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Daily and circadian melatonin release *in vitro* by the pineal organ of two nocturnal teleost species: Senegal sole (*Solea senegalensis*) and tench (*Tinca tinca*)

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ABSTRACT

This research aimed to investigate melatonin rhythms in the pineal organ of two nocturnal fish species, sole and tench, which show high sensitivity to light. Pineal organs were cultured *in vitro* under an LD (12 h light:12 h dark) cycle to study the daily rhythmicity of melatonin release. In addition, the *in vitro* culture was performed under conditions of constant darkness (DD) to study the endogenous control of the rhythm. In the pineal organs cultured under an LD cycle, rhythmic melatonin release was evident in both species, with low values observed during the photophase (15.6 ± 7.2 and 22.6 ± 2.6 pg/mL for sole and tench, respectively) and high values coinciding with the scotophase (74.0 ± 8.2 and 82.1 ± 9.1 pg/mL, for sole and tench, respectively). Under LD, the rhythm had a period of 24 h ($p<0.001$) and presented similar acrophases for both species, located around 9–10 h after lights off (2 and 3 h before the end of the dark phase). When the pineal organs were cultured under DD, the results differed between the species studied. A marked circadian rhythm in melatonin release by the pineal was registered in tench, with lower values during the subjective day, i.e. the period that was previously day (6.2 ± 1.6 pg/mL) and higher values during the subjective night, i.e. the period that was previously night (20.4 ± 5.5 pg/mL). The rhythm had a mean *tau* of 24.1 h ($p<0.01$) and the acrophase was located around 12 h after lights off (the beginning of the subjective day), slightly later than that registered under LD conditions. In contrast, melatonin values in sole remained high during darkness (around 92.0 ± 6.9 pg/mL) for four consecutive days, including subjective day periods. In short, these findings revealed that the rhythm of melatonin release in tench is under endogenous control by a circadian oscillator within the pineal organ, while no such pacemaker was evident in sole, which melatonin rhythm appeared to be exclusively light-driven.

1. Introduction

Biological rhythms in animals are regulated by periodical changes in the environment which entrain the internal biological clock (Aschoff, 1981). Among these environmental factors, light is one of the most important, particularly the daily change between light and darkness (LD cycle), which directly entrains the molecular circadian oscillator (Carr et al., 2006; Ziv and Gothilf, 2006).

The pineal organ is a key component of the circadian system of vertebrates, since it is responsible for transducing environmental cues (such as LD) into a hormonal signal, melatonin, which is produced in high amounts during the night and immediately secreted to the bloodstream (Falcón et al., 2007). In mammals, this organ has lost its photosensitive capacity and the circadian clock is located in the suprachiasmatic nucleus (SCN), which receives photic information from the lateral eyes and regulates melatonin production in the pineal organ via a multisynaptic pathway (Yu and Reiter, 1992). In fish, the circadian clock seems to be located in the pineal organ, since this tissue maintains its photosensitive capacity and presents a self-sustainable oscillator (Iigo et al., 1994; Ekström and Meissl, 1997). In the case of birds, the pineal represents an intermediary state between the directly photosensitive fish pineal and the indirectly photosensitive mammalian pineal, since both the circadian clocks in the SCN and in the pineal organ are involved in the control of melatonin synthesis (Takahashi et al., 1989). Thus, the teleostean pineal organ provides an excellent model for analysing the circadian clock mechanism since it is not under the control of the circadian clock in the SCN (Iigo et al., 2004).

In vitro studies have suggested that most teleost species possess endogenous intrapineal oscillators that drive the rhythm of melatonin production (Iigo et al., 1991, 2003, 2004; Bolliet et al., 1994, 1996; Cahill 1996; Bayarri et al., 2004a). However, such an endogenous control is lacking in salmonids, suggesting that ancestral salmonids lost the circadian regulation of melatonin production after the divergence from osmerid teleosts (Iigo et al., 2007; Migaud et al., 2007).

The species studied in this investigation are Senegal sole, *Solea senegalensis* (Kaup, 1858), a marine pleuronectiform of the Soleidae family, and tench, *Tinca tinca* (Linnaeus, 1758), a freshwater cyprinid. Sole is of high commercial interest in many parts of Europe, and its aquaculture production is the subject of many investigations (Dinis

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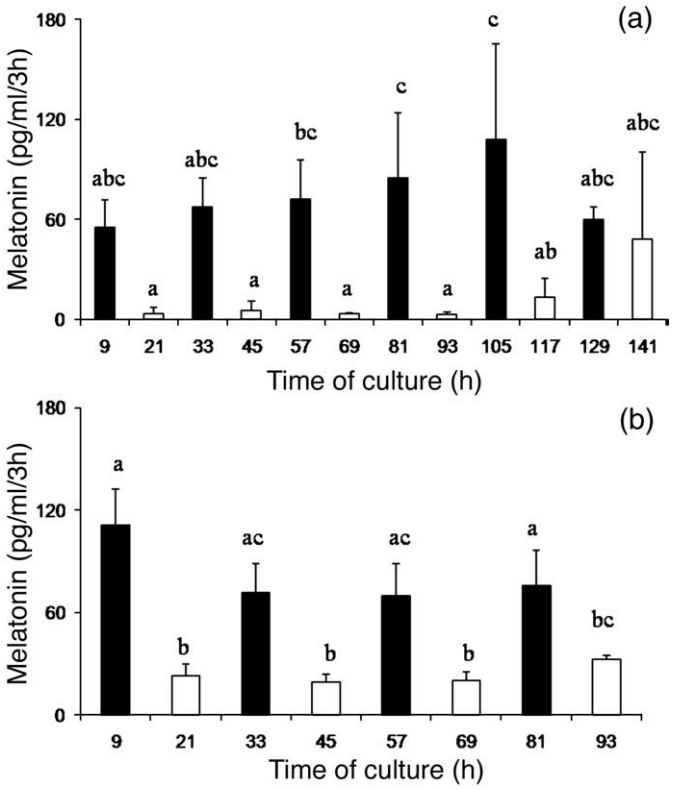

Fig. 1. Mean values produced by sole (a) and tench (b) pineals at mid-light, ML (white bars) and mid-dark, MD (black bars). Error bars represent the standard error of mean. Different letters indicate statistically significant differences (ANOVA, Duncan's test, $p<0.001$).

et al., 1999; Agulleiro et al., 2006). The pineal organ of this species is well developed compared to other species and shows a marked asymmetry in its position, reflecting an adaptation to the benthonic lifestyle of this flatfish (Confente et al., 2008). Tench is a species sold on the European market as a food, for stocking open water systems (especially for recreational fishing) and for ornamental purposes (Arlinghaus et al., 2003). Both species are nocturnal and have been seen to display high activity during the dark phase and low activity during the light phase in laboratory conditions (Herrero et al., 2003; Bayarri et al., 2004b). Furthermore, they are both very sensitive to nocturnal light, having low light intensity thresholds, as a 1 h light pulse of only 5.3 and 3.3 $\mu W/cm^2$ (less than 1 lx) for sole and tench, respectively, can bring melatonin production down to daytime values (Vera et al., 2005; Oliveira et al., 2007). However, no *in vitro* studies on the rhythmic production of melatonin by the pineal organ, or on the endogenous origin of these rhythms, have been carried out in either species.

The objective of this investigation was to characterize the daily rhythms of melatonin release in individual pineals from both species cultured under controlled conditions (20 °C, LD 12:12), and, to check whether an endogenous oscillator existed in the pineal organ that produces a circadian melatonin rhythm under constant darkness (DD) conditions.

2. Material and methods

2.1. Animals and housing

Two different groups of animals were used in this research: one group of 16 sole with a mean body mass of 498 ± 65 g, obtained from the "Instituto Español de Oceanografia", IEO (Mazarrón, Spain), and other group of 10 tench with a mean body mass of 21 ± 7 g, obtained

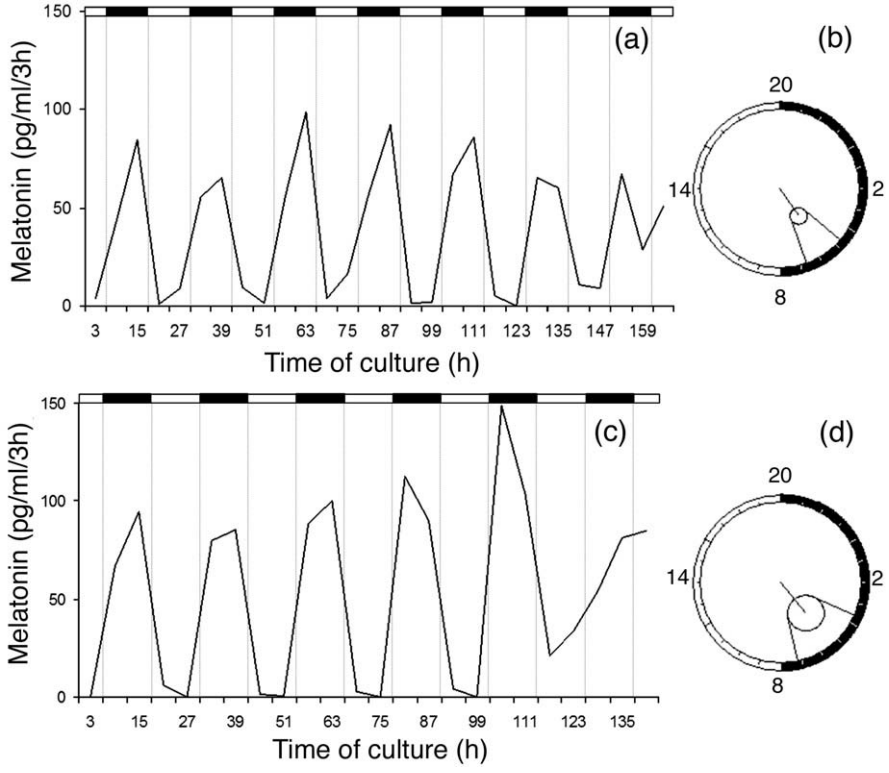

Fig. 2. Daily melatonin rhythms produced by two representative Senegal sole pineal organs (a and c) cultured under LD conditions for seven days (values of melatonin expressed in pg/mL/3 h of culture, as function of time of culture in hours). Horizontal black and white bars at the top of the graphs represent night and day, respectively. Polar representation of the COSINOR analysis, depicting clockwise the daily cycle (b and d). The white and black bars represent the light and the dark phase, respectively. The vectors point to the moment of the acrophase, and its length represents the amplitude. The ellipses represent the confidence interval.

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from the hatchery "Tencas de Casaseca" (Zamora, Spain). All fish were transferred to the facilities of the University of Murcia two weeks prior to the experiments. Sole were reared in 500 L tanks and tench in 60 L aquaria, both well aerated and equipped with biological and mechanical filters in a closed circuit and under LD conditions (12L:12D).

2.2. Experimental design

2.2.1. Daily melatonin rhythms under LD cycle

Both sole and tench were anesthetized with 40 ppm natural clove essence, *Syzygium aromaticum* (GUINAMA, Valencia, Spain) previously dissolved in ethanol. Once they had lost their equilibrium, they were killed by decapitation and the pineal organ was immediately removed and kept in a culture medium bath with gentamicin (25 mL/L) for 10 min. The culture medium used was D-MEM supplemented with glucose (1 g/L) (Gibco — Invitrogen, Barcelona, Spain), with BSA (0.75 g/L), 5-hydroxytryptophan (0.022 g/L), ascorbic acid (17.2×10^{-3} g/L), gentamicin (0.05 g/L) (Sigma-Aldrich Chemie GmbH, Steinheim, Germany), $NaHCO_3$ (2 g/L) (Merck Darmstadt, Germany), Na_2HPO_4 (0.8 g/L) (Panreac, Barcelona, Spain), and HEPES (25 mL/L) (Gibco — Invitrogen, Barcelona, Spain) as described elsewhere (Alonso-Gómez et al., 1995). Pineal organs were placed individually in a sterile glass wool "bed" in a 3 mL culture chamber. The culture system consisted of a superfusion system to mimic the physiological conditions. The culture medium was constantly pumped to the culture chamber by a peristaltic pump (Minipuls 3, Gilson, Middleton, USA) at a flow rate of 0.3 mL/h. The culture medium flowing out of the culture chamber was collected every 3 h by a refrigerated fraction collector (FC203B, Gilson, Middleton, USA) and kept at 4 °C. The culture set-up was placed inside a light-tight chamber in the chronolab, which allowed photoperiod and temperature control. The temperature was kept constant at 20 °C, and the light was provided by a metal halide bulb (GRO-LUX, 40 W, Germany) with a mean light intensity of 400 lx and a photoperiod of 12L:12D with lights on at 8 h. Every 24 h the samples were stored at −80 °C and the culture medium was renewed. Pineal organs from sole ($n=8$) were maintained under LD conditions for 7 days, while the pineals from tench ($n=5$) were maintained under these conditions for 5 days.

2.2.2. Circadian melatonin rhythms under DD conditions

The external synchronizer (light) was suppressed to study the endogenous character of the daily melatonin rhythm produced by the pineal organ, performing the *in vitro* culture under DD conditions. The procedure of pineal extraction and setting of the culture was performed as described above. In this experiment, culture chambers were covered with aluminium foil to further ensure complete darkness. At the beginning of the experiments the pineal organs were subjected to an LD cycle (12L:12D) during the first day, and then under DD during the five consecutive days. Finally, LD conditions were again set on the seventh and last day of culture to test whether the pineal organs were still alive and capable of responding to light changes with daily variations in melatonin production. This procedure was performed for both species being studied, sole ($n=8$) and tench ($n=5$).

2.2.3. Melatonin analysis

Melatonin levels in culture medium samples were measured by a Radioimunoassay commercial Kit (Melatonin Direct RIA, Biosource, Belgium), with a lower limit of quantification (LLOQ) of 2 pg/mL. The intra-assay coefficient of variation (CV) was 9.8–12.3% and inter-assay

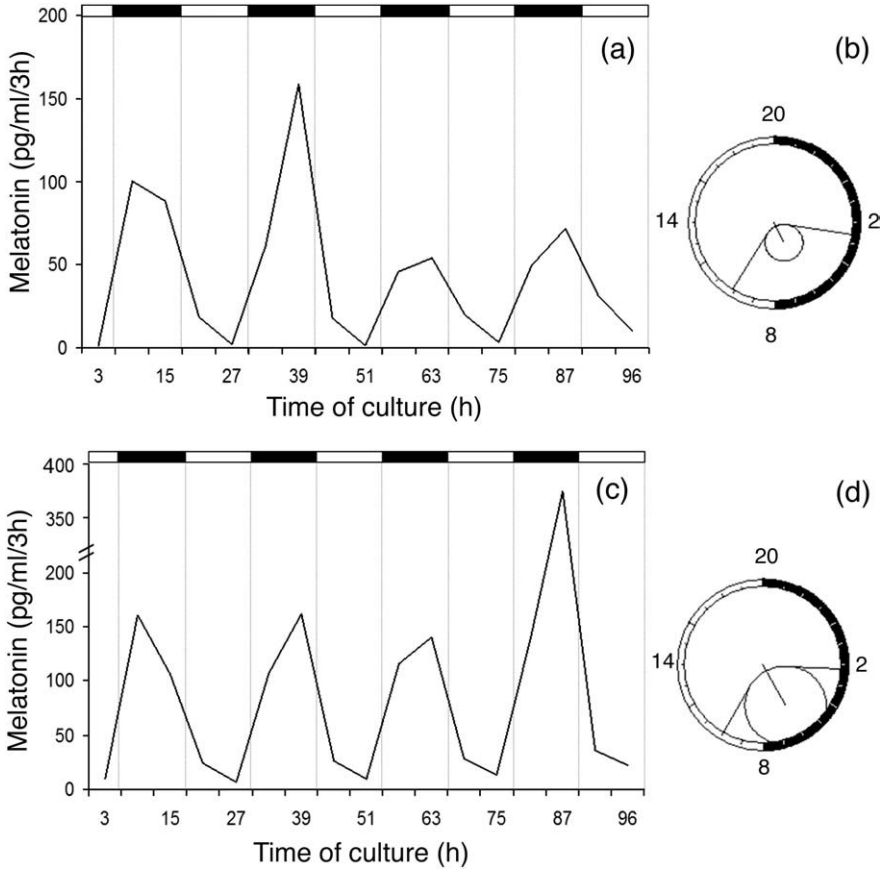

Fig. 3. Daily melatonin rhythms produced by two representative tench pineal organs (a and c) cultured under LD conditions for five days (values of melatonin expressed in pg/mL/3 h of culture, as function of time of culture in hours). Horizontal black and white bars at the top of the graphs represent night and day, respectively. Polar representation of the COSINOR analysis, depicting clockwise the daily cycle (b and d). The white and black bars represent the light and the dark phase, respectively. The vectors point to the moment of the acrophase, and its length represents the amplitude. The ellipses represent the confidence interval.

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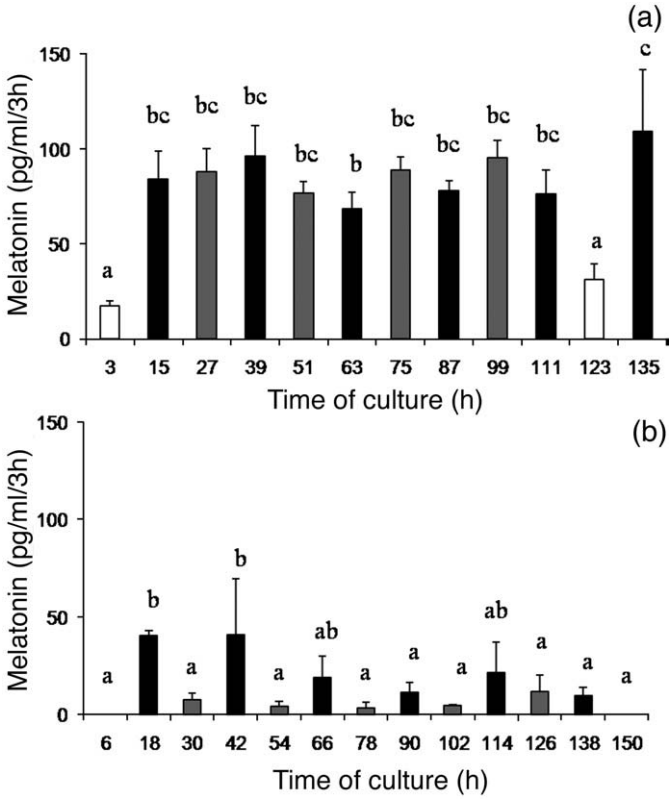

Fig. 4. Mean values produced by sole (a) and tench (b) pineal organs cultured under DD conditions during the day (white bars), subjective day (grey bars) and subjective night (black bars). Error bars represent the standard error of mean. Different letters indicate statistically significant differences (ANOVA, Duncan's test, $p<0.001$).

CV was 9.6–16.2%. Samples were defrosted and 100 µL of each sample was placed in a polystyrene tube with 10 µL of calibrator A. Enzyme solution was added, and the mixture was centrifuged and incubated for 1 h at room temperature, after which assay buffer and melatonin antiserum were added and mixed, before incubating again for 1 h at room temperature. Then, I^{125} melatonin was added and mixed, and the tubes were centrifuged and incubated for 20 h. On the second day, the precipitating reagent was added before centrifugating the tubes, and the supernatant was removed by a vacuum pump. Finally, radioactivity was measured in a γ counter (WALLAC 1470 Automatic Gamma Counter, Perkin Elmer).

2.2.4. Data analysis

The COSINOR analysis was used to determine the existence of daily rhythms of melatonin secretion by means of the chronobiology software "CRONOBIO" and "El Temps" (by Prof. Díez-Noguera, University of Barcelona, Spain). This analysis calculated the "amplitude" (one-half the peak-to-trough variation), "mesor" (the time series mean), and "acrophase" (peak time relative to the time scale) of the rhythm by the least squares approximation of cosine function (Díez, 2007). In addition, data of melatonin secretion were subjected to one way ANOVA, followed by Duncan's post-hoc test, to search for statistically significant differences in the melatonin concentration values between different hours of culture.

3. Results

3.1. Daily melatonin rhythms under LD cycle

In both species, the presence of a daily rhythm of melatonin release was evident in the pineal organs cultured under an LD light cycle. Low melatonin contents were released during the photophase, while high values coincided with the scotophase. In sole, the average amount of melatonin released by the pineal organ at mid-light (ML) was 15.6 ± 7.2 pg/mL and at mid-dark (MD) 74.0 ± 8.2 pg/mL. For the tench pineal organ, the mean amount of melatonin released was 22.6 ± 2.6 pg/mL at ML and 82.1 ± 9.1 pg/mL at MD. Significant statistical differences were found between the mean melatonin values at different times during culture for both sole and tench (ANOVA, $p<0.05$) (Fig. 1).

COSINOR analysis of the melatonin released by pineals pointed to a significant daily rhythmicity in the pineals studied. For sole, the melatonin production rhythm of both pineals (Fig. 2), fitted significantly ($p<0.0001$) a sinusoidal function with a 24 h periodicity and a mean mesor of 45 pg/mL, a mean amplitude of 48.7 pg/mL and an acrophase located at 12:32 h of culture (9:32 h after lights off). In the case of tench pineal organs (Fig. 3), data for rhythmic melatonin release fitted significantly ($p<0.001$) a sinusoidal function with a period of 24 h, a mean mesor of 69.2 pg/mL, a mean amplitude of 76.4 pg/mL and an acrophase at 13:10 h of culture (10:10 h after lights off). Furthermore, in both species the melatonin rhythms were in phase, since the acrophases were similar: at around 9 and 10 h after the end of the light phase, for sole and tench, respectively.

3.2. Circadian melatonin rhythms under DD conditions

During the first day, under the LD cycle, pineal organs of both species showed a secretory profile of melatonin similar to that observed in the first experiment, with low values during the light phase (19.2 ± 2.9 for sole and 0 ± 0 pg/mL for tench), and high values during the dark phase (99.3 ± 13.9 for sole and 26.0 ± 9.6 pg/mL for tench) (Fig. 4). However, during subsequent days under DD conditions, the melatonin values in Senegal sole pineals remained high during both the subjective day, (92.0 ± 6.9 pg/mL) and subjective night (90.0 ± 5.6 pg/mL) (i.e. the periods that were previously day and night, respectively), and no free-running rhythm in melatonin secretion was observed. On the last day, exposure to LD conditions re-

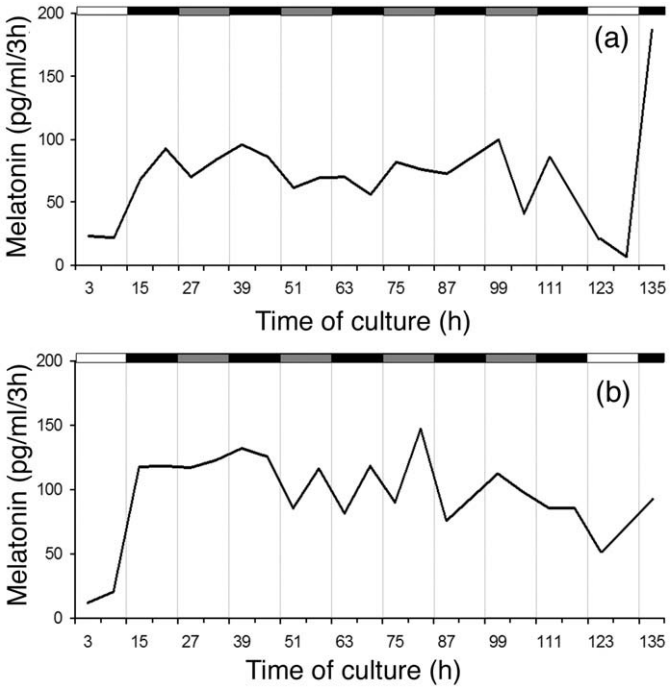

Fig. 5. Melatonin rhythms produced by two representative Senegal sole pineal organs (a and b) cultured under DD conditions for seven days (values of melatonin expressed in pg/mL/3h of culture, as function of time of culture in hours). Horizontal black, grey and white bars at the top of the figures represent night, subjective day and day hours, respectively.

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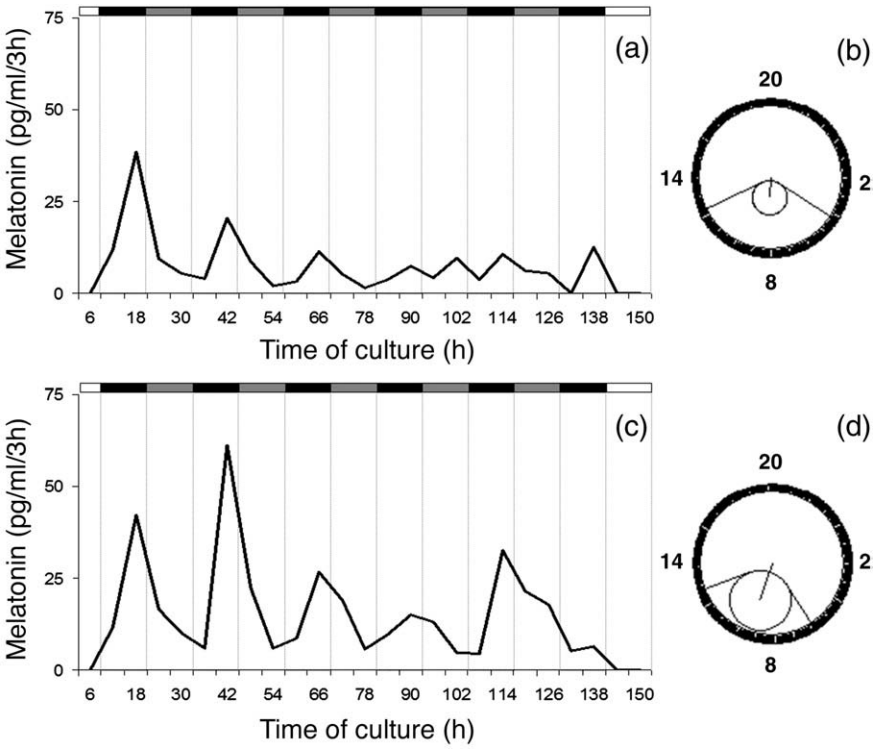

Fig. 6. Circadian melatonin rhythms produced by two representative tench pineal organs (a and c) cultured under DD conditions for seven days (values of melatonin expressed in pg/mL/3 h of culture, as function of time of culture in hours). Horizontal black, grey and white bars at the top of the figures represent night, subjective day and day hours, respectively. Polar representation of the COSINOR analysis, depicting clockwise the daily cycle (b and c). The vectors point to the moment of the acrophase, and its length represents the amplitude. The ellipses represent the confidence interval.

established daily variations in melatonin release with low rates during light phase (26.6 ± 16.4 pg/mL) (Fig. 5). The ANOVA analysis (Fig. 4a) revealed significantly lower values at the times when the pineals were exposed to light (3 and 123 h of culture), while the COSINOR analysis did not reveal any notable rhythm ($p<0.05$) (Fig. 5).

In what concerns tench, when the pineal organs were subjected to DD conditions, a circadian melatonin release was observed, with a low amount of melatonin released during the subjective day (mean lower value 6.2 ± 1.6 pg/mL) and a higher amount of melatonin during the subjective night (mean higher value 20.4 ± 5.5 pg/mL) (Fig. 6). During the last day, exposure to LD re-established daily variations, and melatonin levels decreased in the light phase, indicating that the pineal organs remained alive and functional, responding to light. The ANOVA analysis (Fig. 4b) showed significantly higher values of melatonin production at time points 18 and 42, the peaks of the two first subjective nights ($p<0.05$). When data were subjected to a COSINOR analysis (Fig. 6), 50% of the pineal profiles of secretion of melatonin fitted significantly a sinusoidal function, with a free-running period (*tau*) of 24.1 h ($p<0.01$), a mean mesor of 11.3 pg/mL, a mean amplitude of 9.6 pg/mL and an acrophase located at 18:40 h of culture (12:40 h after lights off).

4. Discussion

In this research, the pineal organs of both Senegal sole and tench released melatonin rhythmically in a daily fashion when cultured under LD conditions, with higher amounts of melatonin being produced during the dark phase, and lower values observed during the light phase. Remarkably, both species exhibited similar *in vitro* melatonin production rates during darkness (74–82 pg/mL) and daytime (16–23 pg/mL). This daily pattern of secretion is in accordance with the *in vivo* results previously observed for these species: when the daily rhythm of plasma melatonin was assessed, the concentrations were higher during the night than during the day (Bayarri et al., 2004b; Vera et al., 2005). This evidence suggests that the pineal organ is the main contributor to the circulating melatonin in both Senegal sole and tench. In several teleost species (for example the goldfish (Iigo et al., 1991), the white sucker (Zachmann et al., 1991), the pike (Bolliet et al., 1994), the ayu (Iigo et al., 2003, 2004) the European sea bass (Bayarri et al., 2004a) or the salmonids (Iigo et al., 1998, 2007)), when the secretion of melatonin by the pineal organ was investigated *in vitro*, it was seen that, when submitted to LD cycle, the pineal organs maintained a rhythmic production of melatonin as observed in our study.

The endogenous control of melatonin release by the pineal organ differed between the species under investigation. In tench, circadian melatonin production was observed when pineals were cultured under conditions of constant darkness. The fact that the rhythm persisted in the absence of photoperiod synchronization suggests the presence of an endogenous mechanism or internal clock in the pineal organ controlling melatonin production. In this case, the amplitude was lower than in experiment 1 (9.6 vs 76.4 pg/mL), although this may have been due to the free-running rhythm characteristics, as was observed in the case of European sea bass (Bayarri et al., 2004a). According to Herrero et al. (2003), tench sustained locomotor rhythmicity under DD conditions with *tau* ranging from 21 to 26 h, which indicated the presence of an endogenous pacemaker, the same being the case with our results. The circadian locomotor activity rhythm may have been controlled by the presence of a circadian pineal oscillator, giving the animal daily information through melatonin rhythms, although there was no LD signaling. Besides, light during the night was previously seen to inhibit both melatonin production and locomotor activity in this species (Vera et al., 2005), so the persistence of the rhythm can only be verified under constant darkness conditions, as it was in goldfish (Iigo et al., 1991), pike (Bolliet et al., 1994) and ayu (Iigo et al., 2004). Constant light suppressed the circadian rhythm in these species, although their pineals showed circadian melatonin production when cultured *in vitro* under DD conditions. In almost all teleost species studied to date when the pineal organs were cultured

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under constant darkness (DD), melatonin secretion follows a circadian rhythm, with higher levels during the subjective night, as in tench in the present study (Iigo et al., 1991; Bolliet et al., 1994, 1996; Cahill 1996; Iigo et al., 2003, 2004; Bayarri et al., 2004a). This strongly suggests that most fish possess endogenous intra-pineal oscillators which drive the rhythm of melatonin production.

In the case of Senegal sole, however, when the pineal organs were cultured under DD conditions, the melatonin values remained high during both subjective night and day and a circadian rhythm could not be described. This lack of endogenous control has been observed before in salmonids, where *in vitro* culture in the absence of light did not produce circadian periodicity of melatonin release from the pineal in any of the species studied. Melatonin levels remained high during both subjective day and subjective night, suggesting that the pineal organ of this group of species lacks the circadian regulation of melatonin production (Iigo et al., 2007).

In our experiments, when the lights were switched on following DD, melatonin production was suppressed and fell to values statistically similar to that produced during the first day under LD conditions, which suggests photic regulation of melatonin production, rather than an endogenous control. However, the fact that the sole pineal organ showed no circadian production of melatonin may be due to a different location of the biological clock, since this species sustained spawning rhythmicity during two days under constant lightning conditions (Oliveira et al., in press), hinting at the presence of some endogenous mechanism controlling the biological rhythms of this species. Information about the existence of a circadian clock in the pineal organ of other species of pleuronectiformes is not available, so this could even be a characteristic of this group of fish. Flatfish are a particular group of teleosts, due to the early metamorphosis of larvae and the anatomical/physiological modifications that this phenomenon brings about. The location of the circadian clock in another component of the circadian system could reflect an adaptation of this group of fish. In the case of Senegal sole, the pineal organ is modified, being larger than in other species and showing a marked asymmetry in its position, reflecting an adaptation to its benthonic life (Confente et al., 2008). Another factor that could have disrupted the rhythm is a possible seasonal effect, since in this species an annual rhythm of nocturnal melatonin has been observed *in vivo*, with melatonin daily rhythms changing seasonally (Vera et al., 2007).

Briefly, we may conclude from the results of this research that both Senegal sole and tench pineal organs produce melatonin rhythmically in a daily fashion when cultured *in vitro* under LD conditions. However, only tench showed circadian melatonin release by this organ when the LD signaling was removed, under DD conditions, pointing to the existence of an endogenous clock sited in the pineal that controls such a rhythm. The Senegal sole pineal failed to maintain the rhythm, with pineal organs producing high amounts of melatonin in both subjective night and day, which suggests that a circadian oscillator may be lacking in the pineal of some fish in addition to salmonids.

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