Monthly day/night changes and seasonal daily rhythms of sexual steroids in Senegal sole (*Solea senegalensis*) under natural fluctuating or controlled environmental conditions

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Abstract

Research on sexual steroids has usually focus on seasonal changes, but little attention has been paid to the existence of daily rhythms. A better knowledge of such daily fluctuations is required to asses accurately their plasma concentrations. To this end, in this paper we described the monthly day/night concentrations and seasonal daily rhythms of plasma sexual steroids (17 β -estradiol, E₂ and testosterone, T) in Senegal sole females, reared under natural photo- and thermo-period. In addition, the influence of the natural annual fluctuation of the water temperature on the plasma concentration of these steroids was investigated, using one group of Senegal sole under a natural photoperiod, but with an attenuated thermal cycle (around 17 - 20 °C) for one year. No significant day/night differences were detected in monthly samplings, but the existence of an annual rhythm of E₂ and T (p<0.01) with an acrophase in February was revealed by COSINOR analysis. Maximum values were reached in

March for both steroids ($6.1 \pm 1.7 \text{ ng ml}^{-1}$ at mid-dark, MD and $4.0 \pm 0.6 \text{ ng ml}^{-1}$ at mid-light, ML for E2 and $1.4 \pm 0.4 \text{ ng ml}^{-1}$ at MD and $0.8 \pm 0.1 \text{ ng ml}^{-1}$ at ML for T) in anticipation of the spawning season (May-June). As regards seasonal daily rhythms, at the spring solstice (21st March) a daily rhythm was observed for both steroids (COSINOR, $p < 0.01$), with an acrophase at 20:00 h (E₂) and at 21:08 h (T). When Senegal sole females were submitted to an attenuated annual thermal cycle, the steroid rhythm disappeared (there was no surge in spring, as in the control group) and these fish did not spawn, despite being subjected to natural photoperiod conditions. This result underlined the importance of the natural annual fluctuation of water temperature and photoperiod on the synchronization of the spawning season and on the onset of steroidogenesis.

Key words: Sexual steroids, 17 β -estradiol, testosterone, day/night concentrations, annual rhythm, seasonal daily rhythms, temperature, *Solea senegalensis* female.

Introduction

The biological rhythms in animals are synchronized by external factors, light being one of the most important. In most fish species from temperate zones, the seasonally changing pattern of daylength seems to be the responsible for the cuing and timing of reproduction. In salmonids, the predominant environmental cue for timing reproduction and spawning is the photoperiod (Bromage et al., 2001), whereas in many cyprinids, the predominant environmental cue is temperature and photoperiod combined (Hontela and Stacey, 1990). Seasonal changes in both photoperiod and temperature are transduced by the pineal organ into melatonin rhythm (producing high levels during the night and low levels during the day) which works as a Clock/Calendar signal. The nocturnal increase lasts longer under a short than under a long photoperiod, the photoperiod signals being transduced by melatonin (Reiter, 1993; Lincoln, 2006), which acts on the hypothalamic-pituitary-gonad axis (Amano et al., 2000; Bromage et al., 2001; Bayarri et al., 2004b; Falcon et al., 2007). This axis, in turn, controls reproduction timing and by means of neuroendocrine signals, regulates the production of sexual steroids and growth factors in the gonads.

Nevertheless, reproduction can not be considered an exclusively annual phenomenon, since daily changes in oocyte maturity and in the secretion of sexual steroids or gonadotropins have been described in species such as red seabream, *Pagrus major* (Matsuyama et al., 1988), snapper, *Pagrus auratus* (Carragher and Pankhurst, 1993), kisu, *Sillago japonica* (Kobayashi

et al., 1988) and European sea bass, *Dicentrarchus labrax* (Bayarri et al., 2004b). Furthermore, the effect of lunar cycle on reproduction has also been observed in various species of rabbitfishes (Takemura et al., 2004), in gilthead seabream, *Sparus aurata* (Saavedra and Pousão-Ferreira, 2006) and in Senegal sole, *Solea senegalensis* (Oliveira et al., 2008).

In females, sexual steroids are produced in the ovary and act both on the brain and the pituitary to regulate luteinizing hormone (LH) release through a combination of positive and negative feedback actions (Yaron et al., 2003). Studies in goldfish suggest that elevated levels of both testosterone (T) and 17 β -estradiol (E₂) are important in the induction of the preovulatory LH surge (Kobayashi et al., 1989). Concentration of gonadal steroids in the blood stream provides information about the status of the brain-pituitary-gonad (BPG) axis and therefore, its measurement, is a useful tool to assess the reproductive status of the animal (Merson et al., 2000).

The Senegal sole (*Solea senegalensis*) is a species of high commercial interest, and the object of investigation by several groups in Europe. Spawning season takes place mainly in spring, with a secondary spawning in autumn (Anguis and Cañavate, 2005). However, one of the curbs on its production in captivity is the lack of control of its reproduction rhythms. Water temperature seems to play a crucial role in the reproductive cycle of both *Solea senegalensis* (Dinis et al., 1999; Anguis and Cañavate, 2005; García-López et al., 2006a) and *Solea solea* (Baynes et al., 1993), acting on gamete maturation, ovulation and spawning (Anguis and Cañavate, 2005). In Senegal sole recent studies have focused at sexual steroids, vitellogenin levels and gonad development (Aguillero et al., 2006; García-López et al., 2006a; García-López et al., 2006b; García-López et al., 2007; Guzmán et al., 2008), and melatonin rhythms and the influence of environmental factors, such as the characteristics of light and the temperature, on these rhythms (Bayarri et al., 2004a; Vera et al., 2007; Oliveira et al., 2007), but no evidences has been offered concerning the existence of changes in plasma steroid concentrations during the day in different seasons. A better knowledge of such daily changes is needed to avoid misinterpretation of the data when studding steroid levels in fish, since different values may be obtained if sampling takes place at different moments of the day.

To this end, we investigated monthly day/nigh changes in 17 β -estradiol (E₂) and testosterone (T) concentrations, and seasonally (during both solstices and equinoxes) the existence of daily rhythms in the levels of these hormones in female Senegal sole maintained under natural conditions of photo- and thermo-period. In addition, the role of the natural annual fluctuation of the water temperature in annual steroid rhythm synchronization is observed.

Material and Methods

Animals and housing

For this study two different groups of Senegal sole from the same lot of an F1 generation with 4 years of age were used (n=120). Both groups were maintained in similar rearing conditions, differing only in the water temperature regimen, which was the environmental factor we most wanted to test. Although both groups were not kept in the same place, the latitude was similar and the photoperiod conditions were exactly the same, so that the comparison can be regarded as reliable.

The first group, composed of 60 fish with a mean body weight of $1,369.1 \pm 32.4$ g and a mean body length of 43.3 ± 0.2 cm, was reared in the facilities of the research centre “Instituto de Acuicultura de Torre la Sal” (IATS) (40°6'N, 0°9'E, Castellón, Spain), in cylindrical 3,000 L tanks equipped with egg collectors. The water was provided in a constant flow of running marine water from the Mediterranean Sea, with a natural annual cycle of temperature. The tanks were covered with a raffia cloth, which provided a natural photoperiod, with maximum light intensity of 600 lux at the water surface during the day. Fish were fed *ad libitum* in the morning five days a week, using a dry pellet (ProAqua) feed and natural food, consisting of chopped fresh mussels and frozen squid.

The second group consisted of 60 Senegal sole with a mean body weight of $1,039.5 \pm 39.3$ g. Fish were reared in a local fish farm, Piscimar S.L. (39°53'N, 0°5'E, Castellón, Spain), in a cylindrical 16,000 L tank equipped with an egg collector. The water temperature fluctuation in this case was minimal (17-20 °C), since water was provided from a well, whose water temperature was almost constant throughout the year. The tank was placed in the outside, and the 90% of the surface was covered with a grey plastic cloth; there was a natural photoperiod, with a maximum light intensity of 870 lux at the surface of the water, during the day. The feeding regime was comprised of five weekly meals of mussels and squids and two of a dry pellet (ProAqua) feed, both *ad libitum*.

In both groups sole were individually labelled with an internal passive integrated transponder (PIT) tag system, enabling identification of each fish in repeated samplings. The egg collectors of both groups were checked daily to verify the presence of eggs, and on spawning days, the total number of eggs was estimated volumetrically.

Handling of the fish was in accordance with the European Union Directive (EEC, 1986) for the protection of animals used for experimental and other scientific purposes.

Experimental design

Experiment 1: Monthly day/night differences in plasma sexual steroids (E₂ and T)

This experiment was intended to ascertain whether day/night differences in the plasma concentration of sexual steroids (E₂ and T) existed in Senegal sole female, and how they varied during the year in natural conditions of temperature and photoperiod. For this, 12 sole (7 female and 5 male) from the first group (IATS) were used. Female were blood sampled around the 25th of each month for a year (except July and October due to the incidence of bacterial infection in the broodstock), at Mid-Dark (MD) and at Mid-Light (ML). During the dark phase, sampling was carried out under a dim red light and after covering the fish head with aluminium foil. Sole were previously anesthetized with 2-phenoxyethanol (0.5 ml L⁻¹), individually weighed and identified with a “pit-tag” lector, and then sampled by caudal puncture, with 1 ml heparinized syringe. Blood was collected in heparinized ependorf tubes on ice, and later centrifuged (3,000 g, 4 °C, 15 min) to separate plasma samples, which were frozen at -20 °C.

Spawning was monitored by collecting eggs, which were classified as viable (floating) or not. Viable eggs were incubated in 5 L sieves submerged in 250 L tanks of filtered and aerated sea water maintained between 16 and 18 °C.

Experiment 2: Daily rhythms of plasma sexual steroids (E₂ and T) in different seasons

This experiment set out to describe the seasonal differences in daily rhythms of plasma E₂ and T. For this, 48 soles also from the first group (IATS), were used. Sampling took place at both equinoxes (March 20th, spring equinox and 22nd September, autumn equinox) and at both solstices (June 21st, summer solstice, and December 21st, winter solstice). In each of these days, four to six female were blood sampled every three hours until a 24h cycle was complete. The same fish were not sampled twice and they were transferred to a recovery tank after blood collection. The procedure of sampling was the same as described above.

Experiment 3: Influence of an attenuated thermal cycle on the seasonal production of plasma sexual steroids (E₂ and T)

To study the importance of the natural fluctuation of temperature during the year in the annual steroid rhythm in Senegal sole female, the second group of fish (PISCIMAR, S.L.) was reared under an attenuated water temperature cycle throughout the year (17-20 °C). Special care was taken to maintain similarity in the rearing conditions (e.g. photoperiod, sex-

ratio, feeding regimen) between this group and the control group, to avoid introducing disrupting factors to the results. In each season, four to six females were blood sampled (as described before) at MD and ML. Then, the values of plasma concentration of E₂ and T were compared with those obtained in the same month in the first experiment, in order to determine whether rhythms persisted in the absence of temperature synchronization.

Data analysis

Sex Steroid (E₂ and T) ELISA

In order to assess the plasma concentrations of E₂ and T, samples were analysed in the facilities of the research institute IATS, by enzyme-linked immunosorbent assay (ELISA) using a protocol previously validated for Senegal sole plasma samples (Guzmán et al., 2005; Guzmán et al., 2008).

Sex steroids were first extracted in ice cold methanol and then reconstituted in 120 µl of potassium phosphate buffer (0.1 M, pH 7.4), and stored at -20 °C for analysis. In the steroid ELISA, microtiter plates (Maxisorp, 96-well microplates, Nunc) were coated with mouse anti-rabbit IgG monoclonal antibody (Cayman Chemical) and incubated (overnight at 4 °C) with the following mixture (0.2 ml per well): standard (E₂ and T) (Sigma-Aldrich) or sample, specific steroid antibody and the acetylcholinesterase tracer (Cayman Chemical). Colour development was then achieved by addition of Ellman's reagent in complete darkness at room temperature for 4 h and the absorbance measured at 405 nm, using a microplate reader. The sensitivities of the ELISAs were 5.19 pg ml⁻¹ and 8.78 pg ml⁻¹ for the E₂ and T ELISA, respectively. The intra- (n=4) and inter-assay (n=8) coefficients of variation (CV %), at 50% binding, were 5.8% and 6.3% for E₂ and 6.1% and 11.3% for T ELISA (Guzmán et al., 2008).

Statistical analysis

Statistical tests were performed using Excel[®], SPSS[®] and a Chronobiology software ("El Temps", by Prof. Díez Noguera, University of Barcelona, Spain).

Data are expressed as mean ± standard error of the mean (S.E.M.). To determine the existence of significant differences in the plasma concentration of E₂ and T between sampling points, data were subjected to one-way ANOVA, followed by a Duncan post hoc test (with a degree of significance of p<0.05). To determine the significance of annual and daily sex steroid rhythms, the COSINOR analysis was used. This analysis calculated the sinusoidal function that fitted our data, in which the mesor is the mean value around which the variable

oscillates, the amplitude the oscillation between the mesor and the maximum value, and the acrophase the moment when the function reaches its maximum (Díez, 2007).

The ELISA data analysis was performed according to Guzmán et al. (2008).

3. Results

Experiment 1: Monthly day/night differences in plasma sexual steroids (E₂ and T)

In the first experiment, which aimed to investigate differences in steroid plasma concentrations between day and night during one year, the results pointed to no such fluctuations in either E₂ or T, although a significant annual rhythm was observed for both steroids. Maximal concentrations for both E₂ and T were obtained in spring, before spawning period. In the case of E₂ (Fig. 1a), in the first sampling months (December and January) the plasma concentrations remained low both for MD and ML (around $1.1 \pm 0.2 \text{ ng ml}^{-1}$). In February they started to increase, reaching a maximum in March ($6.1 \pm 1.7 \text{ ng ml}^{-1}$ at MD and $4.0 \pm 0.6 \text{ ng ml}^{-1}$ at ML). In April, the values started to fall again, reaching a minimum in August ($0.3 \pm 0.3 \text{ ng ml}^{-1}$ at MD and $0.2 \pm 0.1 \text{ ng ml}^{-1}$ at ML), when water temperature was highest, remaining low the rest of the months. Statistically significant differences among sampling months were found, but not between ML and MD samplings within the same month (ANOVA, Duncan's test, $p < 0.05$). The COSINOR analysis revealed the existence of a significant sinusoidal rhythm of annual plasma E₂ in female sole ($p < 0.01$), with a mesor of 1.4 ng ml^{-1} , an amplitude of 1.5 ng ml^{-1} and an acrophase in February (fig 1c_[c1]).

The values of T for samplings varied between 0.1 and 1.4 ng ml^{-1} (fig 1b). The rhythm was similar to that of E₂, the difference in this case being that the concentrations started to rise one month earlier, in January ($0.8 \pm 0.2 \text{ ng ml}^{-1}$ at MD and $0.9 \pm 0.4 \text{ ng ml}^{-1}$ at ML). Values remained high until March ($1.4 \pm 0.4 \text{ ng ml}^{-1}$ at MD and $0.8 \pm 0.1 \text{ ng ml}^{-1}$ at ML), and in April started to descend. During the rest of the months T remained basal. Statistically significant differences among sampling months were found, but not between ML and MD samplings within the same month (ANOVA, Duncan's test, $p < 0.05$). The COSINOR analysis revealed the existence of a significant sinusoidal rhythm of annual plasma T in female sole ($p < 0.01$), with a mesor of 0.4 ng ml^{-1} , an amplitude of 0.4 ng ml^{-1} and an acrophase in February (fig 1c).

A total of six spawning events were observed, from 12th May to 9th June, when temperature ranged from 20 to 21 °C, with a mean volume of $69.2 \pm 44.8 \text{ cm}^3$. The total volume of eggs recovered during the spawning period was 415 cm^3 . In all cases, there was a total absence of egg fertilization.

Experiment 2: Daily rhythms of plasma sexual steroids (E₂ and T) in different seasons

As to the daily rhythms of E₂ and T, high values were only registered for both at Spring Equinox (fig. 2 and 3). In the other three 24h-samplings (summer and winter solstices and autumn equinox) values were low, and no daily rhythms could be described. In the case of E₂, at the spring equinox, values varied from $2.1 \pm 1.5 \text{ ng ml}^{-1}$ at 6:30 h to $6.6 \pm 2.6 \text{ ng ml}^{-1}$ at 18:30 h (fig 2a), a three-fold increase in 12 hours. The COSINOR analysis revealed the existence of a significant sinusoidal rhythm of daily plasma E₂ in female sole ($p < 0.01$), with a mesor of 4.4 ng ml^{-1} , an amplitude of 2.0 ng ml^{-1} and an acrophase at 20:00 h (fig 2e_[c2]). As regards to T, values varied between $0.7 \pm 0.2 \text{ ng ml}^{-1}$ at 9:30 and $1.5 \pm 0.6 \text{ ng ml}^{-1}$ at 21:30 h at the spring equinox (fig 3a), a two-fold increase within the same day. The COSINOR analysis revealed the existence of a significant sinusoidal rhythm of daily plasma T in female sole at the spring equinox ($p < 0.01$) with a mesor of 1.0 ng ml^{-1} , an amplitude of 0.4 ng ml^{-1} and an acrophase at 21:08 h (fig 3e_[c3]).

Experiment 3: Influence of an attenuated thermal cycle on the seasonal production of plasma sexual steroids (E₂ and T)

In the third experiment, the influence of the natural annual fluctuation of water temperature on the annual rhythm of steroids was studied. For both E₂ and T, a strong influence was seen (fig 4). In the case of the E₂, the concentration pattern was arrhythmic in the group maintained under an attenuated thermal cycle throughout the year (well-water group), unlike the group maintained under the natural thermocycle (surface water group) (fig 4a). For well-water group, values remained low in all samplings (between $0.2 \pm 0.1 \text{ ng ml}^{-1}$ in autumn and $1.4 \pm 0.4 \text{ ng ml}^{-1}$ in spring), and did not increase in spring like surface water group ($5 \pm 0.9 \text{ ng ml}^{-1}$). No significant statistical differences were observed between any of the sampling points in well-water group.

As regards T, once more the concentration rhythm differed between the two groups. There were no statistical differences within the well-water group and consequently no concentration surge in winter and spring as in the surface water group. In the winter sampling, plasma levels were significantly lower ($0.3 \pm 0.1 \text{ ng ml}^{-1}$) than those in surface water group ($0.9 \pm 0.2 \text{ ng ml}^{-1}$), while in spring the T concentration rose to reach similar levels to those registered in surface water group. In summer, the values were higher, but not statistically different in well-water group, and finally in autumn, both groups showed low levels of T_[c4].

Discussion

This research attempted to detect within-day fluctuations of plasma sex steroids in Senegal sole female, studying the day/night concentrations each month and the seasonal daily rhythms of E₂ and T. The fact that ML/MD differences in steroid concentrations could not be detected in the first experiment does not necessarily mean that they did not exist; they may well have been located at another moment of the day. Actually, the acrophases of the daily rhythms were located at 20:00h in E₂ and at 21:08h in T, half way between ML and MD. However, this kind of experimental design (the use of two sampling points with 12h interval) does permit the acquisition of reliable mean daily values, even though the daily rhythm may shift seasonally.

An annual rhythm for E₂ and T was observed, with maximum values registered in March, before the spawning period that occurred in May-June, and minimal values in August, when the temperature was highest (25 °C). It is important to note that our samplings were performed around the 25th day of each month, so that high steroid concentrations observed in March corresponded to the end of the month, and probably continued into April. A similar pattern of sex steroids in F1 Senegal sole female was obtained by Guzmán et al. (2008): both steroids reached maximum concentrations in the pre-spawning period (March and April, respectively, for T and E₂) and decreased thereafter. As in our study, T concentrations rose earlier than E₂, which is normal since T is known to be a biosynthetic precursor of E₂ (Kagawa et al., 1982). The peak of E₂ in anticipation of the spawning season is in accordance with the established role of E₂ in initiating and maintaining vitellogenesis and regulating vitellogenin synthesis (Wallace, 1985). In the case of a Senegal sole wild broodstock, the maximum values of E₂ and T were registered slightly earlier, in February, but in this case the spawning season also started earlier, in January in one year and in March the next (García-López et al., 2006b). These results are basically in accordance with ours, despite the slight differences in timing, which may have been due to different years, different geographic or climate conditions, and the origin of the broodstock.

In other teleost species, annual steroid rhythms have been detected. For example, in common dentex (*Dentex dentex*), E₂ and T levels peaked in April, coinciding with the spawning season (Pavlidis et al., 2000), or in European sea bass (*Dicentrarchus labrax*) in which sex steroids reached maximum values from December to February (Prat et al., 1990, Mañanós et al., 1997). These results support the known seasonality of fish reproduction and

the synchronization of this phenomenon to seasonal environmental changes, according to species requirements.

In the second experiment, a daily steroid rhythm was described in Senegal sole. Daily rhythms of both E₂ and T plasma concentrations were observed during the spring equinox, with acrophases occurring in the evening. In the case of E₂ concentrations, a three-fold increase was observed within 12 h, while T concentrations increased two-fold. These results highlight the importance of the time of the day when sampling for steroids. To avoid inconsistencies, these fluctuations must be considered, as well as their seasonality. Furthermore, for hormonal induction studies, the time of the day when the hormone is administered should be carefully considered, since different timing may lead to different responses.

The timing of the acrophases of both steroids was 20:00 h (E₂) and 21:08 h (T), which curiously approached to the acrophase of the daily spawning rhythm in this species. Recent research from our group, revealed a daily spawning rhythm in senegal sole during spring, its acrophase occurring at night, around 23:00h (Oliveira et al., 2008). These findings suggest a strong nocturnal pattern in the reproduction of this species, matching the nocturnal behavioral patterns of sole (Bayarri et al 2004a), which has never been regarded to date.

The daily variations in sexual steroids in fish are short-term fluctuations, which are associated with the maturation of different groups of oocytes at intervals of days during the spawning season (Peter and Yu, 1997). In other fish species, daily patterns of sexual steroids have been described. According to Bayarri et al. (2004b), in sea bass, *Dicentrarchus labrax*, besides a daily rhythm of plasma T concentration in males, there was also evidences of daily rhythms of plasma LH and of pituitary LH and GnRH. In the case of plasma LH, the daily rhythm was in phase with the plasma melatonin rhythm, presenting higher values during the night, after the nocturnal increase of melatonin. In red sea bream (*Pagrus major*) females, plasma E₂ levels reached a peak at 7:00 h in fish with pre-mature stage oocytes, out of phase with spawning, which mainly occurred between 18:00 h and 19:00 h. (Matsuyama et al., 1988). Also in snapper (*Pagrus auratus*) there was evidence that levels of both E₂ and T changed according to the time of day during the spawning season (Carragher and Pankhurst, 1993). Finally, the Japanese whiting (*Sillago japonica*) also presented a diurnal pattern of plasma E₂ (Matsuyama et al., 1990).

Curiously enough, the acrophases in the annual and daily rhythms of steroids preceded those of melatonin by Vera et al. (2007). In the annual rhythm the acrophase of the melatonin rhythm was located in July, which was 5 months later than the corresponding acrophase of E₂

and T. As regards the spring daily rhythm, the same pattern was repeated: the melatonin acrophase was located at 0:30 h, exactly 4.5 and 5.5 h after E₂ and T acrophases respectively. This appears to be the repetition of the same phenomenon at different scales.

Seasonal changes in water temperature seemed to be a key player in synchronizing annual rhythms of steroids in female sole. We observed that the steroid rhythm disappeared in conditions of attenuated thermo cycle (well-water group), since the annual pattern of plasma E₂ and T did not present the typical spring surge. This agrees with the fact that well-water group did not spawn during the experimental period. In this group the values of E₂ and T did not increase more than 1.4 ± 0.4 and 0.7 ± 0.1 ng ml⁻¹, respectively, while in surface water group (natural fluctuating temperature) they reached values of 5 ± 0.9 and 1.1 ± 0.2 ng ml⁻¹, respectively. According to Patiño and Thomas (1990) and Patiño et al. (2001), a certain threshold of E₂ and T plasma levels is required to trigger final oocyte maturation, which may explain the lack of spawning events in well-water group. In addition, water temperature is known to have direct effects on certain aspects of steroidogenesis and rates of steroid hormone metabolism and clearance in teleosts (Van Der Kraak and Pankhurst, 1997; Pankhurst and Porter, 2003), which, under suboptimal conditions, may have the potential to disrupt oocyte development. In a recent study (García-López et al., 2006a), a group of sole were maintained under natural photoperiod and attenuated thermo-cycle (18-20 °C), and in this case females did present E₂ and T rhythms, with a premature reduction and lower values in comparison with the control group, but with a parallel surge and a peak in both steroid concentrations. The same did not happen in our study, since values remained statistically low during all seasons, and no surge was observed in any of the steroid concentrations. These differences could be justified by differences in temperature fluctuations, rearing conditions, origin of sole or geographic location (Mediterranean vs Atlantic). Although the steroid rhythm differed between these two sole groups maintained under conditions of minimal water temperature fluctuation, in both cases animals failed to spawn, which highlights the importance of the temperature cycle for the resumption of spawning.

According to Vera et al. (2007), the water temperature modulates nocturnal melatonin rhythm in Senegal sole, and in the absence of the natural fluctuating regime of temperature, nocturnal plasma concentrations of this hormone were constant during the whole year. As this hormone is known as a “Clock/Calendar” hormone (Reiter, 1993), this could explain the absence of steroid rhythm: without the melatonin signal, fish do not receive seasonal information, and can not synchronize rhythms, including reproduction. Furthermore, Amano et al. (2000) studied the possibility of mimicking a short photoperiod through melatonin

administration. Melatonin treatment had a stimulatory effect on the gonadosomatic index, follicle stimulating hormone contents, and plasma T and 11-ketotestosterone concentrations.

In summary, we may conclude that sole revealed an annual rhythm in plasma E₂ and T, their acrophases being located in February, anticipating the spawning season (May-June). At the spring equinox, a daily rhythm of both these steroids was described, with an acrophase located at 20:00 h for E₂ and 21:08 h for T, preceding again spawning, which occurs during the night. In addition, the annual fluctuating water temperature seemed to be a crucial synchronizer of the reproduction rhythms in Senegal sole female, since, in the absence of this environmental signal, the steroid rhythm disappeared, and spawning did not occur.

Acknowledgments

This research was funded by the MEC (Spanish Ministry of Education and Science) project *Reprosolea* (AGL 2004-07984-C02-01) to FJSV and by the MAPA (Ministry of Agriculture, Fisheries and Food) project JACUMAR 2006 (II National plan for the cultivation of sole) to EM. CO received a FPI Fellowship from the MEC. The authors would also like to thank PISCIMAR S.L. for the use of its facilities and fish during sampling.

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Figure Captions

Figure 1. Annual rhythm of plasma estradiol (a) and testosterone (b) levels at mid-dark (black bars) and mid-light (white bars); and polar representation of the COSINOR analysis, depicting clockwise the annual cycle (c). In (a) and (b), the months are indicated at the bottom and grey horizontal bars indicate spawning period. Upper line represents water temperature and different letters indicate statistically significant differences between sampling months (MD and ML values pooled) (ANOVA, Duncan's test, $P < 0.05$). No differences were detected between MD and ML within the same month. In (c), the vectors point to the moment each acrophase occurred, and its length represents the amplitude. The ellipses represent the confidence interval.

Figure 2. Daily plasma estradiol rhythm at the spring equinox (a), summer solstice (b), autumn equinox (c) and winter solstice (d), and polar representation of the Cosinor analysis for spring equinox, depicting clockwise the daily cycle (e). In (a) to (d) the black and white

bars correspond to sampling points during the day or night, respectively. The continuous line in Spring equinox graph represents the adjustment of the sinusoidal rhythm. Water temperature on the sampling day is indicated at the bottom. In (e) the vector points to the moment the acrophase occurred, and its length represents the amplitude. The ellipse represents the confidence interval.

Figure 3. Daily plasma testosterone rhythm at the spring equinox (a), summer solstice (b), autumn equinox (c) and winter solstice (d) and polar representation of the Cosinor analysis for spring equinox, depicting clockwise the daily cycle (e). In (a) to (d) the black and white bars correspond to sampling points during the day or night, respectively. The continuous line in Spring equinox graph, represents the adjustment of the sinusoidal rhythm. Water temperature on the sampling day is indicated at the bottom. In (e) the vector points to the moment the acrophase occurred, and its length represents the amplitude. The ellipse represents the confidence interval.

Figure 4. Comparison between plasma estradiol (a) and testosterone (b) levels from fish submitted to the environmental temperature cycle (mid-dark: black bars, mid-light: white bars) and reared under attenuated thermo-cycle (mid-dark: dotted black bars, mid-light: dotted white bars). The upper continuous and dotted lines represent the water temperature of fish reared under natural and attenuated thermo-cycle, respectively. Different letters indicate statistically significant differences (ANOVA, Duncan's test, $P < 0.05$).

E2
Acrophase 20:00h
Mesor 4.4 ng/ml
Amplitud 2.0 ng/ml
p 0,005

(e)

	T
Acrophase	21:08h
Mesor	1.0 ng/ml
Amplitud	0.4 ng/ml
p	0,008