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Daily locomotor activity and melatonin rhythms in Senegal sole (*Solea senegalensis*)

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Abstract

The daily locomotor and melatonin rhythms of the Senegal sole, a benthonic species of increasing interest in aquaculture, are still unknown, despite the fact that such knowledge is of prime importance for optimising its production. The aim of the present research was therefore to investigate the daily rhythms of locomotor activity and melatonin in the Senegal sole. For this purpose, the individual locomotor activity rhythms of fish were registered using a photocell. Plasma and ocular melatonin rhythms were studied in animals reared in circular tanks placed in earth under an LD 12:12 light regime and 16–18 °C temperature range (spring equinox). Blood and eye samples were taken every 3 h during a complete 24-h cycle. The impact of a light pulse in the middle of the dark period (MD) on plasma melatonin was also studied. Locomotor activity was mainly nocturnal, with 84.3% of the total activity occurring during darkness. The levels of plasma melatonin were higher at night (55 pg/ml) than during the day (2 pg/ml), while ocular melatonin levels appeared to be arrhythmic. Both weight and melatonin content were found to be significantly higher in the left eye in relation to the right eye. A light pulse in MD provoked a significant decrease in plasma melatonin levels. In summary, photoperiod is a key factor in synchronising locomotor activity and melatonin rhythms in the Senegal sole, whose nocturnal habits should be taken into account for their rearing by aquaculture.

Keywords: Melatonin; Locomotor activity rhythms; Daily rhythms; *Solea senegalensis*; Light pulse

1. Introduction

Melatonin, a hormone synthesised by the pineal organ at high levels during the dark period, provides animals with information concerning the time of day and also the time of year [1]. Because melatonin and the pineal organ are thought to be involved in the integration of photoperiod information and the coordination of internal rhythmic functions [2], they are both considered to play an important role in the circadian system.

Photoperiod seems to be the most powerful factor in entraining animals' rhythms and, in all species investigated to date, plasma melatonin levels are high during the night and low during the day. The retina also produces melatonin, but rhythms are not so consistent, and they vary depending on the species [3]. In European sea bass, the retina synthesises melatonin during the day [4,5].

The effects of a light pulse given in the middle of the dark phase have been studied in several fish species, including the European sea bass [6] and the brook trout [7]. In both studies, melatonin production by the pineal organ measured in plasma was seen to be directly proportional to light intensity.

The pleuronectiform Senegal sole (*Solea senegalensis*), a species with a great commercial interest, is commonly cultured off in southern Portuguese and Spanish coasts [8]. In the past years, many studies carried out in different species of sole have increased our knowledge on their sexuality, distribution, embryonic development and oogenesis [9–12], age, growth and population [13], spawning and nursery conditions [14]. Many of the above events occur rhythmically, and are synchronised to light–dark cycles, probably by means of melatonin [15]. Recent neuroanatomical and morphofunctional studies performed in Senegal sole have also

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provided information on brain cytoarchitecture and distribution of gonadotrophin-releasing hormone and serotonin-immunoreactive systems, revealing that the characteristic body asymmetry of adult flatfishes is also reflected in rostral forebrain asymmetry [16–18].

Furthermore, melatonin studies have already been carried out in several pleuronectiform fish species, such as the flounder [19] or turbot [20], in which plasma melatonin levels during the photophase and scotophase were described. However, the daily melatonin and locomotor activity rhythms of the Senegal sole have not been reported yet. In addition, ocular melatonin rhythms and putative differences in the melatonin content of left and right eyes (which become interesting because of pleuronectiform asymmetry) have still not been described. This study was therefore directed to elucidate the existence of behavioural (locomotor activity) and endocrine (plasma and ocular melatonin) daily rhythms of the Senegal sole.

2. Materials and methods

2.1. Animals and housing

For this study, Senegal sole specimens with body weight around 300 g were used. Animals came from two different fish farms: PROMAN (Carchuna, Granada, Spain), and CICEM El Toruño (Puerto de Santa María, Cádiz, Spain). In both cases, the animals were reared under natural conditions of photoperiod and temperature.

2.2. Experiment 1: recording of locomotor activity rhythms

The objective of this experiment was to study the daily rhythms in the locomotor activity of sole. Five fish were transferred to our indoor chronolab at Murcia University when natural photoperiod was LD 12:12, and kept individually in 60l aquaria, the sides of which were covered with black paper, in order to prevent fish from being disturbed. The aquaria were filled with artificial seawater (Premium Sera, Toronto, Canada) which was well aerated and continuously recycled through skimmer, biological and UV filters. Environmental conditions (photoperiod, temperature and salinity) were maintained constant at LD 12:12, 23 °C and 30 ‰, respectively. Illumination was supplied by a white fluorescent tube (GRO-LUX, 30 W, Germany), directed downwards and providing 300 lx at the

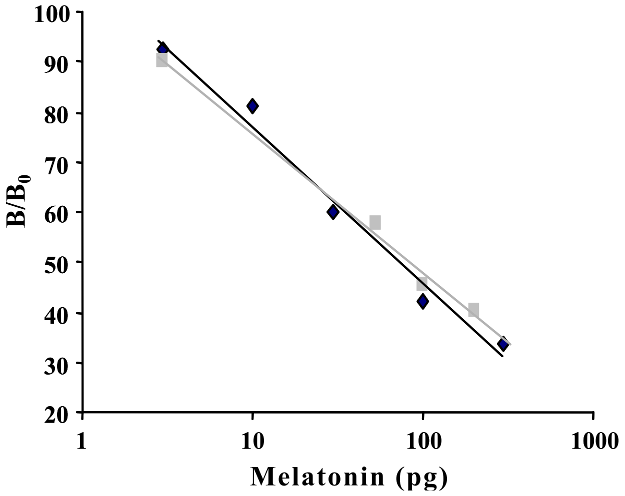

Fig. 1. Validation of the ELISA for sole plasma. The parallelism between standard curve (black points) and melatonin-added pooled plasma samples (grey points) shows that the assay is valid for sole.

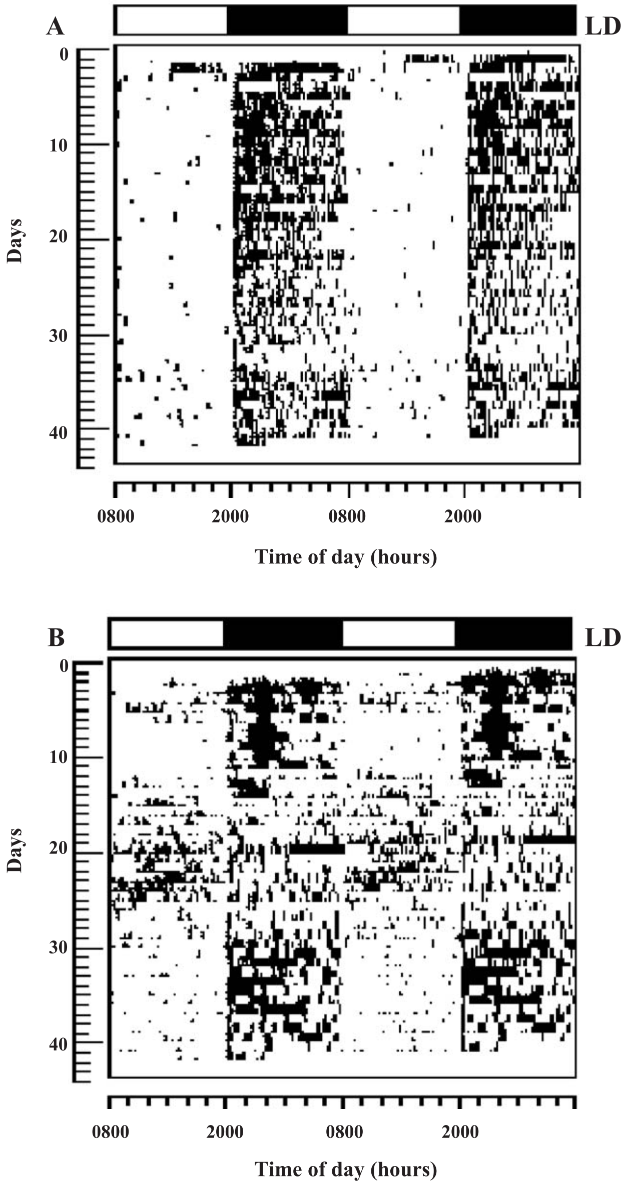

Fig. 2. Locomotor activity records of two soles (A and B) under constant laboratory conditions of temperature (23 °C), photoperiod (LD 12:12) and salinity (30 ‰). Horizontal bar at the top of the actogram represents day (in white) and night (in black) hours of two consecutive days.

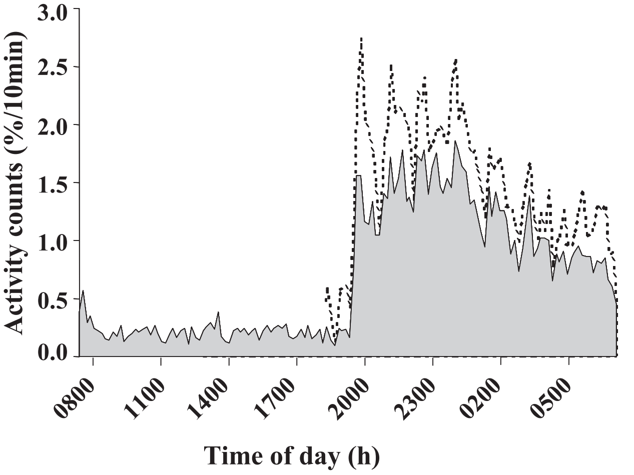

Fig. 3. Average (thick line) + S.D. (dotted line) percentage of light- and dark-time locomotor activity in sole kept under laboratory conditions.

water surface. Animals were fed a commercial diet (Proaqua, Palencia, Spain) by hand once a day at nonscheduled times. A photoswitch (Omron, model E3S-AD62, Japan) connected to a computer was attached to the front of each aquarium, approximately 2.5 cm from the bottom. The photoswitch continuously emitted an infrared light beam, and every interruption caused by fish was registered in a computer. The activity rhythms of fish were recorded during 44 days.

2.3. Experiment 2: plasma and ocular daily melatonin rhythms

This study was designed to describe the daily melatonin rhythm in plasma and eye of sole, which were reared in circular earth tanks, under natural conditions. The experiment took place during the spring equinox (March 20–21), when the natural photoperiod was 12:12 and water temperature was 16.6 °C. For the experiment, fish ($n = 48$) were taken from a tank every 3 h during a complete 24-h cycle ($n = 6$ in each hour group). The fish were anaesthetised on ice and weighed. Blood samples (2–3 ml) were taken by caudal puncture with heparinised syringes, and were kept on ice until centrifugation at 3000 rpm for 15 min at 4 °C. Plasma was then separated and frozen at − 80 °C. Both eyes were dissected out and frozen immediately in solid CO_2. Fish were killed by decapitation. Sampling during the dark was performed under a dim red light, covering the fish head with aluminium foil.

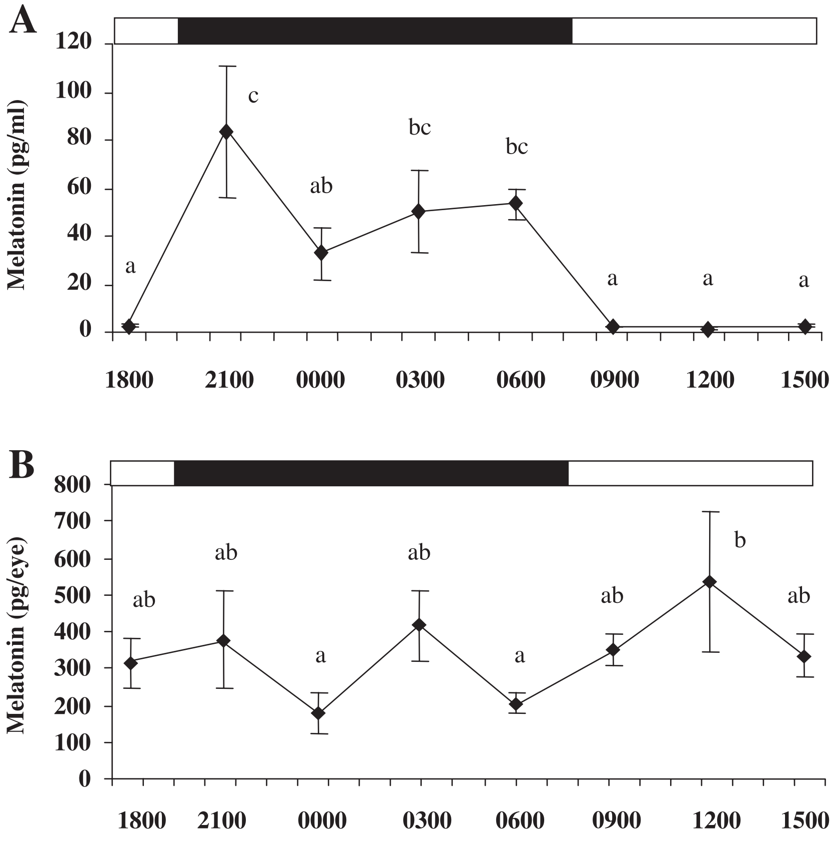

Fig. 4. Daily rhythm of plasma (A) and ocular (B) melatonin of sole. Plasma melatonin rhythm showed low values during the day and higher values during the night. The ocular rhythm of melatonin in the right eye seemed arrhythmic. Black and white bars at the top of the figures represent night and day hours, respectively. The different letters indicate significant ($P < .05$) differences between sampling points.

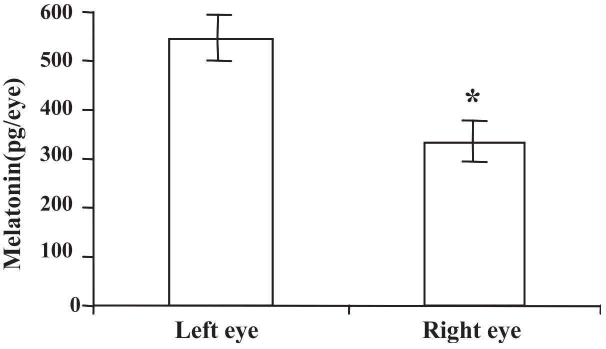

Fig. 5. Differences in melatonin contents of the right and left eye of sole, pointing to the significantly higher concentration in the left eye (* ANOVA, $P<.05$).

2.4. Experiment 3: response to a light pulse at midnight in ocular and plasma melatonin

The aim of this experiment was to investigate the response in the plasma melatonin of sole to a light pulse provided in the middle of the dark period (MD). Six animals were reared in a 400-l tank in the laboratory, filled with artificial sea water (Premium Sera), under constant conditions of temperature (25 °C), photoperiod (LD 12:12) and salinity (30 ‰). Fish received a 1-h light pulse in MD (GRO-LUX, 30 W), and immediately afterwards, 0.8- to 1-ml blood samples were taken. Fish were allowed to recover during 10 days, after which control blood samples were taken in the middle of the light period (ML). After the same recovery period, control MD blood samples were also extracted. Samples were treated in the same way as in Experiment 1.

2.5. Melatonin analysis

Eye samples were thawed, individually weighted and homogenised, discarding cornea and crystalline lens, at 4 °C in 1-ml phosphate-buffered saline. Plasma and homogenised eyecup samples were extracted and purified using C_{18} phase extraction columns (Waters, Massachusetts, USA) in the centrifuge, after which a commercial ELISA was performed, as described by Bayarri et al. [6]. Homogenised eyecup samples were diluted four times with commercial buffer before assay.

2.6. Data analysis

Data are expressed as mean ± S.E.M. values. Excel and SPSS were used for data analysis. Some plasma and ocular outlier melatonin values were discarded for being below or above twice the mean plus the standard deviation. The statistical differences between mean melatonin levels were determined by one-way analysis of variance (ANOVA) followed by Duncan's test, with $P<.05$ taken as the statistically significant threshold. A paired t test was performed to determine significant differences between weight of left and right eyes. Activity data were collected regularly from the data acquisition microcomputer, and analysed using chronobiological software ("El Temps," by Prof. Díez-Noguera, University of Barcelona, Spain). For convenient visualization of the activity records, data were double-plotted on a 48-h time scale. The average daily waveform was calculated for all the fish.

To validate the ELISA for sole samples, certain known amounts of melatonin were added to melatonin-free pooled plasma samples. Fig. 1 shows parallelism between the standard curve and melatonin-added samples, demonstrating that the assay is perfectly valid for sole plasma samples.

3. Results

3.1. Experiment 1: locomotor activity rhythms

Actograms representing locomotor activity rhythms of the Senegal sole are shown in Fig. 2. Number of records

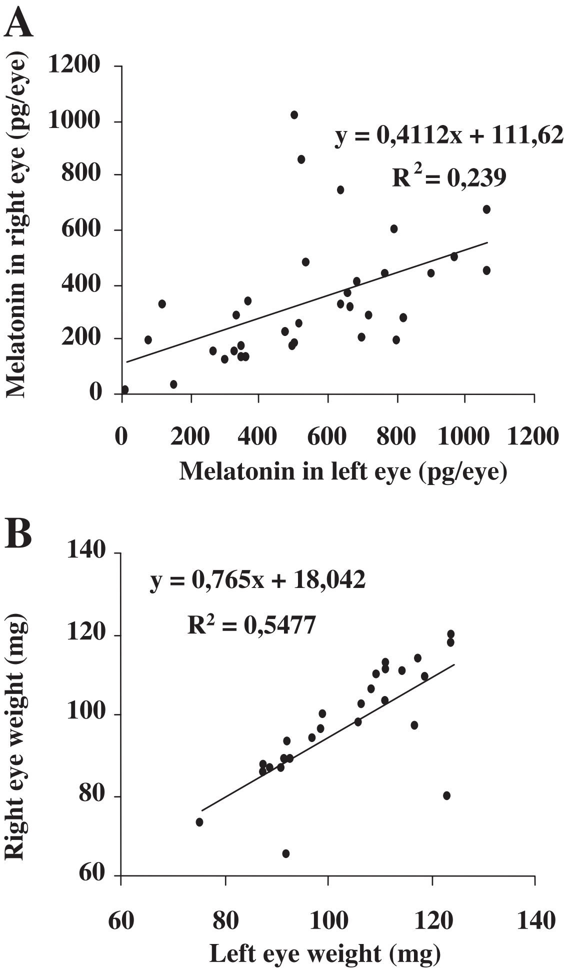

Fig. 6. Correlation between melatonin concentrations (A) and weight (B) of right and left eye of sole. The figures show that the left eye has a higher concentration of melatonin, and also a higher, but not proportional weight.

registered every 10 min determined the height of daily activity line in the actogram.

Under LD 12:12 conditions in the laboratory, the sole locomotor activity rhythms recorded at the bottom of the aquaria appeared to be strongly entrained by the photoperiod. These rhythms showed a typical nocturnal pattern (Fig. 2A and B), although some patterns were occasionally unclear. The average percentage of nocturnal activity in the daily activity counts was 84.3% (Fig. 3), being more intense during the first half of the dark period.

3.2. Experiment 2: daily plasma and ocular melatonin rhythms

Plasma melatonin (Fig. 4A) showed low levels during the daytime (1800, 0900, 1200 and 1500 h) and high levels during the night (2100, 0000, 0300 and 0600 h). Curiously, melatonin peaked at 2100 h (first dark sampling time) but fell in a significant manner at 0000 h, in which plasma melatonin levels did not differ statistically from daytime values (Fig. 4A).

The melatonin content in the eye did not seem to be rhythmic because differences in melatonin levels were inconsistent between light and dark hours (Fig. 4B). Nevertheless, statistically significant differences were observed between middark (0000 h) and midday (1200 h) values, the former being lower than the latter ones.

Furthermore, melatonin content from a pool of 48 sole in Experiment 2 appeared to be higher in the left eye, as Figs. 5 and 6A show. The weight of the left eye was also greater, although in a nonproportional relationship (Fig. 6B).

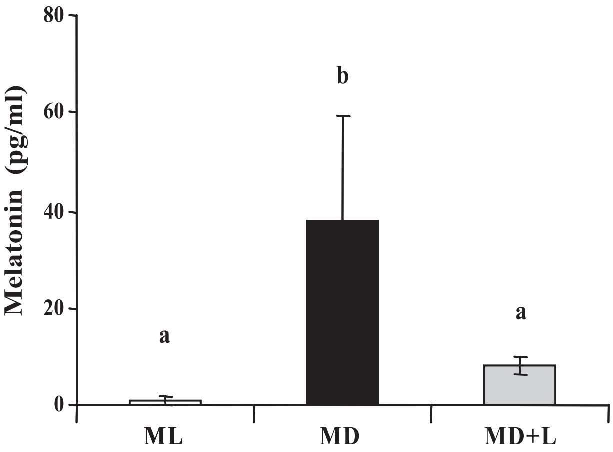

Fig. 7. Plasma response to a 1-h light pulse in MD. The melatonin levels of ML are represented by white columns and MD levels by black columns. The grey columns represent values after a light pulse given at MD (MD + light). The different letters indicate significant (P<.05) differences between groups.

3.3. Experiment 3: impact of a light pulse at MD on plasma melatonin rhythms

In this experiment, the response of a light pulse given in MD on plasma melatonin concentration was studied. The plasma melatonin levels of control groups were low at ML and high at MD. The light pulse given at MD significantly decreased the plasma melatonin concentration to values similar to those obtained at ML (Fig. 7).

4. Discussion

Our results provide the first information on melatonin and activity rhythms in the Senegal sole, a species of growing interest in aquaculture but which has not been widely investigated. The sole appeared to be strongly influenced by photoperiod, as seen from their clearly nocturnal activity pattern, and their daily plasma melatonin rhythm, similar to the typical melatonin profile, with low values during the day and high values during the night. The ocular melatonin rhythm, however, did not reflect this marked influence of photoperiod because it displayed a very irregular pattern, with inconsistent melatonin levels during light and dark hours. In addition, the Senegal sole responded as expected to a light pulse at MD, showing a decrease in its plasma melatonin concentration.

Locomotor activity rhythms have been studied in many vertebrate species, in which rhythmicity, like many other circadian functions, is usually entrained by light–dark cycles [21]. In laboratory conditions with an artificial photoperiod of 12:12, the Senegal sole was clearly nocturnal in most cases, although a few fish did not show well-defined activity rhythms. These results resemble those obtained for catfish [22,23] and tench [24], which have been described as nocturnal species. In sole, the activity was maximal in the first part of the dark period, progressively decreasing during the night. A similar behavioural pattern has been described for the self-feeding activity of the same species (Boluda-Navarro et al., in preparation), when an anticipatory feeding activity was detected in sole reared in the laboratory.

The daily plasma melatonin rhythms recorded in the Senegal sole are in accordance with those of other fish species investigated, because melatonin levels were low during the daytime and high during the nighttime, the high levels lasting as long as the dark period [15]. During the spring equinox, when this study took place, under a natural photoperiod of 12:12, half of the sampling times were under darkness and half under light conditions. However, our results showed an acute melatonin peak at the first dark sampling time (2100 h), followed by a decrease (0000 h), with significant differences between both values, although the levels still remained higher than those obtained during the day. This profile is not common in other species, although in Atlantic cod, circulating melatonin rhythm displays a single peak near the end of the dark period [25].

Nevertheless, in other teleost species, such as Atlantic salmon [26] and European sea bass [27], any increase in melatonin levels reflects the duration of the dark period. It should be noted that the peak of plasma melatonin found in sole at the beginning of the dark phase coincided with the night period exhibiting the highest locomotor activity. Interestingly, and according to the melatonin profiles, the sole seems to exhibit a dampening of activity throughout the night, rather than an abrupt cessation with lights on. Further research, however, is required to ascertain whether there is a correlation between these findings.

In the Senegal sole eye, melatonin rhythms appeared to be inconsistent with the photoperiod, and there were no significant differences between the light and dark phases. These results differ to those obtained in other teleost fish, like the European sea bass or the goldfish [5,28], both of which showed great day–night differences in melatonin levels. However, as it occurred in the European sea bass, middark (0000 h) values appeared lower than those of midday (1200 h) [28]. In species such as sea bass, the time of year may affect ocular melatonin, so that in certain seasons, daily rhythms may disappear for still unknown reasons [27]. Seasons might also affect ocular melatonin rhythms in the Senegal sole, and further studies should be performed to confirm this hypothesis.

This species, like the rest of the fish belonging to the Pleuronectiformes order, displays asymmetry during adult life, although they are externally symmetric until metamorphosis [29–31]. The left eye migrates to the right side, which faces upward [31]. This asymmetry implies differences between the left and right brain hemispheres in shape and size, such as in the winter flounder [32] and in turbot [33], the right hemisphere of the former being bigger, and smaller in the latter. In the Senegal sole, we observed that the left eye was significantly bigger than the right eye, and that the melatonin content was accordingly higher in the former. Nevertheless, the relationship between size and melatonin content did not seem to be proportional because slopes in the regression lines (Fig. 6A and B) were not parallel. No references to differences in size of the right and left optic lobes, or to one eye projecting more than the other (thus, being more exposed to light) have been mentioned for the Senegal sole, and thus, further research would be valuable in this respect. Nevertheless, the existence of structural asymmetries in the epithalamus of several teleost species and in dorsal/ventral crossing at the optic chiasm of flatfishes [34] has been described. Furthermore, NADPH diaphorase activity in the optic tectum of metamorphic turbots [35] and [^{14}C]2-deoxyglucose uptake by neurons of the octavolateralis complexes of adult flatfishes [36] also exhibit a distinct bilateral asymmetry. A clear asymmetry in the number and location of catecholaminergic perikarya and fibers was also observed in the olfactory bulbs of the Senegal sole [37]. An asymmetry has also been demonstrated in the brain of the lizard *Anolis carolinensis*, in which a high degree of melatonin binding was present in the left habenular nucleus, but no binding was observed in the habenulum of the right brain hemisphere [38].

Despite the fact that, in some species, ocular melatonin is thought to have a local paracrine role related to photic adaptation functions [39], this has not been demonstrated for the Senegal sole.

With regard to the effects of a 1-h light pulse in MD, previous studies performed in teleost fish, such as the European sea bass [6,28] and brook trout [7], showed that light inhibits melatonin production in the pineal organ, and thus circulating levels of the hormone. Our results in the Senegal sole agree with both studies because a light pulse in MD provoked a decrease in melatonin concentration, which reached similar levels to those obtained at ML.

Photoperiod has been shown to exert an important effect on the reproductive system of fish [15]. These effects have also been demonstrated in the European sea bass [40], in which the application of long photoperiods determined a reduction in gonadal development. As in other fish species, melatonin production in the Senegal sole is strongly influenced by the light regime and could be involved in the mediation of physiological effects of photoperiod and in the control of rhythmic processes, such as reproduction.

In conclusion, this study on the Senegal sole, a species with growing commercial interest in Southern Europe, provides information concerning its activity and melatonin rhythms, and relates them with the photoperiod, the strongest synchroniser of animal rhythms. This knowledge could be relevant for some important aspects of aquaculture, such as feeding or reproduction.

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