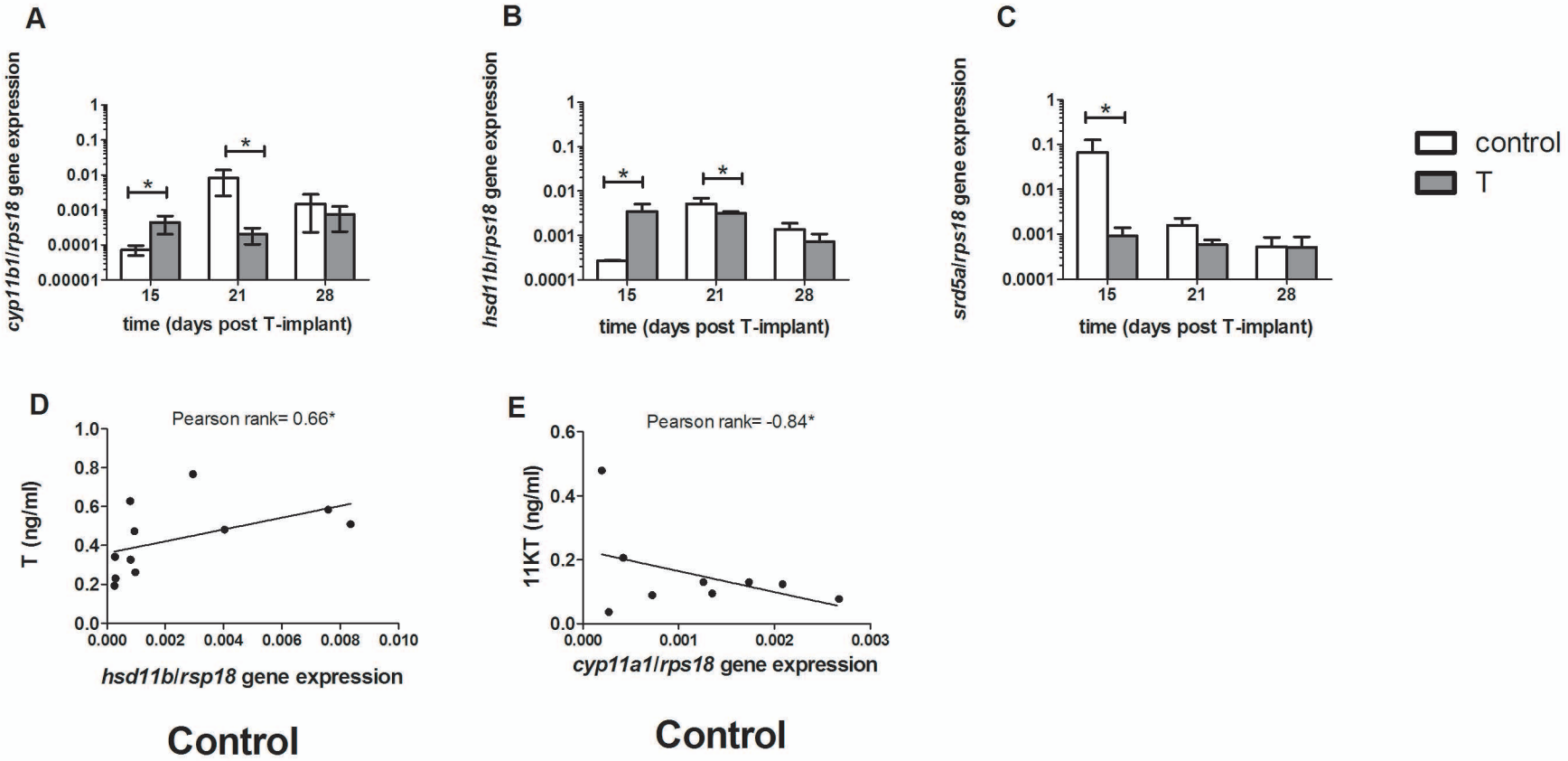

A
cyp11b1/rps18 gene expression
1
0.1
0.01
0.001
0.0001
0.00001
*
*
15
21
28
time (days post T-implant)
B
hsd11b/rps18 gene expression
1
0.1
0.01
0.001
0.0001
*
*
15
21
28
time (days post T-implant)
C
srd5a/rps18 gene expression
1
0.1
0.01
0.001
0.0001
*
15
21
28
time (days post T-implant)
control
T
D
Pearson rank= 0.66*
T (ng/ml)
1.0
0.8
0.6
0.4
0.2
0.0
0.000
0.002
0.004
0.006
0.008
0.010
hsd11b/rsp18 gene expression
Control
E
Pearson rank= -0.84*
11KT (ng/ml)
0.6
0.4
0.2
0.0
0.000
0.001
0.002
0.003
cyp11a1/rps18 gene expression
Control

Figure 3

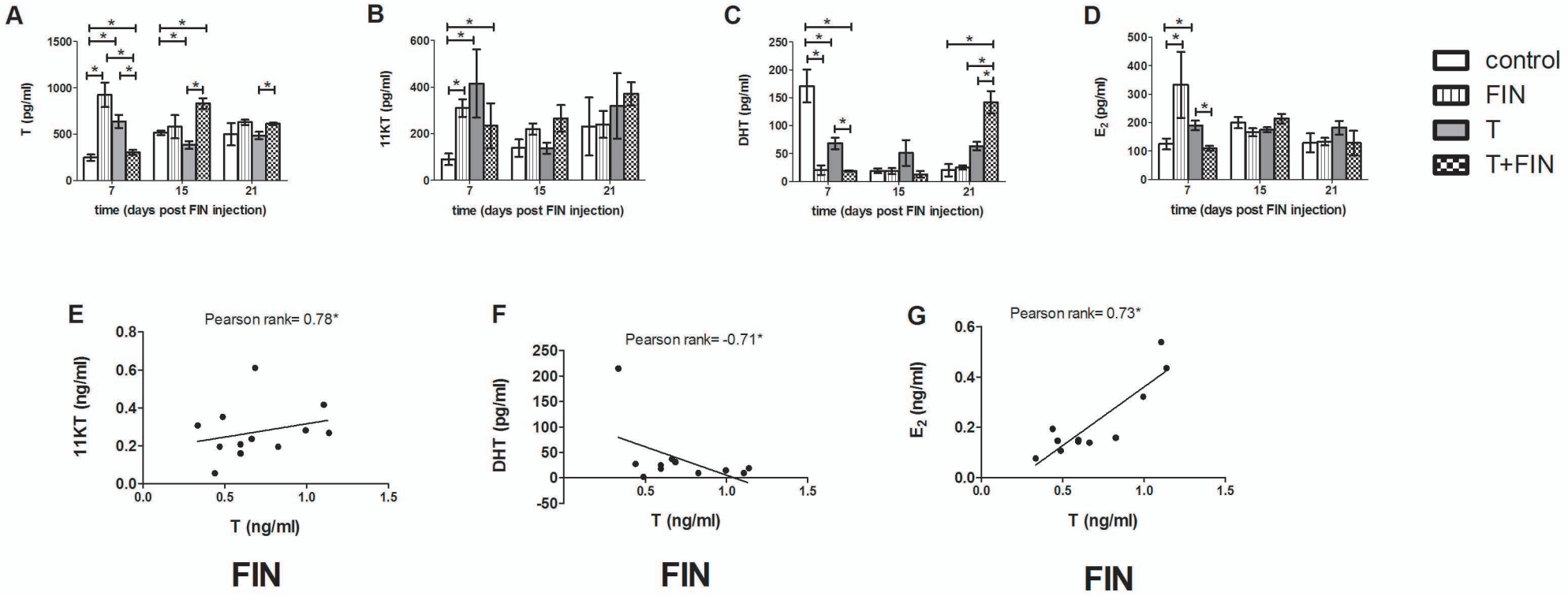

A
T (pg/ml)
time (days post FIN injection)
B
11KT (pg/ml)
time (days post FIN injection)
C
DHT (pg/ml)
time (days post FIN injection)
D
E2 (pg/ml)
time (days post FIN injection)
control
FIN
T
T+FIN
E
Pearson rank= 0.78*
11KT (ng/ml)
T (ng/ml)
FIN
F
Pearson rank= -0.71*
DHT (pg/ml)
T (ng/ml)
FIN
G
Pearson rank= 0.73*
E2 (ng/ml)
T (ng/ml)
FIN

Figure 4

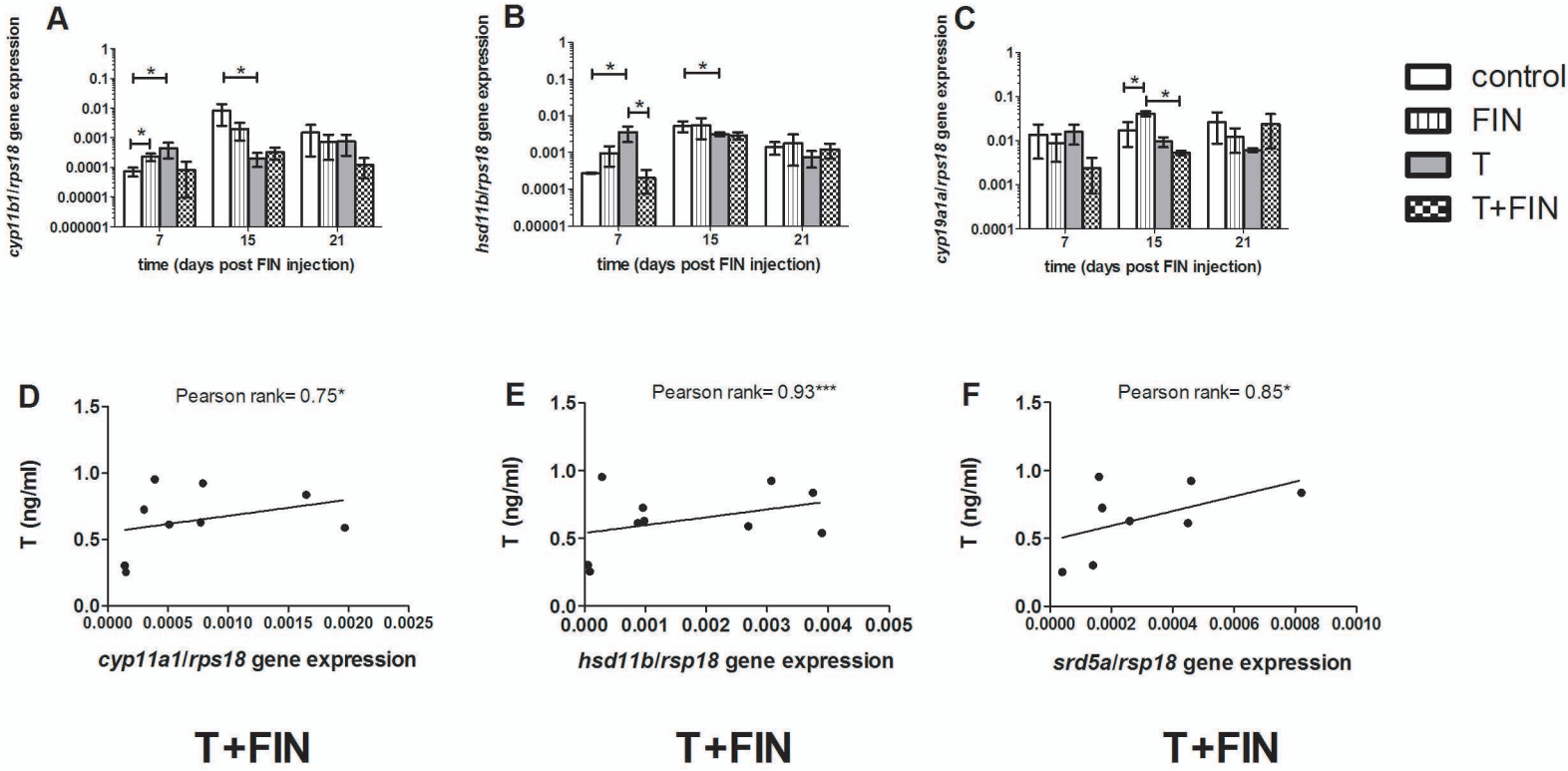

A
B
C
cyp11b1/rps18 gene expression
hsd11b/rps18 gene expression
cyp19a1a/rps18 gene expression
time (days post FIN injection)
control
FIN
T
T+FIN
D
E
F
Pearson rank= 0.75*
Pearson rank= 0.93***
Pearson rank= 0.85*
T (ng/ml)
cyp11a1/rps18 gene expression
hsd11b/rsp18 gene expression
srd5a/rsp18 gene expression
T+FIN
T+FIN
T+FIN

Figure 5

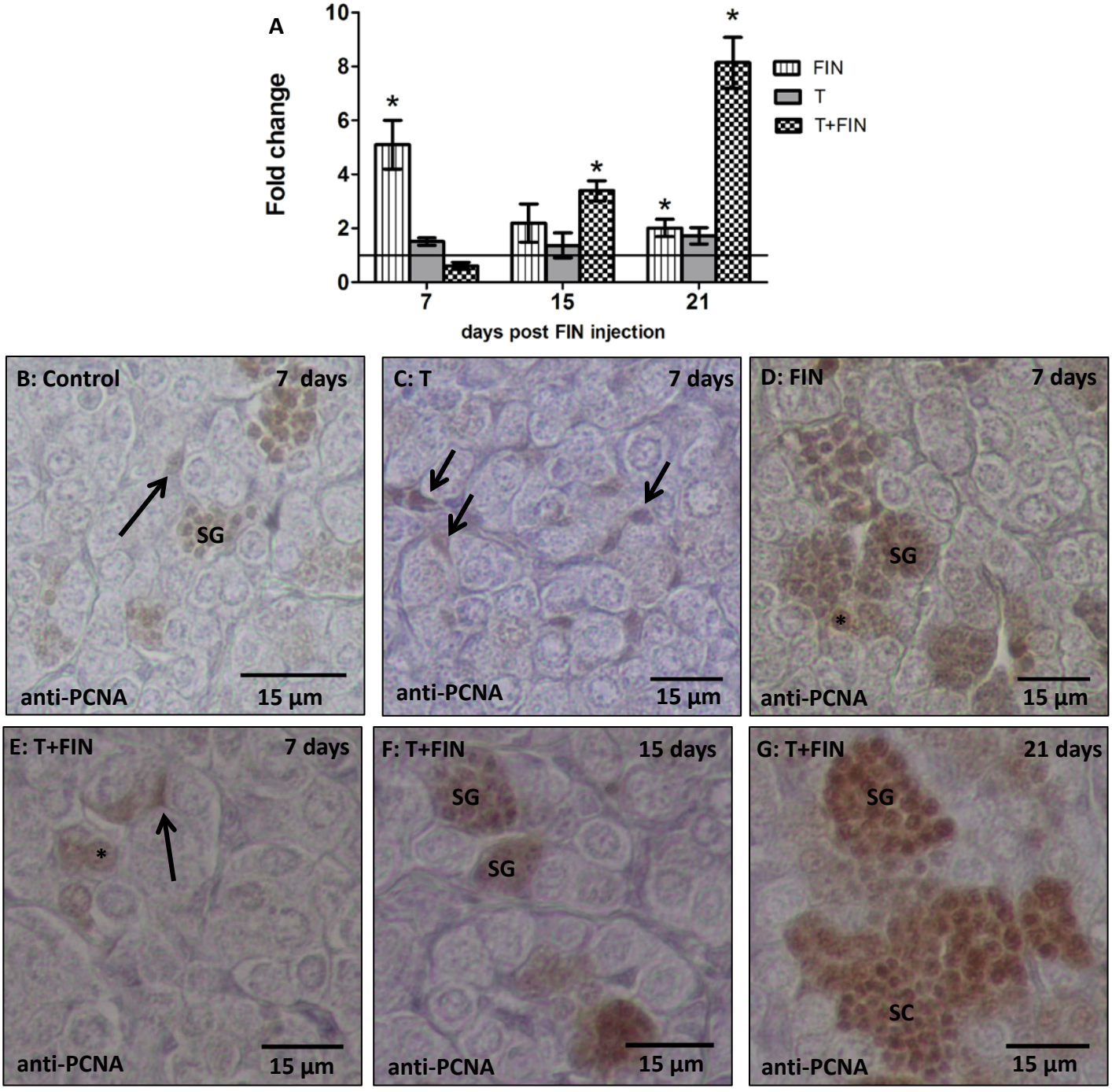

A
Fold change
10
8
6
4
2
0
7
15
21
days post FIN injection
FIN
T
T+FIN
*
B: Control
7 days
SG
anti-PCNA
15 µm
C: T
7 days
anti-PCNA
15 µm
D: FIN
7 days
SG
*
anti-PCNA
15 µm
E: T+FIN
7 days
*
anti-PCNA
15 µm
F: T+FIN
15 days
SG
SG
anti-PCNA
15 µm
G: T+FIN
21 days
SG
SC
anti-PCNA
15 µm

Figure 6

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

Gene	Accession number	Name	Sequence (5'-3')	Use	Firstly used reference
cyp11a1	FM159974.1	F	CGCTGCTGTGGACATTGTAT	Real time PCR	(Sánchez-Hernández et al., 2013)
		R	CATCATGTCTCCCTGGCTTT		
cyp11b1	FP332145	F	GCTATCTTTGGACCCCATCA	Real time PCR	
		R	CTTGACTGTGCCTTTCAGCA		
hsd11b	AM973598	F	AGACATGGGCAACGAGTCAG	Real time PCR	
		R	TCCACATCTCCCTCCCACAT		
srd5a	AM958800	F	TGCACTTTCGTGACTCTGCT	Real time PCR	
		R	TTTCGCACAAGACGTCCAGA		
cyp19a1a	AF399824	F2	CAATGGAGAGGAAACCCTCA	Real time PCR	
		R2	ATGCAGCTGAGTCCCTGTCT		
rps18	AM490061	F	AGGGTGTTGGCAGACGTTAC	Real time PCR	(Chaves-Pozo et al., 2008)
		R	CTTCTGCCTGTTGAGGAACC		

Highlights

1. T serum levels regulate DHT serum levels by down-regulating the transcript levels of *srd5a*.
2. DHT triggers the onset of the meiotic phase of spermatogenesis.
3. DHT is involved in the regulation of E_2 serum levels.