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Differential action of androgen implants on the spermatogenesis of pre-pubertal sea bass exposed to a continuous light regime

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ABSTRACT

This study investigated the influence of long-term T and 11-KT administration (by means of an implant) on the synthesis and release of gonadotropins (Fsh, Lh) and spermatogenesis progression in juvenile male sea bass exposed to either a natural photoperiod (NP) or continuous light (LL). The results in the control group indicated a clear role for Fsh and 11-KT in the recruitment of type-A spermatogonia (SPGA) towards more advanced stages of spermatogenesis under the NP regime. Fsh promoted androgen synthesis and SPGA differentiation into spermatocytes, while no significant changes in pituitary Gnrh1 and plasma Lh levels were detected. On the other hand, the LL regime inhibited Fsh synthesis, and although no effects were observed on Gnrh1 and Lh, the progression of spermatogenesis was arrested. T and 11-KT administration increased the Gnrh1 and Lh content and reduced the pituitary Fsh content, regardless of the photoperiod regime, although the inhibitory effect of T on pituitary Fsh was stronger than that of 11-KT. Our results show that exogenous 11-KT administration in fish under the LL regime was able to partially restore the progression of spermatogenesis, thus stimulating gonadal development and eliciting spermiation in a few fish, while males that received T implants remained sexually immature under LL. In addition, our results provided new knowledge about the importance of Fsh bioactivity modulation in the regulation of this gonadotropin function, and in turn, on spermatogenesis control in fish.

1. Introduction

Gametogenesis is a biological process under the influence of numerous factors that act in a hierarchical manner along the brain-pituitary-gonadal axis (BPG), with the production of neuropeptides and neurotransmitters in the brain, gonadotropin hormones (Gths) in the pituitary and sexual steroids in the gonad and brain, all of this regulated by complex feedback mechanisms. Besides this extraordinary performance, there is a strong interaction with environmental factors (photoperiod) in fish that contributes to the activation of the BPG axis and triggers sexual maturation (Schulz et al., 2010; Levavi-Sivan et al., 2010; Carrillo et al., 2015; Mazón et al., 2015). Accordingly, the study of gonadal development represents an opportunity for a better understanding of the regulatory pathways involved in the control of fish reproduction. It is known that in many perciform fish, three different gonadotropin releasing hormone (Gnrh) forms are expressed in the brain. Of these, Gnrh1 represents the main hypophysiotrophic hormone that elicits the release of Gths, the follicle stimulating hormone (Fsh) and the luteinizing hormone (Lh) to regulate gonadal physiology (Kah et al., 2007; Mazón et al., 2015). Gonadotropins interact with their receptors (Fshr and Lhr) in a highly specific manner in the gonads, although some cross-activation has been reported in several species, thus evidencing that the biological activities of fish gonadotropins seem to be less clearly separated (Kah et al., 2007; Schulz et al., 2010; Molés et al., 2020). Data available on gonadotropin plasma levels from most of the teleost analyzed so far have shown that plasma Fsh levels increase during the early stages of gametogenesis, and then decrease before the final stages of fish gamete maturation and spermiation. On the other hand, circulating Lh levels are low or undetectable at the start of gonadal development, and they increase in the final stages of gonadal growth (Schulz et al., 2010; Molés et al., 2020; Mazón et al., 2015). In light of this, piscine Gths have also been shown to be potent steroidogenic hormones. Interestingly, Fsh is able to rouse steroidogenesis during the initiation of spermatogenesis, thus inducing the production of 11-ketotestosterone

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(11-KT), the major androgen of teleosts that stimulates spermatogonial proliferation towards meiosis (Miura et al., 1991a, 1991b; Kamei et al., 2005; Ohta et al., 2007; Molés et al., 2008; García-López et al., 2009; Mazón et al., 2014), although it is believed that its action might be also mediated by growth factors (Schulz et al., 2010).

The European sea bass (*Dicentrarchus labrax* L.) is a highly valuable marine fish for Mediterranean and European aquaculture. In wild populations in the Mediterranean Sea, males reach sexual maturation at the age of 2 years, while females are mature at 3–4 years of age. However, under intensive culture conditions, males outnumber females, reaching up to 70% in the population, in which 20–30% of 1-year-old males are precocious fish (Carrillo et al., 1995, 2015; Felip and Piferrer, 2018). This situation leads to unwanted side effects that particularly affect sea bass male growth during their second year of life (Felip et al., 2006). As a result, this species has been the subject of various studies on the hormonal and environmental control of puberty, with a special emphasis on males (Carrillo et al., 2009a, 2009b, 2015; Rodríguez et al., 2012, 2019). Although the mechanisms of action on the onset of early puberty are not completely understood, significant progress has been made in recent years in this species. Along these lines, it has been demonstrated that sex steroid treatment increases brain Gnrh2 levels in juveniles, thus inducing the positive feedback of steroids at the brain and/or pituitary level that accelerates gonadal differentiation and stimulates spermatogenesis in pre-pubertal sea bass (Zanuy et al., 2001). Moreover, the administration of steroid implants to adult sea bass during their resting period also exerts different actions on gonadotropin expression, inhibiting Fsh, while stimulating Lh synthesis (Mateos et al., 2002). Gnrh administration is also able to stimulate Lh synthesis and release, but it has no effect on Fsh synthesis (Mateos et al., 2002). On the other hand, a vast number of studies on fish indicate that piscine Gths are potent steroidogenic hormones. In this sense, both in vivo and in vitro experiments support the proliferative effect of Fsh on spermatogonia (Ohta et al., 2007; Kazeto et al., 2008; Kobayashi et al., 2010), although further results provide evidence that the main function of Fsh in fish spermatogenesis is to stimulate androgen production (Miura et al., 1991a, 1991b, 1996; Kamei et al., 2005; Ohta et al., 2007; Molés et al., 2008; García-López et al., 2009; Mazón et al., 2014). In light of this, in European sea bass, a stimulatory effect of native Fsh has been shown on the production of 11-KT in testicular tissue cultured in vitro (Molés et al., 2008). Furthermore, the in vivo administration of Fsh is able to trigger spermatogenesis in prepubertal male sea bass, initiating the proliferation of spermatogonia to meiosis and evoking the reduction of anti-müllerian (*amh*) expression (Mazón et al., 2014). This study also demonstrated that Fsh prepares the sea bass testes for Lh activity through the upregulation of *lhr* expression (Mazón et al., 2014). These results are in agreement with previous studies on juvenile male European sea bass (Rodríguez et al., 2005; Felip et al., 2008; Rodríguez et al., 2019) and adult Atlantic cod (*Gadus morhua*) (Almeida et al., 2011), where continuous light (LL) produced a significant downregulation of pituitary *fshβ* mRNA expression, 11-KT plasma levels and an inhibition of testicular growth. All these results suggest that continuous light reduces precocity by impairing Fsh synthesis. Additionally, pharmacokinetic studies of Gths in European sea bass (Molés et al., 2011a) suggest that constitutive plasma levels of Fsh seem to be necessary for gonadal development, whereas short-term increases in Lh levels have a specific effect throughout the reproductive cycle, and therefore are likely to be cleared more quickly. On the other hand, the pituitary Fsh bioactivity profile in European sea bass females suggests that Fsh isoforms with different potencies may be present throughout the gonadal development stages in this species (Molés et al., 2011b). The aim of this study is to elicit the effect that high circulating steroid levels exert in juvenile male sea bass under a continuous light regime (LL). Accordingly, the action of testosterone (T) and 4-androsten-3,11,17-trione, the precursor for 11-ketotestosterone (11-KT), on Gnrh content and the regulation of sea bass gonadotropins are analyzed in order to determine the differential action of T and 11-KT on testicular growth under a LL regime.

2. Material and methods

2.1. Animal housing: photoperiodic regime and sex-steroid implantation

The experiment was conducted at the facilities of the Instituto de Acuicultura Torre de la Sal (IATS; Castellón, Spain, 40°N 0°E. Juvenile male sea bass (average weight:3.5 g) were obtained from Aquanord (Gravelines, France) in May and evenly distributed into six 2000-l capacity tanks at an initial stocking density of 2.5 kg m^3. Four of them were exposed to continuous light (24 h light/day; LL), and the other two to a simulated natural photoperiod (NP). The temperature ranged naturally between 12 and 25 ± 1 °C. Tanks were provided with well-aerated running seawater (salinity = 37–38‰), and photoperiodic regimes were regulated by means of electronic clocks that controlled tungsten bulbs (650–700 lx; Philips, PAR38Pro) located at the water's surface. A total of 312 fish were used for implantation. Steroids were implanted in October, once the fish attained a weight of 70 ± 3.0 g and a length of 17.7 ± 0.33 cm. Steroid solid silastic implants (DowCorning, Midland, MI) were prepared as previously described for sea bass (Zanuy et al., 1999) and administered via a small 2- to 3-mm incision in the abdomen. All fish were treated with povidone iodine (Betadine®) after implantation. Testosterone (T) and 4-androsten-3,11,17-trione, a precursor of 11-ketotestosterone (hereafter called 11-KT) were purchased from Sigma (St. Louis, MO). Accordingly, fish in these six groups (n = 52 fish/tank) were either i) kept under NP and administered empty implants (control group), ii) kept under LL and administered empty implants (control group for photoperiod manipulation), iii) kept under NP and administered T implants, iv) kept under NP and administered 11-KT implants, v) kept under LL and administered T implants or vi) kept under LL and administered 11-KT implants. Males received one silastic implant, either empty or containing T or 11-KT at a dose of 80 μg/g of fish. The day on which the implants were inserted was considered to be the start of the experiment (day 0). The experiment lasted for a total of five months, starting in October and ending in February, coinciding with the reproductive period in this species. The feeding regime was adjusted according to temperature and fish size, following standard procedures (Barnabé, 1995a, 1995b). Twice a day, fish were hand fed pellets from Proaqua Nutrición, S.A. (Palencia, Spain) (protein 54–45%, lipid 20–12%, carbohydrate 9–25%, ash 11%, moisture 1–3%, DE 22.4–19.7 MJ kg^{-1}). The sex ratio (100% males) was confirmed by histological examination throughout the experiment at each sampling interval. All animal experiments were conducted in accordance with the guidelines for animal experimentation established by European legislation (ETS No. 123, 01/01/91).

2.2. Sampling procedures

From October to February, coinciding with testicular recrudescence in this species, fish were periodically sampled on various days after implantation (DAI): 0 (October 6), 60 (December 6), 107 (January 22) and 143 (February 25). Animals were anaesthetized with MS-222 before sampling and were subsequently weighed (W) and measured (L). Blood samples were collected from the caudal vein using 1-ml heparinized syringes, transferred to 0.5-ml Eppendorf tubes treated with heparin, and placed on ice. Tubes were subsequently centrifuged at 3000*g* for 30 min at 4 °C. The plasma was stored at −20 °C until analyzed. At each sampling point, 10–13 fish per group were sacrificed and their gonadosomatic index (GSI) was calculated according to the formula: gonad weight/body weight x 100. The pituitary was separated from the brain, frozen on dry ice and stored at −80 °C until later use for the determination of gonadotropin content.

2.3. Hormonal analysis

The pituitary content of Gnrh1 was determined using a competitive enzyme-linked immunosorbent assay (ELISA), as described by Holland

et al. (1998). The assay had a sensitivity of 6 pg/well and the intra- and inter-assays coefficients of variation (CV) were 6% and 7%, respectively. Protein levels were measured according to Bradford (1976). In order to analyze Fsh levels, the following two methods were used. In the pituitary, Fsh content was determined by a specific immuno-dot-blot assay according to Molés et al. (2011b). The sensitivity of the immuno-dot-blot assay was 162.8 ng/ml and the intra-and inter-assay CV were 9.8% and 11.5%, respectively. In plasma, circulating Fsh levels were determined by homologous ELISA, as described by Molés et al. (2012). The sensitivity of the assay was 1.59 ng/ml and the intra- and inter-assay CV were 2.2% and 5.4%, respectively. The levels of Fsh bioactivity in plasma were estimated using the method previously described by Molés et al. (2011b). The sensitivity of the in vitro bioassay was 0.104 ng/ml and the intra and inter-assay coefficients of variation were 9.3% and 10.9%, respectively. Due to the use of different methodological approaches and standards, a relative representation of the values was implemented to normalize Fsh data and thus avoid differences in the representation of the concentrations. More specifically, the values were expressed as fold times the levels of the control group (NP) obtained at 60 DAI (NP60 = 1). The levels of Lh in pituitary and plasma were measured by homologous competitive ELISA, as described by Mateos et al., 2006. The sensitivity of the assay was 0.65 ng/ml, and the intra- and interassay coefficients of variation were 11.7% and 11%, respectively. Plasma T was determined using a specific immunoassay (EIA) as described by Rodríguez et al. (2000), and plasma 11-KT was analyzed using an EIA developed for Siberian sturgeon (Cuisset et al., 1994) and modified for European sea bass (Rodríguez et al., 2005). The sensitivities of the assays were 5–0.009 ng/ml and 1.75 pg/well for T and 11-KT, respectively, and the interassay CV was 9.5% in both cases.

2.4. Histology analysis

A small piece of gonad tissue was fixed for 24 h in 4% formaldehyde:1% gluteraldehyde buffered saline (McDowell and Trump, 1976), dehydrated in a 70–96% ethanol series, and then embedded in glycol methacrylate resin (Technovit 7100; Heraeus, Kulzer, Germany). 2-μm-thick sections were stained with methylene blue/azure II/basic fuchsin (Bennett et al., 1976). Identification and characterization of the stage of testicle development was determined according to Begtashi et al. (2004). Based on this, stages I and II were considered to be indicators of mitotic proliferation of spermatogonia towards meiosis (i.e., psm stage), including the presence of spermatogonial mitoses and the initiation of the meiotic phase with primary spermatocytes by stage II. On the other hand, stages III, IV and V were considered to be indicators of gonadal maturity, including the presence of secondary spermatocytes and the spermiogenic phase with haploid spermatids emerging and differentiating into spermatozoa (i.e., the spermiogenesis stage towards sperm maturation, ssm). In any case, fish at stage V were considered as running males (early maturing fish), and thus animals were gently stripped to evaluate the percentage of spermiation. The number of type-A spermatogonia (SPGA) was identified and counted in 18 standard areas (11,439 μm^2/standard area) per fish using a Nikon microscope at 1000×. To this end, 2-μm-thick sections were distributed on one slide as follows: 6 standard areas at three different depths: x, +75, +75 μm, with x being the first histological section to be analyzed.

2.5. Statistics

A two-way ANOVA test was used to compare the effects of the implants and photoperiodic regimes on reproductive parameters, including the number of SPGA, the percentage of fish in stages I + II and III + IV + V and hormonal analyses of juvenile male sea bass at different sampling points. When necessary, normality was assessed by applying the Kolmogorov–Smirnoff test after a logarithmic transformation of data. Barlett's test was used to establish homogeneity of variance. A Tukey HSD multiple range test was used to examine significant differences between means. Nominal data of the percentage of early maturing fish versus immature fish under the different photoperiodic regimes was calculated using the Chi-Square statistic and applying the Yates correction when v = 1, or the Bonferroni inequality for P values when performing a multiple test. The analyses were conducted using the statistical software SigmaStat version 3.5 (SYSTAT Software Inc.). All data were expressed as the mean ± SEM. Differences were accepted as significant when $P < 0.05$ (Sokal and Rohlf, 1981).

3. Results

3.1. Steroid plasma levels

Blood samples from juvenile male sea bass implanted intraperitoneally with sham, T or 11-KT silastic implants were analyzed at 0, 60, 107 and 143 days after implantation. T plasma levels in sham-implanted fish showed a similar hormonal profile independently of the photoperiodic regime, with a significant increase from 107 DAI onwards (Fig. 1A). Of note, plasma T levels in T-implanted fish steadily increase from 60 DAI onwards regardless of the photoperiod, reaching higher values than those seen in sham-implanted fish (Fig. 1B). These results demonstrated that T silastic implants were able to maintain high plasma levels of this steroid throughout the entire experiment. In the case of 11-KT-implanted fish, plasma T levels showed a significant increase at 60 DAI under both photoperiods, followed by a decrease at 107 DAI, and then increased again at 143 DAI, albeit the differences were only significant for the LL group (Fig. 1C). When implantation groups were compared, significant T level differences were observed between 11-KT-implanted and sham-implanted animals under both photoperiod regimes (Fig. 1A and C), as well as between 11-KT-implanted and T-implanted fish, with the latter group displaying significantly higher plasma T values (Fig. 1B and C). With regard to 11-KT plasma levels, NP sham-implanted fish showed a significant increase at 60 DAI, which peaked at 107 DAI, and then decreased at 143 DAI; whereas those exposed to LL displayed low levels of this hormone throughout the entire experimental period, with a maximum at 107 DAI (Fig. 1D). In the case of T-implanted fish, 11-KT plasma levels remained constantly low throughout the experiment under both photoperiods, with only a slight increase at 60 DAI (Fig. 1E). On the contrary, 11-KT plasma levels in 11-KT-implanted fish exhibited a large, significant increase at 60 DAI, and remained high until the end of the experiment, thus proving that 11-KT silastic implants were able to maintain high plasma levels of this androgen throughout the experiment (Fig. 1F). Interestingly, the profile of circulating 11-KT levels was very similar for under both photoperiod regimes. 11-KT values in these fish were also significantly higher as compared to sham-implanted fish from 60 DAI onwards regardless of the photoperiod (Fig. 1D and F). Finally, significant differences between 11-KT-implanted and sham-implanted fish exposed to NP and LL were also observed with respect to their counterparts carrying T implants (Fig. 1D, E and F).

3.2. Effects of steroid implants on gonadal development

High numbers of SPGA were found in sham-implanted juvenile fish at day 0 under both photoperiods. SPGAs remained constantly high for the LL group at all time points, whereas they significantly decreased from January (107 DAI) onwards for the NP group (Fig. 2A). A similar photoperiod-dependent pattern of SPGA numbers was also seen in T-implanted fish (Fig. 2B), while 11-KT-implanted fish evidenced a significant decrease in the number of SPGA from 60 DAI onwards, regardless of the photoperiodic treatment (Fig. 2C). It is interesting to note that the percentage of fish showing mitotic proliferation of spermatogonia towards meiosis (psm: stages I + II) significantly decreased at 107 and 143 DAI in comparison to day 0 in NP sham-implanted fish, but no changes were seen in the group of male sea bass kept under LL conditions (Fig. 2D). With regard to T-implanted fish, the percentage of

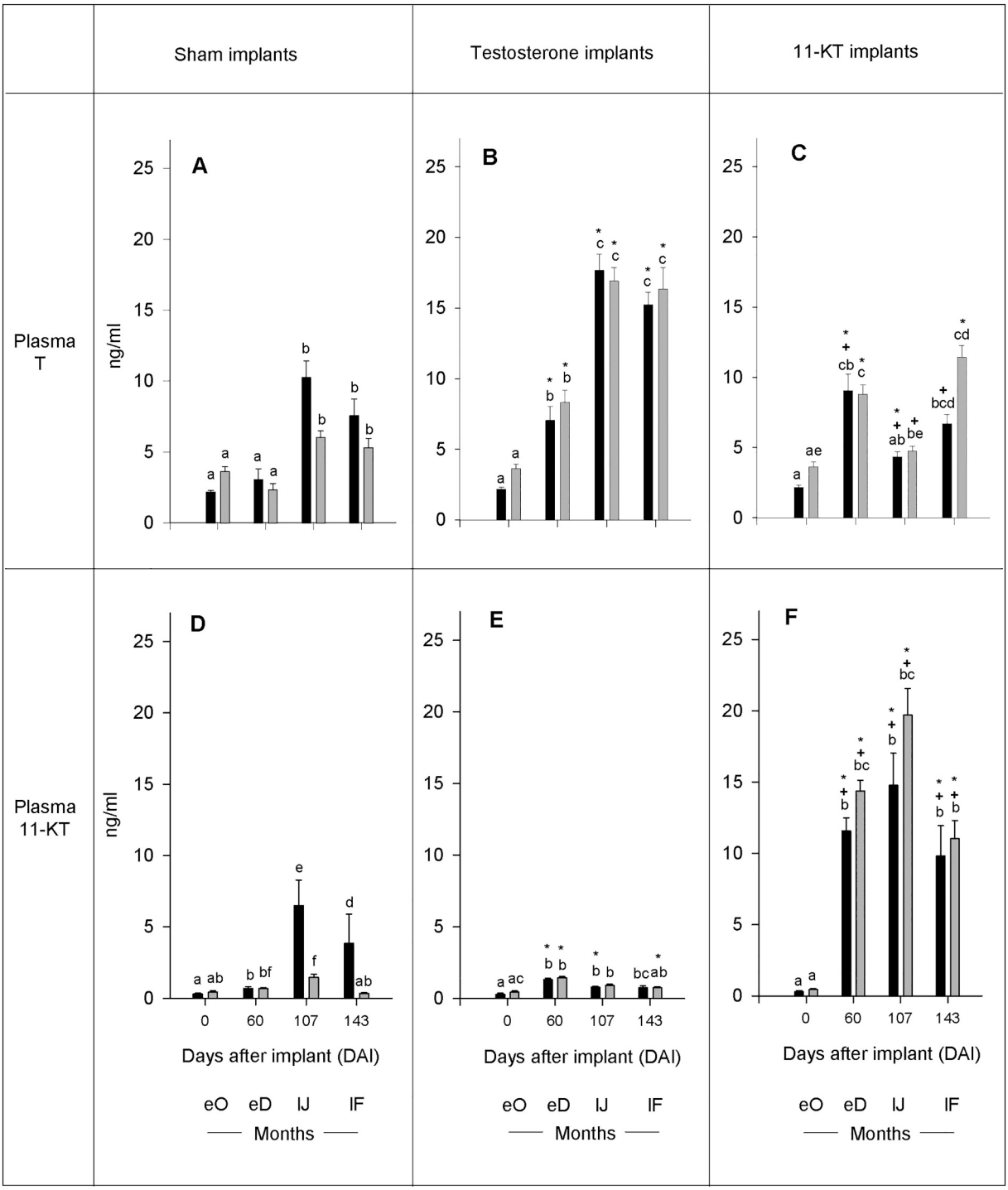

Fig. 1. Evolution of T (A, B, C) and 11-KT (D, E, F) plasma levels in male sea bass kept under a simulated natural photoperiod (NP; black bars) or continuous light (LL; grey bars) conditions and implanted with either an empty (sham) silastic implant or one containing testosterone (T) or 11-ketotestosterone (11-KT) at a dose of 80 μg/g of fish. The day on which the implants were inserted was considered to be the start of the experiment (day 0), which lasted until day 143 (February) post-treatment, coinciding with advanced gametogenesis in this species. Different letters indicate significant differences in the same photoperiod over time and between photoperiods in the same implant treatment. (*) Asterisks indicate significant ($P < 0.05$) differences as compared to the corresponding group of sham-implanted fish; (+) indicates differences as compared to the corresponding group of T-implanted fish. Level of significance: $P < 0.05$. Values of 10–13 individuals were analyzed at each sampling point per treatment. Data are expressed as the mean ± SEM. eO = early October; eD = early December; lJ = late January; lF = late February.

psm-stage fish in the NP group significantly decreased at 107 DAI and then reached values similar to those of day 0; meanwhile, those subjected to LL exhibited a similar percentage to that observed in LL sham-implanted animals (Fig. 2E). On the other hand, 11-KT-implants evoked a drastic decrease in the percentage of psm-stage fish from 60 DAI onwards in both photoperiodic groups (Fig. 2F). Conversely, the percentage of ssm-stage fish (stages III + IV + V) significantly increased from 107 DAI onwards in the NP sham-implanted group, reaching values as high as 80%; while only around 20% of the LL group was in the ssm-stage (Fig. 2G) at all sampling points. In the case of T-implants, the percentage of fish at the ssm-stage significantly increased in the NP group at 107 DAI as compared to day 0, while no changes were observed

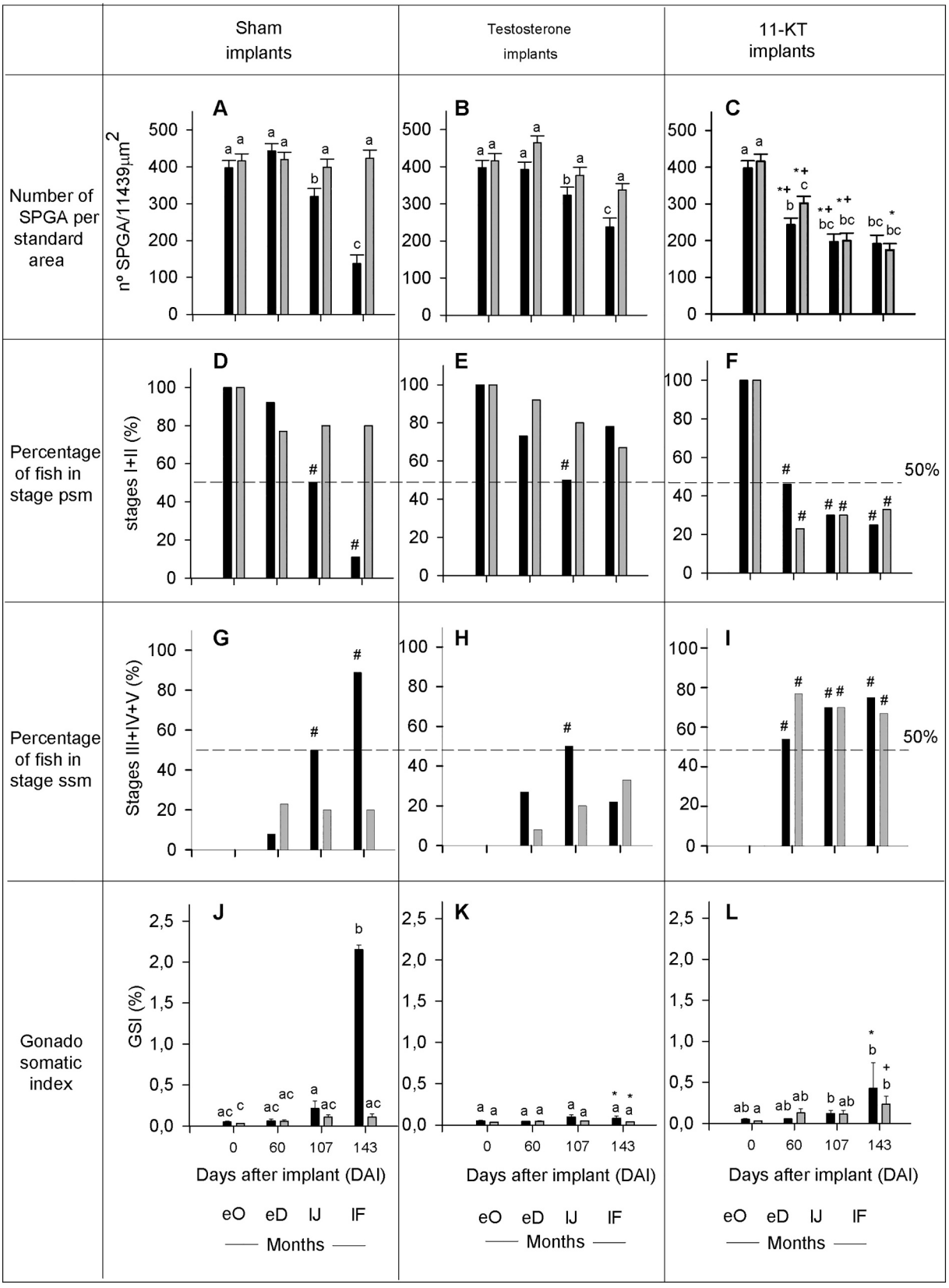

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Fig. 2. Evolution of the number of type-A spermatogonia (SPGA) (A, B, C), percentage of fish showing mitotic proliferation of spermatogonia towards meiosis (i.e., psm; stages I + II) (D, E, F), percentage of fish showing spermiogenesis stage towards sperm maturation (i.e, ssm; stages III + IV + V) (G, H, I) and gonadosomatic index (GSI) (J, K, L) of male sea bass kept under a simulated natural photoperiod (NP; black bars) or continuous light (LL; grey bars), and implanted with either an empty (sham) silastic implant or one containing testosterone (T) or 11-ketotestosterone (11-KT) at a dose of 80 μg/g of fish. The day on which implants were inserted was considered to be the start of the experiments (day 0), which lasted until day 143 (February) post-treatment, coinciding with advanced gametogenesis in this species. Different letters indicate significant differences in the same photoperiod over time and between photoperiods in the same implant treatment. (*) Asterisks indicate significant ($P < 0.05$) differences as compared to the corresponding group of sham-implanted fish; (+) indicates differences as compared to the corresponding group of T-implanted fish, and (#) represents differences from the corresponding group at 0 days after implantation. Level of significance: $P < 0.05$. Values of 10–13 individuals were analyzed at each sampling point per treatment. Data are expressed as the mean ± SEM and horizontal broken lines indicate 50%. eO = early October; eD = early December; lJ = late January; lF = late February.

in the LL group, where this percentage remained below 30% (Fig. 2H). On the other hand, 11-KT-implanted fish displayed a significant increase in ssm-stage percentage from 60 DAI onwards, independently of the photoperiod (Fig. 2I). Finally, while sham-implanted fish in the NP group had significantly higher GSI values (2.15%) at 143 DAI, those in the LL group displayed consistently low values (GSI ≤ 0.1; Fig. 1J) at all time points. GSI values were also very low for T-implanted fish throughout the entire experiment under both light regimes (GSI ≤ 0.09; Fig. 2K), while 11-KT-implanted fish exhibited a steady increase at 143 DAI with GSI values of 0.43% and 0.23% for the NP and LL group, respectively (Fig. 2L), although to levels that were not statistically different from the previous sampling point. In addition, the highest incidence of early maturing males, assessed by stripping, was observed in the NP sham-implanted group (Fig. 3A), with 12.6% of spermiating fish in February (143 DAI). By contrast, the LL group exhibited 0% maturity (Table 1, Fig. 3B). Moreover, T-implanted fish under both light regimes showed a clear inhibition of gonadal maturation, with 0% of spermiating fish in February (143 DAI), while a slight increase was seen in the rate of spermiation of 11-KT-implanted fish (2.1% in the NP group and 2.5% in the LL group), albeit not as large as that of sham-implanted animals (Table 1, Fig. 3C).

3.3. Pituitary levels of Gnrh1

Pituitary Gnrh1 levels remained constant during the sampling period in both photoperiodic groups receiving sham implants, with just one non-significant transient increase at 60 DAI (Fig. 4A). Administration of T implants resulted in a significant rise in Gnrh1 content at 60 DAI, which then gradually decreased during the experiment, independently of the photoperiod (Fig. 4B). Interestingly, 11-KT-implanted fish showed a progressive increase in Gnrh1 levels that peaked at 107 DAI, and thereafter reverted to levels similar to those observed at 60 DAI under both light regimes (Fig. 4C). No significant differences between the NP and LL groups were found for any of the hormonal treatments applied throughout the experiment. Nevertheless, it should be noted that under LL conditions, Gnrh1 levels were statistically higher in 11-KT-implanted fish than in sham-implanted fish at 107 DAI (Fig. 4A, and C).

Table 1
Percentage of early maturing fish assessed by stripping juvenile male sea bass during their first annual cycle of life (late February) that were maintained under six different experimental conditions.

Experimental group	Maturing fish (%)
NP + sham	12.6
LL + sham	0.00
NP + T	0.00
LL + T	0.00
NP + 11-KT	2.07
LL + 11-KT	2.50

Abbreviations: animals exposed to a simulated natural photoperiod (NP) or continuous light (LL) and carrying either an empty silastic implant (sham) or one containing testosterone (NP + T, LL + T) or 11-ketotestosterone (NP + 11-KT, LL + 11-KT) at a dose of 80 μg/g of fish. The day on which implants were inserted was considered to be the start of the experiment (day 0), which lasted until day 143 (February) post-treatment, coinciding with advanced gametogenesis in this species.

3.4. Pituitary and plasma levels of Lh

An increase in the pituitary Lh content of NP sham-implanted fish was observed at 60 DAI that remained high until the end of the experiment (143 DAI). Although not statistically significant, this increase at 60 DAI was also present in the LL group, after which pituitary Lh progressively decreased to day 0 levels (Fig. 4D). Overall, administration of T or 11-KT implants under NP conditions (Fig. 4E and F) did not significantly alter the patterns and levels of the pituitary Lh content seen in sham-implanted fish (Fig. 4D). Under LL conditions, however, the pituitary Lh content of T- and in 11-KT-implanted fish at 143 DAI (Fig. 4E and F) was significantly higher than that of sham-implanted fish at the same time point (Fig. 4D). Interestingly, the plasma Lh profiles for the different implants were similar to those of pituitary Gnrh1 content. Regardless of the photoperiod, sham-implanted fish showed a peak in plasma Lh secretion at 60 DAI, which coincided with the increase in pituitary Gnrh1 and Lh content, only to further decrease until the end of

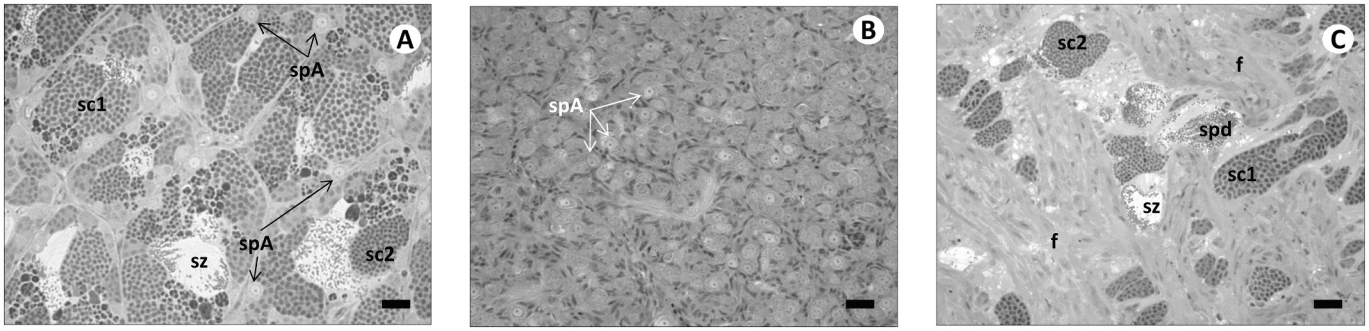

Fig. 3. Micrographs showing the stage of testes development in male sea bass carrying empty implants and exposed to a natural photoperiod (NP, control group) (A) or continuous light (LL) (B), and those under LL carrying 11-KT implants (C). Micrographs were taken at 123 days after implantation. Abbreviations: type-A spermatogonia (SpA), primary spermatocytes (sc1), secondary spermatocytes (sc2), spermatids (spd), spermatozoa (sz) and fibrocytes (f). Scale bars = 10 μm.

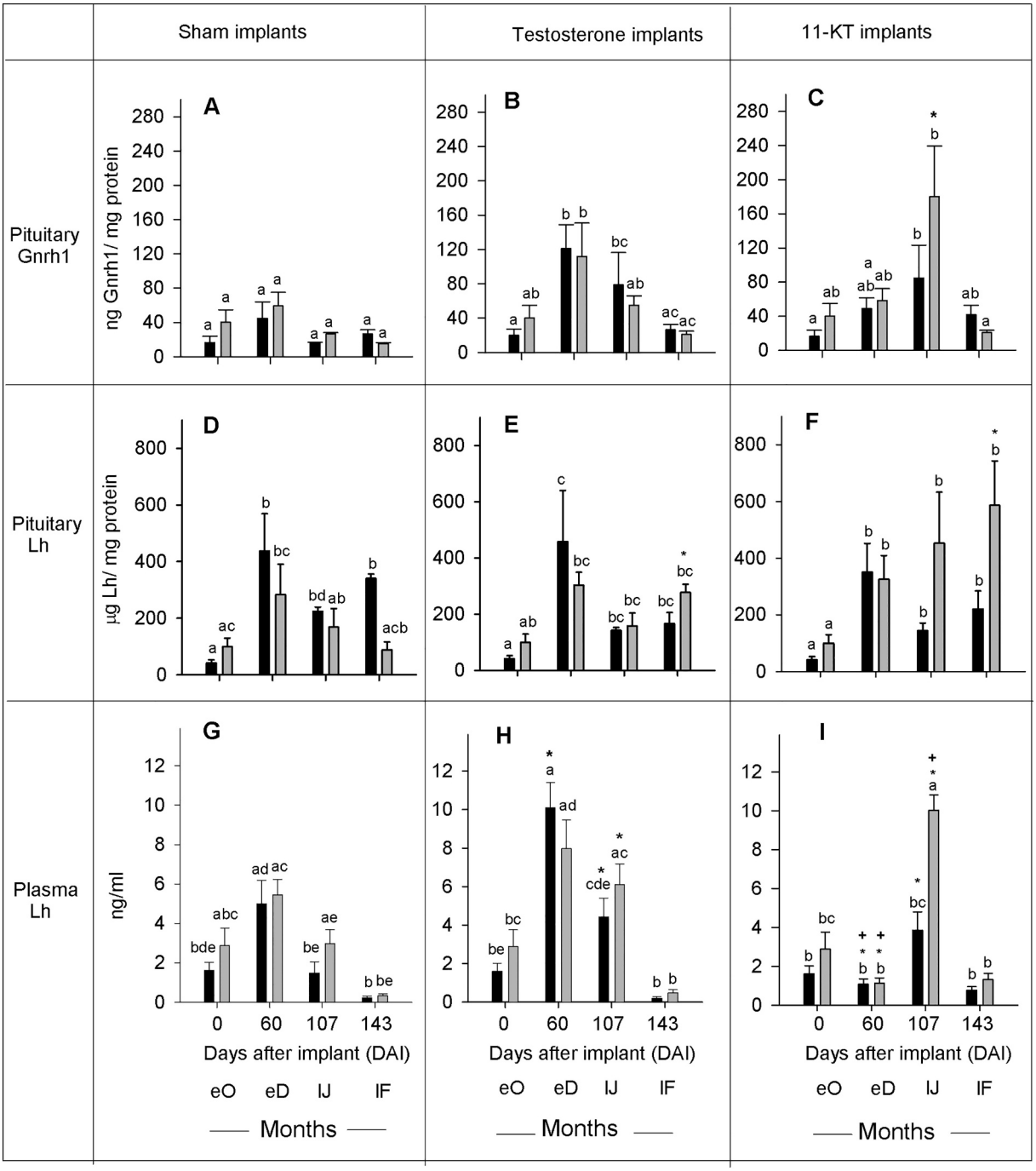

Fig. 4. Evolution of pituitary Gnrh1 content (A, B, C), Lh content (D, E, F) and Lh plasma (G, H, I) in male sea bass kept under a simulated natural photoperiod (NP; black bars) or continuous light (LL; grey bars) conditions and implanted with either an empty (sham) silastic implant or one containing testosterone (T) or 11-keto-testosterone (11-KT) at a dose of 80 µg/g of fish. The day on which the implants were inserted was considered to be the start of the experiment (day 0), which lasted until day 143 (February) post-treatment, coinciding with advanced gametogenesis in this species. Different letters indicate significant differences in the same photoperiod over time and between photoperiods in the same implant treatment. (*) Asterisks indicate significant ($P < 0.05$) differences as compared to the corresponding group of sham-implanted fish; (+) indicates differences as compared to the corresponding group of T-implanted fish. Level of significance: $P < 0.05$. Values of 10–13 individuals were analyzed at each sampling point per treatment. Data are expressed as the mean ± SEM. eO = early October; eD = early December; lJ = late January; lF = late February.

the experiment (Fig. 4G). This was also the pattern for T-implanted fish, although Lh levels at 60 and 107 DAI in both the NP and LL groups were significantly higher with respect to the sham-implanted groups (Fig. 4H). On the other hand, fish carrying 11-KT implants showed a more delayed plasma Lh peak at 107 DAI, with significant differences between the NP and LL groups, and from sham-implanted fish (Fig. 4I). The highest levels of plasma Lh in T and 11-KT-implanted fish coincided with the highest levels of pituitary Gnrh1 content in the same fish (Fig. 4B-C and H-I).

3.5. Pituitary and plasma levels of Fsh

Pituitary Fsh levels in NP sham-implanted fish showed a progressive and significant increase at 107 and 143 DAI. In LL sham-implanted fish,

pituitary Fsh levels remained low throughout the entire experiment, and were significantly lower than those of the NP group at 107 and 143 DAI (Fig. 5A). In T-implanted fish, pituitary Fsh levels remained low throughout all sampling points under both photoperiods, with significantly lower values than those of their counterparts in the sham-implanted group at 107 and 143 DAI (Fig. 5B). Furthermore, 11-KT-implanted fish also showed constant low levels of Fsh throughout the experiment, regardless of the light regime, with values for the NP group being significantly lower than those of the NP sham-implanted group at 143 DAI (Fig. 5C). As for plasma Fsh levels, a significant increase in NP sham-implanted fish was observed only at 143 DAI (Fig. 5D), while no significant changes in plasma Fsh levels were detected in the LL group at any sampling point (Fig. 5D). Similarly, no changes were observed between the NP and LL regime in T- or 11-KT-implanted fish (Fig. 5E and F), except for T-implanted fish at 143 DAI, which displayed higher plasma Fsh levels under NP (Fig. 5E). On the other hand, an analysis of plasma Fsh bioactivity was performed on sham-implanted fish according to Molés et al. (2011b), using the HEK293 cell line stably expressing the sea bass Fsh receptor and the firefly luciferase reporter gene under the control of a promotor with cAMP-responsive element binding sites (pCRE-LUC). The results showed an increase in Fsh bioactivity in sham-implanted fish under NP conditions at 107 and 143 DAI (Fig. 6A). The same trend was observed under the LL regime, but in this case it was not statistically significant. Finally, with the data obtained from plasma Fsh bioactivity (Fig. 6A) and quantity (Fig. 5D), a ratio (B:I) expressing the bioactivity of Fsh per unit of mass was calculated. The ratio showed an increase in relative Fsh biopotency under NP at 107 DAI (Fig. 6B).

4. Discussion

In the present study, we investigated the long-term effect of T and 11-KT administration on the synthesis and release of Gths, gonadal growth (GSI) and spermatogenesis progression in juvenile male sea bass exposed to either NP or LL photoperiod regimes during their early sexual maturation (Supplementary Fig. 1). Extended exposure to LL has been reported to be effective in reducing early sexual maturation of juvenile male sea bass (Begtashi et al., 2004; Felip et al., 2008; Rodríguez et al., 2012, 2019). However, the mechanisms of action of the LL photoperiod on gametogenesis, as well as sex steroid feedback mechanisms on gonadotropin regulation, still need further clarification. Previous studies

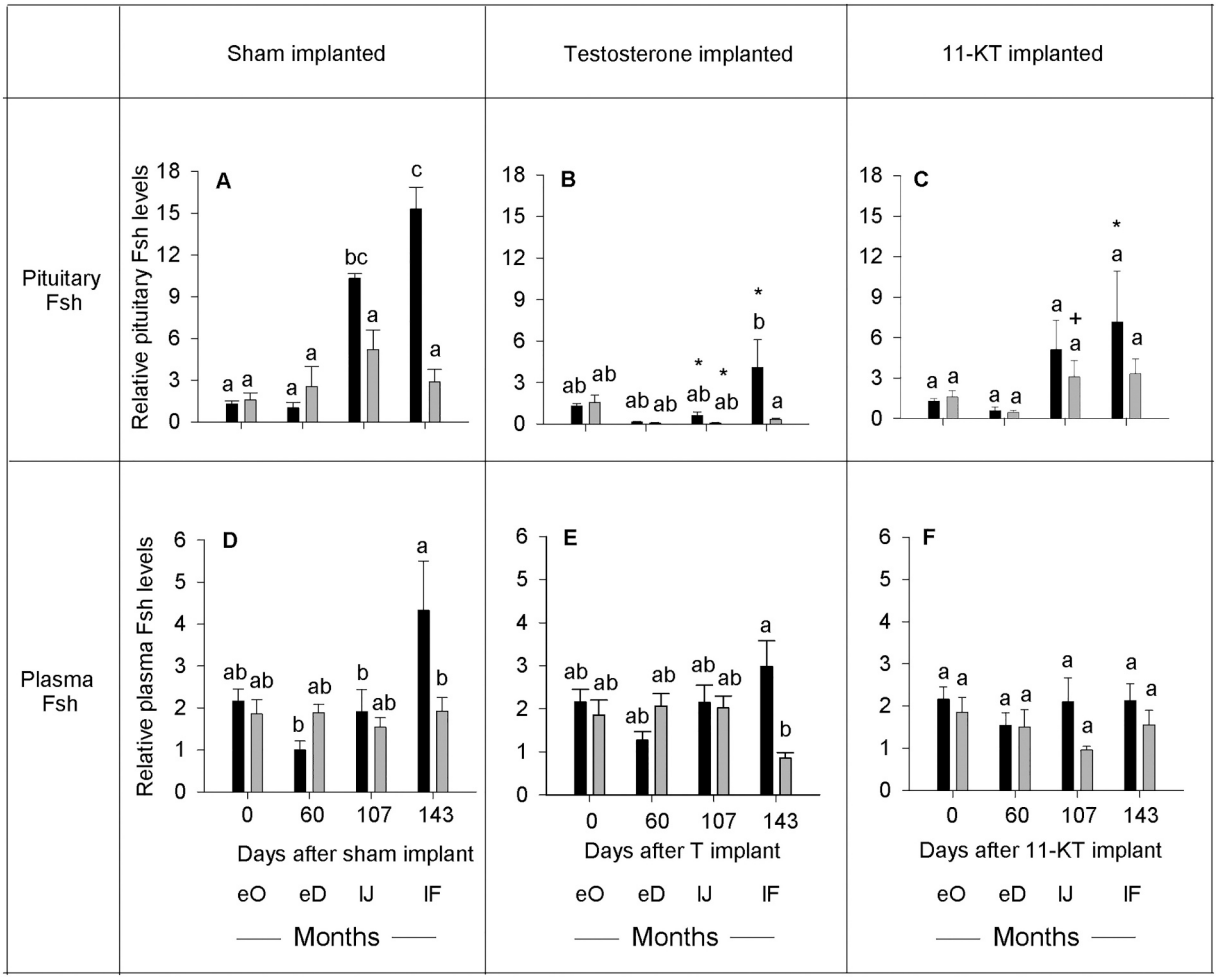

Fig. 5. Evolution of pituitary Fsh (A, B, C) and Fsh plasma (D, E, F) content of male sea bass kept under a simulated natural photoperiod (NP; black bars) or continuous light (LL; grey bars) conditions and implanted with either an empty (sham) silastic implant or one containing testosterone (T) or 11-ketotestosterone (11-KT) at a dose of 80 μg/g of fish. The day on which implants were inserted was considered to be the start of the experiment (day 0), which lasted until day 143 (February) post-treatment, coinciding with advanced gametogenesis in this species. A relative representation was expressed as fold times the levels of the control group (NP), obtained at 60 DAI (NP60 = 1). Different letters indicate significant differences in the same photoperiod over time and between photoperiods for the same implant treatment. (*) Asterisks indicate significant ($P < 0.05$) differences as compared to the corresponding group of sham-implanted fish; (+) indicates differences as compared to the corresponding group of T-implanted fish. No differences were found from the corresponding group at 0 days after implantation. Level of significance: $P < 0.05$. Values for 5–8individuals were analyzed at each sampling point per treatment. Data are expressed as the mean ± SEM. eO = early October; eD = early December; lJ = late January; lF = late February.

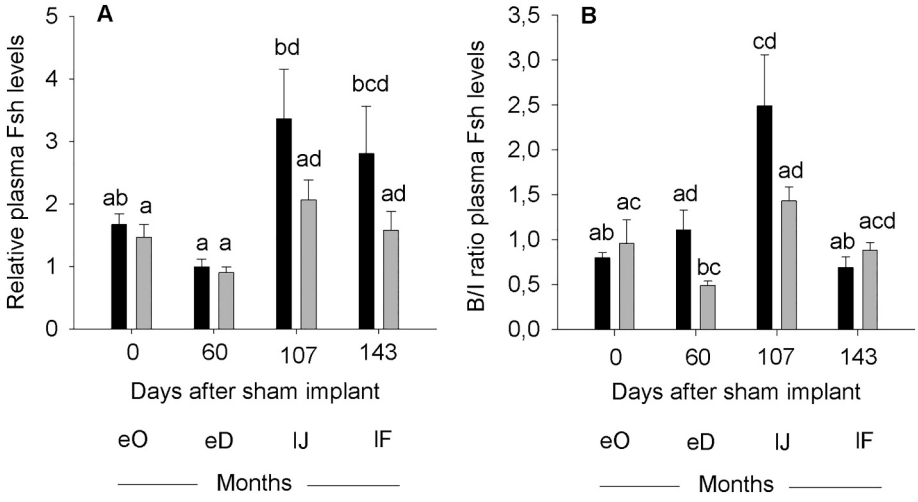

Fig. 6. Analysis of plasma Fsh biological activity in sham-implanted fish. (A) Plasma Fsh Bioactivity levels. A relative representation was expressed as fold times the levels of the control group (NP) obtained at 60 DAI (NP60 = 1). (B) Biological to immunological (B/I) ratios in plasma samples. Different letters indicate significant differences in the same photoperiod overtime and between photoperiods. Level of significance: $P < 0.05$. Values for5–8 individuals were analyzed at each sampling point. Data are expressed as the mean ± SEM. eO = early October; eD = early December; lJ = late January; lF = late February.

on male sea bass have shown that plasma sex steroids, i.e. T and 11-K, peak in December–January (Rodríguez et al., 2000, 2001; Rocha et al., 2009), which agrees with the high levels of these steroids that we observed in January in the control group (NP sham implants). In this context, 11-KT is considered to be one of the main androgens involved in the initiation of spermatogonial proliferation towards meiosis, and its administration triggers spermatogenesis in several teleost species (Schulz et al., 2010). In this study, we observed an increase in T and 11-KT in January–February in the control group. This was paralleled by a decrease in the number of SPGA and in the proportion of fish in the "psm" stage (I + II), as well as by an increase in the percentage of fish in the "ssm" stage (III + IV + V) and higher GSI values. Of note, while an elevation in pituitary and plasma Fsh levels was observed in the NP group during this period of time, no significant changes in pituitary Gnrh1 and plasma Lh levels were detected. These findings suggest a clear recruitment of SPGA cells towards more advanced stages of spermatogenesis, with Fsh promoting the synthesis of androgens and the differentiation of types of SPGA into spermatocytes. In this line, previous works on male sea bass have shown that Fsh is able to stimulate the production of 11-KT in vitro and in vivo, thus initiating germ cell proliferation (Molés et al., 2008; Mazón et al., 2014). In our study, the percentage of fish undergoing spermiogenesis and spermiation (i.e., maturing fish) was limited to 12.6%, which might be associated with the low levels of pituitary Gnrh1 and plasma Lh observed in the control group between January and February. Similarly, these results support the idea that Fsh regulates the early phases of gametogenesis in sea bass, while Lh would be responsible for the final maturation processes (Mazón et al., 2015). On the other hand, these low percentages of precocious fish may be also explained by the reduced body size and length (around 120 g and 20 cm) of the animals by the culmination of their first sexual maturation. According to Rodríguez et al. (2012), low weight and small size may be a limiting factor for the onset and/or completion of gametogenesis, and consequently, fish populations with animals that have reached larger sizes also exhibit a higher percentage of precocious fish.

Of note, the administration of T and 11-KT silastic implants clearly increased the plasma levels of these steroids, with values above the physiological levels seen in the control group (NP Sham). This increase in the plasma values was similar in both light regimes, triggering a significant increase in the levels of pituitary Gnrh1 that elicited an elevation of Lh plasma levels in prepubertal male fish. Interestingly, the high levels of Lh observed in plasma under LL carrying 11-KT in comparison to NP might be a response to the high levels of Gnrh1 observed in these treated fish. Thus, the 11-KT administration resulted in an elevation of Gnrh1 pituitary content. Although this elevation occurred in both photoperiods, it was slightly higher in LL and could explain the differences observed in the plasma Lh levels. On the contrary, steroid treatments inhibited the rise of pituitary and plasma Fsh levels in comparison to those observed in the NP control group (107 and 143 DAI). This illustrates the positive and negative feedback effects that sex steroids exert at the brain and pituitary level. Similar observations have been described in adult sea bass, where treatment with T showed a clear increase in lhβ mRNA levels and a reduction in those of fshβ (Mateos et al., 2002). Thus, it seems that no apparent differences exist in the response to T between sexually immature juvenile fish and mature adult males in sea bass. Positive and negative feedback effects have also been reported in other teleost fish, mainly salmonids (Crim and Evans, 1979; Magri et al., 1985; Schreibman et al., 1986; Dubois et al., 2001; Larsen and Swanson, 1997; Dickey and Swanson, 1998; Saligaut et al., 1998). However, information on gonadotropin regulation by steroids is still scarce in other orders, especially with regard to Fsh protein levels, due to the fact that assays for the determination of Fsh have been restricted to a reduced number of fish species for a long time. In this study, T and 11-KT administration resulted in an elevation of Gnrh1 pituitary content, independently of the photoperiod. This agrees with previous studies on this species in which the annual rhythms of Gnrh1 were unaffected by artificially long photoperiods (Rodríguez et al., 2004; Carrillo et al., 2010). Although this elevation in Gnrh1 pituitary content was associated with an increase in the synthesis and release of Lh, but not Fsh, it remains unclear whether it is influenced by the high levels of T or 11-KT or by a lack of effect of Gnrh1 on Fsh cells. A study on quiescent male sea bass has shown that a differential regulation of Gth synthesis and secretion exists in this teleost species, where GnRHa treatment stimulated Lh release and pituitary expression of lhβ, but had no effect on fshβ mRNA levels (Mateos et al., 2002). In our study, the administration of both androgens reduced the pituitary content of Fsh, regardless of the photoperiod, although the inhibitory effect of T was stronger than that of 11-KT.

Additionally, no recruitment of SPGA cells towards more advanced stages of spermatogenesis was observed under the LL photoperiod, which led to reduced gonadal development (low GSI). The increasing levels of 11-KT and Fsh seen in the control group (Sham; 107 and 143 DAI) were inhibited under LL, while T, Gnrh1 and Lh levels were practically unaltered. Moreover, this inhibition of the synthesis and release of Fsh under LL was enhanced with the administration of T implants. Previous studies on sea bass (Felip et al., 2008; Rodríguez et al., 2019) have demonstrated that fshβ expression is affected by LL, suggesting that this down-regulation might be responsible for the arrest in the progression of spermatogenesis in sea bass. Furthermore, the administration of LL during a photolabile period in this species reduces precocious maturation in sea bass (Rodríguez et al., 2012), suggesting

that the natural photoperiodic change occurring after the summer solstice in this marine teleost fish serves as an external signal that might induce the synthesis and release of Fsh in order to trigger the onset of gametogenesis. In our study, we observed a clear inhibition of pituitary and plasma Fsh levels and a blockage of the onset of spermatogenesis under LL conditions, corroborating the previous studies. However, our results show that exogenous 11-KT administration in this fish under a LL regime was able to partially restore the progression of spermatogenesis, thus stimulating gonadal development and eliciting spermiation in a few fish, while males that received T implants remained sexually immature. These findings are in agreement with the role that 11-KT plays in the initiation of spermatogonial proliferation towards meiosis described in other teleosts (Miura and Miura, 2003; Schulz et al., 2010), while T does not seem to have a direct action on gonadal maturation. In this regard, a previous work on juvenile sea bass showed that T-implants failed to enhance gonadal growth after 76 days of treatment (Zanuy et al., 1999). Nevertheless, the administration of 11KT was by itself insufficient to fully restore the blockage, and the reduced percentage of spermiation seen in 11-KT-implanted fish under LL conditions might be due to the low Fsh levels observed in these animals. Although it has been shown that Fsh may act at early stages of spermatogenesis via the production of 11-KT in other fish species (Ohta et al., 2007; Kazeto et al., 2008), treatments with recombinant Fsh in zebrafish have revealed that Fsh promotes spermatogenesis not only by stimulating androgen production, but also by modulating the production of paracrine factors through androgen-independent pathways (Nóbrega et al., 2015; Safian et al., 2019). Similarly, in rainbow trout, Fsh exerts steroid-independent regulatory functions on many genes that are important for the onset of spermatogenesis (Sambroni et al., 2013), and in sea bass, Fsh prepares the testes for Lh activity by upregulating Lhr expression (Mazón et al., 2014). It is worthy to note that the first significant surge of pituitary Fsh and plasma Fsh bioactivity in the control group (NP Sham) occurred concomitantly with active spermatogonial mitosis and the onset of meiosis (107 DAI), supporting the role of Fsh in the onset of gametogenesis in sea bass (Mazón et al., 2014; this study) and other fish (Almeida et al., 2011; Chauvigne et al., 2017; Sanchís-Benlloch et al., 2017). Although Fsh plasma levels were not significantly high at 107 DAI, the elevated Fsh bioactivity suggests higher Fsh potency at the onset of gonadal recrudescence, which was confirmed by the high B:I ratio. Assuming that immunoreactivity reflects the amount of Fsh, and bioactivity reflects its ability to elicit a biological response, the B:I ratio might represent the average quality of those Fsh molecules in a biological sample at a given time. Thus, the use of two different assays for Fsh determination and their combination in this study has allowed us to evaluate the relative bioactivity of defined amounts of Fsh during sea bass spermatogenesis. Our data support the idea of an additional level of gonadotropin regulation based on the modulation of their bioactivity, which may vary throughout the reproductive cycle. Gths are glycoproteins that are not produced as unique molecules, but rather as a collection of isoforms that differ from each other mainly in the type of glycosylation. In mammals, the composition of these oligosaccharides is partly controlled by the hypophysial hormonal milieu. It is known that the extent and type of glycosylation can influence gonadotropin secretion, half-life in circulation and receptor binding, among others (Cahoreau et al., 2015; Ulloa-Aguirre and Lira-Albarrán, 2016), thus modulating the biological function of these reproductive hormones.

Taken together, our results support the hypothesis that Fsh and 11-KT are needed for the onset of spermatogenesis in sea bass. The data suggest that Fsh acts mainly via the production and secretion of 11-KT, which evokes the recruitment of SPGA towards more advanced stages of gametogenesis, although Fsh might also modulate other factors via androgen-independent pathways. In addition, the present study provides novel knowledge regarding the importance of Fsh bioactivity in the regulation of Gths function. Our results confirm that the LL photoperiod disrupts testicular development, as it inhibits the synthesis and release of Fsh, but not Lh, which affects 11-KT levels and, consequently, spermatogenesis. This supports the idea that photoperiod is an essential signal to trigger spermatogenesis. The exogenous administration of 11-KT was shown to be able to partially restore the progression of spermatogenesis under a LL regime. The exogenous administration of both androgens triggers negative feedback on pituitary Fsh synthesis, but the effect of T was stronger than that of 11-KT.

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Authors' contributions

SZ and MC conceived and supervised the investigation. GM, AF and OY participated in sampling, analyses and data processing. SZ participated in histological analysis. MC participated in statistical analysis. GM, AF, SZ and MC contributed to paper writing. All authors read and approved the final manuscript.

Declaration of Competing Interest

Nothing to declare.

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