General and Comparative Endocrinology 169 (2010) 58–64

Contents lists available at ScienceDirect

General and Comparative Endocrinology

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

# 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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ARTICLE INFO

*Article history:*
Received 24 March 2010
Revised 22 June 2010
Accepted 21 July 2010
Available online 27 July 2010

*Keywords:*
Photoperiod manipulation
*Dicentrarchus labrax*
Reproduction
Variations
Melatonin receptors
Kd
Bmax

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.



## 1. 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 signaling. 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 above mentioned 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

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doi:10.1016/j.ygcen.2010.07.007

(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 focused 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).

## 2. Materials and methods

### 2.1. Animals and housing

Six-month-old sea bass fingerlings (approximately 3.5 g), originating from L'Ecloserie Marine (Gravelines, France), were raised at the Instituto de Acuicultura de Torre la Sal (Castellón, Spain), at 40°N, 0°E. Fish were distributed into four identical 2000-l light-proof fiberglass 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 (11–25.5 °C), with daily oscillations within a range of 0.5 °C, 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).

### 2.2. Experimental procedure

Every 3 h 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 four physiological stages during the reproductive cycle: PSpg (September), Spg (November), Spm (February) and PSpm (May). Blood was collected by caudal punction with heparinized syringes; plasma was separated by centrifugation and frozen at −80 °C 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.

### 2.3. 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 h with assay buffer, 2-[$^{125}$I]-melatonin ($^{125}$IMel) and antibody. Radioactivity was counted using a γ-counter for 1 min after addition of precipitating antiserum, centrifugation and aspiration of supernatant.

### 2.4. 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 (50 mM/$CaCl_2$ 4 mM/PMSF (phenylmethylsulphonyl fluoride, a serine protease inhibitor) 1 mM, pH 7.4), using three pulses of 3 s 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 (50 mM/$CaCl_2$ 4 mM, pH 7.4), and a final protein concentration of 1 mg/ml was used in the binding assays. Membranes were manipulated at 4 °C during the process and stored at −80 °C until assayed. Proteins were determined using the Bradford assay (Bio-Rad, California, USA).

### 2.5. 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 min. The reaction was stopped by the addition of cold Tris buffer and immediate vacuum filtration using a harvester (Brandel tygon 48 standard 220 V, 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 1 min. Specific binding, which was expressed as fmol/mg of protein, was calculated by subtracting non-specific binding from total binding.

### 2.6. 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).

## 3. Results

### 3.1. 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, $p < 0.05$;