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IDENTIFICATION OF A PHOTOLABILE PERIOD FOR REDUCING SEXUAL MATURATION IN JUVENILE MALE SEA BASS (*Dicentrarchus labrax*) BY MEANS OF A CONTINUOUS LIGHT REGIME

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Photoperiodic Control of Early Onset Puberty in Sea Bass

Abstract

The objective of this study was to identify a photolabile period suitable for reducing precocious gonadal maturation in juvenile male sea bass (*Dicentrarchus labrax*) through the exploration of discrete windows of continuous light (LL), 1-2 months in duration, in late summer-early autumn. Somatic growth, 11-ketotestosterone plasma levels and the rates of testicular maturation and spermiation were analyzed to evaluate the effect of the applied photoperiodic regimes. Three LL treatments, with duration of 2 months each, were previously screened between the months of August and November. Administration of LL during the October-November period failed to show any differential effects in reducing early maturation, as compared to the simulated natural photoperiod. However, the August-September period was considered to be a likely candidate for photolability. To define this photolabile period, four LL treatments with duration of 1 month were then screened within the same late summer period. Our results demonstrate that the time interval including the month of September is the most sensitive photolabile period in order to reduce precocious gametogenesis in sea bass.

Keywords: Photosensitive period, continuous light, precocity, 11-KT, gonadal stages, sperm release, sea bass.

Abbreviations:

Bw: Body weight

E_2: 17-β Estradiol

GSI: Gonadosomatic index

Gw: Gonad weight

G1LL: Constant continuous light

G2AS: Continuous light from August 1 to September 30

G3AA: Continuous light from August 1 to August 30

G3SO: Continuous light from September 1 to October 30

G4ON: Continuous light from October 1 to November 30

G415AS: Continuous light from August 15 to September 15

G5SS: Continuous light from September 1 to September 30

SNP: Simulated natural photoperiod

T: Testosterone

11-KT: 11-Ketotestosterone

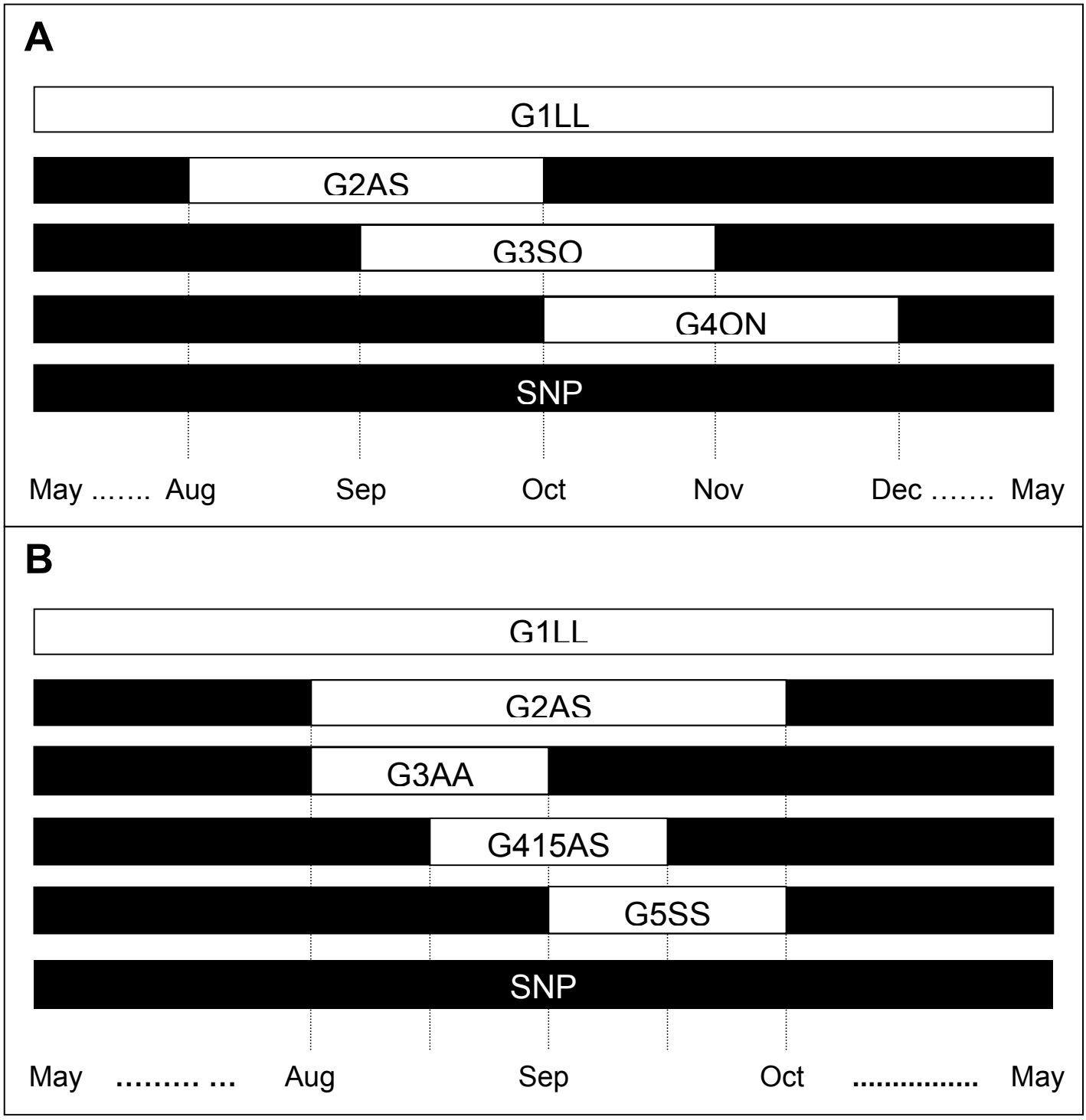

Fig. 1 Photoperiod regimes applied in the present study. **(A)** Trial 1. G1LL: constant continuous light (LL); G2AS: LL from August 1 to September 30; G3SO: LL from September 1 to October 30; G4ON: LL from October 1 to

November 30; SNP: constant simulated natural photoperiod. **(B)** Trial 2. G1LL: constant continuous light (LL); G2AS: LL from August 1 to September 30 (the photolabile period identified in trial 1); G3AA: LL from August 1 to August 30; G415AS: LL from August 15 to September 15; G5SS: LL from September 1 to September 30; G6: constant SNP.

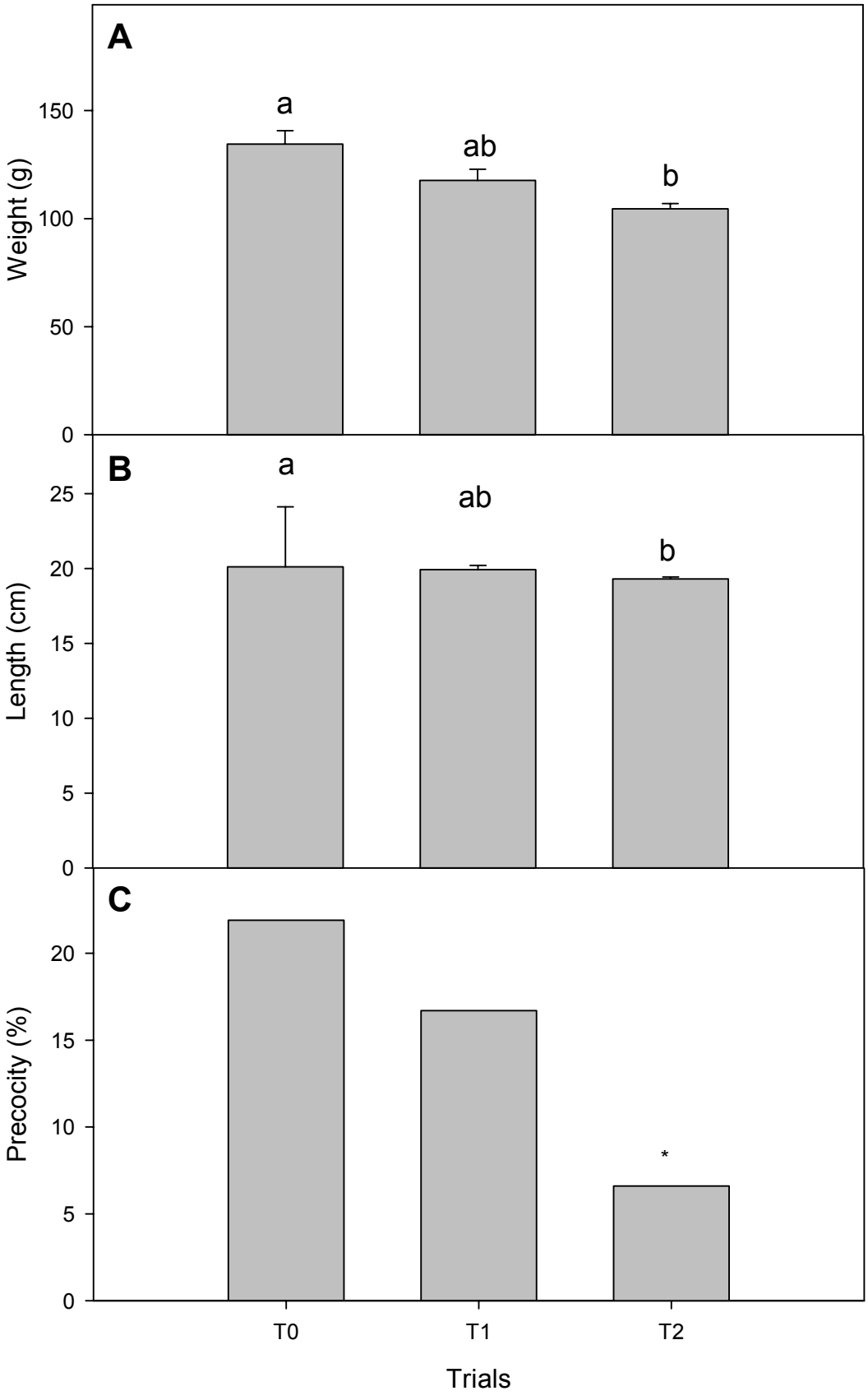
A
a
ab
b
Weight (g)
150
100
50
0
B
a
ab
b
Length (cm)
25
20
15
10
5
0
C
Precocity (%)
20
15
10
5
0
*
T0
T1
T2
Trials

Fig. 2 Average weight (A), length (B) and precocity (C) of different fish populations (T0 from Begtashi et al. 2004; T1 and T2 from the present study) during the month of February (maximum spermiation period). Different letters indicate significant (P<0.05) differences among groups. The asterisk above T2 indicates significant differences with respect to the T1 and T0 trials.

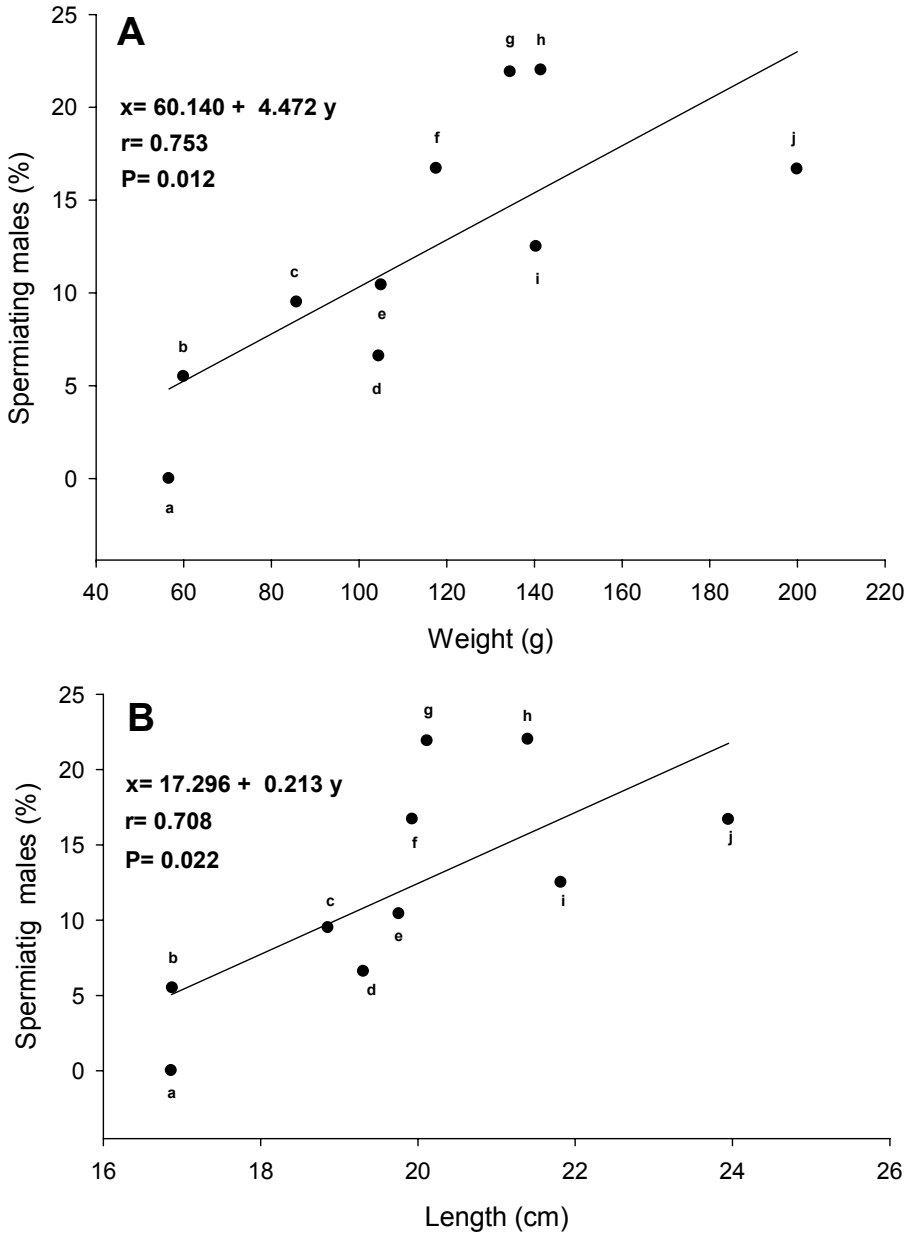

Fig. 3 Linear regression for the rate of spermiating males *versus* fish weight **(A)** and length **(B)** for prepubertal male sea bass assessed during the period of maximum spermiation. The data points come from the following sources: a = Zanuy, personal communication; b = Rodríguez et al. (2001); c = Zanuy, personal communication; d = Present study (T2); e = Bayarri et al. (2009); f = Present study (T1); g = Begtashi et al. (2004); h = Bayarri et al. (2009) i = Felip et al. (2008); j = Bayarri et al. (2009).

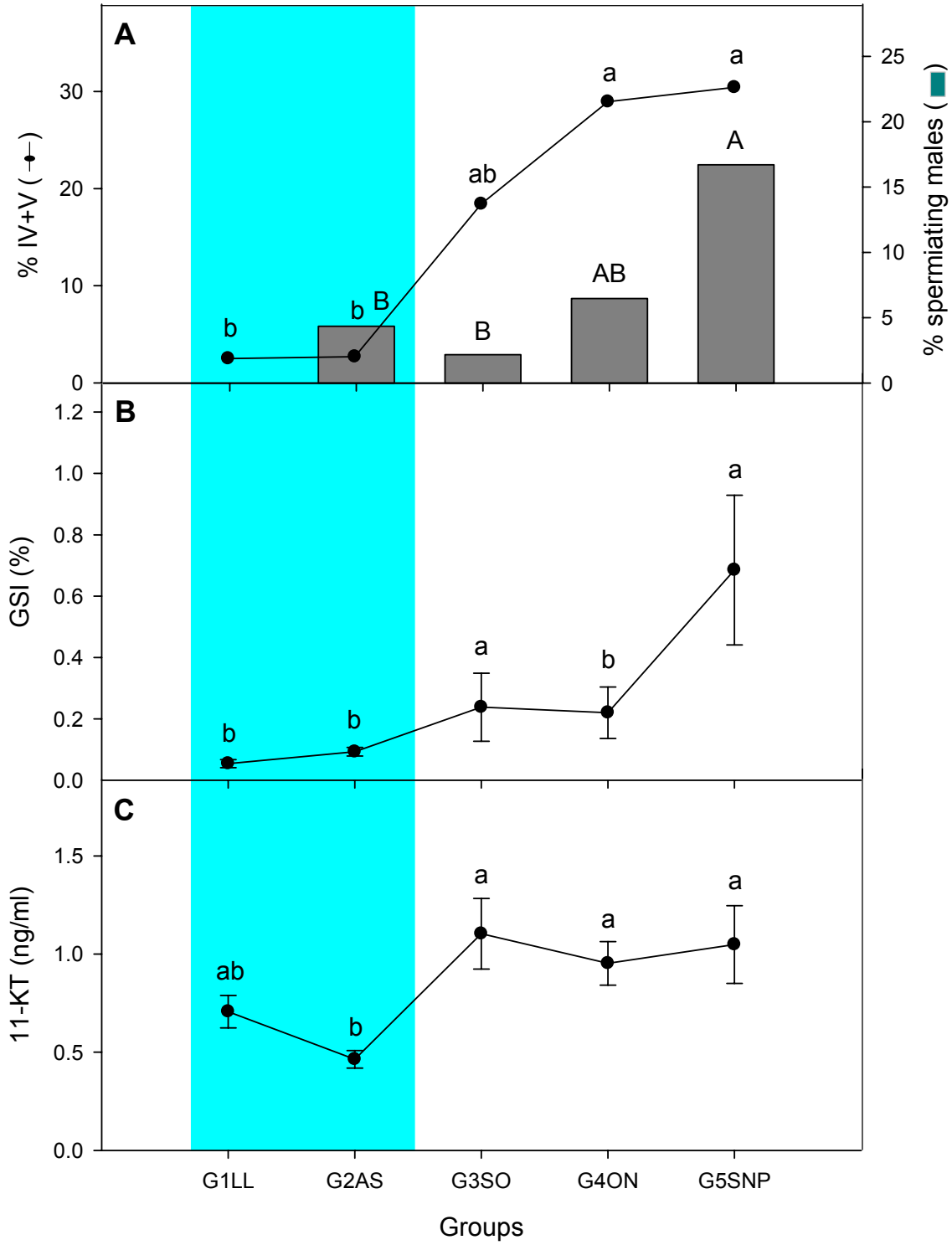

Fig. 4 Indicators of the maturation process in juvenile male sea bass kept under different 2-month photoperiod regimes of LL. **(A)** Total rates of spermiating fish (*solid bars*) throughout the sexual cycle, from October to May, and fish at stage IV+V (*black filled circles and solid line*) in February and March. **(B)** Gonadosomatic index (GSI) in February. **(C)** 11-KT plasma levels in February.

The photoperiodic regime references shown in this figure are explained in Fig. 1A. Different letters indicate significant (P<0.05) differences among treatments. The shaded area includes the group displaying the maturation indicator values most similar to those of the G1LL group.

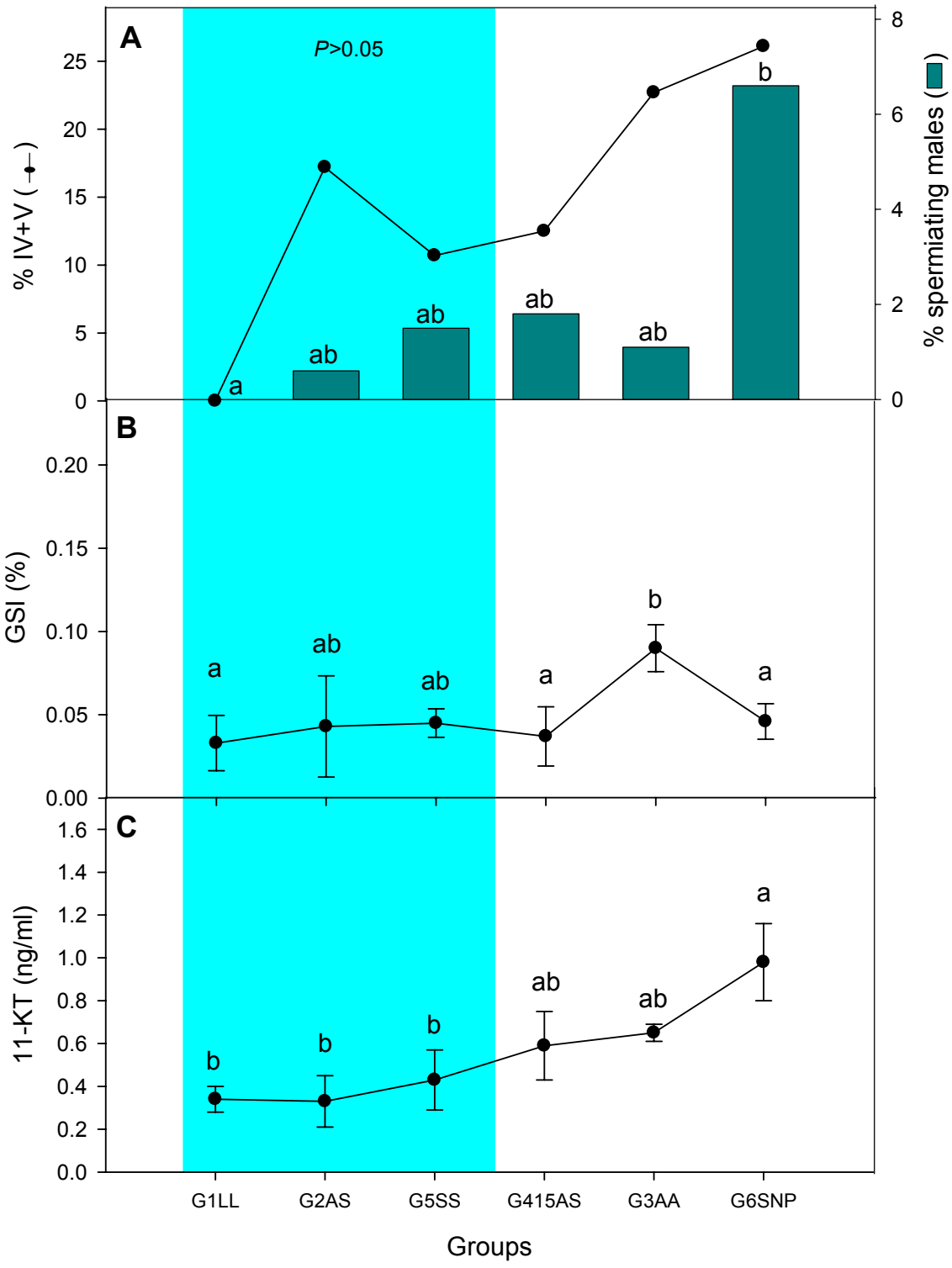

Fig. 5 Indicators of the maturation process in juvenile male sea bass kept under different 1-month photoperiod regimes of LL, assessed during February. **(A)** Total rates of spermiating fish (*solid bars*) throughout the sexual cycle from June to May, and fish at stage IV+V (*black filled circles and solid line*) in February and

March. **(B)** Gonadosomatic index (GSI) in February. **(C)** 11-KT plasma levels during February. The photoperiodic regime references shown in this figure are explained in Fig. 1B. Different letters indicate significant (P<0.05) differences among treatments. The shaded area includes those groups displaying the maturation indicator values most similar to those of the G1LL group.

Introduction

The early onset of puberty is a common feature in species of interest in aquaculture, such as *Oreochromis niloticus* (Longalong et al. 1999), *Sparus aurata* L. (Ginés et al. 2003), *Hippoglossus hippoglossus* (Weltzien et al. 2003), *Gadus morhua* L. (Karlsen et al. 2006), *Perca flavescens* (Shewmon et al. 2007), *Salmo salar* (McClure et al. 2007), and *Dicentrarchus labax* L. (Felip et al. 2008). Precocious maturation before the fish are marketed affects the production parameters, as it changes the appearance of the fish and causes a general decrease in their somatic growth (Carrillo et al. 2010; Taranger et al. 2010).

The mechanisms that regulate puberty in fish are not yet fully understood. However, it is known that genetic factors, metabolic signals and environmental stimuli (Nocillado et al. 2008) trigger a hormonal cascade along the brain-pituitary-gonad axis. Steroids [testosterone (T), 17-β estradiol (E_2) and 11-ketotestosterone (11-KT)] produced and released by the gonads are the last link in this hormonal cascade, which plays an important role in gonadal growth. Among these hormones, 11-KT is the major androgen produced in the testicles of teleost fish. It is important because it promotes spermatogonial proliferation, leading to meiosis and the later stages of spermatogenesis (Miura et al. 1991; Schulz et al. 2002). For this reason, this androgen has been proposed as a possible trigger for the onset of puberty in sea bass (Rodríguez et al. 2005).

Among environmental stimuli, the photoperiod is considered to be one of the most important in terms of modulating the onset of puberty in fish. In fact, it

has been proposed that fish species living in temperate zones perceive the photoperiodic change occurring after the summer solstice and this serves as an external signal that triggers the onset of puberty (Bromage et al. 2001). On the other hand, the masking of this external signal by a continuous light regime prevents the onset of sexual maturation in these fish (Davie et al. 2007). Consequently, continuous light regimes have been effective in reducing precocious maturation in salmonids (Taranger et al. 1998), Gadidae (Davie et al. 2007), flatfishes (Imsland et al. 2003; García-López et al. 2006) and Perciforms, including sea bass (Migaud et al. 2004; Begtashi et al. 2004; Felip et al. 2008).

In sea bass, one of the most important species in European aquaculture, sexual maturity occurs in nature during the second year of life in males and during the third year of life in females. However, under aquacultural conditions, there is a high proportion (20-30%) of males that exhibit precocious maturation during the first year of life, before they can be sent to the market. Additionally, the sex ratio between males and females in aquacultural facilities is usually 3:1 (Carrillo et al. 1995). Therefore, almost 70% of the fish populations are male individuals, 20% of which exhibit precocity. This seriously affects the aquacultural profitability of the species, because precocious males are significantly larger than non-precocious males during the first year of life, but they exhibit lower growth rates (18%) by the second year (Felip et al. 2008).

Photoperiodic control has also been used to reduce early puberty in sea bass (Carrillo et al. 2009). In fact, male sea bass precocity was reduced to < 3%

when a regime of continuous light was applied throughout the entire first year of life (Begtashi et al. 2004). Chronic exposure to continuous light, however, can produce a permanent stress condition that may diminish their immune response to bacterial infections (M. Carrillo, unpublished data). Furthermore, like other marine species of interest in aquaculture, sea bass can be raised in outdoor facilities where it is not always easy to maintain long-term regimes of continuous light (Taranger et al. 2010). Therefore, it would be interesting to discover shorter photoperiod regimes that permit both decreasing male precocity and reducing the negative impact of continuous light on sea bass health. Felip et al. (2008) showed that continuous light regimes applied for 4 and 6 months during pregametogenesis and gametogenesis, respectively, were able to reduce the rate of male precocity in sea bass to a value similar to that obtained by Begtashi et al. (2004) in males maintained under continuous light conditions (LL) year round. Moreover, Felip et al. (2008) suggest that there may be a shorter period of sensitivity to LL between August and November that might be used to significantly reduce sea bass precocity.

The aim of the present work was to screen out a period with continuous light exposure between August and November, capable of reducing sea bass male precocity. To accomplish this goal, we conducted a trial (Trial 1) using a 2-month LL window to identify an ample photolabile period of time between August and November. Based on this trial results, we conducted a second trial (Trial 2), using a 1-month LL window, in order to screen out and refine the 2-month period previously identified in Trial 1 (August-September). As indicators of the effects of continuous light on the reduction of precocity, the

gonadosomatic index (GSI), 11-KT plasma levels, rates of spermiating fish, and individuals at stage IV+V were determined, during the period of maximum spermiation, for prepubertal male sea bass subjected to the different light regimes. In addition, a linear regression analysis was performed for the rate of spermiating males *versus* biometric parameters (i.e., weight and length).

Materials and methods

Animals and photoperiodic regimes

Artificial continuous light regimes of varying durations were applied using tungsten light bulbs (Philips, PAR38Pro), controlled by means of electronic clocks and located at the water surface of each tank. This light source produced 650–700 lx at surface level. Two experimental trials were carried out. The first trial consisted of 400 juveniles with an average weight of 4.5 g, obtained from Aquanord (Gravelines, France). In May, the specimens were evenly distributed among five lightproof tanks with a capacity of 2,000 l and subjected to the following light regimes: constant continuous light (LL) (G1LL), LL from August 1 to September 30 (G2AS); LL from September 1 to October 30 (G3SO); LL from October 1 to November 30 (G4ON); and constant simulated natural photoperiod (SNP) (Fig. 1a). The second trial consisted of 3,000 juvenile fish with an average weight of 4.0 g, obtained from the same hatchery during a different year. In May, these fish were evenly distributed into 12 lightproof tanks with a capacity of 2,000 l. Six experimental groups were organized in duplicates as follows: constant continuous light (LL) (G1LL); LL from August 1 to September 30 (G2AS); LL from August 1 to August 30 (G3AA); LL from August 15 to September 15 (G415AS); LL from September 1 to September 30 (G5SS); and

SNP (Fig. 1b). In both trials, a SNP was also used outside the LL window proper for all those groups subjected to a temporal window of constant light.

Morphological and precocity analysis

The fish were anesthetized with ethylene glycol-monophenyl ether (0.5 ml/l of water) and killed by a quick cut at the level of the medulla oblongata in February and March (Trials 1 and 2). Gonads were collected, weighed, and kept for further histological analysis. Size (with a precision 0.1 cm) and weight (with a precision 0.01 g) were recorded on a monthly basis from the beginning of the experiment. Every month from December onwards, the rates of spermiating males (assessed by a gentle abdominal massage) were determined and blood sample were obtained by caudal puncture. The GSI was calculated as: GSI = 100 x Gw/Bw, where Bw = body weight and Gw = gonad weight.

All fish were maintained under natural temperature conditions, which ranged between 12 and 25 ± 1°C, and 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 $MJ.kg^{-1}$). The feed doses were adjusted according to temperature and biomass, following the instructions provided by the suppliers.

Hormonal analysis

Blood samples were collected from the caudal vein using heparinized syringes. They were then centrifuged at 3,000 rpm for 30 min at 4°C and stored at −20°C until further analysis. 11-KT plasma levels were determined by an EIA,

as described by Rodríguez et al. (2000).

Testis histology

Testes were fixed for 24 h in 4% formaldehyde/1% glutaraldehyde 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). Using the Cleveland- Wolf technique, 4-µm sections were stained (Herland 1960). Identification and characterization of the stage of testicle development were performed according to Begtashi et al. (2004). Stages IV and V were taken as indicators of maturity, as one of their distinctive characteristics is the presence of spermatozoids; by stage V, the spermiogenesis process has finished, and the fish are considered as running.

Statistical analysis

A one-way ANOVA after logarithmic transformation of the data was performed to determine possible significant differences between the effects of the different photoperiodic regimes on 11-KT plasmatic levels and GSI. A Kolmogorov–Smirnoff Barlett's test was used to verify the normality of the data. The data for both variables were expressed as mean ± SEM, and the differences were considered to be significant when $P<0.05$ (Sokal and Rohlf 1981). A chi-square test was used to compare the percentages of spermiating males and individuals in advanced gonadal stages among the different groups. A linear regression analysis was applied to the relationship between the percentage of spermiating males and both biometric parameters, taking into account the results of the present study and others previously conducted in our

laboratory. Statistical analyses were carried out using the SPSS 15.0 for Windows package (SPSS Inc., Chicago, IL, USA) and Sigma Plot 11.0 for Windows (Systat Software Inc., Germany).

RESULTS

Average weight, size, and rates of precocity recorded in Trials 1 and 2 during February, the period of maximum spermiation, are depicted in Fig. 2. The data used for Trial T0 were taken from Begtashi et al. (2004) in order to establish comparisons with Trials T1 and T2. High weight and length values were associated with elevated precocity. Consequently, the fish in T1, with greater weights and sizes than those in T2, also presented higher rates of precocity. Linear regressions between the rate of spermiating males and biometric parameters (weight and length), taken from different authors, are shown in Fig. 3 Significant (P=0.012, P=0.022) positive correlation (r=0.753, r=0.708) was observed between the spermiation rate and the fish weight and size, respectively

Trial 1

The percentages of spermiation and gonadal stage IV+V (Fig. 4a) run roughly parallel, with G1LL and G2AS presenting very low values for both variables (0.00%, 4.34% and 2.5%, 2.70% respectively) (shaded area in Fig. 4a), while the SNP and G4ON groups registered the maximum values (16.70%, 6.47% and 30.43%, 28.95%, respectively). Despite exhibiting a percentage of spermiation (2.17%) significantly lower than that of the G4ON and SNP groups, the percentage of gonadal stage IV+V for the G3SO group was relatively high

(18.42%), but failed to show any significant differences with respect to the rest of the groups. Groups G1LL and G2AS also presented the lowest GSI values (0.05 and 0.09%, respectively) (shaded area in Fig. 4b), while the other groups presented high values that were closer to SNP (0.68%). The lowest values for plasma 11-KT were once again exhibited by groups G1LL and G2AS (0.71 ng/ml and 0.46 ng/ml) (shaded area in Fig. 4c), and the highest values by the rest of the groups. Collectively, these data indicated that the August-September period (G2AS group) was the time interval in which the fish were more sensitive to the photoperiod treatment; for this reason, it was chosen as the candidate to be used in Trial 2, where a further screening was performed to refine the localization of the photolabile period.

Trial 2

As it was the case in Trial 1, the spermiation rates roughly correlated with the rates of fish at stage IV+V, with the lowest (0.00 and 0.00%) and highest (6.60 and 26.10%) values being exhibited by G1LL and SNP, respectively. The rest of the groups presented intermediate positions (Fig. 5a). However, the G2AS and G5SS groups showed values (0.60%, 17.20 % and 1.50%, 10.70%, respectively), which were closer to those of G1LL than the rest of the groups (shaded area in Fig. 5a).

All groups showed similar GSI values (nearly 0.04%), the only exception being G3AA, whose values were significantly higher (0.09%) than those of the control group (0.05%) (Fig. 5b). 11-KT plasma levels are shown in Fig. 5c. The lowest values (0.34, 0.33 and 0.43 ng/ml) were found in groups G1LL, G2AS,

and G5SS, respectively (shaded area in Fig. 5c), while the highest value (0.98 ng/ml) corresponded to the SNP group. The other groups occupied intermediate positions and failed to show significant differences. Figure 2 shows average size and weight values in February for the entire fish population during Trials T1 and T2. Also included is another trial (T0) from a different author, for comparison purposes only. Our study supports the hypothesis that larger fish produce more precocious fish than smaller individuals, since the larger weight and size of the T1 population compared to the T2 population (117.63 ± 5.26 g and 19.93 ± 0.28 cm vs. 104.54 ± 2.23 g and 19.31 ± 0.13 cm, respectively) was also matched by a higher precocity rate (16.70 and 6.60% for T1 and T2, respectively). Figure 3 shows the linear regression of the relationship between the percentage of spermiating fish and the average weight and length of different fish populations assessed during the month of February. The regression lines indicate a strong, significant correlation in both cases.

Discussion

This is the first study to have successfully localized a precise photolabile period for the inhibition of precocity in sea bass by screening specific periods with continuous light exposure within a 4-month time interval falling between August and November. Many studies evidence that masking the autumnal reduction in photoperiod by means of continuous light (LL) inhibits the onset of early puberty in different species of fish that are of interest in aquaculture (Taranger et al. 2010). In the case of sea bass, the first evidence that LL exposure effectively reduces gonadal development, thereby preventing the appearance of precocious males, was obtained by Begtashi et al. (2004) using

a 12-month consecutive treatment with LL. A few years later, Felip et al. (2008) presented evidence that a 4- or 6-month exposure to LL either during pregametogenesis (June-September) or gametogenesis (October March) was equally effective in reducing the number of early maturing males. Furthermore, these authors have also hypothesized that a potential photolabile period may exist in the case of sea bass, possibly located sometime during the autumn, between the months of August and September. Our studies have long supported this hypothesis. As a first approach, the proposed 4-month period between August and November was screened using 2-month windows of LL. This led to the identification of a photolabile period (associated with significantly lower rates of gonadal maturation, GSI values, and 11-KT plasma levels) shorter than the one previously identified and positioned between August and September. Other periods, such as October-November (G4ON) or September-October (G3SO) were unresponsive or not so responsive to the LL treatment and therefore, were discarded as possible targets for therapies aimed at reducing precocity. Once this photolabile period was identified, a second approach was used to refine the localization of this period, screening the time interval of August-September with LL windows of 1 month in duration. The results, based on the effects of the different LL treatments on 11-KT plasma levels, permitted restricting the photolabile period to September, as this appeared to be the most sensitive period in which application of LL might significantly reduces the precocious gametogenic process in prepuberal sea bass. Other groups, such as G3AA and G415AS, were discarded as suitable photolabile candidates because most of their maturation indicators were very close to the values of the SNP group or occupied intermediate positions, which

suggested that LL exposure during these specific periods did not induce any relevant reduction of the gametogenetic process. It is interesting to note that Molés et al. (2007) also reported a peak in pituitary GnRHs, GnRH-R, and gonadotropin mRNA expression during the month of September (200 days after hatching) in prepubertal male sea bass. Rocha et al. (2009) further described relatively high mRNA levels of gonadotropin receptor FSHR and mCYP11B1 (a gene that encodes an enzyme required for the final steps of 11-KT synthesis) in fish at stage I of gonadal maturation, again during September. Collectively, these data support the hypothesis that September may be the key period in which the onset of the gametogenesis process occurs. Our more precise identification of the photolabile period in September by examining the effects of LL on gonadal maturation and 11-KT levels is in agreement with previous works conducted on prepubertal fish, in which longer LL treatments including the September-October period consistently and significantly decreased 11-KT plasma levels (Rodríguez et al. 2005; Felip et al. 2008).

Despite the general reduction in fish precocity under continuous light in all groups, Trial 1 presented higher rates of precocious fish than Trial 2. This difference can be attributed to the different fish size of the populations; the fish were larger in T1 than in T2 and consequently presented higher rates of precocious fish. This characteristic pattern has been confirmed throughout a series of studies conducted on populations of distinct average size or weight, which presented different rates of precocious fish at the time of maximum spermiation (February). Furthermore, the lineal regression equation for both variables may provide further support for the hypothesis that a low body weight

or size could be a limiting factor for the onset of gametogenesis in sea bass.

Previous works have suggested that the first gonadal maturation is an extremely demanding process in terms of energy and only occurs when the virgin fish attain a certain degree of somatic growth or reach an energy reserve threshold (Rowe et al. 1991). Begtashi et al. (2004) have hypothesized that the rate of precocious sea bass is associated with the size and weight the fish attain in November, with reported average values of 107.80 g and 19.35 cm, respectively, for the maximum precocity rate (21.9%).

In the present work, the groups with the lowest numbers of individuals in advanced gonadal stages also registered the lowest 11-KT and GSI values. However, the effectiveness of continuous light on all the parameters studied was evident in the groups subjected to constant LL, which failed to register spermiating males in any trial. In addition, the percentages of individuals in advanced gonadal stages and 11-KT levels were also lower (2.5% and 0.71 ng/ml in Trial 1 and 0.0% and 0.34 ng/ml in Trial 2) than in the SNP groups (30.43% and 1.05 ng/ml in Trial 1 and 26.1% and 0.98 ng/ml in Trial 2). These results are in agreement with those obtained in sea bass by Begtashi et al. (2004), which showed a greater reduction in precocity under constant light (0.0%) *versus* SNP (21.9%) during the month of February. In a similar work on sea bass published by Rodríguez et al. (2005), there was evidence that the 11-KT profile for the LL group was very low and remained unchanged throughout the entire spermatogenesis process, while the SNP group showed a peak in 11-KT plasma levels during February (around 5 ng/ml). Another study on sea bass

by Felip et al. (2008) evidenced that 4- or 6-month exposures to LL during pregametogenesis and gametogenesis, respectively, produced similar lower rates of precocity (< 3%) as compared to fish maintained under LL conditions all year. Similarly, fish exposed to LL registered constant low levels of 11-KT (around 0.5 ng/ml), in contrast to those in the SNP group, which attained values of around 4 ng/ml on these same dates.

The ultimate effect of continuous light on gonadal maturation may be inhibiting the synthesis and release of 11-KT, a hormone that would appear to be essential for the development of all stages of maturation (Miura et al. 1991). This would explain the low levels of this hormone in LL-treated fish, which were always immature. On the other hand, elevated levels of 11-KT have been associated with the initiation of spermiogenesis in males (Rodríguez et al. 2004; Weltzien et al. 2002). Finally, as previously mentioned, there is mounting evidence that the low levels of androgens induced by continuous light may be due to insufficient gonadotropic stimulation. These low levels of androgen would not only reduce the number of spermatogenic cysts recruited during spermatogenesis, they would also potentially increase spermatogonial apoptosis, as has been observed in other species of teleost fish (Almeida et al. 2009). However, this is still pending confirmation and is one of the objectives in our present lines of research.

In conclusion, these results demonstrate the existence of a critical photolabile period for LL exposure in order to reduce precocity in sea bass. An accurate localization of this period suggests that September may be the time for

the beginning of gametogenesis in fish kept under natural photoperiod and temperature conditions, at least at our geographical latitude (40ºLN). Furthermore, 11-KT plasma levels would also seem to be a valuable indicator of gonadal maturation in sea bass. The reduction from the 12, 6 or 4 months of continuous light exposure reported in previous works to just 1 or 2 months in the present study, with practically no loss of effectiveness, is extremely important in terms of the welfare of the animals being subjected to LL therapies in order to reduce precocity. Another key advantage of using short LL treatments is the potential reduction in the artificial light needed to suppress maturation.

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