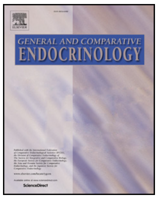
General and Comparative Endocrinology 275 (2019) 82–93

Contents lists available at ScienceDirect

General and Comparative Endocrinology

journal homepage: www.elsevier.com/locate/ygcen



Advanced puberty triggered by bi-weekly changes in reproductive factors during the photolabile period in a male teleost fish, *Dicentrarchus labrax* L.

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A R T I C L E I N F O

Keywords:
Precocity
Spermatogenesis disruption
Continuous light
Reproduction
Teleost

A B S T R A C T

This study evaluated the impact of continuous light (LL) within the photolabile period on advanced puberty in juvenile male European sea bass. The exposure to an LL regime for 1 month, from August 15 to September 15 (LLa/s), was compared to a constant simulated natural photoperiod (NP) and constant continuous light conditions year-round (LLy). Somatic growth, hormone plasma levels, rates of testicular maturation and spermiation, as well as the mRNA levels of some reproductive genes were analyzed. Our results demonstrated that both LLa/s and LLy treatments, which include LL exposure during the photolabile period, were highly effective in inhibiting the gametogenesis process that affects testicular development, and clearly reduced the early sexual maturation of males. Exposure to an LL photoperiod affected body weight and length of juvenile fish during early gametogenesis and throughout the first year of life. Interestingly, LL induced bi-weekly changes in some reproductive factors affecting Gnrh1 and Gnrh2 content in the brain, and also reduced pituitary *fshβ* expression and plasmatic levels of 11-KT, E_2, Fsh throughout early gametogenesis. We suggest that low levels of E_2 in early September in the LL groups, which would be concomitant with the reduced number of spermatogonial mitoses in these groups, might indicate a putative role for estrogens in spermatogonial proliferation during the early gonadal development of this species. Furthermore, a significant decrease in *amh* expression was observed, coinciding with low plasma levels of 11-KT under LL regimes, which is consistent with the idea that this growth factor may be crucial for the progress of spermatogenesis in male sea bass.

1. Introduction

Puberty is a complex biological process in which the brain-pituitary-gonad (BPG) axis acquires functional competence, which results in the ability of an individual to sexually reproduce for the first time (Taranger et al., 2010). In the aquaculture of fin fish species, the occurrence of advanced puberty (precocity or early maturation) is common in males, although it can also affect females (Brown et al., 2014). When this happens, it reduces fish growth before harvest and, consequently, it entails important losses for the aquaculture industry (Felip et al., 2006; Carrillo et al., 2009; Taranger et al., 2010). Several studies have reported that photoperiod is one of the main environmental cues for the control of reproductive maturation in many temperate fish species and seasonal breeders (Carrillo et al., 1993; Bromage et al., 2001; Taranger et al., 2010; Migaud et al., 2010). A vast number of studies have assessed the effect of different photoperiod regimes on fish maturation and growth, and they have reported that some reproductive factors are critical for modulating the reproductive axis in teleosts (Taranger et al., 2010; Carrillo et al., 2015; Espigares et al., 2015a,b, 2017).

In the case of European sea bass (*Dicentrarchus labrax* L.), an economically important marine species and valued fish by consumers, the use of different photoperiod regimes to control male maturation has been widely investigated. While compressed photoperiods have been shown to advance the first sexual maturation (Rodríguez et al., 2001a), these constant long light regimes delay spermatogenesis in this species (Rodríguez et al., 2001b). On the other hand, exposure to continuous light (LL) lasting for 12 consecutive months (Begtashi et al., 2004) or less before or during gametogenesis (Felip et al., 2008) is equally effective in reducing early maturation in underyearling fish. Along these lines, a photolabile period that includes the month of September has been shown to exist in this teleost fish. The application of LL during this period reduces the number of early maturing males (Rodríguez et al., 2012), although LL has no effect on the gonadal maturation of the animals during the second annual life cycle (Felip et al., 2017). Accordingly, further physiological studies aimed at gaining deeper

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https://doi.org/10.1016/j.ygcen.2019.02.008
Received 23 November 2018; Received in revised form 16 January 2019; Accepted 6 February 2019

knowledge of the endocrine regulation of spermatogenesis in sea bass have shown that the temporal profile of both seabream and salmon GnRH forms in the brain and pituitary, as well as the GnRH-R-2A receptor, play an important role during gonadal differentiation and first spermiation in males of this species (Rodríguez et al., 2000a,b; Molés et al., 2007). Furthermore, recent data have revealed that photoperiod signalling modulates the expression of *kiss1* and chicken *gnrh* in the brain, which may be involved in the translation of environmental stimuli to trigger the reproductive axis (Espigares et al., 2017). Moreover, plasma follicle-stimulating hormone (Fsh) levels increase during sex differentiation and testicular growth, but they decrease just before the start of full spermiation. Circulating luteinizing hormone (Lh) levels, on the other hand, show a moderate increase during spermatogenesis, with a significant elevation from late gonadal recrudescence onwards (Molés et al., 2007). Male sea bass under different photoperiods exhibit shifts in hormone profiles that are crucial for gonadal maturation. In fact, LL regimes affect both the follicle-stimulating hormone beta subunit (*fshβ*) expression and 11-ketotestosterone (11-KT) plasma levels in prepubertal fish (Felip et al., 2008). These results are in agreement with recent studies on this same species (Espigares et al., 2017) suggesting that the unbalanced production of this androgen, which is regulated by Fsh (Mazón et al., 2014), might be limiting the stimulation of germ cell proliferation at the testicular level. In zebrafish (*Danio rerio*), Fsh stimulates spermatogonial proliferation and differentiation regulating a stimulatory factor, namely the insulin-like growth factor type 3 (Igf3) (Nóbrega et al., 2015). Also in adult zebrafish, Fsh down-regulates testicular anti-müllerian hormone (*amh*) expression, and mitigates the inhibitory effects of Amh on spermatogenesis and steroidogenesis (Skaar et al., 2011). However, other experiments have shown that *amh* is involved in the maintenance of mitotic proliferation of germ cells during early sex differentiation in medaka (*Oryzias latipes*) (Shiraishi et al., 2007; Morinaga et al., 2007) and black porgy (*Acanthopagrus schelegeli*) (Wu et al., 2010), although it apparently represses the proliferation of steroidogenic cells in prepubertal testes of Atlantic salmon (*Salmo salar*) (Maugars and Schmitz, 2008), which demonstrates that *amh* can exhibit multiple regulatory functions in fish gonadal development. Along these lines, the role of other genes involved in the control of spermatogenesis has also been explored. In Atlantic cod (*Gadus morhua*), for example, changes in the mRNA levels of steroidogenic acute regulatory protein (*star*) have shown a direct relationship with changes in 11-KT levels (Kortner et al., 2008). Sea bass Amh (Halm et al., 2007) and Star (Rocha et al., 2009) may play important roles in testicular development, although their relationship to steroid production and expression of estrogen receptor (*er*) genes on prepubertal fish is unknown. A number of studies have shown the existence in fish of predictive mechanisms that use photoperiod as a proximal factor for the recruitment of individuals into gonadogenesis. Continuous artificial lighting tricks the fish, which fail to maintain the circadian variations in certain reproductive hormones that are necessary for the normal process of reproduction (Falcón et al., 2010). Here we report on bi-weekly changes in the expression of *fshβ*, *amh*, *star* and *er*, brain content of GnRH forms and circulating levels of sex steroids and gonadotropins in males maintained under LL conditions throughout the photosensitive period, in order to decipher a possible mechanism of endocrine control over maturity during early gametogenesis in this teleost fish.

2. Materials and methods

2.1. Animals and photoperiodic regimes

A total of 1500 juvenile fish with a high percentage of males were obtained from Aquanord (Gravelines, France). Fish with an average weight of 4.0 g were distributed in six 2000-l lightproof circular fiberglass tanks (n = 250 fish/tank). Sex-ratio (100% males) was confirmed by histological examination throughout the experiment at each sampling interval. In May, three experimental groups were organized in duplicate as follows: simulated natural photoperiod (NP), constant continuous light (LL) from August 15 to September 15 (LLa/s for 1 month) under otherwise NP conditions, and constant continuous light all the year round (LLy). Artificial continuous light regimes were applied using tungsten lightbulbs (650–700 lx; Philips, PAR38Pro), controlled by means of electronic clocks, and located at the water surface of each tank. Temperature naturally ranged between 12 and 25 ± 1 °C. Fish were fed to apparent satiety, using automatic feeders with pellets from Proaqua Nutrición, S.A. (Palencia, Spain) (protein 54–45%, lipids 20–12%, carbohydrates 9–25%, ash 11%, moisture 1–3%, and DE 22.4 19.7 MJ kg^{-1}), according to temperature and fish size, and using standard procedures (Barnabé, 1995).

2.2. Growth, gonadal maturation and sample collection

Animals were treated according to the guidelines for animal experiments set out in European legislation (ETS No. 123, 01/01/91). Fish were anaesthetised with 2-phenoxyethanol (0.5 ml l^{-1} of seawater) before sampling. All fish from each tank were periodically weighed (body weight, BW) and measured (fork length, FL), and their condition factor was calculated as $100xBW/FL^3$. The percentage of early maturing males was periodically assessed by abdominal massage from December to April. Twenty fish per photoperiod regime (n = 10 fish/replicate) were sacrificed by a quick cut at the level of the medulla oblongata every fifteen days from August 1 to October 15, during early gametogenesis. The gonads were quickly removed and weighed to determine the gonadosomatic index (GSI), according to the formula: GW (g)/BW (g) × 100, where GW = gonad weight. Fragments of testes were frozen on dry ice and stored at −80 °C until later use for total RNA extraction. Small pieces of testes were also fixed in 4% formaldehyde: 1% glutaraldehyde buffered saline for histological analysis (Bennett et al., 1976; McDowell and Trump, 1976). The stages of testicular development were assessed according to the procedure described by Begtashi et al. (2004). Stages I (immature) and IV (late recrudescence) were taken as indicators of sexual maturation, as one of their distinctive characteristics in the presence of spermatogonia and spermatozoids, respectively. The number of mitotic cell divisions of spermatogonia A was evaluated at 50x magnification in twelve optical areas (2500 μm^2) per fish, according to the method described by Escobar et al. (2014) for taking cell counts in histological samples.

2.3. Hormonal analyses

Blood samples were collected from the caudal vein using 1-ml heparinized syringes; these were then transferred to 0.5-ml Eppendorf tubes treated with heparin and placed on ice. Tubes were subsequently centrifuged at 3000 rpm for 30 min at 4 °C. The plasma was stored at −20 °C until it was analyzed for certain reproductive hormones, using validated enzyme immunoassays (EIA). Bi-weekly changes in the circulating levels of certain reproductive hormones were analyzed from August 1 to October 15, during early gametogenesis. Plasmatic levels were determined according to Rodríguez et al. (2000a) for testosterone (T), Rodríguez et al. (2001a) for 11-ketotestosterone (11-KT), Molés et al. (2008) for oestradiol-17b (E_2) and Molés et al. (2012) for Fsh. Similarly, the content in the brain of three forms of gonadotropin-releasing hormones was measured according to Holland et al. (1998) for seabream (Gnrh1) and chicken (Gnrh2) GnRH, and Kah et al. (1994) for salmon GnRH (Gnrh3).

2.4. Relative expression of *fshβ* in the pituitary and *amh*, *star*, *erβ1* and *erβ2* in the gonad as assessed by quantitative real-time PCR

A total of 20 pituitaries per treatment were analyzed at every sampling point in each group for *fshβ*, using a real-time qPCR approach as described by Felip et al. (2008). The gene expression in the extracted gonads was quantified according to Crespo et al. (2013) for anti-

Table 1

Gene-specific primers and Taqman and SybGreen fluorogenic probes, when necessary, used in this study for qRT-PCR analysis.

Gen	Detection method	5′to 3′sequence	Size (bp)[b]	nM[c]	Efficiency (%)[d]
18S rRNA (AM419038)[e] Felip et al. (2008)	SYBR Green-based	[f]F: GCATGCCGGAGTCTCGTT [g]R: TGCATGGCCGTTCTTAGTTG	70	200 200	103
fshβ (AF543314) Felip et al. (2008)	SYBR Green-based	F: TCGATAGTACGTGTCTGTGCT R: CACTTGGATGCTGATGTTGG	131	200 200	99
erβ1 (AJ489523) Blázquez et al. (2008)	SYBR Green-based	F: CTGTCCTCACAGAGTAGAACAC R: GCAGGTGCACCTGGTCCAAG	295	200 200	99
erβ2 (AJ489524) Blázquez et al. (2008)	SYBR Green-based	F: GTGGACTCCAGACTCGGGAC R: ATCATGCTAGCCTCGGTGAAG	246	200 200	98
amh (AM232701) Crespo et al. (2013)	Probe-based	F: TCCAAACACTGCTAACATCAACAA R: TGGCGTGGTTCTTGGGATT [h]pr:[6 ~ FAM]CCATGGCTCATGTGCTTTCCCCCT[TAMRA]	74	50 300 125	90
star (EF409994) Rocha et al. (2009)	Probe-based	F: GGCTGGATCCCGAAGACAA R: CCTGAGGTGGTTGGCAAAGT pr:[6 ~ FAM]CATAAACAAAGTGCTCTCTCAGACGCAGGTG[TAMRA]	72	900 900 75	98

[a]Primers were obtained from Invitrogen Corp. (Carlsbad, CA) and fluorogenic probes were purchased from Operon Biotechnologies GmbH (Cologne, Germany).

[b] Fragment size.

[c] Amount of primer or probe in the PCR.

[d] PCR efficiency.

[e] GenBank accession number for sea bass genes.

[f] F: forward primer.

[g] R: reverse primer.

[h] pr: Taqman fluorogenic probe.

Müllerian hormone (*amh*), Rocha et al. (2009) for the steroidogenic acute regulatory (*star*) protein, and Blázquez et al. (2008) for estrogen receptors (*erβ1* and *erβ2*) (Table 1). To this end, pooled gonad samples (2 gonads/pool) were prepared from fish exhibiting similar plasma levels of 11-KT as an indicator of the maturation stage in juvenile male sea bass (Rodríguez et al., 2012). A total number of 6–10 pools per treatment were analyzed at every sampling point in each group. Total RNA was prepared using TRI-REAGENT solution (Sigma, St. Louis, MO, USA) and each sample was DNase-treated (RQ1 RNase-Free DNase, Promega, Madison, WI, USA) prior to the reverse-transcription procedure. The RNA samples were resuspended in 5 or 20 µl of DEPC water for pituitaries and gonads, respectively. We ran 1 µl of total RNA through a 0.8% agarose gel and used a 0.24–9.5 Kb RNA ladder (Invitrogen, Life Technologies) for quantification by densitometry, using image analysis software (Densitometer GS-710 and Quantity One Software, BioRad). A total volume of 20 µl of reaction, including 2 µg of total RNA and random hexamer primers, was used for reverse-transcription reactions (SuperScript II Reverse Transcriptase, Invitrogen, Life Technologies). The PCR mixture consisted of 1 µl of cDNA sample in a final volume of 25 µl, except in the case of *18S* rRNA and *fshβ*, in which the template was 1 µl of a 1:500 dilution. The qRT-PCR was performed using Mastermix for Syber Green I (Platinum SYBR Green qPCR SuperMix UDG, Invitrogen, Life Technologies) or Absolute qPCR Mix (ABgene, UK) for TaqMan probe-based assays. The amount of primer or TaqMan fluorogenic probe, when necessary, in the PCR reactions of each gene is detailed in Table 1. The thermocycling conditions for SYBER Green analyses were as follows: initial denaturation at 95 °C for 3 min, followed by 40 cycles of denaturation at 95 °C for 15 sec and annealing-extension at 60 °C for 1 min. A melt-curve analysis was run after each real-time experiment for each gene, thus evidencing the specificity of the real time qPCR. For TaqMan probe-based assays, the thermocycling conditions were: 95 °C for 15 min, 40 cycles of 95 °C for 15 sec and 60 °C for 1 min. All amplifications were carried out in an iQCycler iQ Multi-Color Real Time PCR Detection System (Bio-Rad, Hercules, CA, USA), using 96-well plates (Abgene, UK). The data were analyzed with the iCycler IQ Optical System Software (Version 3.0a) provided by the manufacturer. All transcripts were quantified using standard curves for each gene analyzed, which were generated by a 10-fold serial dilution of known concentrations of the plasmid containing the target transcripts. PCR efficiency was calculated for each gene in each real-time PCR experiment (Table 1). All standards and experimental samples were run in duplicate. The mRNA levels were normalized to the amount of sea bass *18S* rRNA. Data were expressed as relative values of mRNA of each target gene/mRNA *18S* rRNA (starting quantity mean ± the standard error of the mean, SEM). Negative controls were also run for each real-time experiment.

2.5. Statistical analysis

A two-way ANOVA test was used to compare the effects of the three photoperiodic regimes on growth and reproductive performance, including biometric parameters, number of mitotic cell divisions of spermatogonia A, hormonal analyses and relative gene expression of juvenile male sea bass at different sampling times during early gametogenesis. When necessary, normality was assessed by applying the Kolmogorov–Smirnoff test after the logarithmic transformation of data. Barlett's test was used to establish homogeneity of variances. 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 three photoperiodic regimes was calculated by using the Chi-Square statistic and applying the Yates correction when $v = 1$, or Bonferroni inequality for P values when performing a multiple test. 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. Growth and male maturation

Differences in growth performance between fish under NP and LL conditions were found (Fig. 1). Mean weight of fish under NP gradually

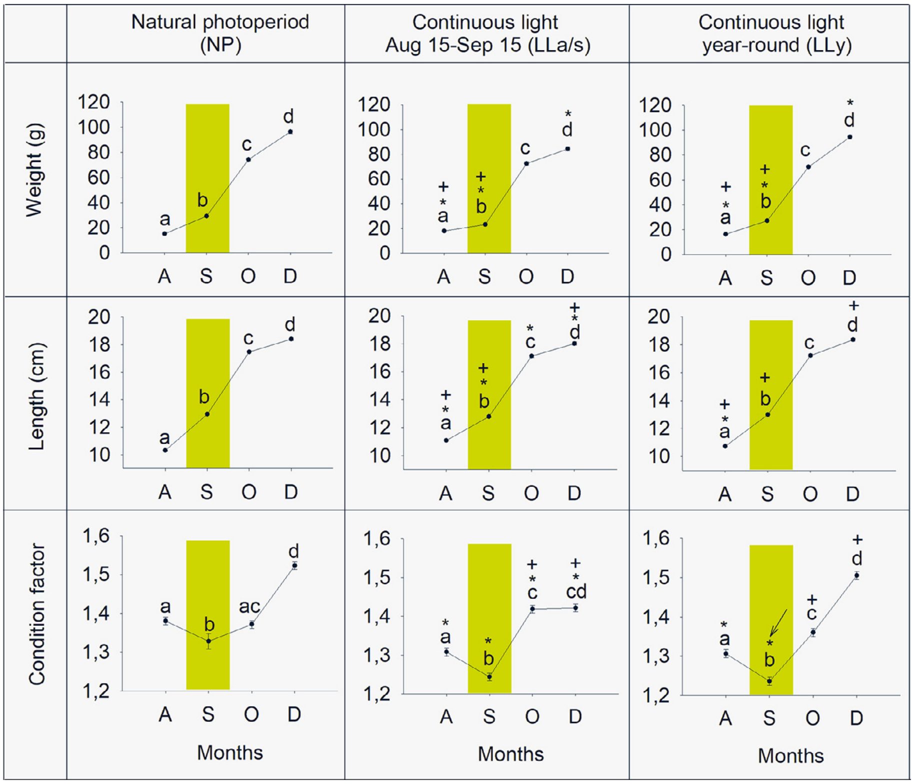


Fig. 1. Evolution of somatic growth and condition factor (FC) in juvenile male sea bass kept under a natural photoperiod (NP), continuous light (LL) from August 15 to September 15 under otherwise NP conditions (LLa/s), and LL year-round (LLy). Asterisks (*) by either LLa/s or LLy groups indicate significant differences ($P < 0.05$) for the corresponding dates of the NP group. Positive symbols (+) indicate significant differences ($P < 0.05$) between the LLy group and the LLa/s group for the same date. Arrows indicate the first significant decrease in the values with respect to either the NP group on the same date or the previous values of the same group. Abbreviations: A, August; S, September; O, October, D, December.

increased from 15.29 ± 0.20 g in August to 96.35 ± 1.54 g in December, whereas their length increased from 10.31 ± 0.05 cm to 18.40 ± 0.1 cm during the same period of time. The mean weight of fish under LLa/s was approximately 12.42% lower than that of those kept under NP, and 10.87% lower than that of the LLy treatment in the month of December. Meanwhile, the fish mean length under LLa/s was approximately 2% shorter than that under NP and 1.9% shorter than those kept under LLy for the same period of time. The mean weight and length values of fish under LLy conditions were approximately 1.81% and 0.22% lower, as compared to the control group. The condition factor varied between 1.38 and 1.52 in fish under NP, while it was around 1.24–1.42 in the LLa/s group. Fish under LLy exhibited condition factor values ranging from 1.24 to 1.51. The mean condition factor of fish under LLy was approximately 5.9% higher than under LLa/s in December. Of note, the mean condition factor values for fish under LLy were 0.66% lower than those of the NP group, while significant differences were observed in the LLa/s for the same period of time (Fig. 1). The percentages of gonadal stage I (Fig. 2A), II (Fig. 2B) and III (Fig. 2C) run roughly parallel in the LL groups. Of note, the percentage value of gonadal stage I (immature) in the LL groups varied in the range of 50–73.33% in the LLa/s group and 21.43–63.64% in the LLy group during the winter (January-March). Meanwhile, the NP group registered low values that varied from around 0 to 46.15% throughout this period. On the other hand, the percentages of gonadal stage IV (late recrudescence) (Fig. 2D) for the NP group peaked in March (36.36%), while the other groups presented very low values (0%). We also observed that the relative spermiation rate in the NP group (100%) was significantly higher than that observed in the LLa/s (46.5%) and LLy (0%) groups (Fig. 2E), although GSI values were comparable among the groups in February (0.04%), and no significant differences were observed.

3.2. Seasonal changes in the brain content of three forms of GnRH and *fshβ* expression levels in the pituitary

While GnRH1 levels in the NP group ranged from 0.68 to 2.39 ng/ml during the early gametogenesis (August-mid October), we observed a significant decrease in GnRH1 levels in the LLa/s (0.59 ± 0.09 ng/ml) and LLy (1.15 ± 0.08 ng/ml) groups, as compared to those in the NP group (1.34 ± 0.15 ng/ml) in early September (Fig. 3 top panel). On the other hand, GnRH2 levels in the NP group remained unchanged throughout the mid-August and early October period (1.94–2.47 ng/ml); this contrasted with the findings for the LL groups, which showed a steady decrease from mid-September onwards, resulting in GnRH2 levels of less than 1.36 ± 0.14 ng/ml for the LLa/s group and 2.03 ± 0.22 ng/ml for the LLy group (Fig. 3, middle panel). Finally, the levels of GnRH3 reached during mid-September were unaffected by the photoperiod regimes, showing values of 0.93 ± 0.09, 0.90 ± 0.07 and 0.87 ± 0.07 ng/ml for the NP, LLa/s and LLy groups, respectively (Fig. 3, bottom panel). *fshβ* expression in the pituitary (Fig. 4, top panel) increased significantly in the NP group during mid-September and declined thereafter. In contrast, the expression levels of *fshβ* remained low and unchanged in LLa/s and LLy groups throughout this period of early gametogenesis.

3.3. Seasonal changes in *amh*, *star*, *erβ1* and *erβ2* expression levels in the gonad and evolution of spermatogonial mitoses in males

In the NP group, *amh* expression significantly decreased in early September (Fig. 4, middle panel), concomitantly with the increase in the number of spermatogonial mitoses (9 mitoses/mm^2) (Fig. 4, bottom panel) and the increased expression of *fshβ* in the pituitary (Fig. 4, top panel). *amh* expression was low ($P < 0.05$) during early and mid-September, with a slight and significant increase in October that coincided with a significantly lower number of spermatogonial mitoses (1.67 mitoses/mm^2) and *fshβ* mRNA levels. On the other hand, exposure to different LL treatments evoked a slight, but not significant decrease in *amh* mRNA from early August to mid-September/early October, proceeding to further increase thereafter. Neither of the LL groups exhibited any differences in the number of mitoses of spermatogonial cells and *fshβ* mRNA levels, which remained low and constant during this entire period (Fig. 4, top panel). In the case of the LLa/s

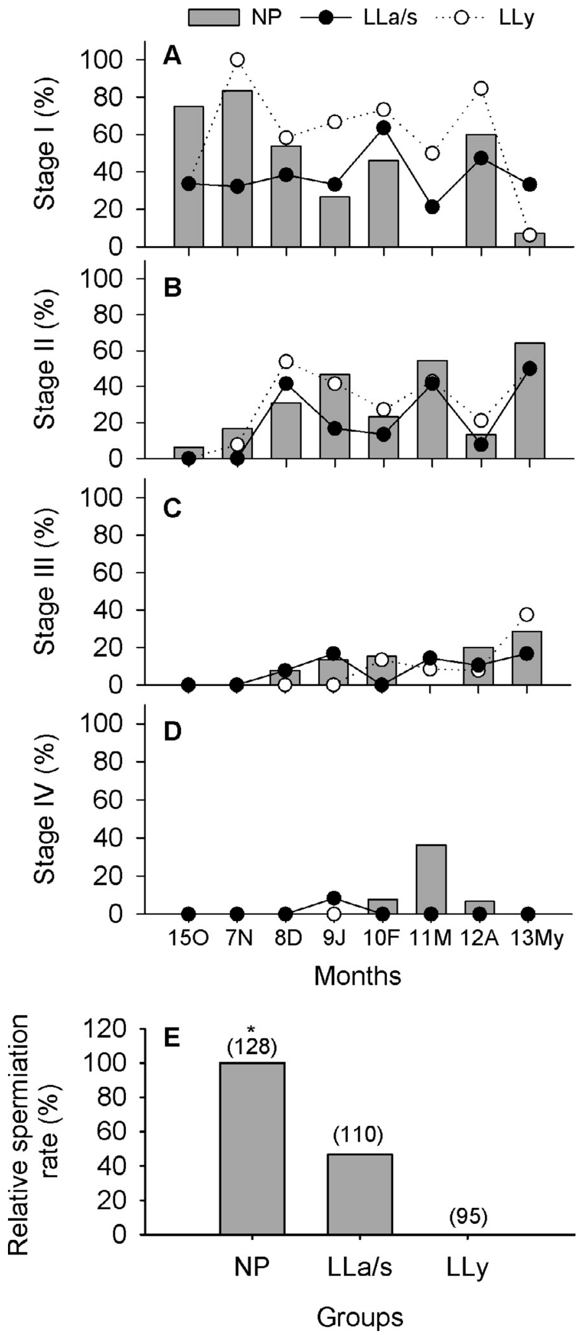


Fig. 2. Evolution of testicular development in juvenile male sea bass kept under a natural photoperiod (NP, solid bars), continuous light (LL) from August 15 to September 15 under otherwise NP conditions (LLa/s, solid lines and closed circles) and LL year-round (LLy, broken lines and open circles). Evolution is shown in percentages of gonadal stage I (immature) (A), II (B), III (C) and IV (late recrudescence) (D) throughout the first year of life of the fish, following previously established histological criteria (Begtashi et al., 2004). (E) Cumulative rates of spermiation (assessed by abdominal massage) per experimental group are expressed as relative spermiation rate. Numbers in brackets indicate the number of fish counted. Asterisks (*) indicate significant differences ($P < 0.05$) in the NP group with respect to LLa/s and LLy groups Abbreviations: O, October; N, November; D, December; J, January; F, February; M, March; A, April; My, May.

group, the number of spermatogonial mitoses peaked during early October (7.83 mitoses/mm^2), and then decreased during mid-October (4.17 mitoses/mm^2), although to levels not statistically different from the previous period. The number of spermatogonial mitoses in the LLy group ranged from 1.7 to 3.6 from September onwards. As observed in *amh* expression, changes in *star* mRNA significantly decreased during early and mid-September, to further increase in October, although to levels similar to those observed in August in the NP group (Fig. 5, top panel). The LLa/s group showed a gradual decrease in *star* mRNA from early August to mid-September, and then increased in October to values similar to those observed in August. *star* mRNA levels in the LLa/s group were significantly higher than those observed in the NP group. Meanwhile, *star* expression significantly decreased in the LLy group, and these low levels were maintained until the end of the early gametogenesis. On the other hand, *erβ1* and *erβ2* gonadal expression profiles throughout early gametogenesis among the three experimental groups were apparently not affected by light regimes (Fig. 5, middle-bottom panels).

3.4. Seasonal changes in hormonal plasma levels

The E_2 levels in the NP group displayed high levels from August to September (up to 1.16 ± 0.10 ng/ml), with a slight decrease thereafter. Interestingly, E_2 levels in the LL groups were significantly lower than those of the NP group in early September, with values of 0.49 ± 0.04 ng/ml in the LLa/s group and 0.51 ± 0.05 ng/ml in the LLy group (Fig. 6, top panel). On the other hand, both plasma 11-KT (around 1.0 ± 0.1 ng/ml) and T (around 4.04 ± 0.37 ng/ml) showed constant levels throughout the August 15 to September 15 period in the NP group (Fig. 6, middle panel), whereas these values were affected by the LL treatment. In the LLa/s group, average values of plasma 11-KT were around 0.59 ± 0.04 ng/ml during mid-September and decreased to levels that were not statistically significant in October. At the same time, levels of T were around 1.88 ± 0.37 ng/ml during mid-September and decreased to levels significantly lower than the previous period during this time interval. In the LLy group, we observed that plasma 11-KT levels (around 0.57 ng/ml) never reached the high levels of the NP group, while the average values of plasma T were around 2.62 ng/ml, with maximum levels of 3.79 ± 0.38 ng/ml in early September. Finally, the NP group Fsh levels showed a significant increase at the beginning of September (24.58 ± 1.94 ng/ml). The levels progressively increased thereafter, and peaked in October (44.25 ± 2.04 ng/ml). For this same period, Fsh levels in LLa/s increased somewhat later, during mid-September, and were maintained throughout mid-October, with values around 36.54 ± 1.72 ng/ml. In the LLy group, the increase in Fsh was comparable to that in the NP group, appearing in early October (47.33 ± 3.16 ng/ml) (Fig. 6, bottom panel).

4. Discussion

This work demonstrates how the bi-weekly changes in some reproductive factors may be altered by the application of continuous light within the photolabile period, leading to disturbances in the testicular development of European sea bass, which in turn results in a clear reduction in the numbers of precocious males. The present results provide further support for the photoperiod as an external cue to which organisms have been adapted (Falcón et al., 2010), and consequently, the alteration of the lighting regime within the boundaries of the photolabile period impacts reproduction. In juvenile male sea bass, exposure to LL affected the rhythmicity of the hormone and gene profiles in a different way, inhibiting seasonal patterns or significantly decreasing their amplitude. Although differences in the intensity of the impact on reproductive factors were observed (Fig. 7), this gives us an early glimpse into the endocrine control of the early events of gametogenesis in a teleost fish.

The fact that the growth performance of juvenile males was different between LL and NP fish agrees with previous studies on this species (Begtashi et al., 2004; Felip et al., 2017), although the work of Felip et al. (2008) showed no differences among treatments. It is

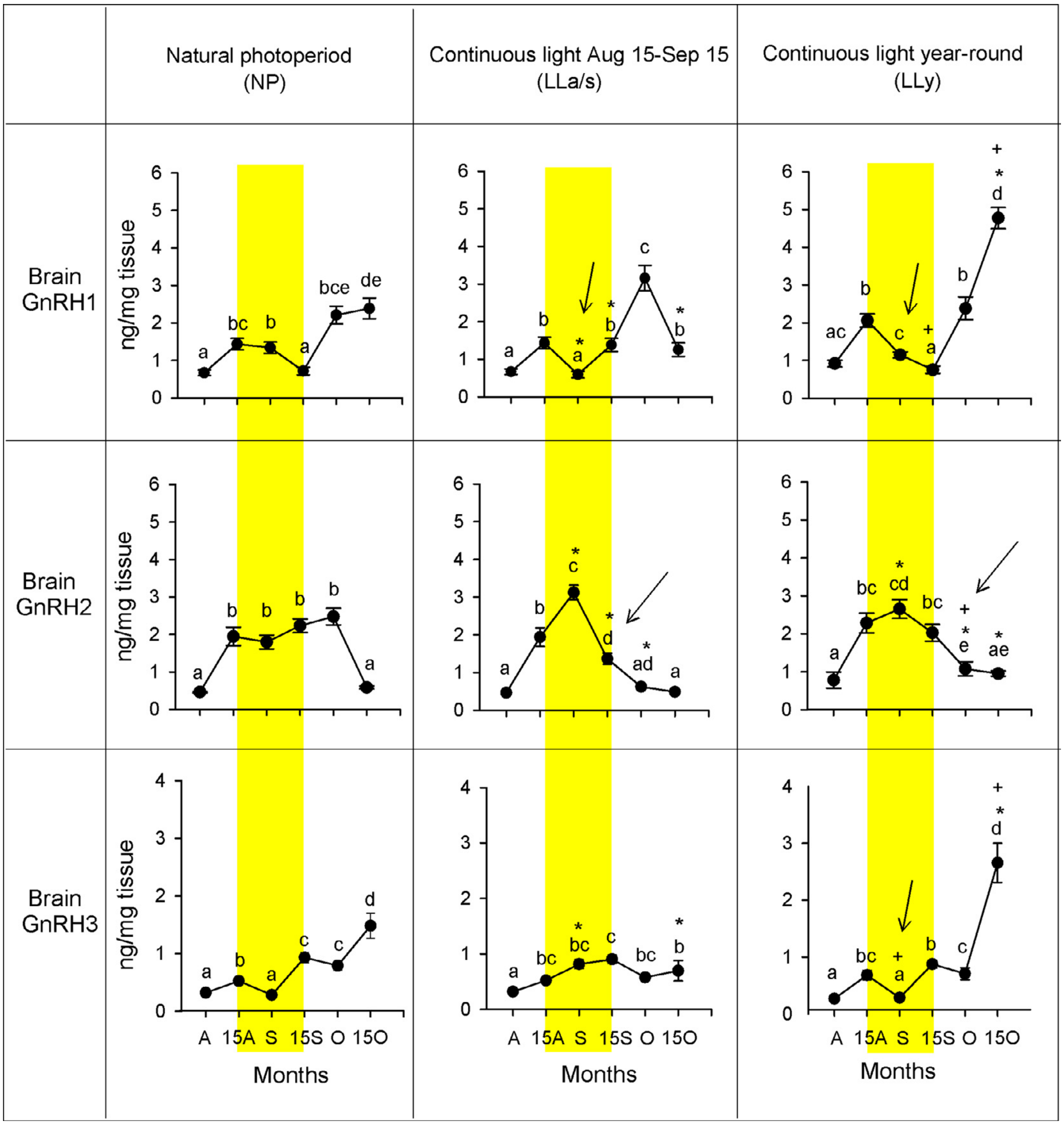


Fig. 3. Brain seabream (GnRH1), chicken-II (GnRH2) and salmon (GnRH3) GnRH content in juvenile male sea bass, kept under a natural photoperiod (NP), continuous light (LL) from August 15 to September 15 under otherwise NP conditions (LLa/s) and LL year-round (LLy), and sampled during their first year of life. Different letters represent significant differences between months ($P < 0.05$) for a given variable. Asterisks (*) by either LLa/s or LLy groups indicate significant differences ($P < 0.05$) for the corresponding dates of the NP group. Positive symbols (+) indicate significant differences ($P < 0.05$) of the LLy group with respect to the LLa/s group for the same date. Arrows indicate the first significant decrease in the values with respect to either the NP group on the same date or to the previous value of the same group. Abbreviations: A, August; S, September; O, October.

interesting to note that LL fish exhibited lower condition factor values than their counterparts, and this was concomitant with reduced gonadal development in less corpulent fish. In other relevant species, LL treatment has been demonstrated to have a stimulatory effect on somatic fish growth (Kashyap et al., 2015; Navarro et al., 2015; Biswas et al., 2016; Veras et al., 2016; Imsland et al., 2018), most likely by stimulating the animals' feed intake and/or food conversion efficiency (Boeuf and Le bail, 1999) or by modulating some hormones that may contribute to the changes in food intake and growth rate in fish (Volkoff et al., 2010). However, in juvenile turbot (*Scophthalmus maximus*) continuous light caused a suppression in growth performance (Imsland et al., 1995; Stefánsson et al., 2002). It is possible that fish under LL regimes may require greater quantities of feed to maintain basic metabolic processes, as they are more active and stressed under these environmental conditions (Stefánsson et al., 2002). It is even possible that temperature variations might alter the impact of light on fish growth (Imsland et al. 1995; Jonassen et al., 2000; Døskeland et al., 2016).

In addition, it has been observed that central injection of Gnrh2 has an inhibitory effect on food intake in goldfish (*Carassius auratus*), which is mediated in part by inhibition of the hypothalamic orexin system that regulates appetite in fish (Hoskins et al., 2008; Matsuda et al., 2008). In European sea bass, Gnrh2 is known to stimulate melatonin (Servili et al., 2010), which exhibits an inhibitory effect on food intake and affects somatic growth in this marine teleost (Rubio et al., 2004; Alvarado et al., 2015). It is also worthy to note that Gnrh1 enhances *fshβ* gene expression during gonadal differentiation of sea bass, thus suggesting that the brain's GnRH system may trigger both sex differentiation and the onset of puberty in this fish (Molés et al., 2007). In fact, a moderate Gnrh1 and Gnrh2 pituitary content has been observed at the onset of puberty in male sea bass (Rodríguez et al., 2000b). Data collected in the present study show that the Gnrh1 content in fish exposed to LL during the photosensitive period was lower than that of the NP fish during early gametogenesis (September). This suggests that low levels of this hormone in a critical period may block the hormonal regulation of the reproductive axis. Furthermore, NP fish showed a

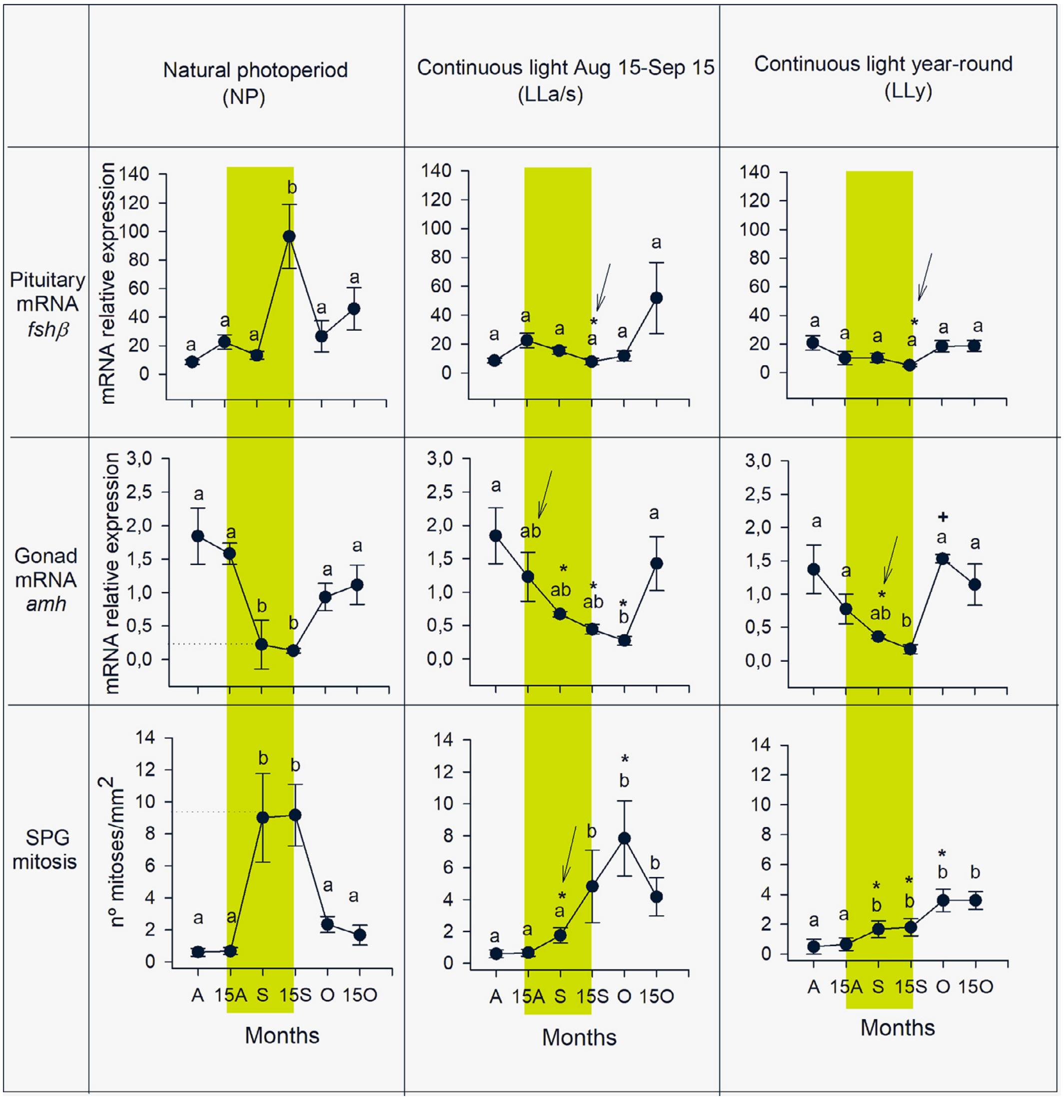


Fig. 4. Evolution of *fshβ* and *amh* expression and mitotic index of spermatogonia type A (SPGA) at early gametogenesis in juvenile male sea bass, kept under a natural photoperiod (NP), continuous light (LL) from August 15 to September 15 under otherwise NP conditions (LLa/s) and LL year-round (LLy). Relative changes in expression of *fshβ* in the pituitary and *amh* in the gonad of fish sampled during their first year of life. All expression values are normalized to *18S* rRNA. Changes in the mitotic index of SPGA in fish sampled during their first year of life. Different letters represent differences between months ($P < 0.05$) for a given variable. Asterisks (*) by either LLa/s or LLy groups indicate significant differences ($P < 0.05$) for the corresponding dates of the NP group. Positive symbols (+) indicate significant differences ($P < 0.05$) in the LLy group with respect to the LLa/s group for a same date. Arrows indicate the first significant decrease in the values with respect to either the NP group on the same date or to the previous value for the same group. Abbreviations: A, August; S, September; O, October.

constant Gnrh2 content during the early phases of gametogenesis, in contrast to the presence of a Gnrh2 peak in LL fish in September, which was followed by a decrease afterwards. All these data suggest that both Gnrh1 and Gnrh2 are essential hormonal factors for the onset and progression of spermatogenesis in sea bass, and their disturbances are most likely the reason why individual fish lack functional competence to launch spermiogenesis. Interestingly, a lack of correlation of the hypophysiotropic Gnrh forms, Gnrh1 and Gnrh2, with those of Fsh plasma levels should be contextualized in light of the European sea bass mode of gonadal development. Sea bass is a group-synchronous spawner and Gnrh levels might indicate the parallel maintenance of spermatogenesis in several batches of gametes present in the testes of this species (Carrillo et al., 1993). In goldfish, continuous light induces variations in both Gnrh forms and kisspeptin 1, a neuroendocrine factor regulating GnRH secretion in the hypothalamus, demonstrating that photoperiod regulates sex maturation by GnRH and Kiss1 in the brain of this species (Shin et al., 2014). Recent studies in sea bass have indicated that LL downregulates *gnrh1* and *kiss2* (Martins et al., 2015) and that this photoperiod modulates the expression of *kiss1* and *gnrh2* in the midbrain-forebrain, affecting normal testicular development during the onset of puberty (Espigares et al., 2017). These findings suggest that both kisspeptin and gnrh systems are involved in the translation of the light stimulus to activate the reproductive axis of this marine teleost fish. In addition, their co-regulation has clear effects on *fhsβ* transcription at the pituitary level during early spermatogenesis, thus modulating the onset and progression of the entire gametogenesis process (Alvarado et al., 2015). In this sense, it has been well established that long-term exposure to LL regimes diminishes *fshβ* expression (Rodríguez et al., 2005; Felip et al., 2008), and a strong reduction in *fshβ* expression and low plasmatic levels have been also found in the LLa/s and LLy groups in this study, thus supporting the importance of photoperiod signalling in the activation of the reproductive axis. These results reinforce the role that Fsh play in the regulation of early-mid phases of spermatogenesis in sea bass, where Lh would be more involved with later stages of gonadal development.(measurements not included in this study) (Rodríguez et al., 2000b; Mateos et al., 2003).

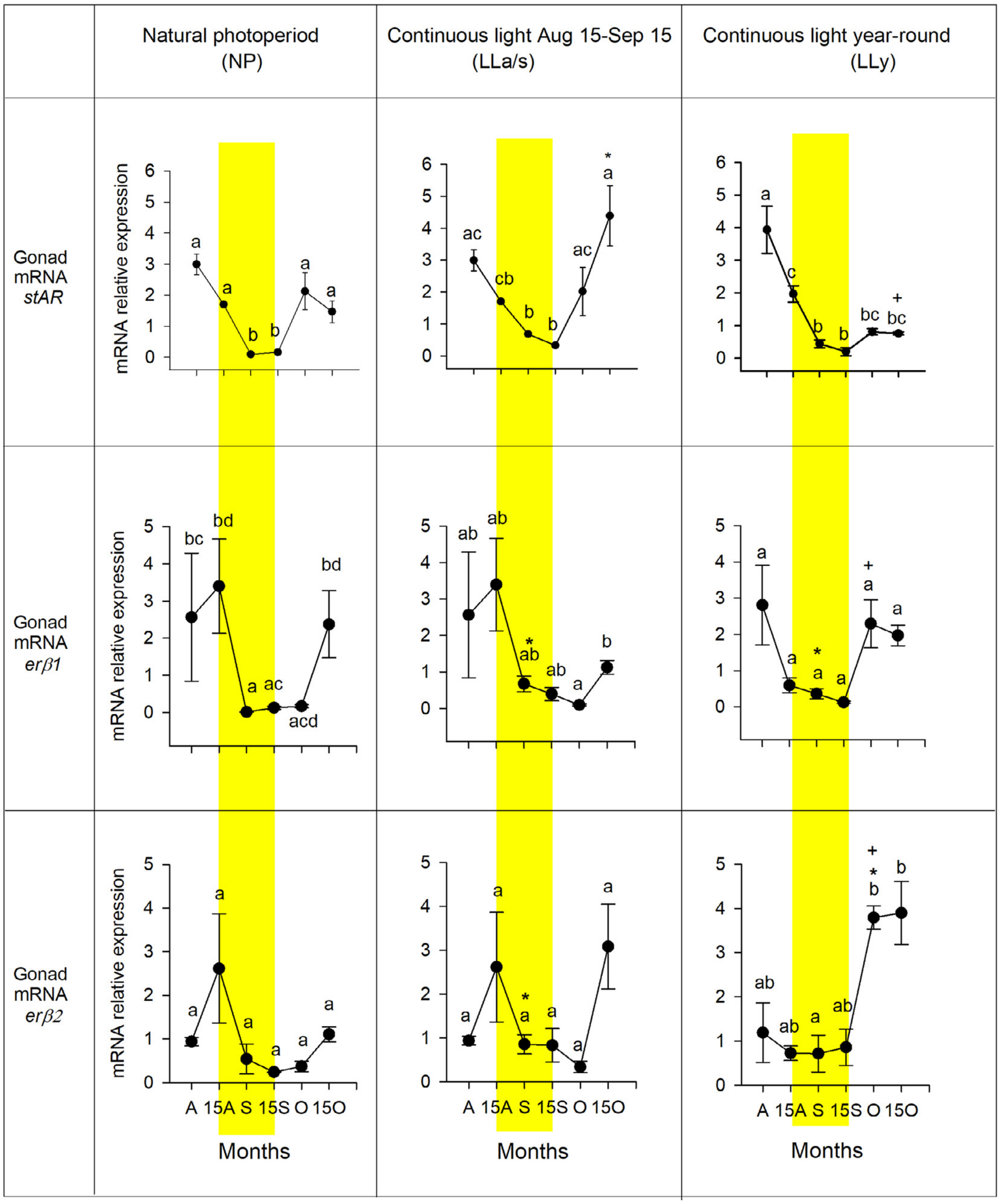


Fig. 5. Relative changes in the expression of *star*, *erβ1* and *erβ2* in the gonad of juvenile male sea bass, kept under a natural photoperiod (NP), continuous light (LL) from August 15 to September 15 under otherwise NP conditions (LLa/s) and LL year-round (LLy), and sampled during their first year of life. Different letters represent significant differences between months ($P < 0.05$) for a given variable. Asterisks (*) by either LLa/s or LLy groups indicate significant differences ($P < 0.05$) for the corresponding dates of the NP group. Positive symbols (+) indicate significant differences ($P < 0.05$) in the LLy group with respect to the LLa/s group for the same date. Abbreviations: A, August; S, September; O, October.

On the other hand, it is also accepted that piscine gonadotropins are steroidogenic (Planas and Swanson, 2008), and their main function during fish spermatogenesis is the stimulation and production of steroids (Schulz et al., 2010). Nevertheless, it has been observed that gonadotropins show different potencies and responses which could be based on the spermatogenic stage, and therefore in the presence of their corresponding receptors (Mazón et al., 2015). *In vitro* tissue culture of adult sea bass testis, Lh was more potent than Fsh in stimulating 11-KT production in late spermatogenesis, however, administration of Fsh *in vivo* in juvenile males of this species caused a significant increase of 11-KT and initiated germ cell proliferation and spermatogenesis (Mazón et al., 2014). In fact, this androgen has been proposed as a possible trigger for the onset of puberty in sea bass (Rodríguez et al., 2005), suggesting that the unbalanced production of 11-KT regulated by *fshβ* might be limiting the stimulation of germ cell proliferation at the testicular level, and hence the onset and progression of gametogenesis in juvenile fish (Felip et al., 2008), as shown in this study. In yellowtail (*Seriola quinqueradiata*), 11-KT plays an important physiological role in gonadal development and stimulates early mitotic divisions of spermatogonia toward meiosis (Higuchi et al., 2017).

In addition, our results also revealed that LL evoked changes on the plasmatic levels of sex steroids as previously observed in this species (Begtashi et al., 2004; Rodríguez et al., 2005; Felip et al., 2008) and others (Ammar et al., 2015). Studies on fish report that estrogens evoke germ cell proliferation (Amer et al., 2001; Song and Gutzeit, 2003; Kobayashi et al., 2011) and even inhibit spermatogonia proliferation

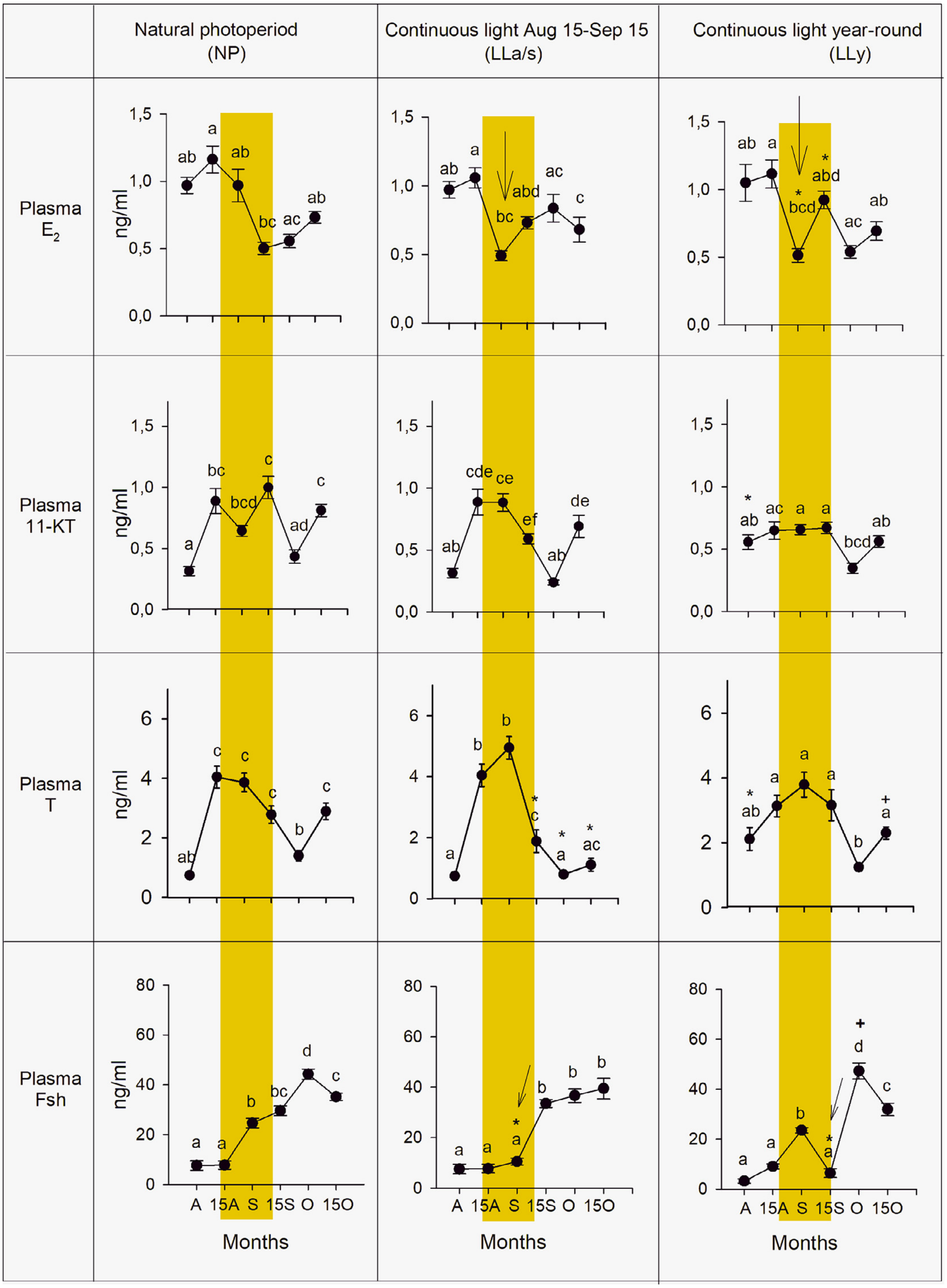
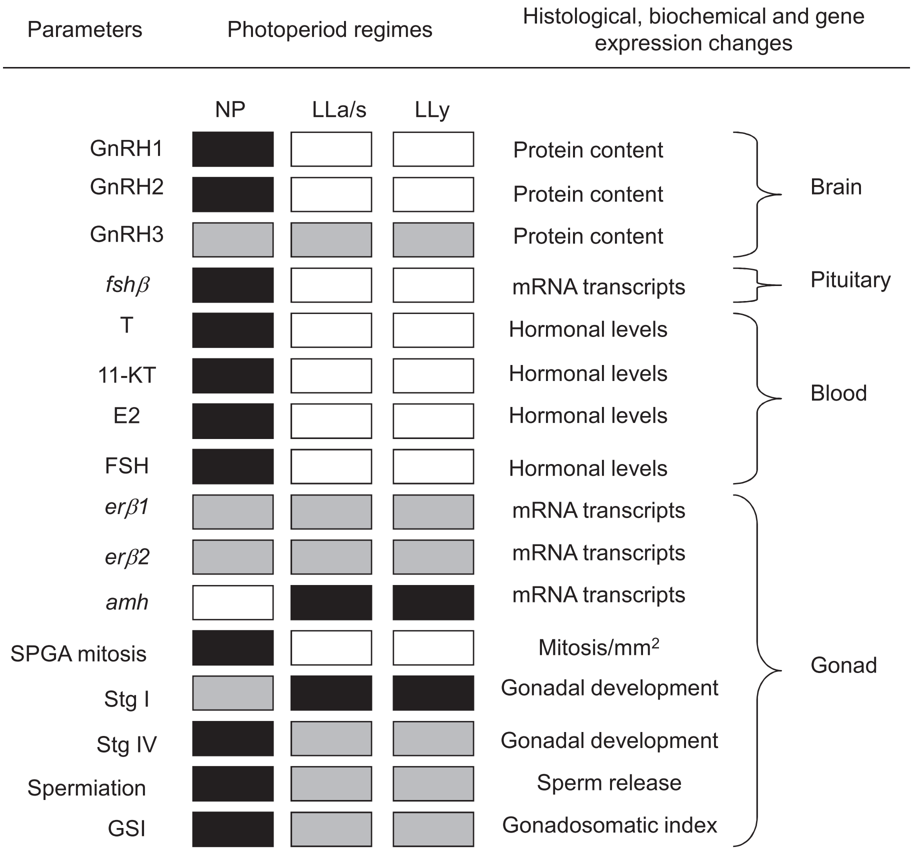


Fig. 6. Changes in plasma levels of E_2, 11-KT, T and Fsh in juvenile male sea bass, kept under a natural photoperiod (NP), continuous light (LL) from August 15 to September 15 under otherwise NP conditions (LLa/s) and LL year-round (LLy) and sampled during their first year of life. Different letters represent significant differences between months ($P < 0.05$) for a given variable. Asterisks (*) by either LLa/s or LLy groups indicate significant differences ($P < 0.05$) for the corresponding dates of the NP group. Positive symbols (+) indicate significant differences ($P < 0.05$) in the LLy group with respect to the LLa/s group for the same date. Arrows indicate the first significant decrease in the values with respect to either the NP group on the same date or to the previous value of the same group. Abbreviations: A, August; S, September; O, October.

and induce the post-spawning process (Chaves-Pozo et al., 2007). In sea bass under LLa/s and LL, the low levels of E_2 in early September were concomitant with a reduced number of spermatogonial mitoses as compared to the NP group, suggesting that E_2 might be related to spermatogonial proliferation during early gonadal development in this species. In addition, while testicular *erβ1* and *erβ2* expression was similar among groups during early gametogenesis, a significant decrease in *amh* expression was observed with the concomitant decrease of circulating levels of 11-KT and Fsh under LL regimes. This may suggest a possible mechanism of endocrine control over early events of gametogenesis in male sea bass, in which the lack of increasing 11-KT levels presumably induced by Fsh are unable to block *amh* expression. Consequently, Amh may act as a meiosis inhibiting factor and prevent spermatogenesis progression, as previously demonstrated in male sea



Parameters	Photoperiod regimes NP	LLa/s	LLy	Histological, biochemical and gene expression changes	
GnRH1	■	□	□	Protein content	Brain
GnRH2	■	□	□	Protein content	
GnRH3	▨	▨	▨	Protein content	
fshβ	■	□	□	mRNA transcripts	Pituitary
T	■	□	□	Hormonal levels	Blood
11-KT	■	□	□	Hormonal levels	
E2	■	□	□	Hormonal levels	
FSH	■	□	□	Hormonal levels	
erβ1	▨	▨	▨	mRNA transcripts	Gonad
erβ2	▨	▨	▨	mRNA transcripts	
amh	□	■	■	mRNA transcripts	
SPGA mitosis	■	□	□	Mitosis/mm^2	
Stg I	▨	■	■	Gonadal development	
Stg IV	■	▨	▨	Gonadal development	
Spermiation	■	▨	▨	Sperm release	
GSI	■	▨	▨	Gonadosomatic index	

Fig. 7. Summary of the effects of continuous light during the photolabile period in early gametogenesis on histological, biochemical and gene expression changes of some relevant parameters related to the maturation process in juvenile male sea bass kept under a natural photoperiod (NP), continuous light (LL) from August 15 to September 15 under otherwise NP conditions (LLa/s) and LL year-round (LLy). The shading in each box represents the levels of the variable being considered: white, significantly low levels; grey, low-intermediate levels with no significant differences and black, significantly high levels.

bass (Mazón et al., 2014; Rocha et al., 2016; Blázquez et al., 2017). In this respect, several studies have shown that *amh* expression in the testes is downregulated by 11-KT in eel (Miura et al., 2002) and also in zebrafish (Skaar et al., 2011) and Atlantic salmon (Melo et al., 2015), although its biological function needs to be further investigated in fish. Interestingly, a positive regulation of Fsh on *star* expression has been described in mammals (Balasubramanian et al., 1997; Sekar et al., 2000). In adult male sea bass, *star* expression increases as spermatogenesis proceeds, reaching a peak at advanced gonadal stages during the first sexual maturation, supporting the involvement of the coded protein in the synthesis of sea bass progestins (Rocha et al., 2009). In this regard, the relationship among these profiles in juvenile males needs to be further investigated in order to gather a better understanding of the rapid delivery of cholesterol substrate, which requires the Star protein, during early gametogenesis.

In conclusion, this study demonstrates that, over the course of the photosensitive period, the LL regime modulates the rhythm and/or amplitude of some important reproductive factors (Fig. 7) during early gametogenesis in male sea bass, which results in disturbances that drastically affect gonadal maturation and reduce precocity in a marine teleost fish.

Acknowledgements

We would like to thank C. Marín and X. Simó for their technical assistance in rearing and sampling the fish, J. Monfort and L. Rodríguez for their help with histological procedures and S. Ibáñez for her assistance with sampling analysis. This research was supported by grants Q5RS-2002-01801 (PUBERTIMING) from the UE, Generalitat Valenciana Grupos 04/80 and PROMETEO 2010/003-2014/051, Ministerio de Ciencia y Tecnología, with special action No. AGL2002-12470-E and AGL2009-11086 and AGL2016-75400-R from the Ministerio de Ciencia e Innovación (MICINN) of Spain. R.R. was supported by an FPI fellowship from MICINN.

References

Alvarado, M.V., Carrillo, M., Felip, A., 2015. Melatonin-induced changes in kiss/gnrh gene expression patterns in the brain of male sea bass during spermatogenesis. Comp. Biochem. Physiol. Part A: Mol. Integ. Physiol. 185, 69–79.

Amer, M.A., Miura, T., Miura, C., Yamauchi, K., 2001. Involvement of sex steroid hormones in the early stages of spermatogenesis in Japanese huchen (*Hucho perryi*). Biol. Rep. 65 (4), 1057–1066.

Ammar, I.B., Teletchea, F., Milla, S., Ndiaye, W.N., Ledoré, Y., Missaoui, H., Fontaine, P., 2015. Continuous lighting inhibits the onset of reproductive cycle in pikeperch males and females. Fish Physiol. Biochem. 41 (2), 345–356.

Balasubramanian, K., LaVoie, H.A., Garmey, J.C., Stocco, D.M., Veldhuis, J.D., 1997. Regulation of porcine granulosa cell steroidogenic acute regulatory protein (StAR) by insulin-like growth factor I: synergism with follicle-stimulating hormone or protein kinase A agonist. Endocrinology 138 (1), 433–439.

Barnabé, G., 1995. The sea bass. In: Nash, C.E., Novotny, A.J. (Eds.), Production of Aquatic Animals. Elsevier, Oxford, pp. 269–287.

Begtashi, I., Rodríguez, L., Molés, G., Zanuy, S., Carrillo, M., 2004. Long-term exposure to continuous light inhibits precocity in juvenile male European sea bass (*Dicentrarchus labrax*, L.). I. Morphological aspects. Aquaculture 241, 539–559.

Bennett, H.S., Wyrick, A.D., Lee, S.W., McNeil, J.H., 1976. Science and art in preparing tissues embedded in plastic for light microscopy, with special reference to glycol methacrylate, glass knives, and simple stains. Stain Technol. 51, 71–94.

Biswas, A., Takaoka, O., Kumai, H., Takii, K., 2016. Combined effect of photoperiod and self-feeder on the growth performance of striped knifejaw, *Oplegnathus fasciatus*. Aquaculture 452, 183–187.

Blázquez, M., González, A., Papadaki, M., Mylonas, C., Piferrer, F., 2008. Sex-related changes in estrogen receptors and aromatase gene expression and enzymatic activity during early development and sex differentiation in the European sea bass (*Dicentrarchus labrax*). Gen. Comp. Endocrinol. 158, 95–101.

Blázquez, M., Medina, P., Crespo, B., Gómez, A., Zanuy, S., 2017. Identification of conserved genes triggering puberty in European sea bass males (*Dicentrarchus labrax*) by microarray expression profiling. BMC Genomics 18 (1), 441.

Boeuf, G., Le Bail, P.Y., 1999. Does light have an influence on fish growth? Aquaculture 177 (1–4), 129–152.

Bromage, N., Porter, M., Randall, C., 2001. The environmental regulation of maturation in farmed finfish with special reference to the role of photoperiod and melatonin. Aquaculture 197, 63–98.

Brown, C., Miltiadou, D., Anastasiades, G., 2014. Precocious female maturation in seacage populations of European seabass (Dicentrarchus labrax) in Cyprus. Poster, Aquaculture Europe 2014, San Sebastián, Spain.

Carrillo, M., Zanuy, S., Prat, F., Serrano, R., Bromage, N., 1993. Environmental and hormonal control of reproduction in sea bass. In: Bromage, N., Donaldson, E.M., Carrillo, M., Zanuy, S., Planas, J. (Eds.), Recent Advances in Aquaculture IV. Blackwell Scientific Publications, Oxford, Great Britain, pp. 43–54.

Carrillo, M., Zanuy, S., Felip, A., Bayarri, M.J., Molés, G., Gómez, A., 2009. Hormonal and environmental control of puberty in perciform fish. Annals of the. New York Acad. Sci. 1163 (1), 49–59.

Carrillo, M., Espigares, F., Felip, A., Escobar, S., Molés, G., Rodríguez, R., Alvarado, M.V., Gómez, A., Zanuy, S., 2015. Updating control of puberty in male European sea bass: a holistic approach. Gen. Comp. Endocrinol. 221, 42–53.

Chaves-Pozo, E., Liarte, S., Vargas-Chacoff, L., García-López, A., Mulero, V., Meseguer, J., Mancera, J.M., García-Ayala, A., 2007. 17Beta-estradiol triggers postspawning in spermatogenically active gilthead seabream (*Sparus aurata* L.) males. Biol. Rep. 76 (1), 142–148.

Crespo, B., Gómez, A., Mazón, M.J., Carrillo, M., Zanuy, S., 2013. Isolation and characterization of Ff1 and Gsdf family genes in European sea bass and identification of early gonadal markers of precocious puberty in males. Gen. Comp. Endocrinol. 191, 155–167.

Døskeland, I., Imsland, A.K.D., Fjelldal, P.G., Stefansson, S.O., Roth, B., Eriksen, K.E., Mikalsen, B., Handeland, S., 2016. The effect of low temperatures and photoperiods on growth and vertebra morphometry in Atlantic salmon. Aquacult. Internat. 24 (5), 1421–1434.

Escobar, S., Felip, A., Salah, M., Zanuy, S., Carrillo, M., 2014. Long-term feed restriction in prepubertad male sea bass (*Dicentrarchus labrax* L.) increases the number of apoptotic cells in the testis and affects the onset of puberty and some sperm quality parameters. Aquaculture 433, 504–512.

Espigares, F., Rocha, A., Molés, G., Gómez, A., Carrillo, M., Zanuy, S., 2015a. New insights into the factors mediating the onset of puberty in sea bass. Gen. Comp. Endocrinol. 224, 176–185.

Espigares, F., Zanuy, S., Gómez, A., 2015b. Kiss2 as a regulator of Lh and Fsh secretion via paracrine/autocrine signaling in the teleost fish European sea bass (*Dicentrarchus labrax*). Biol. Rep. 93 (5), 1–12.

Espigares, F., Rocha, A., Gómez, A., Carrillo, M., Zanuy, S., 2017. Photoperiod modulates the reproductive axis of European sea bass through regulation of *kiss1* and *gnrh2* neuronal expression. Gen. Comp. Endocrinol. 240, 35–45.

Falcón, J., Migaud, H., Muñoz-Cueto, J.A., Carrillo, M., 2010. Current knowledge on the melatonin system in teleost fish. Gen. Comp. Endocrinol. 165, 469–482.

Felip, A., Zanuy, S., Carrillo, M., 2006. Comparative analysis of growth performance and sperm motility between precocious and non-precocious males in the European sea bass (*Dicentrarchus labrax* L.). Aquaculture 256 (1–4), 570–578.

Felip, A., Zanuy, S., Muriach, B., Cerdá-Reverter, J.M., Carrillo, M., 2008. Reduction of sexual maturation in male *Dicentrarchus labrax* by continuous light both before and during gametogenesis. Aquaculture 275, 347–355.

Felip, A., Rodríguez, R., Zanuy, S., Carrillo, M., 2017. Effect of combining different photoperiod regimes on the sexual maturation of male sea bass (*Dicentrarchus labrax* L.) during the first two years of life. J. Aquacult Mar. Biol. 5 (4), 1–9.

Halm, S., Rocha, A., Miura, T., Prat, F., Zanuy, S., 2007. Anti-Müllerian hormone (AMH/AMH) in the European sea bass: its gene structure, regulatory elements, and the expression of alternatively-spliced isoforms. Gene 388 (1), 148–158.

Higuchi, K., Gen, K., Izumida, D., Kazeto, Y., Hotta, T., Takashi, T., Aono, H., Soyano, K., 2017. Changes in plasma steroid levels and gene expression of pituitary gonadotropins, testicular steroidogenesis-related proteins and insulin-like growth factors during spermatogenesis of the yellowtail *Seriola quinqueradiata*. Fish. Sci. 83 (1), 35–46.

Holland, M.C.H., Gothilf, Y., Meiri, I., King, J.A., Okuzawa, K., Elizur, A., Zohar, Y., 1998. Levels of the native forms of GnRH in the pituitary of the gilthead seabream, *Sparus aurata*, at several stages of the gonadal cycle. Gen. Comp. Endocrinol. 112, 394–405.

Hoskins, L.J., Xu, M., Volkoff, H., 2008. Interactions between gonadotropin-releasing hormone (GnRH) and orexin in the regulation of feeding and reproduction in goldfish (*Carassius auratus*). Horm. Behav. 54 (3), 379–385.

Imsland, A.K., Folkvord, A., Stefansson, S.O., 1995. Growth, oxygen consumption and activity of juvenile turbot (*Scophthalmus maximus* L.) reared under different temperatures and photoperiods. Netherlands. J. Sea Res. 34 (1–3), 149–159.

Imsland, A.K., Jonassen, T.M., Hangstad, T.A., Stefansson, S.O., Elvegård, T.A., Lemmens, S.C., Urskog, T.C., Nytrøc, A.V., Reynholds, P., 2018. The effect of continuous light and compressed photoperiods on growth and maturation in lumpfish *Cyclopterus lumpus*. Aquaculture 485, 166–172.

Jonassen, T.M., Imsland, A.K., Kadowaki, S., Stefansson, S.O., 2000. Interaction of temperature and photoperiod on growth of Atlantic halibut *Hippoglossus hippoglossus* L. Aquac. Res. 31 (2), 219–227.

Kah, O., Zanuy, S., Pradelles, P., Cerdá, J.L., Carrillo, M., 1994. An enzyme immunoassay for salmon gonadotropin-releasing hormone and its application to the study of the effect of diet on brain and pituitary GnRH in the sea bass, *Dicentrarchus labrax*. Gen. Comp. Endocrinol. 95, 464–474.

Kashyap, A., Chandra Pathak, B., Awasthi, M., Serajuddin, M., 2015. Effect of different photoperiods on the growth and survival of juvenile of Indian major carp, *Catla*. Iranian J. Fish. Sci. 14 (4), 946–955.

Kobayashi, Y., Nozu, R., Nakamura, M., 2011. Role of estrogen in spermatogenesis in initial phase males of the three-spot wrasse (*Halichoeres trimaculatus*): Effect of aromatase inhibitor on the testis. Dev. Dyn. 240 (1), 116–121.

Kortner, T.M., Rocha, E., Silva, P., Castro, L.F.C., Arukwe, A., 2008. Genomic approach in evaluating the role of androgens on the growth of Atlantic cod (*Gadus morhua*) previtellogenic oocytes. Comp. Bioch. Physiol. Part D 3, 205–218.

Martins, R.S., Gomez, A., Zanuy, S., Carrillo, M., Canário, A.V., 2015. Photoperiodic modulation of circadian clock and reproductive axis gene expression in the prepubertal European sea bass brain. PLoS One 10 (12), e0144158.

Mateos, J., Mañanós, E., Martínez-Rodríguez, G., Carrillo, M., Querat, B., Zanuy, S., 2003. Molecular characterization of sea bass gonadotropin subunits (α, FSHβ, and LHβ) and their expression during the reproductive cycle. Gen. Comp. Endocrinol. 133 (2), 216–232.

Matsuda, K., Nakamura, K., Shimakura, S.I., Miura, T., Kageyama, H., Uchiyama, M., Shioda, S., Ando, H., 2008. Inhibitory effect of chicken gonadotropin-releasing hormone II on food intake in the goldfish, *Carassius auratus*. Horm. Behav. 54 (1), 83–89.

Maugars, G., Schmitz, M., 2008. Gene expression profiling during spermatogenesis in early maturing male Atlantic salmon parr testes. Gen. Comp. Endocrinol. 159 (2–3), 178–187.

Mazón, M.J., Gómez, A., Yilmaz, O., Carrillo, M., Zanuy, S., 2014. Administration of follicle-stimulating hormone in vivo triggers testicular recrudescence of juvenile European sea bass (*Dicentrarchus labrax*). Biol. Rep. 90 (1), 1–10.

Mazón, M.J., Molés, G., Rocha, A., Crespo, B., Lan-Chow-Wing, O., Espigares, F., Muñoz, I., Felip, A., Carrillo, M., Zanuy, S., Gómez, A., 2015. Gonadotropin actions in European sea bass: endocrine roles and biotechnological applications. Gen. Comp. Endocrinol. 221, 31–41.

McDowell, E.M., Trump, B.F., 1976. Histologic fixatives suitable for diagnostic light and electron microscopy. Arch. Pathol. Lab. Med. 100, 405–414.

Melo, M.C., van Dijk, P., Andersson, E., Nilsen, T.O., Fjelldal, P.G., Male, R., Nijenhuis, W., Bogerd, J., de França, L.R., Taranger, G.L., Schulz, R.W., 2015. Androgens directly stimulate spermatogonial differentiation in juvenile Atlantic salmon (*Salmo salar*). Gen. Comp. Endocrinol. 211, 52–61.

Migaud, H., Davie, A., Taylor, J.F., 2010. Current knowledge on the photoneuroendocrine regulation of reproduction in temperate fish species. J. Fish Biol. 76 (1), 27–68.

Miura, T., Miura, C., Konda, Y., Yamauchi, K., 2002. Spermatogenesis-preventing substance in Japanese eel. Development 129 (11), 2689–2697.

Molés, G., Carrillo, M., Mañanós, E., Mylonas, C.C., Zanuy, S., 2007. Temporal profile of brain and pituitary GnRHs, GnRH-R and gonadotropin mRNA expression and content during early development in European sea bass (*Dicentrarchus labrax* L.). Gen. Comp. Endocrinol. 150 (1), 75–86.

Molés, G., Gómez, A., Rocha, A., Carrillo, M., Zanuy, S., 2008. Purification and characterization of follicle-stimulating hormone from pituitary glands of sea bass (*Dicentrarchus labrax*). Gen. Comp. Endocrinol. 158, 68–76.

Molés, G., Gómez, A., Carrillo, M., Zanuy, S., 2012. Development of a homologous enzyme-linked immunosorbent assay for European sea bass FSH. Reproductive cycle plasma levels in both sexes and in yearling precocious and non-precocious males. Gen. Comp. Endocrinol. 176, 70–78.

Morinaga, C., Saito, D., Nakamura, S., Sasaki, T., Asakawa, S., Shimizu, N., Mitani, H., Furutani-Seiki, M., Tanaka, M., Kondoh, H., 2007. The hotei mutation of medaka in the anti-Müllerian hormone receptor causes the dysregulation of germ cell and sexual development. Proceed. Nat. Acad. Sci. 104 (23), 9691–9696.

Navarro, R.D., Souza, S.C., Bizarro, Y.W.S., Silva, R.F., Navarro, F.K.S.P., 2015. Effects of photoperiod on somatic growth and gonadal development in male Nile tilapia. Acta Scientiarum Tech. 37 (4), 381–385.

Nóbrega, R.H., Morais, R.D.V. de S., Crespo, D., de Waal, P.P., de França, L.R., Schulz, R. W., Bogerd, J., 2015. Fsh stimulates spermatogonial proliferation and differentiation in zebrafish via Igf3. Endocrinology 156(10);3804–3817.

Planas, J.V., Swanson, P., 2008. Physiological function of gonadotropins in fish. In: Fish Reproduction. Rocha, M.; Arukwe, A.; Kapoor, B.G., eds. Science Publishers, Inc. Enfield, New Hampshire. ISBN: 978-1-57808-331-2, pp. 37–68.

Rocha, A., Zanuy, S., Carrillo, M., Gómez, A., 2009. Seasonal changes in gonadal expression of gonadotropin receptors, steroidogenic acute regulatory protein and steroidogenic enzymes in the European sea bass. Gen. Comp. Endocrinol. 162 (3), 265–275.

Rocha, A., Zanuy, S., Gómez, A., 2016. Conserved anti-Müllerian hormone: anti-Müllerian hormone type-2 receptor specific interaction and intracellular signaling in teleosts. Biol. Rep. 94 (6), 1–13.

Rodríguez, L., Begtashi, I., Zanuy, S., Carrillo, M., 2000a. Development and validation of an enzyme immunoassay for testosterone: Effects of photoperiod on plasma testosterone levels and gonadal development in male sea bass (*Dicentrarchus labrax* L.) at puberty. Fish Physiol. Biochem. 23, 141–150.

Rodríguez, L., Carrillo, M., Sorbera, L.A., Soubrier, M.A., Mañanós, E., Holland, M.C.H., Zohar, Y., Zanuy, S., 2000b. Pituitary levels of three forms of GnRH in the male European sea bass (*Dicentrarchus labrax* L.) during sex differentiation and first spawning season. Gen. Comp. Endocrinol. 120, 67–74.

Rodríguez, L., Begtashi, I., Zanuy, S., Saw, M., Carrillo, M., 2001a. Changes in levels of reproductive hormones during first maturation in European sea bass (*Dicentrarchus labrax* L.) under artificial day light. Aquaculture 202, 235–248.

Rodríguez, L., Zanuy, S., Carrillo, M., 2001b. Influence of day length on the age at first maturity and somatic growth in male sea bass *Dicentrarchus labrax*, L. Aquaculture 196, 159–175.

Rodríguez, L., Begtashi, I., Zanuy, S., Carrillo, M., 2005. Long-term exposure to continuous light inhibits precocity in juvenile male European sea bass (*Dicentrarchus labrax* L.). II. Hormonal aspects. Gen. Comp. Endocrinol. 140, 116–125.

Rodríguez, R., Felip, A., Cerqueira, V., Hala, E., Zanuy, S., Carrillo, M., 2012. Identification of a photolabile period for reducing sexual maturation in juvenile male sea bass (*Dicentrarchus labrax*) by means of a continuous light regime. Aquacult. Internat. 20, 1071–1083.

Rubio, V.C., Sánchez-Vázquez, F.J., Madrid, J.A., 2004. Oral administration of melatonin reduces food intake and modifies macronutrient selection in European sea bass (*Dicentrarchus labrax* L.). J. Pineal Res. 37 (1), 42–47.

Schulz, R.W., De França, L.R., Lareyre, J.J., LeGac, F., Chiarini-Garcia, H., Nobrega, R.H., Miura, T., 2010. Spermatogenesis in fish. Gen. Comp. Endocrinol. 165 (3), 390–411.

Sekar, N., Garmey, J.C., Veldhuis, J.D., 2000. Mechanisms underlying the steroidogenic synergy of insulin and luteinizing hormone in porcine granulosa cells: joint amplification of pivotal sterol-regulatory genes encoding the low-density lipoprotein (LDL) receptor, steroidogenic acute regulatory (stAR) protein and cytochrome P450 sidechain cleavage (P450scc) enzyme. Mol. Cel. Endocrinol. 159 (1–2), 25–35.

Servili, A., Lethimonier, C., Lareyre, J.J., López-Olmeda, J.F., Sánchez-Vázquez, F.J., Kah, O., Muñoz-Cueto, J.A., 2010. The highly conserved gonadotropin-releasing hormone-2 form acts as a melatonin-releasing factor in the pineal of a teleost fish, the European sea bass *Dicentrarchus labrax*. Endocrinology 151 (5), 2265–2275.

Shin, H.S., Song, J.A., Choi, J.Y., Kim, N.N., Choi, Y.J., Sung, S.N., Park, M.S., Min, B.H., Choi, C.Y., 2014. Effects of various photoperiods on kisspeptin and reproductive hormones in the goldfish, *Carassius auratus*. Anim. Cells Syst. 18 (2), 109–118.

Shiraishi, E., Yoshinaga, N., Miura, T., Yokoi, H., Wakamatsu, Y., Abe, S.I., Kitano, T., 2007. Mullerian inhibiting substance is required for germ cell proliferation during early gonadal differentiation in medaka (*Oryzias latipes*). Endocrinology 149 (4), 1813–1819.

Skaar, K.S., Nóbrega, R.H., Magaraki, A., Olsen, L.C., Schulz, R.W., Male, R., 2011. Proteolytically activated, recombinant anti-Müllerian hormone inhibits androgen secretion, proliferation, and differentiation of spermatogonia in adult zebrafish testis organ cultures. Endocrinology 152 (9), 3527–3540.

Sokal, R.R., Rohlf, F.J., 1981. Biometry. The principles and practice of statistics in biological research (2nd ed.). W.H. Freeman, NY, pp. 859.

Song, M., Gutzeit, H.O., 2003. Effect of 17-α-ethynylestradiol on germ cell proliferation in organ and primary culture of medaka (*Oryzias latipes*) testis. Develop. Growth Diff. 45 (4), 327–337.

Stefánsson, M.Ö., FitzGerald, R.D., Cross, T.F., 2002. Growth, feed utilization and growth heterogeneity in juvenile turbot *Scophthalmus maximus* (Rafinesque) under different photoperiod regimes. Aquac. Res. 33 (3), 177–187.

Taranger, G.L., Carrillo, M., Schulz, R.W., Fontaine, P., Zanuy, S., Felip, A., Weltzien, F.-A., Dufour, S., Karlsen, O., Norberg, B., Andersson, E., Hansen, T., 2010. Control of puberty in farmed fish. Gen. Comp. Endocrinol. 165 (3), 483–515.

Veras, G.C., Brabo, M.F., Dias, J.A., Abe, H.A., Nunes, Z.M.P., Murgas, L.D.S., 2016. The effect of photoperiod and feeding frequency on larval of the Amazonian ornamental fish Pyrrhulina brevis (Steindachner, 1876). Aquac. Res. 47 (3), 797–803.

Volkoff, H., Hoskins, L.J., Tuziak, S.M., 2010. Influence of intrinsic signals and environmental cues on the endocrine control of feeding in fish: potential application in aquaculture. Gen. Comp. Endocrinol. 167 (3), 352–359.

Wu, G.C., Chiu, P.C., Lyu, Y.S., Chang, C.F., 2010. The expression of *amh* and *amhr2* is associated with the development of gonadal tissue and sex change in the protandrous black porgy, *Acanthopagrus schlegeli*. Biol. Rep. 83 (3), 443–453.