Continuous light and melatonin: daily and seasonal variations of brain binding sites and plasma concentration during the first reproductive cycle of sea bass

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ABSTRACT

The present study reports on the daily and seasonal variations in plasma melatonin concentration, and also in optic tectum and hypothalamus melatonin binding sites, in male European sea bass maintained under natural photoperiod (NP) or continuous light (LL) from early stages of development. Samples were collected on a 24-h cycle, at four physiological phases of their first annual reproductive cycle, *i.e.*, pre-spermatogenesis, spermatogenesis, spermiation and post-spermiation. Under NP, (1) plasma melatonin levels were higher at night than during the day regardless of the year period, and the duration of the signal matched the duration of the dark phase; (2) daily variations in Kd and Bmax were found in the optic tectum, but only during spermiation, with the acrophase being 180° out of phase with the plasma melatonin variations; and (3) significant seasonal Kd and Bmax changes were seen in the hypothalamus. Under LL, (1) plasma melatonin showed no elevation during the subjective night; and (2) Kd and Bmax exhibited seasonal variations in the hypothalamus. These results led to the conclusion that long-term exposure to LL affected both plasma melatonin and receptor oscillations; particularly, LL disrupted the receptor density circadian oscillation found in the optic tectum during spermiation under NP. This oscillation appears to be important for sea bass to pursue gametogenesis until full spermiation. The persistence of both daily and seasonal variation of receptor affinity and density in the hypothalamus under LL indicates that these variations are controlled by internal circadian and circannual clocks that do not involve melatonin.

INTRODUCTION

Reproduction, like many other biological functions, is a rhythmically programmed process that allows the offspring to arrive when the probability of survival is at its highest (Foster and Kreitzman, 2004). This has been extensively studied in teleost fish, in which seasonality shows a very marked effect on the onset of reproduction, and where the photoperiod, rather than temperature or food supply, appears as the most important cue for entraining the reproduction rhythms (Bromage et al., 2001). The pineal organ, through its rhythmic melatonin production, is one of the major transducers of photoperiod signalling. In a majority of cases, the melatonin rhythm is driven by internal clocks synchronized by the photoperiod, and the hormone is considered to be the physiological link between the circadian and reproductive systems (Falcón et al., 2010).

In recent years, interest has focused on the European sea bass (*Dicentrarchus labrax*), a teleost species that is much appreciated in the Mediterranean market. The increasing amount of data that has been collected and the use of new experimental approaches have shed light on the photo-neuroendocrine control of reproduction and growth in this species. Artificial photoperiods have been proved efficient for controlling reproduction by altering spawning time in adult fish (Carrillo et al., 1993, 1995) or by inhibiting/delaying puberty in male sea bass (Zanuy et al., 2001; Begtashi et al., 2004; Rodríguez et al., 2004, 2005; Felip et al., 2008). Applications of constant long (rather than natural) photoperiods to pre-pubertal sea bass (i) induced delayed gonadal development, and (ii) enhanced the occurrence of precocious males (Rodríguez et al., 2001, 2004). Furthermore, continuous light (LL), which disrupts the plasma melatonin rhythm, also caused alterations in the rhythm of reproductive hormones and inhibited precocious puberty of males when applied from early stages of development in sea bass (Begtashi et al., 2004; Rodríguez et al., 2005; Felip et al., 2008; Bayarri et al., 2009).

As a hormonal photoperiod messenger, it is believed that melatonin mediates, at least in part, the abovementioned effects. Recent data on the expression of melatonin receptors and melatonin binding sites support this view (Falcón et al., 2007b, 2010). Melatonin acts through specific binding sites, which are believed to correspond to MT1 receptor subtypes in sea bass brain (Bayarri et al., 2004a,b; Sauzet et al., 2008). The highest density of melatonin receptors has been found in the optic tectum and hypothalamus, two brain areas whose functionality is closely related to photoperiod and reproductive responses, respectively (Bayarri et al., 2004a,b). In sea bass, no significant Kd or Bmax daily variations were found in the optic tectum or hypothalamus (Bayarri et al., 2004b) near the summer solstice, when fish are sexually quiescent (Rodríguez et al., 2001). The present study was designed in order to investigate whether melatonin binding changes in optic tectum and hypothalamus during other stages of the reproductive cycle, at some of which fish showed higher reproductive activity (Bayarri et al., 2009). For this purpose, we chose four physiological stages of the reproductive cycle, namely pre-spermatogenesis in September (PSpg), spermatogenesis in November (Spg), spermiation in February (Spm) and post-spermiation in May (PSpm).

The experimental design was aimed at testing whether the LL-induced suppression of precocity mentioned above (Felip et al., 2008; Bayarri et al., 2009) could be related to alterations in melatonin and/or melatonin receptor variations in sea bass. To that end, we compared the daily and seasonal profiles of plasma melatonin and brain melatonin binding in animals maintained under either natural photoperiod (NP) or LL conditions. Attention was focussed on the optic tectum, known to express a high density of receptors in fish (Mazurais et al., 1999), and on the hypothalamus, a major integrator of external as well as internal information important for the control of pituitary function (Okuzawa et al., 2002).

MATERIALS AND METHODS

Animals and housing

Six-month-old sea bass fingerlings (approximately 3.5g), originating from L'Ecloserie Marine (Gravelines, France), were raised at the Instituto de Acuicultura de Torre la Sal (Castellón, Spain), at 40ºN, 0ºE. Fish were distributed into four identical 2000-litre light-proof fibreglass tanks, 1-m in depth, provided with well-aerated running sea water (salinity 37‰) and subjected to simulated NP or LL conditions from the day of their arrival in May. Light in each tank was supplied by tungsten bulbs (PAR38Pro, Philips, Madrid, Spain), providing 650-700 lux at the surface of the water, with the simulated NP controlled by an electronic clock (ORBIS, Madrid, Spain), set weekly according to the geographical coordinates. Fish were maintained under a natural temperature regime throughout the experiment (11-25.5ºC), with daily oscillations within a range of 0.5ºC, and fed a commercial diet (Proaqua, Dueñas, Palencia, Spain) *ad libitum* twice a day by hand.

The handling of fish and conduct of the experimental procedures were always performed according to the national and institutional regulations and the current European Union legislation on handling experimental animals (EEC, 1986).

Experimental procedure

Every 3 hours during a 24-h cycle, 6 fish reared under NP or LL were anaesthetized with 2-phenoxyethanol (0.3 ppm), weighed and measured. The same procedure was performed at 4 physiological stages during the reproductive cycle: PSpg (Sep), Spg (Nov), Spm (Feb) and PSpm (May). Blood was collected by caudal punction with heparinized syringes; plasma was separated by centrifugation and frozen at -80ºC until the time of analysis. The optic tectum and hypothalamus were dissected out (Bayarri et al., 2004a) and

frozen separately in liquid nitrogen. During periods of darkness, the sampling was performed under a dim red light.

Melatonin analysis

Individual plasma samples were analyzed using a commercial direct RIA kit (IBL, Hamburg, Germany). Briefly, after enzymatic pre-treatment of the samples, these were incubated for 40 hours with assay buffer, 2-[^{125}I]-melatonin (^{125}IMel) and antibody. Radioactivity was counted using a γ-counter for 1 minute after addition of precipitating antiserum, centrifugation and aspiration of supernatant.

Membrane preparation

Membranes were prepared and assayed at the Laboratoire Aragó (Banyuls-sur-Mer, France), as described earlier (Bayarri et al., 2004b). Individual optic tectum samples and pooled (n = 2) hypothalamus samples were sonicated in Tris buffer (50mM / $CaCl_2$ 4mM / PMSF (phenylmethylsulphonyl fluoride, a serine protease inhibitor) 1mM, pH 7.4), using 3 pulses of 3 seconds each (Sonics, Bioblock Scientist, France). Homogenates were centrifuged at 800 g to eliminate melanin granules and thus reduce the non-specific binding (Isorna et al., 2004). The supernatants were centrifuged at 13,000 g for 10 min. The pellets thus obtained were resuspended in 700 µl of Tris-HCl buffer, and the suspension was then centrifuged again for 10 min at 13,000 g. The pellet was resuspended in Tris buffer (50mM / $CaCl_2$ 4 mM, pH 7.4), and a final protein concentration of 1 mg/ml was used in the binding assays. Membranes were manipulated at 4ºC during the process and stored at -80ºC until assayed. Proteins were determined using the Bradford assay (Bio-Rad, California, USA).

Binding assays

Saturation assays were performed on a total volume of 60 µl containing 20 µg of membrane and ^{125}IMel as radioligand at concentrations ranging from 30 to 400 pM. Unlabeled melatonin (150 µM) was used to quantify the non-specific binding. The binding of ^{125}IMel was measured in duplicate, after incubation on an orbital shaker (200 rpm) at room temperature for 90 minutes. The reaction was stopped by the addition of cold Tris buffer and immediate vacuum filtration using a harvester (Brandel tygon 48 standard 220V, Gaithersburg, MD, USA) and glass fiber filters (FPB-248L Whatman GF/C). Filters were washed three times and radioactivity was quantified in a LKB γ-counter for one minute. Specific binding, which was expressed as fmol/mg of protein, was calculated by subtracting non-specific binding from total binding.

Data analysis

Data are expressed as mean ± SEM values. In the variations of Kd and Bmax throughout the reproductive cycle, the value for any given reproductive period was calculated as the average of the eight daily sampling time points for that period. The statistical differences between groups were determined by one-way analysis of variance (ANOVA) followed by Tukey's test, with $P < 0.05$ taken as the statistically significant threshold. The significance of variations was determined by the cosinor method (Halberg et al., 1967) using chronobiology software (Cosinor, by Prof. Díez-Noguera, University of Barcelona, Spain).

RESULTS

Daily and seasonal plasma melatonin levels.

Plasma melatonin displayed day/night variations under NP, with significantly higher values during the dark phase than during the light phase for all four periods studied (ANOVA, $p < 0.05$; Fig. 1). The Cosinor method revealed a significant daily variation ($p < 0.05$) during Spg and Spm, with acrophases at 01:02 and 00:25, respectively. Under LL, melatonin showed no daily variations. However, significant differences were found during PSpg (Sep) and Spm (Feb), with the highest values occurring during the subjective day (14:30 in PSpg and 11:30 in Spm) and the first half of the subjective night in Spm (20:30 and 23:30).

Variations in melatonin concentration were seen at different times of the year under both lighting conditions, beginning to increase in November, during Spg, and reaching maximal values in February, during Spm (>500 pg/ml for NP and 300 pg/ml for LL, which are comparable to those found in the NP group the day-time (14:30, 17:30, 08:30 and 11:30)).

^{125}IMel binding sites

1. - Optic tectum

Daily variations

Significant daily Kd variations (ANOVA, $p < 0.05$) were seen under NP, but only in February (Spm) (Fig. 2A), with the highest values occurring at 14:30 (>300 pM), and the lowest values 12 hours later, *i.e.*, at 02:30 (<150 pM). No daily variations were observed at other times of the year. Under LL, a significant daily variation in Kd was found in May (PSpm), with a peak being observed at the end of the subjective day (ANOVA, $p < 0.05$; not shown). When both light regimes were compared in February (Spm), the highest Kd value observed at 14:30 under NP (318 pM) was significantly higher than that seen under LL (220

pM) at the same time of the day, whereas the lowest value at 02:30 was significantly lower (137 *vs*. 263 pM for NP and LL, respectively) (Fig. 2A).

Bmax also displayed significant daily variations in February (Spm) in fish maintained under NP conditions (ANOVA and COSINOR, $p < 0.05$; Fig. 2B). The lowest values were seen at the end of the night (05:30), and the highest values were observed in the afternoon (14:30). This daily variation was not significant under LL (Fig. 2B), although a significant rhythmic component did appear in September (PSpg; ANOVA, $p < 0.05$, data not shown). When comparing between treatments, the fish maintained under LL conditions had significantly higher Bmax values in February (Spm) at the two last time points of the night (02:30 and 05:30).

Seasonal variations

Kd, but not Bmax, exhibited significant variations under NP, with maximum values in November (Spg) and minimum values in September (PSpg) (ANOVA, $p < 0.05$; Fig. 3A,B). Under LL conditions, neither Kd nor Bmax displayed significant differences throughout the reproductive period. Compared to NP fish, LL fish showed significantly higher Kd values during September (PSpg) and Bmax values during November (Spg) (Kd: 225 pM *vs*. 156 pM; Bmax: 42 *vs*. 34 fmol/mg prot for LL and NP, respectively).

2. - Hypothalamus

Daily variations

In this tissue, Kd did not vary on a daily basis under any of the treatments tested, and no relevant differences were found between light treatments.

Bmax did not show any daily variation in hypothalamic membranes under NP. In contrast, significant daily variations in Bmax were seen under LL in May (PSpm) (ANOVA, $p < 0.05$, data not shown), showing minimum levels at 14:30 (17 fmol/mg prot) and maximum levels at 08:30 (27 fmol/mg prot).

Differences in Bmax between the two treatments were observed at certain time points throughout the reproductive cycle (data not shown).

Seasonal variations

Kd variations were significant (ANOVA, $p < 0.05$; Fig. 4A) in hypothalamic membranes under both NP and LL conditions throughout the reproductive cycle. In both cases, the highest Kd values were detected in November (Spg) (169 pM under NP and 192 pM under LL). No significant differences were found between the light treatments at any time of the year.

Bmax also exhibited significant variations among the reproductive stages studied under both light treatments (ANOVA, $p < 0.05$; Fig. 4B). Under NP conditions, the highest values were seen during May (PSpm) (31 fmol/mg prot), while under LL, maximum values occurred in November (Spg) and February (Spm). When both light treatments were compared, Bmax differences were statistically significant for all the reproductive periods except PSpg (September), with higher values under LL conditions during November (Spg) and February (Spm), but lower during May (PSpm).

DISCUSSION

These results improve our understanding of the melatonin system in sea bass. Aside from this, they provide interesting new information concerning brain melatonin binding sites in relation to the annual reproduction cycle. Not only do they extend previous data indicating that melatonin binding is under circadian control, they also complete the picture by demonstrating that a circadian clock is present in sea bass brain, and that this clock drives an annual rhythm of melatonin binding.

Plasma melatonin profile

Under NP, the daily variations in plasma melatonin showed high levels at night and low levels during the day, thus matching the prevailing photoperiod. This is the usual pattern observed in teleost fish (Falcón, 1999; Falcón et al., 2007a,b, 2010). Under a natural light/dark cycle, melatonin profiles varied from one time of the year to another. The highest amplitudes were seen in November and February, coinciding with Spg and Spm, respectively. In September (PSpg) and May (PSpm), nocturnal levels were lower. Annual rhythms of nocturnal melatonin production have been reported for a number of fish species, including sea bass (García-Allegue et al., 2001), but contrary to what we have observed, these rhythms tend to be of low amplitude/long duration during the winter, and of high amplitude/short duration in the summer (Falcón, 1999). These discrepancies could be due to the differences in the experimental protocols used (months analyzed, strains within the species, ambient light and temperature, etc.).

Light is known to inhibit pineal melatonin production through the inhibition of arylalkylamine N-acetyltransferase2 (AANAT2) activity (Falcón et al., 2007b). It is therefore not surprising that the LL treatment applied here disrupted the plasma melatonin variation. In

fish, many experiments have been performed under constant illumination or darkness. A circadian melatonin rhythm has been shown to persist *in vitro* under constant darkness in several non-salmonid species (Bolliet et al., 1994, 1996a,b), including sea bass (Bayarri et al., 2004c). *In vivo*, however, studies performed on sea bass under DD conditions showed high levels of plasma melatonin without any significant variation after day 2 following the onset of the treatment, most probably as a result of the uncoupling of multioscillatory units (Iigo et al., 1997). In the present study, it might be possible that the daily variations found under LL conditions during September (PSpg) and February (Spm) reflect the presence of some active circadian component controlling AANAT2, as (i) the sea bass pineal gland contains a circadian oscillator (Bayarri et al., 2004c), (ii) in contrast to AANAT2 protein and AANAT2 enzyme activity, AANAT2 mRNA is not light sensitive (Coon et al., 1999), (iii) *Aanat2* gene expression is controlled by the clock machinery (Appelbaum et al., 2006), and (iv) a minor part of the pineal AANAT2 protein pool is photo-stable (Falcón et al., 2010).

Daily and seasonal variations in melatonin binding

Many studies have described the location and distribution of melatonin binding sites in fish brains; however, few of them report on their daily variations, and their results have been contradictory. Furthermore, none have investigated variations throughout the reproductive cycle. Here we provide evidence showing that Kd and Bmax exhibit daily variations in the optic tectum at some times during the year, whereas no such variations may be detected in the hypothalamus. The specific differences related to tissue and/or time-of-year indicates the existence of different mechanisms regulating the binding sites, most probably a multifactorial process. One of these regulators could be melatonin itself, as is the case in mammals (Guerrero et al., 2000). This would explain the differences observed between NP and LL fish in terms of Bmax variations. The observation that under NP conditions, Bmax reached its

minimum level when melatonin was high (*i.e.*, at night) agrees with data other researchers have obtained for pike, goldfish and seabream (Gaildrat et al., 1998; Iigo et al., 2003; Falcón et al., 1996), and with the idea that melatonin could contribute to down regulate its own receptors. However, this is not a general rule, because plasma melatonin, Kd and Bmax are all in phase in masu salmon maintained under NP conditions (Amano et al., 2003a). More studies are needed to elucidate which factors, others than melatonin, are involved in the control of melatonin binding. Nervous inputs from both the retina and the pineal gland are good candidates, since the areas studied here are targeted by both pinealfugal and retinofugal innervations (Ekström and Meissl, 1997). The variations observed under LL also indicate that a circadian control of melatonin binding is at work, as seen in pike (Gaildrat et al., 1998).

One of the new findings of this study is the existence of variations among four reproductive stages, particularly significant in the hypothalamus, in melatonin binding under both NP and LL conditions. This further supports the notion that the melatonin system is involved in annual time measurement, rather than being merely the result of seasonal variations in plasma melatonin levels. Moreover, variations persisted under constant light, which provides strong support for the idea that the brain of sea bass contains a circannual clock, and that melatonin binding sites are one of its outputs.

In summary, it appears that both melatonin production and melatonin binding sites are controlled by circadian and circannual clocks in the brain of sea bass, and that the effects of melatonin depend not only on its rhythmic production, but also on the rhythmic expression of its binding sites. Where the clocks that control the circadian and circannual variations in melatonin binding sites are located, and how they interact with the clocks that control melatonin production are among the questions to be answered in the future.

Variations in melatonin binding and seasonal reproduction

Seasonal reproduction is a well known feature of living organisms. In fish, photoperiod manipulation may advance or delay reproduction. It has long been suspected that melatonin, as a hormonal signal of photoperiod and season, could play a role in the effects of photoperiod on annual reproduction (Falcón et al. 2007b, 2010). In fact, melatonin has been demonstrated to modulate LH secretion in Atlantic croaker by *in vivo* and *in vitro* studies (Khan and Thomas, 1996)

Here we investigated the characteristics of melatonin binding in sea bass optic tectum and hypothalamus and found differences between both tissues throughout the reproductive cycle. These differences most certainly reflect differences in their functions: the optic tectum is tightly linked to integration of light information (Mazurais et al., 1999), which makes it sensitive to circadian changes, while the hypothalamus is more closely related to reproduction (González-Martínez et al., 2002), and therefore to circannual rhythms.

The optic tectum of sea bass showed, under NP, daily variations of both Kd and Bmax in February, during spermatogenesis, one of the periods when reproductive activity is at its peak, supported by the highest levels of expression of pituitary LH and FSH β sub-units (Mateos et al., 2003) and plasma levels of LH and 11-ketotestosterone (Rodríguez et al., 2005; Felip et al., 2008). However, no daily variations were reported in the optic tectum near the summer solstice (Bayarri et al., 2004a,b), a quiescent period for sea bass reproduction. Therefore, the daily fluctuation of Kd and Bmax observed during or before the reproductive stage suggests that this rhythmic behaviour could be very important for the hormonal hierarchy regulation of maturation, as occurs in mammals (Ojeda et al., 2006). In the present experiment, under LL, the period showing significant daily Bmax variations in optic tectum was pre-spermatogenesis (September) instead of spermiation (February). The fact that a lower number of precocious individuals was detected among fish maintained under LL compared to those maintained under NP (Begtashi et al., 2004; Felip et al., 2008; Bayarri et al., 2009)

supports our hypothesis that the daily variations in melatonin receptor density are important for the reproduction process to be completed during the spermiation period. On the other hand, Amano et al. (2006) failed to find any daily Kd or Bmax rhythms under LL or DD conditions for masu salmon, and Iigo et al. (2003) found a significant rhythm only for Bmax in goldfish under DD conditions, with fish being sampled after a few days of constant conditions of illumination in both cases. As suggested above, the long period during which our fish were subjected to LL may have drastically altered their circadian oscillators, changing the affinity and density of melatonin receptors throughout the 24-h cycle.

Regarding variations on melatonin binding during the reproductive stages, only the hypothalamus showed, under NP, significant fluctuation on receptor density, with lowest values during September (PSpg) and highest during May (PSpm). To date, there are very few studies available regarding the annual rhythms of melatonin binding sites in fish. Nevertheless, Amano et al. (2003b) described maturational differences of melatonin binding sites in the whole brain of masu salmon. These authors found a higher density in July in precocious males, which have initiated testicular development, than in October, when fish had spermiated. No maturational differences were evident in the Kd. In the present study, density was highest during May (PSpm), a period with no sexual activity, and Kd was the parameter which showed the highest values during November (Spg).. Considering the well-known differences between the circadian system of salmonid and non-salmonid species (including the presence in the latter, but not in the former, of endogenous intrapineal oscillators), it was not surprising to also find species-dependent differences in the sensitivity of melatonin receptors. However, what appears to be entirely clear is that the maturational status of fish correlates with the daily and seasonal variation characteristics of melatonin binding sites. The effect of treatment with LL throughout the reproductive cycle was observed especially in the hypothalamus, where the Bmax of fish maintained under LL showed significantly higher

values during November (Spg) and February (Spm), but lower values during May (PSpm), as compared to fish kept under NP conditions. The physiological meaning of these differences may need further research, however.

In summary, male European sea bass reared under NP conditions exhibited daily variations in plasma melatonin, with nocturnal elevations that were abolished in fish maintained under LL conditions from early stages of development. This prolonged submission to LL also affected melatonin receptor affinity and density variations. In the optic tectum, a marked role of daily variations of Bmax during the period of maximal sexual activity is suggested, since there was no significant oscillation present during the rest of the reproductive periods or during the same period in fish under LL conditions. The latter group of fish showed a lower percentage of precocity, as seen in previous studies. The presence of seasonal, but not daily, changes in Kd and Bmax in the hypothalamus is thought to be due to the role this tissue plays in the rhythmic control of reproduction. Moreover, the persistence of reproductive stage-related variations of affinity and density in the hypothalamus while under LL conditions may indicate that these variations are controlled by internal clocks, which may not involve melatonin.

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FIGURE LEGENDS

Figure 1. Daily variations in plasma melatonin throughout a 24-h cycle during pre-spermatogenesis (PSpg), spermatogenesis (Spg), spermiation (Spm) and post-spawning (PSpm), in fish maintained under NP (black) and LL (white). Horizontal white and black bars represent day and night, respectively. Different lowercase and capital letters indicate significant variations (ANOVA, Tukey's test, $P < 0.05$) among different time points throughout the 24-hour period and among the four different phases of the reproductive cycle, respectively. Differences between treatments at each time point are represented by an asterisk.

Figure 2. Kd (A) and Bmax (B) daily variations of melatonin binding in optic tectum membranes under NP (black) and LL (white) during Spm. Different letters indicate statistically significant variations, only present under NP (ANOVA, Tukey's test, $p < 0,05$). Differences between light treatments at each sampling point are represented by an asterisk. The significance of the daily Bmax variation was also demonstrated by cosinor analysis ($p < 0.05$). Horizontal bars represent the illumination regime (light period in white and dark period in black).

Figure 3. Kd (A) and Bmax (B) changes of melatonin binding throughout the reproductive cycle in the optic tectum for fish maintained under NP (black) and LL (white) conditions. Different lowercase and capital letters indicate significant variations (ANOVA, Tukey's test, $p < 0.05$) among the four study periods for fish under NP and LL, respectively. Differences between treatments at each sampling period are represented by an asterisk.

534 Figure 4. Kd (A) and Bmax (B) variations of melatonin binding throughout the reproductive
535 cycle in the hypothalamus for fish maintained under NP (black) and LL (white) conditions.
536 Different lowercase and capital letters indicate significant variations (ANOVA, Tukey's test,
537 $p < 0.05$) among the four study periods for fish under NP and LL, respectively. Differences
538 between treatments at each sampling period are represented by an asterisk.

Figure 1

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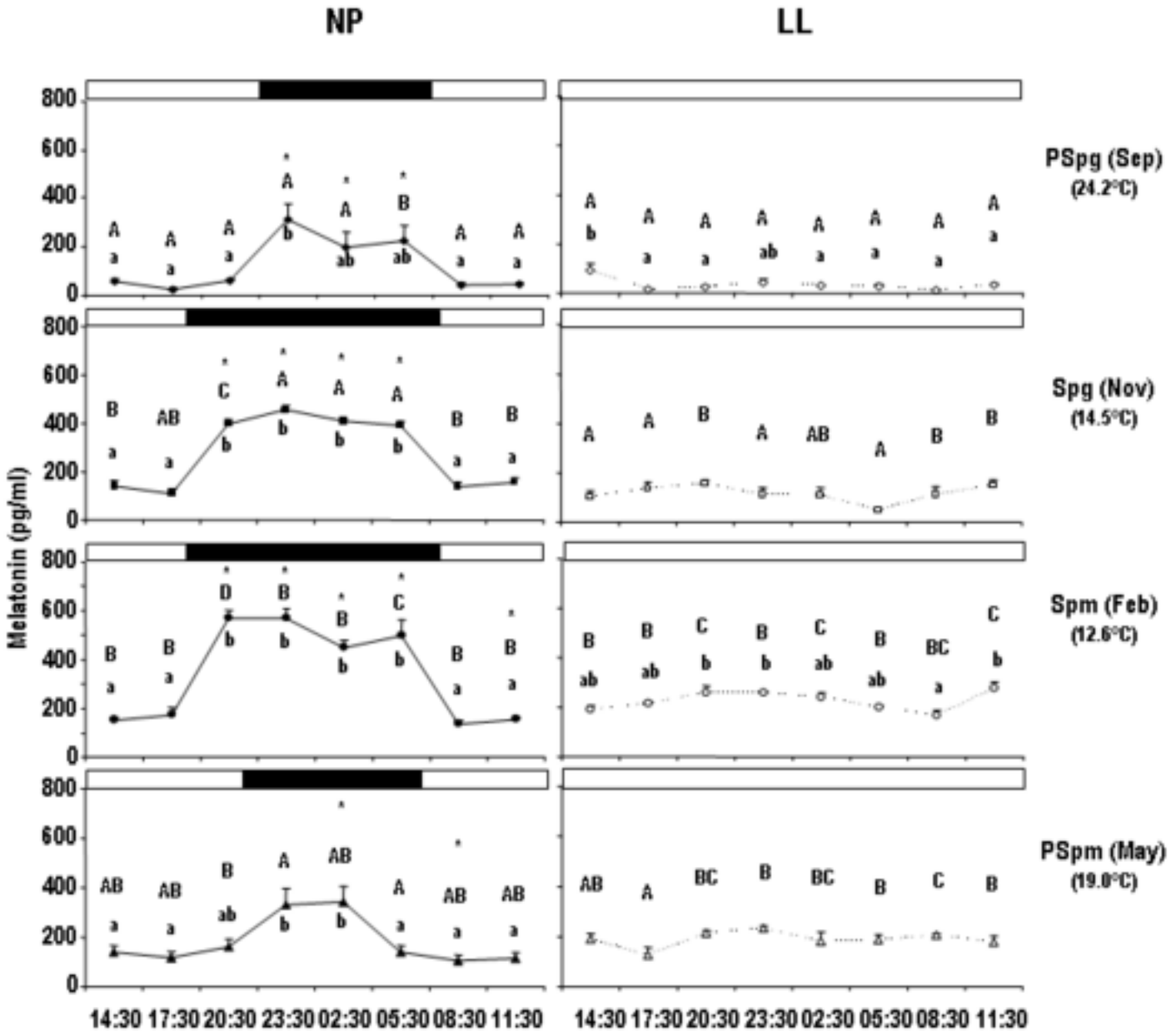

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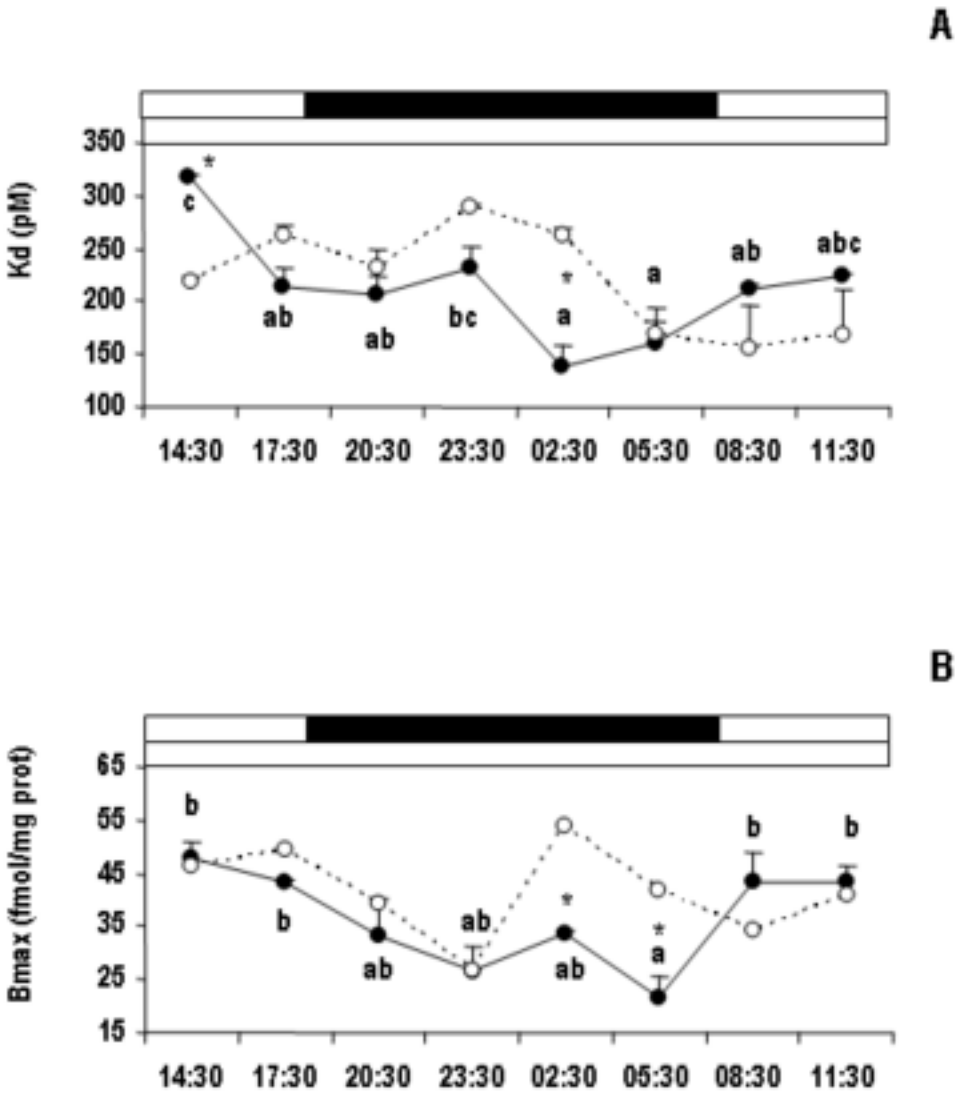

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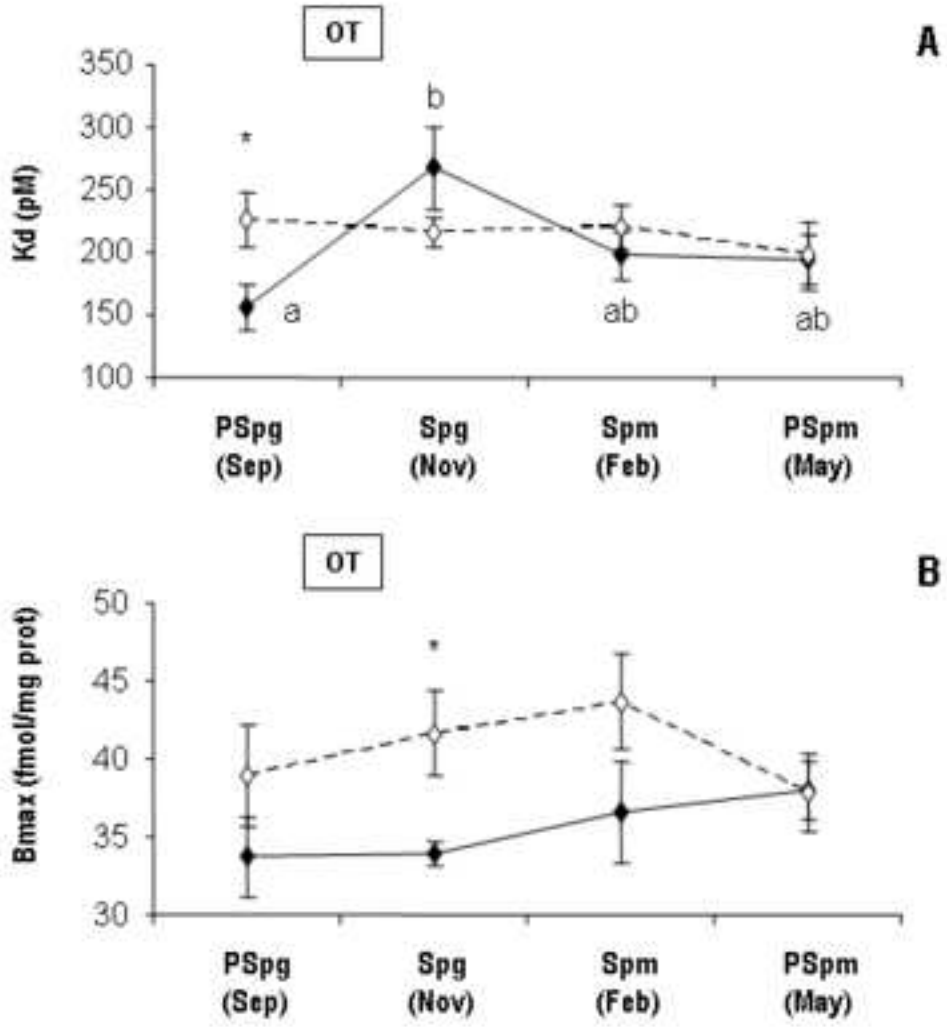

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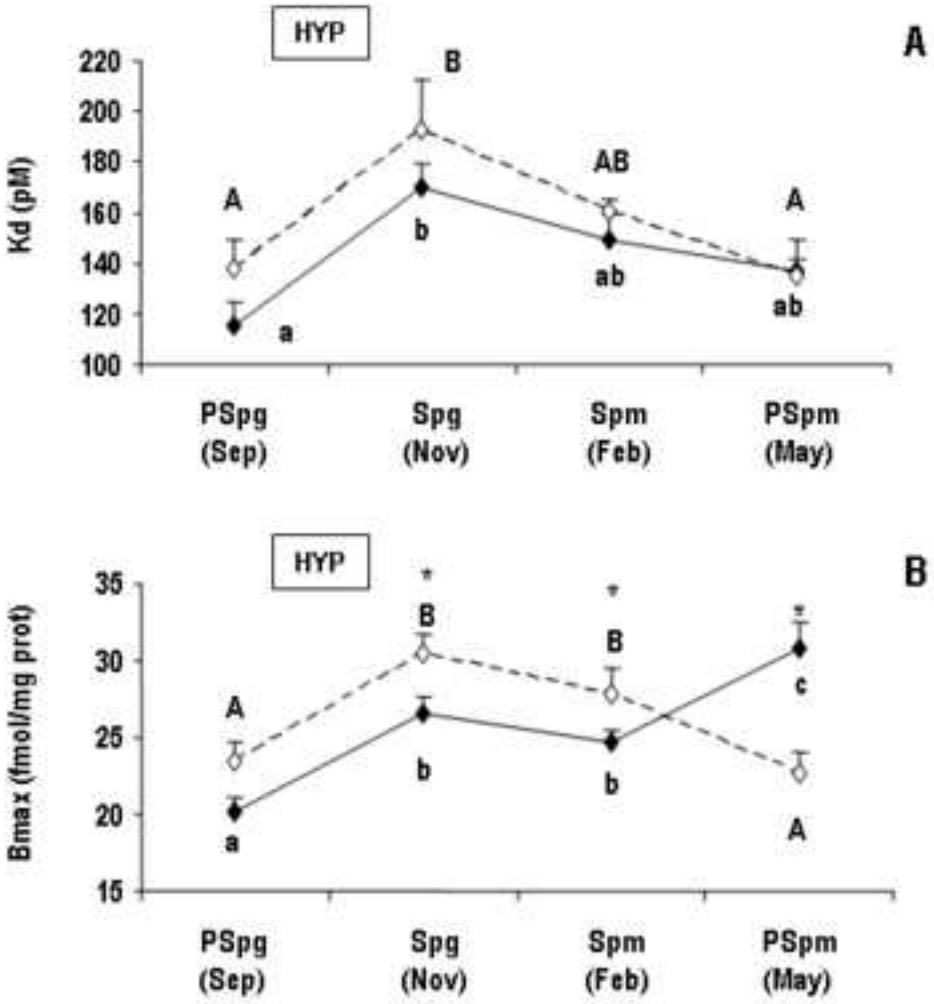

Figure 4