Effects of continuous light on the reproductive system of European sea bass as gauged by alterations of circadian variations during their first reproductive cycle

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ABSTRACT

The European sea bass is a short-day breeder, a characteristic that is highly valued in aquaculture. A high percentage of males of this species mature precociously before reaching commercial size, resulting in economic losses for fish farmers. We investigated the effects of continuous light (LL) on the circadian variations of several reproductive hormones in males of this species in order to understand how the presumed absence of a melatonin rhythm caused by LL affects their daily profile. The study was conducted during four critical stages of the sea bass reproductive cycle (pre-spermatogenesis (PSpg), spermatogenesis (Spg), spermiation (Spm), and post-spermiation (PSpm)). Every 3 h during a complete 24 h cycle, six fish kept under a natural photoperiod (NP) and six under LL were anaesthetized, measured, weighed, and bled. The pituitary was removed and frozen at -80ºC. The pituitary content of sea bream gonadotrophin-releasing (sbGnRH) and luteinizing hormone (LH), as well as plasma content of LH, testosterone, and 11-ketotestosterone (11-KT) were analyzed by ELISA. The percentage of spermiating males (precocity) per group was determined by periodic abdominal massages of the animals. Our results confirm LL treatment, maintained from the early stages of development onward, effectively reduces the percentage of precocious male sea bass. As has already been described for caged sea bass, plasma LH showed a clearly marked nocturnal rise near midnight during Spg and Spm during NP, but which was absent under LL. Pituitary sbGnRH and LH content and plasma LH concentration, under both NP and LL conditions, increased during the second half of the reproductive cycle, while sexual steroids were higher at the beginning of the cycle. LL inhibited steroid secretion, especiallytestosterone secretion, during Spg. In summary, without photoperiod cue, as accomplished by continuous exposure to LL, circadian variations of reproductive hormones appeared altered, causing irregularities in the reproductive process of male sea bass. These findings may have a practical application in aquaculture, namely by applying LL treatment in an effort to reduce the presence of precocious males in a stock. (E-mail correspondence: carrillo@iats.csic.es)

Key words: photoperiod manipulation, *Dicentrarchus labrax*, reproductive hormones, daily and seasonal rhythms, *in vivo*.

INTRODUCTION

The success of current marine aquaculture is based on a sound knowledge of animal biology, the application of new technologies, the development of specific diets, and control over diseases, genetics, and reproduction in fish farming. Controlling reproduction is an important aspect in order to obtain spawns from brood stocks to prevent over-exploitation of wild stocks and to produce fish with faster growth rates by manipulating their natural reproductive rhythms, thus allowing the farmer to obtain higher profits.

The reproductive process, like many others, is rhythmic. The physiological functions of living organisms have adapted to occur at those moments of the day, and also year, when the probability of success is the highest (Foster & Kreitzman, 2004). Cyclic changes in environmental factors are responsible for the generation of biological rhythms in organisms, with photoperiod being the most important cue in entraining these rhythms as exemplified by a recent study involving light synchronization of the circadian rhythm of spawning in gilthead sea bream (Mesequer et al., 2008). Therefore, the study of reproductive hormone circadian variations may provide interesting information that could contribute to a better understanding of reproductive function of fish and other species.

The European sea bass (*Dicentrarchus labrax*) is a teleost fish species that is highly valued in Mediterranean aquaculture. A high percentage of the males of this species mature precociously during their first year of life, before reaching commercial size, which in this species occurs at around 18 months of age (Carrillo et al., 1995). This circumstance results in important economic losses for fish farmers, since the nutrients and energy obtained by feeding during this period are invested in gonadal development, at the expense of somatic growth. Moreover, flesh quality decreases while sexual maturation is taking place (Bromage et al., 2001).

Reproductive control, therefore, is crucial to optimizing or extending the somatic growth period of male sea bass, and photoperiod manipulation seems to be an effective method to achieve such. . A number of studies have been conducted on several cultured fish species with the objective

of delaying or advancing gonadal maturation and spawning, using photoperiod manipulation, among other techniques (Bromage et al. 1995). Artificial photoperiods have been reported to be an effective method for delaying sea bass puberty (Zanuy et al., 2001) and/or altering spawning time (Carrillo et al., 1993). In later studies (Rodríguez et al., 2001a; Bayarri et al, 2004), the application of long photoperiods starting in the early stages of development proved effective for controlling reproduction. Bayarri et al. (2004) studied melatonin and reproductive hormone circadian variations during the first year of life in sea bass raised in sea cages and subjected to an artificially long photoperiod (18L:6D). This study provided the first evidence of the alteration of circadian variations caused by long photoperiod, affecting several different hormones in this species, such as pituitary sbGnRH or plasma LH. Moreover, the final consequence of this light treatment was delayed gonadal development and spawning at the time of puberty (Carrillo, Begtashi, Rodríguez, & Zanuy, unpublished results). Long-term exposure of sea bass to LL proved highly effective in inhibiting early precocity (Beghtashi et al., 2004), and for dramatically reducing the levels of several reproductive hormones (Rodríguez et al., 2005). However, and despite their importance, no data are yet available concerning the daily rhythms of reproductive hormones in fish kept under LL. This is especially relevant considering the great differences that exist between the reproductive effects of long photoperiods, such as that applied in the study by Bayarri et al. (2004), and LL, which delays or inhibits puberty and reproductive hormones, respectively.

The characterization and temporal appearance of male sea bass gonadal stages, as well as their endocrine regulation, have been widely studied (Prat et al., 1990, 1999; Zanuy et al., 1999; Rodríguez et al., 2000a, 2000b, 2001a, 2001b, 2004, 2005; Beghtashi et al., 2004; Molés et al., 2007). These studies have provided valuable information for selecting the relevant times for sampling and studying hormonal daily rhythms, i.e., pre-spermatogenesis (PSpg) in September, with low hormonal levels; spermatogenesis (Spg) in November, with the first significant surge of hormones and morphological indexes; spermiation (Spm) in February, with maximum hormone levels; and post-spermiation (PSpm) in May, with basal hormonal levels.

In a previous study (Bayarri et al., 2004), we tested the effects of a long photoperiod (18L:6D) applied beginning in the early stages of development on male European sea bass. The aim of the present study was to test the effects of LL, a treatment that presumably disrupts the melatonin rhythm, on the circadian variation of a number of reproductive hormones of the brain-pituitary-gonad axis (gonadotrophin releasing hormone, luteinizing hormone, and sexual steroids) during four critical stages of the reproductive cycle of this species: PSpg, Spg, Spm, and PSpm.

MATERIALS AND METHODS

Animals and housing

This study took place at the Instituto de Acuicultura de Torre la Sal (Castellón, Spain), 40ºN, 0ºE. Six-month-old sea bass fingerlings (approx. 3.5g) obtained from L'Ecloserie Marine (Gravelines, France) were distributed into four identical 2000-liter, light-proof fiberglass tanks, supplied with well-aerated running sea water (salinity 37‰), and exposed to either a simulated natural photoperiod (NP) or constant light (LL) from the moment of their arrival in May. (At this latitude, the photoperiod reaches a maximum of 15L:9D in June, and a minimum of 9L:15D in December). The fish were kept under a natural temperature regime throughout the experiment (13-25ºC), with the daily oscillation within a range of 0.5ºC. Light in each tank was supplied by tungsten bulbs (PAR38Pro, Philips, Madrid, Spain) providing 650-700 lux at the water's surface, and the simulated natural photoperiod was controlled by an electronic clock (ORBIS, Madrid, Spain), programmed weekly according to local geographical coordinates.

Fish from both light treatments were fed a commercial diet (Proaqua, Dueñas, Palencia, Spain) *ad libitum* by hand twice a day (i.e, in the morning, around 08:30 and in the afternoon around 13:30 h).

The handling of fish and conduct of the experimental procedures were always performed according to the international ethical standards as outlined in Portaluppi et al. (2008).

Experimental procedure

The objective of this experiment was to analyze the effects of LL on the daily variation of reproductive hormones and precocity of male European sea bass during their first year of life.

Every 3 h during a 24 h period, six male fish reared under the conditions prescribed for each treatment (NP and LL) were simultaneously anaesthetized with 2-phenoxyethanol (0.3 ppm) and then weighed and measured (n=48/treatment), at four critical stages of their reproductive cycle:

PSpg, Spg, Spm, and PSpm. Blood was collected by caudal punction, using heparinized syringes, and plasma was separated by centrifugation and preserved in aliquots at -80ºC until the time of analysis. The pituitary was removed and frozen separately in liquid nitrogen.

Pituitary sbGnRH Immunoassays

The pituitary was individually homogenized with a syringe in 250 ml of phosphate buffered saline tween-20 (PBST), and extracted for 10 min at 80ºC by adding 2N acetic acid to each microcentrifuge tube containing pituitary homogenate. After centrifugation (13000 g for 30 min at 4ºC), the supernatants were dried in a speed vacuum and reconstituted in EIA buffer for analysis. The pituitary sbGnRH content was measured in reconstituted samples using a competitive enzyme-linked immunosorbent assay (ELISA) similar to that described by Holland et al. (1998), which was modified for sea bass. The sensitivity of the assay was 6 pg/well, and cross-reactivity with cII and sGnRH was calculated to be 0.6 and 0.4%, respectively.

LH Immunoassay

A homologous ELISA (Mateos et al., 2006) was used to analyze LH pituitary content and plasma levels. The sensitivity of the assay was approximately 0.6 ng/ml, and the intra and inter-assay coefficients of variation were approximately 11% and 13%, respectively.

Steroid assays

A specific enzyme immunoassay (EIA) developed by Rodríguez et al. (2000a) for sea bass was used to determine plasma testosterone levels. Plasma 11-ketotestosterone levels were analyzed using an EIA originally developed for Siberian sturgeon (Cuisset et al., 1994), which was modified for sea bass, and using a final dilution of 1:320000 for primary antibodies and 1:10 for the tracer (Cayman Chemicals, MI, USA) (Rodríguez et al., 2001a).

Determination of precocity

Every 15 days, from February to May, the fish from both groups were subjected to an abdominal massage to determine how many of them released sperm.

Data analysis

Data are expressed as mean ± SEM values. The statistical differences between the groups were determined by a one-way analysis of variance (ANOVA), followed by a Tukey's test, with $p < 0.05$ considered the threshold for statistical significance.

RESULTS

Continuous light had an inhibiting effect on precocity (data not shown), as indicated by the lower percentage of precocious animals found in the group exposed to LL (2.08% in LL *vs*. 12.50% in NP) at the end of the experiment.

Under NP conditions, the pituitary sbGnRH content only exhibited statistically significant daily variations during PSpm, with smooth elevations at 17:30 and 11:30 h (Figure 1). The application of LL induced the appearance of peaks on the Spg and PSpm daily profiles at 23:30 and 05:30 h, respectively. When both light treatments were compared, significant sbGnRH pituitary content differences were found for PSpg and Spg at 08:30 h, and for PSpm at 14:30, 02:30, 05:30, and 11:30 h. Regardless of the light regime, lowest pituitary sbGnRH levels for the entire reproductive cycle corresponded to PSpg; they increased during the following stages, reaching especially high values in the case of Spm under LL.

The pituitary LH daily profile did not exhibit circadian oscillations under NP in any of the four stages analyzed (Figure 2). On the other hand, significant daily differences appeared under LL during PSpg and PSpm, with maximum LH concentrations occurring at 23:30 and 14:30 h for PSpg and PSpm, respectively. Significant differences were found between both light regimes in the late afternoon and during the first half of the night (from 17:30 to 23:30 h) during PSpg, at 14:30 h during Spm, and at 11:30 h during PSpm. The only significant difference in pituitary LH content among the different stages of the reproductive cycle in the group exposed to NP was observed at 08:30 h, with the highest value being recorded during Spm. In contrast, fish exposed to LL displayed significant differences at most time points, although highest LH levels were also recorded during Spm.

The daily plasma LH concentration profiles in fish maintained under NP conditions displayed statistically significant peaks at 23:30 h during Spg and Spm, and 3 h earlier (20:30 h) during PSpm (Figure 3). Exposure to LL, on the other hand, induced the appearance of daily variations during PSpg, with the peak at 23:30 h, a pattern that was not seen under NP. Besides this,

the daily profiles in fish subjected to LL displayed a significant morning elevation in plasma LH during Spm, and a peak at 17:30 h, which was statistically significant with respect to the 23:30 h time-point value during PSpm. Comparison between the two light treatments yielded significant plasma concentration differences for PSpg around midday, Spg at midnight, Spm at the first time points in the morning, and PSpm at 05:30 h. Finally, the comparison between the different stages within a given light regime also yielded significant differences at certain time points. In this case, highest LH values in the NP group were recorded at night (23:30 and 02:30 h) during Spg, and at 11:30 h during PSpm, whereas under LL the maximum concentration was seen at 08:30 h during Spm.

Significant daily variation in 11-KT was found in the plasma of fish kept under NP only during PSpg and Spg, with peak values being recorded at 11:30 and 14:30 h, respectively (Figure 4). Under LL, the 11-KT concentration profile displayed significant circadian variation during all four stages, with smooth elevations at 14:30 (PSpg), 20:30 (Spg), 02:30 (Spm), and 14:30 and 20:30 h (PSpm). Significant differences were seen at several time points during the daily cycle in the concentration of this hormone in fish exposed to LL *vs.* NP conditions. Thus, 11-KT levels were significantly higher in the NP group at 02:30, 08:30, and 11:30 h during PSpg, 11:30 h during Spg, and during the last half of the scotophase and first half of the photophase in PSpm (i.e., 02:30, 05:30, 08:30, 11:30 h). The highest levels of the plasma 11-KT annual variation for both light treatments occurred during the first half of the reproductive cycle, i.e., PSpg and Spg.

Plasma testosterone levels of fish subjected to NP exhibited significant daily variation during PSpg, with a peak at 11:30 h, and also during PSpm, with a maximum concentration at 02:30 h, followed by a smooth decrease (Figure 5). Under LL, however, it was only during Spg that a significant daily rhythm was found, with the lowest values being recorded at 23:30, 02:30, and 05:30 h. The testosterone levels of fish exposed to LL were significantly lower that those of the NP group at 11:30 h during PSpg, on the entire Spg daily cycle, throughout the entire Spm scotoperiod, and at 20:30, 02:30, 08:30, and 11:30 h during PSpm. Finally, when testosterone levels within each

group and throughout the entire reproductive cycle were considered, significantly higher values were found at all time points during PSpg and Spg, regardless of the light regime.

DISCUSSION

The effect of LL on the circadian pattern of several reproductive hormones has been analyzed for the first time during four critical stages of the reproductive cycle of European male sea bass, corroborating the effectiveness of this treatment for inhibiting precocity, as previously described for this species (Begtashi et al., 2004; Rodríguez et al., 2005). In carp, however, LL resulted in precocious maturation of the testis during the pre-spawning phase, probably due to the age and sexual status of the fish (Bhattacharya et al., 2007). In the present study, some reproductive hormones, particularly plasma LH, appeared to be related to the photoperiod, as evidenced by the daily profile differences detected between the LL and NP treatments. The presence of daily variations under LL conditions leads us to conclude that the light treatment did not inhibit the functioning of circadian oscillators, perhaps due to the persistence of other periodical cues, such as temperature, which were still present. However, the 24 h rhythmicity did appear to be altered.

In the present experiment, pituitary sbGnRH levels under NP conditions did not vary over the 24 h cycle, except in May, i.e., during the PSpm stage. Therefore, our results do not agree with those of Bayarri et al. (2004), who found significant daily variations near spermatogenesis (December) in male sea bass kept in sea cages. This discrepancy might suggest that the daily pituitary sbGnRH profile is not relevant for completing the reproductive process of male sea bass, and that other hormones are ultimately responsible. In the fish that we exposed to LL, pituitary sbGnRH significantly peaked at 23:30 and 05:30 h during Spg and PSpm, respectively, in both cases during the subjective night, demonstrating absence of a photoperiod cue alters this neurohormone circadian variation. Regarding the annual cycle, the pituitary GnRH content has been shown to be related to gonadal maturation (Okuzawa et al., 1990; Amano et al., 1992, 1993; Holland et al., 1998; Andersson et al., 2001; Collins et al., 2001). In the present study, highest pituitary sbGnRH values were found during Spm, especially in fish exposed to LL. Under NP conditions, sbGnRH increased from November onward, reaching maximum level in February, and remaining elevated even in May. This is in contrast with the findings of other studies (Rodríguez et

al., 2000b; 2004), where maximum values were recorded in November, during sexual differentiation and the first spawning season, with a smaller peak occurring in February. In these studies, however, samples were taken at one single time point during the day, where the relative position on the daily profile is not known and, therefore, does not necessarily reflect the daily sbGnRH maximum.

Pituitary LH, in those fish subjected to NP, failed to exhibit statistically significant daily rhythms at any stage of the reproductive cycle. However, the shape of November (Spg) daily profile was somewhat comparable (although slightly shifted) to that reported by Bayarri et al. (2004) for the same reproductive stage (December), with significant decreases at 16:00 and 01:00 h. Neither LL in the present study nor the long photoperiod used in a previous study by Bayarri et al. (2004) induced the appearance of significant pituitary LH rhythms during Spg. In our experiment, the circadian variation in LH under LL was only significant during PSpg (September) and PSpm (May). The highest values throughout the reproductive cycle were recorded during Spm (10-15 μg/mg prot) under both light treatments. The lack of studies regarding daily and annual pituitary LH rhythms in other species makes it difficult to undertake any further comparisons with our results.

Plasma LH peaked under NP at 23:30 h during Spg and Spm, the most critical stages of the reproductive cycle. Similar results were reported by Bayarri et al. (2004), who found a relationship between the onset of the scotophase and the appearance of the nocturnal LH rise in December, when a long photoperiod (18L:6D) was tested on sea bass. In fish exposed to this long photoperiod, the LH peak appeared after a 5 h delay, coinciding with the delay in the beginning of the dark period, with the ultimate consequence being a delay in puberty (Carrillo et al., unpublished results). In our case, no nocturnal peaks were detected during the Spg or Spm stages in fish exposed to LL. The lack of plasma LH daily rhythmicity under LL at such important reproductive times may partially explain the inhibition of precocity, since LL prevented the hormonal cascade from being completed. The nocturnal LH surges found in pre-pubertal sea bass (Bayarri et al., 2004) may resemble in certain aspects the LH pattern described in pre-pubertal humans, according to a study

conducted by Wu (1995). This pattern of nocturnal plasma LH peaks appears again in the results we have now obtained for male sea bass during their first year of life. According to Zohar (1988), the profile of the fish LH surge depends on the physiology of the gonad. For instance, in female sea bream (*Sparus aurata*), a species with non-synchronous ovarian development, a daily pre-ovulatory plasma LH peak was reported to appear 6 h before spawning (Zohar, 1988). In male carp, on the other hand, some authors have described a time interval of 12 h between gonadotropin (GtH) rise and spermiation (Courtois et al., 1986; Saad & Billard, 1985). The lowest concentration of LH over the reproductive cycle was found during PSpg in September. This is similar to the findings of Rodríguez et al. (2000b, 2001a, 2004), who observed lowest plasma LH concentrations at their first sampling point (October-November). In female sea bass, Navas et al. (2004) once again found lowest plasma LH values in October, and highest levels during ovulation, corroborating the general agreement that LH could be the maturational hormone, whose secretion induces the production of maturation-inducing steroids (Swanson et al., 1989; Swanson, 1991; Prat et al., 1996). In mammals, such as the mink (*Mustela vison*), the lowest values for amplitude, frequency, and mean LH secretion occur during the months of quiescence (Jallageas et al., 1994). These findings reinforce the role of LH during the last stage of the reproductive cycle in short-day breeders.

Under NP, daily plasma 11-KT variations were found only during PSpg and Spg, at the beginning of the reproductive cycle. The maximum concentrations in both stages were detected near midday. In fish exposed to LL, significant circadian variations were evident during all four stages, with a progressive delay in the appearance of the peaks throughout the reproductive cycle, similar to what occurs in a free-running rhythm. Theconcentration of this hormone tended to increase at the beginning of the day under NP, with values being significantly higher than those obtained under long photoperiods (Bayarri et al., 2004), and under LL (present results). This steroid is of importancein reproduction; 11-KT appears to be higher in dominant male rainbow trout than in subordinate fish (Cardwell et al., 1996), and it induces typical male-type spawning behavior in male goldfish (Kobayashi & Nakanishi, 1999). Therefore, absence of high levels of 11-KT during part of

the daily cycle under LL might probably be sufficient to modify sea bass reproduction, inhibiting precocity, for example. Maximum 11-KT concentration in our study was detected at the beginning of the cycle, during PSpg and Spg, which agrees with previous published results, and indicating the maximum effect of 11-KT on this species occurs during spermiogenesis (Rodríguez et al., 2000b). Under LL, 11-KT levels were also high during PSpg, although significantly lower than under NP.

Under NP conditions, testosterone showed circadian variation during PSpg and PSpm. In fish exposed to LL, however, a daily rhythm was found only during Spg, with lowest values attained during the subjective night. Bayarri et al. (2004) also described a significant Spg rhythm under both natural and long photoperiods. In that study, none of the rhythms was affected by light, since both showed a peak around 07:00 h, coinciding with the minimum water temperature. In our case, testosterone peaks under NP conditions were detected near midday during PSpg, and near midnight during PSpm, showing an undefined pattern of rhythmicity. Daily testosterone variations have been also described for other teleost species, such as catfish (Lamba et al., 1983), carp (Santos et al., 1986), and Japanese eel char (Yamada et al., 2002). Moreover, , the highest testosterone concentration over the course of the reproductive cycle occurred during PSpg and Spg, that is, at the beginning of the reproductive cycle, which is similar to that found for 11-KT. Rodríguez et al. (2004) also found highest concnetration of this hormone in November, during the period of testicular differentiation and growth(the first reproductive season for sea bass). Differences between treatments were especially appreciable during Spg, when all time points throughout the day proved to be statistically different.

In conclusion, our results derived under a natural photoperiod showed certain similarities with those found in previous studies on European sea bass, especially regarding the daily variation in plasma LH (Bayarri et al., 2004) as well as the concentrations of plasma LH throughout the reproductive cycle (Rodríguez et al. 2000b, 2001a, 2001b, 2004, 2005). However, in contrast with the study by Bayarri et al. (2004), which was performed during spermatogenesis, differences were detected in the daily profile of certain reproductive hormones under NP conditions. Therefore, it

might be concluded that the circadian variation in those hormones, whose daily profile differs in the two studies, are not very important for sexual development. Nevertheless, plasma LH levels under NP peaked during the first half of the night in both studies, which demonstrates the relevance of this peak for sexual maturation. In fact, when this peak shifted to the second half of the night in fish housed under the long photoperiod (Bayarri et al. 2004), the consequence was delayed puberty (Carrillo et al., unpublished results), and when it was not present under LL (as in the present study), precocity was inhibited. Exposure to LL, on the other hand, altered the circadian and annual profile of several reproductive hormones, with the ultimate consequence being reduction in the incidence of precocity. As a result, it would appear that LL treatment would have a practical application in Aquaculture.

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FIGURE LEGENDS

Figure 1. Daily pituitary sbGnRH content variations at various stages of the reproductive cycle in sea bass maintained under NP (black symbols) and LL (white symbols) conditions. Horizontal white and black bars represent day and night time, respectively. Different lower case and capital letters indicate significant differences (ANOVA, Tukey's test, $p < 0,05$) among different time points throughout the 24 h period, and among the different stages of the reproductive cycle, respectively. Significant differences between treatments at each time point are indicated by an asterisk (*). PSpg = pre-spermatogenesis, Spg = spermatogenesis, Spm = spermiation, PSpm = post-spawning.

Figure 2. Pituitary LH content variations over the 24 h cycle at various stages of the reproductive cycle in sea bass maintained under NP (black) and LL (white) conditions. See the legend in Figure 1 for details.

Figure 3. Plasma LH concentration changes over the 24 h cycle at various stages of the reproductive cycle in sea bass maintained under NP (black) and LL (white) conditions. See the legend in Figure 1 for details.

Figure 4. Concentration of 11-ketotestosterone over the 24 h cycle at various stages of the reproductive cycle in sea bass maintained under NP (black) and LL (white) conditions. See the legend in Figure 1 for details.

Figure 5. Daily plasma testosterone variation in fish maintained under NP (black) and LL (white) conditions at various stages of the reproductive cycle. See the legend in Figure 1 for details.

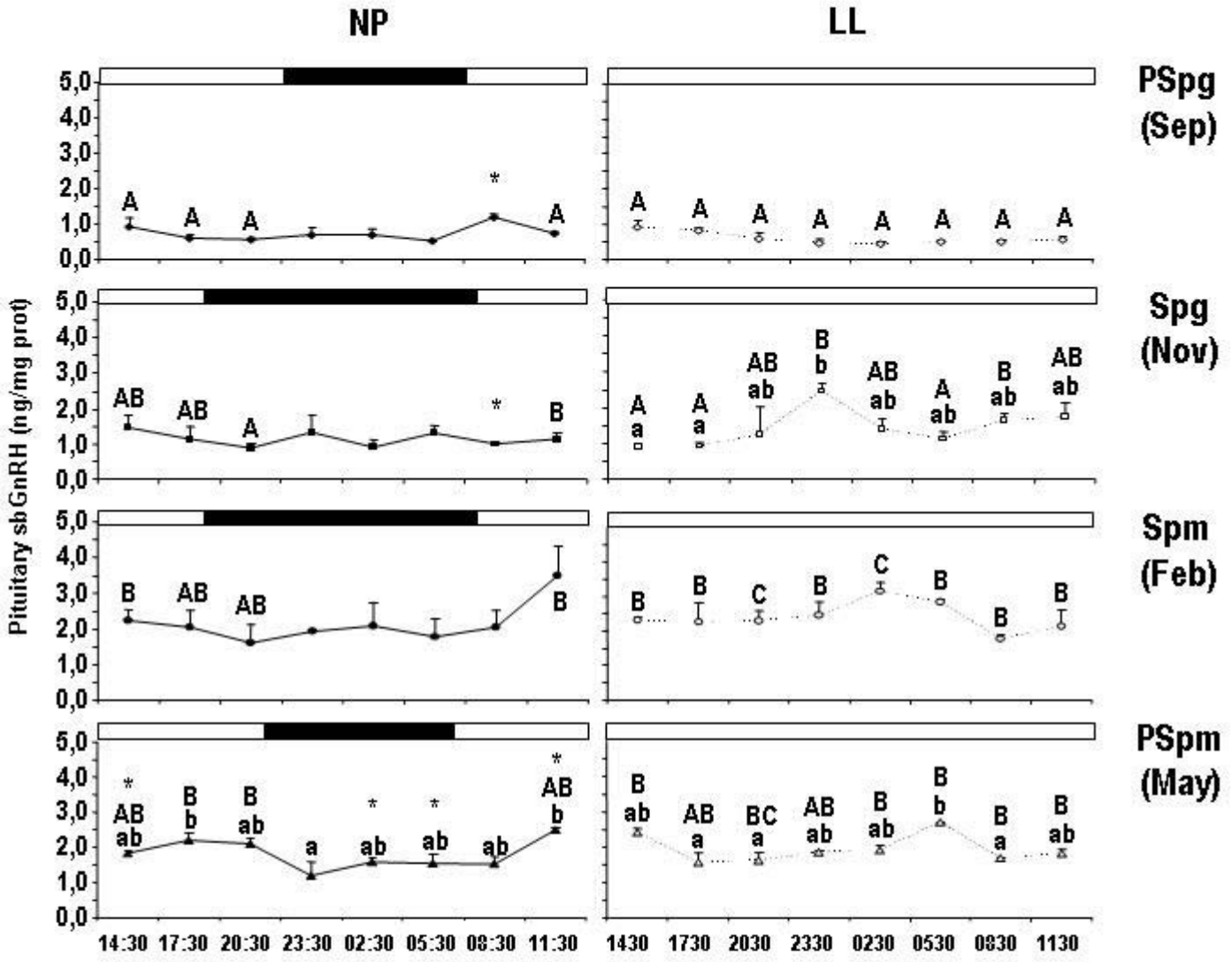
NP
LL
PSpg (Sep)
Spg (Nov)
Spm (Feb)
PSpm (May)
Pituitary sbGnRH (ng/mg prot)
5,0
4,0
3,0
2,0
1,0
0,0
14:30 17:30 20:30 23:30 02:30 05:30 08:30 11:30
1430 1730 2030 2330 0230 0530 0830 1130

Figure 1

1

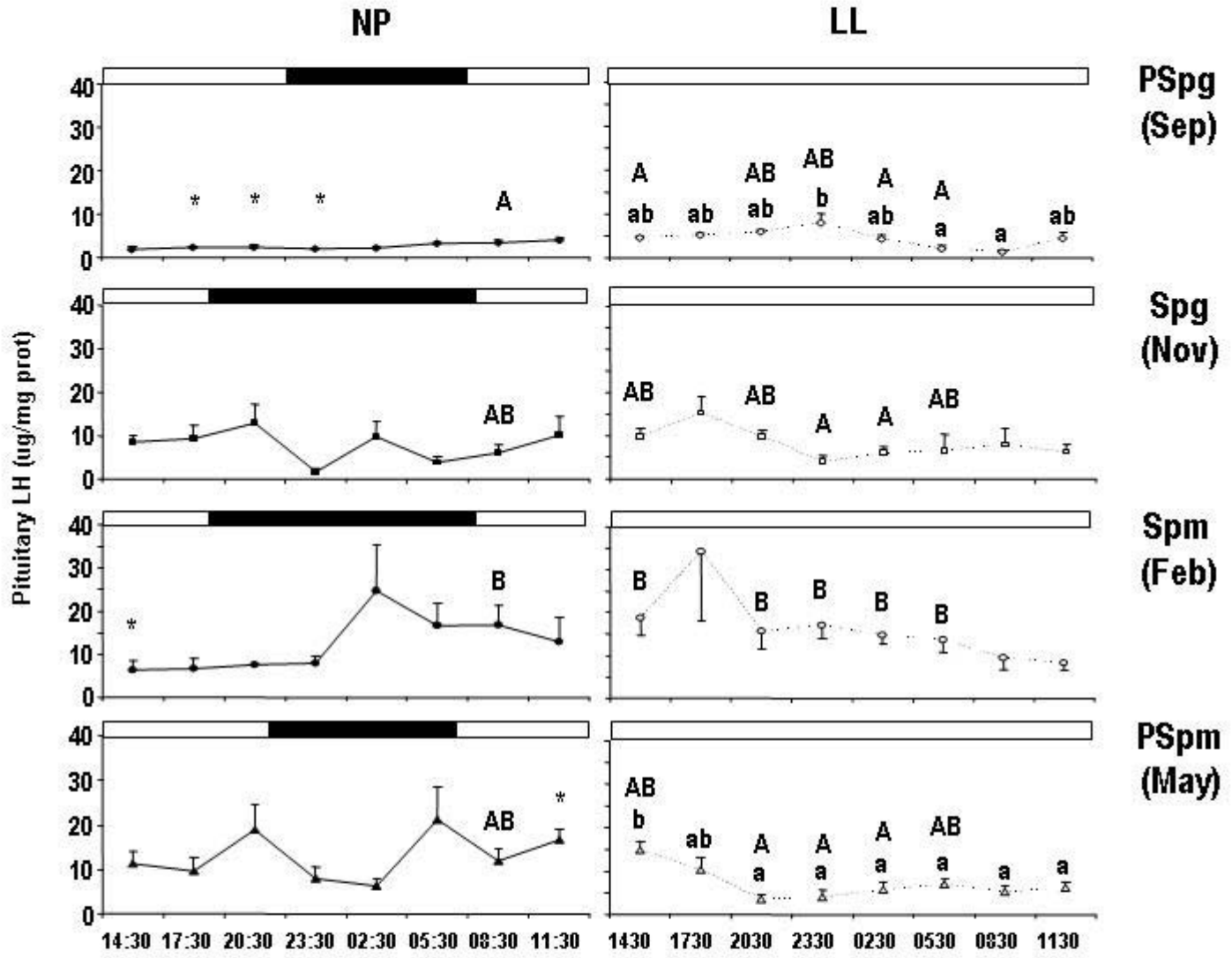
NP
LL
PSpg (Sep)
Spg (Nov)
Spm (Feb)
PSpm (May)
Pituitary LH (ug/mg prot)
14:30 17:30 20:30 23:30 02:30 05:30 08:30 11:30
1430 1730 2030 2330 0230 0530 0830 1130

Figure 2

1

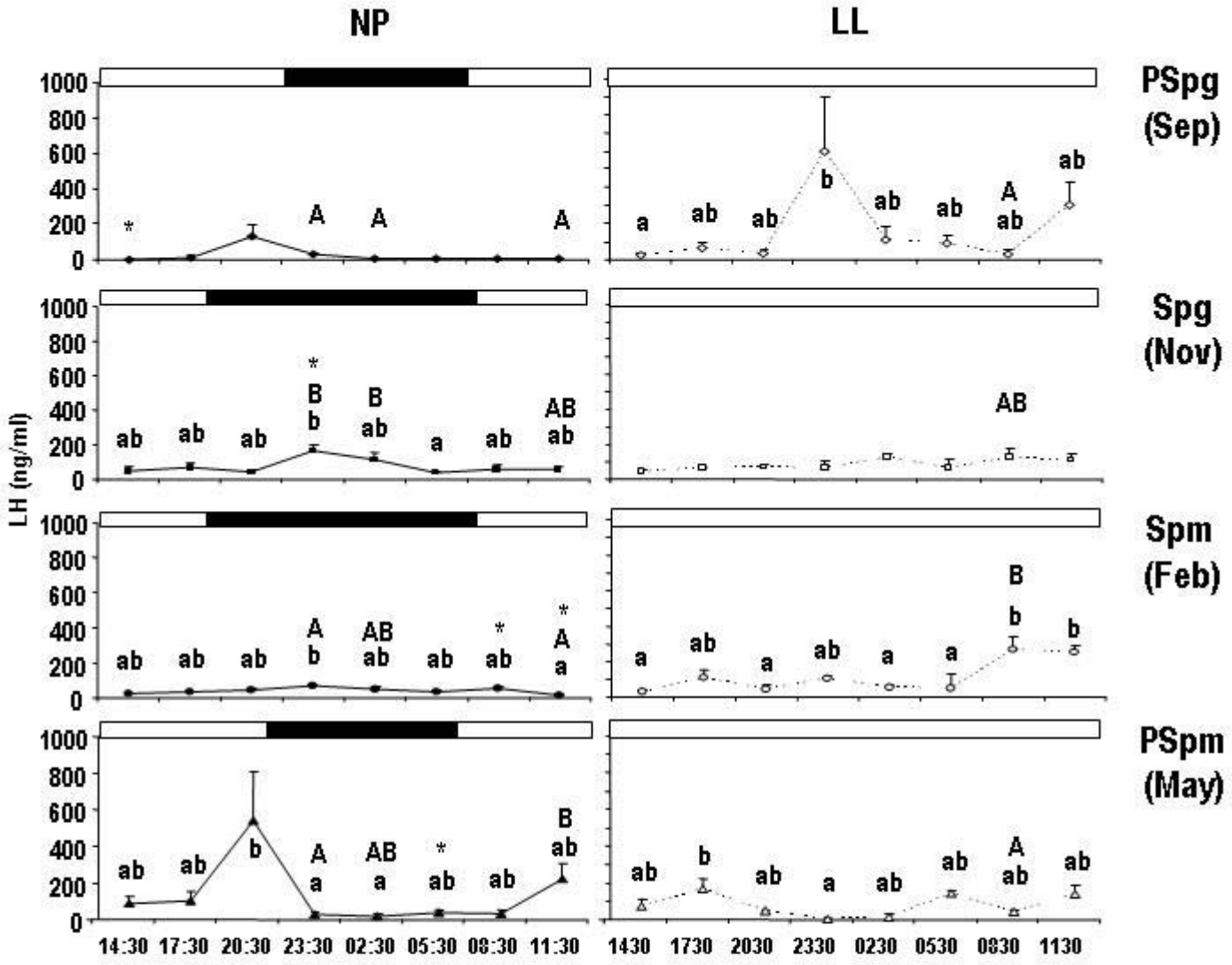
NP
LL
PSpg
(Sep)
Spg
(Nov)
Spm
(Feb)
PSpm
(May)
LH (ng/ml)
1000
800
600
400
200
0
14:30 17:30 20:30 23:30 02:30 05:30 08:30 11:30
1430 1730 2030 2330 0230 0530 0830 1130

Figure 3

1

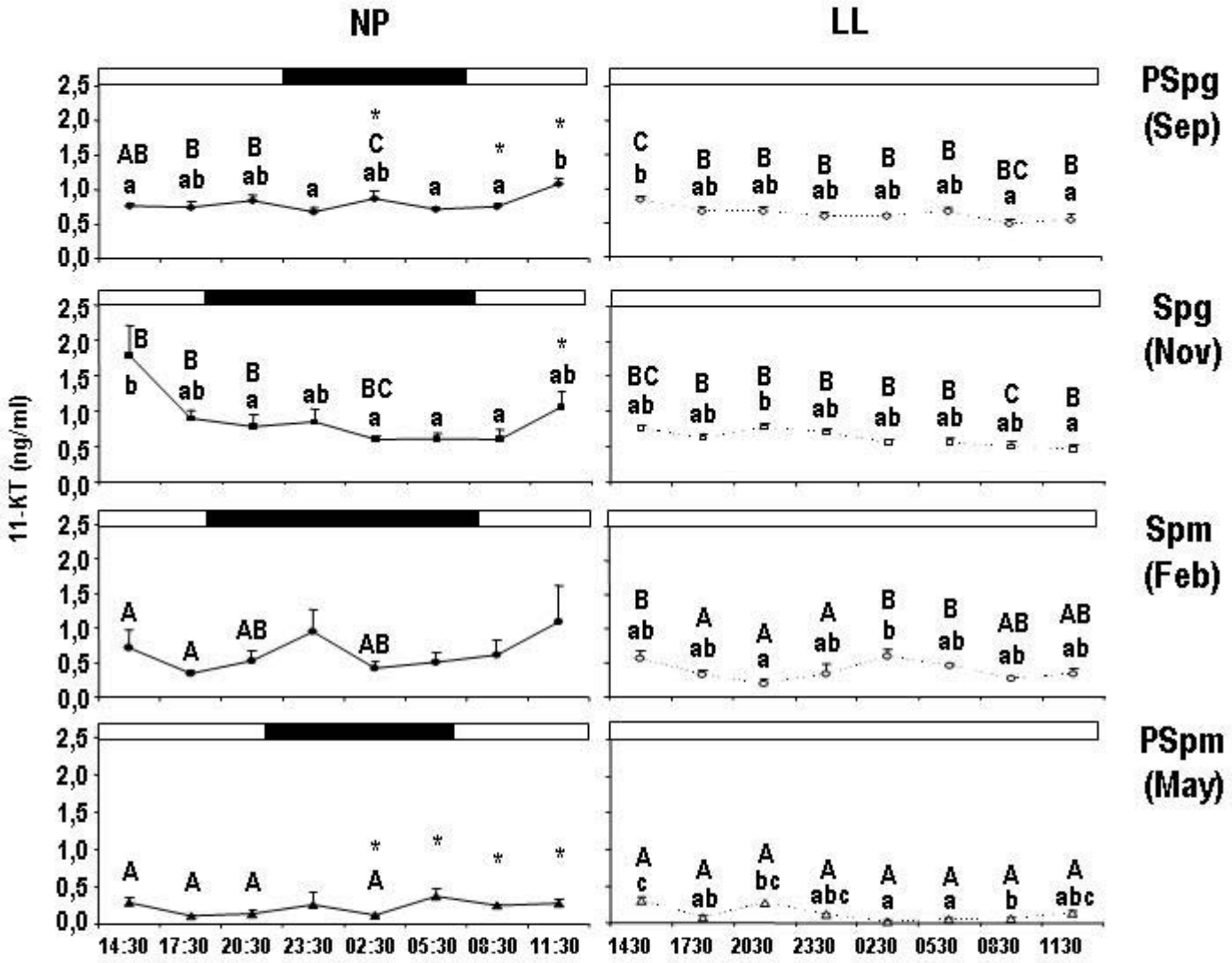
NP
LL
PSpg (Sep)
Spg (Nov)
Spm (Feb)
PSpm (May)
11-KT (ng/ml)
2,5
2,0
1,5
1,0
0,5
0,0
14:30 17:30 20:30 23:30 02:30 05:30 08:30 11:30
1430 1730 2030 2330 0230 0530 0830 1130

Figure 4

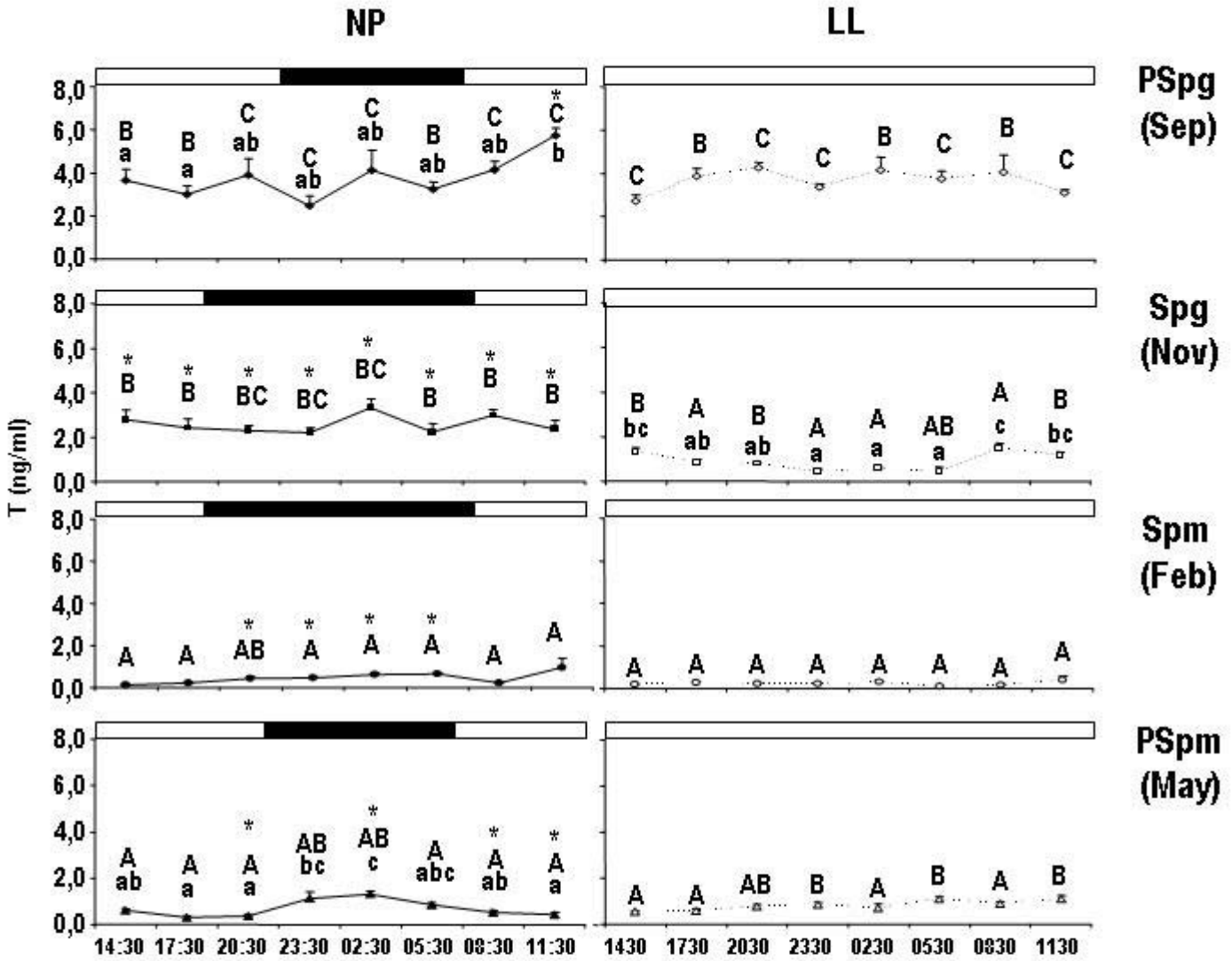
NP
LL
PSpg (Sep)
Spg (Nov)
Spm (Feb)
PSpm (May)
T (ng/ml)
8,0
6,0
4,0
2,0
0,0
14:30 17:30 20:30 23:30 02:30 05:30 08:30 11:30
1430 1730 2030 2330 0230 0530 0830 1130

Figure 5