SEASONAL AND DAILY PLASMA MELATONIN RHYTHMS AND REPRODUCTION IN SENEGAL SOLE KEPT UNDER NATURAL PHOTOPERIOD AND NATURAL OR CONTROLLED WATER TEMPERATURE

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ABSTRACT

The melatonin daily rhythm provides the organism with photoperiod-related information and represents a mechanism to transduce information concerning time of day. In addition, the duration and amplitude of the nocturnal elevation gives information about duration and thus the time of year. In this study we investigate the existence of an annual rhythm of plasma melatonin in the Senegal sole. Differences in plasma melatonin levels between fish kept at a controlled temperature (17-20 ºC) and those exposed to the environmental temperature cycle (11.5-25 ºC) were also examined throughout the year. Spawning was registered in both groups to determine the time of year in which reproductive rhythms occurred. Our results pointed to the existence of an annual rhythm of plasma melatonin at mid-darkness (MD), with the highest levels (203 ± 44 pg/ml) observed when water temperature reached 25 ºC. Water temperature influenced nocturnal, but not diurnal melatonin. Daily melatonin rhythms showed seasonal differences, with higher mean nocturnal levels during the summer solstice (138 ± 19 pg/ml) and autumn equinox (149 ± 49 pg/ml). When animals were kept at a constant temperature throughout the year, plasma melatonin levels differed from those observed in fish exposed to the environmental temperature cycle. Regarding the reproductive rhythms, spawning was observed at the end of spring in sole kept under natural temperature conditions, whereas no spawning at all was registered in sole reared at a constant temperature. In short, both photoperiod and temperature affected melatonin

production in the Senegal sole, transducing seasonal information and controlling annual reproductive rhythms.

INTRODUCTION

Animals receive photoperiod information through the pineal gland, which transduces environmental light cycles into a chemical signal, melatonin. In all the vertebrates studied to date, melatonin is produced during the dark phase, as light inhibits its secretion by the pineal. Melatonin biosynthesis is regulated by both photoreceptors that receive environmental light stimuli, and endogenous oscillators. In several non-mammalian species, the pineal gland functions as an independent photoneuroendocrine system, since it is involved in photoreception, endogenous rhythm generation and the production of melatonin [1]. Daily melatonin rhythms inform the organism about the time of day, whereas the duration of the nocturnal elevation provides information concerning the time of year, since photoperiod length varies with the season [2].

Besides light, temperature has also been reported to modulate melatonin production in ectotherms, influencing the amplitude of the melatonin rhythm and so helping to provide the organism with accurate seasonal information [3]. For instance, in Atlantic salmon, plasma melatonin was significantly higher in fish maintained at 12ºC than in those kept at 4ºC [4] while in European sea bass, the photoperiod length controlled the duration of the nocturnal melatonin increase, and the water temperature determined the amplitude of the melatonin rhythm [5].

In animals, seasonal changes in both photoperiod and temperature synchronize annual rhythms, including reproduction. In fish, the optimal spawning time of long-day breeders is during spring or summer, and this can be stimulated by extending the photoperiod. However, in salmonids and other species (short-day breeders), spawning takes place in autumn and winter, under a reducing photoperiod [6]. The pineal gland transduces environmental information into a melatonin rhythm, which act on the hypothalamic-pituitary-gonadal axis, controlling the timing of reproduction [7,8].

The sole, *Solea senegalensis*, is a marine fish of great aquacultural interest, especially in southern Portuguese and Spanish coasts [9]. Daily rhythms of plasma melatonin have been investigated previously in this species. They showed the typical plasma melatonin profile of vertebrates: low levels during the day time and high values during the night. Indeed, when a light pulse was applied at mid-darkness (MD), plasma melatonin decreased [10]. Other studies on the Senegal sole have revealed that temperature seems to play an important role in controlling reproduction [11]. Nevertheless, seasonal melatonin rhythms have not been studied in this species, and nor has the influence of temperature.

The aim of this study was to investigate the annual rhythms of plasma melatonin and seasonal variations in daily rhythms in Senegal sole kept under a natural photoperiod and in water at environmental temperature (11.5-25 ºC) or under the same photoperiod and water maintained at 17-20 ºC throughout the year. Finally, spawning was registered to determine the time of spawning in both groups.

MATERIALS AND METHODS

Animals and housing

For this study a total of 269 sole of 1,284 ± 53 g bw were used. Most of them (204) were housed at the research centre “Instituto de Acuicultura de Torre la Sal” (40º N 0º E,

Castellón, Spain), where the animals were kept in round 3,000 L fibreglass tanks located in a shed and provided with a constant flow of running marine water from the Mediterranean Sea. The fish were exposed to attenuated natural sun light and maximum light intensity on the water surface was 600 lux. Animals were fed with a mixture of mussels and squid four days a week, at a rate of 1.5% of the tank biomass.

The rest of the animals (64) were reared in a local fish farm (Piscimar S.L., Castellón, Spain), where they were kept in a round 16,000 L fiber glass tank covered with a light retention plastic. Maximum light intensity on the water surface was 870 lux. The water temperature in this case was maintained at 17-20ºC throughout the year, since the water was obtained from a well, the water of which had an almost constant temperature. Fish were fed *ad libitum* with both commercial feed (Proaqua Solea immunofeed, PROAQUA S.A., Spain) and a mixture of mussels and squid. The first was provided five days a week whereas the second was given twice a week.

Each sole was labeled with an internal passive integrated transponder (PIT) tag system, enabling the individual identification of fish in repeated samplings. Each tank was provided with an egg collector that was checked daily and, when necessary, the total number of eggs was estimated volumetrically.

Experimental design

Annual rhythm of plasma melatonin. For this experiment, twelve animals (7 females and 5 males) from the "Instituto de Acuicultura de Torre la Sal" were used. Blood samples were taken at mid-light (ML) and mid-night (MD) every month during a year. During the dark phase, sampling was carried out under a dim red light and after covering the fish head with aluminium foil. Before blood withdrawal, animals were individually anaesthetized with water containing 0.5 ml/l 2-phenoxiethanol. As soon as the fish lost their equilibrium, they were

weighed and 1 ml of blood was withdrawn from the caudal vein using heparinized syringes. Blood samples were transferred to polypropylene Eppendorf tubes for centrifugation at 4 ºC, and the resulting plasma samples were frozen and stored at -80 ºC until analysis.

Spawning was monitored by collecting eggs, which were classified as viable or not, and the percentage of viability was calculated as:

$$\% V = (\text{volume of floating eggs} / \text{total volume of eggs}) \times 100$$

Floating eggs were incubated in 5 L sieves submerged in 250 L tanks of filtered and aerated sea water maintained between 16 and 18 ºC.

Seasonal variations of daily rhythms of plasma melatonin. At the winter and summer solstices and at the spring and autumn equinoxes, fish from the "Instituto de Acuicultura de Torre la Sal" were sampled. A total of 48 x 4 fish were used and blood samples were taken every 3 h for 24 h to study daily plasma melatonin rhythm. These samples were taken and processed as described above. Special care was taken to avoid fish being exposed to light at night, so sampling was performed under a dim red light and after covering the fish head with aluminium foil.

Influence of constant water temperature on plasma melatonin**.** In this experiment, we looked at plasma melatonin levels in sole maintained under controlled water temperature. To this end, we used fish from a local fish farm, Piscimar S.L., where animals were provided with sea water at 17-20 ºC throughout the year. Each season, eight fish were sampled at ML and MD (group I), and their plasma melatonin concentrations were compared with those obtained on the same date in animals reared with a natural water temperature (group II). Blood samples were taken and stored until melatonin analysis as described above.

Melatonin analysis

Melatonin was determined using a commercial radioimmunoassay (RIA) kit (IBL, Hamburg, Germany) [12].

Data analysis

Data analysis was performed with the aid of the statistical program SPSS and Microsoft Excel. Statistical differences between mean melatonin concentrations were analyzed by one-way analysis of variance (ANOVA) followed by a Duncan's test, with $P < 0.05$ taken as the statistically significant threshold. To test the existence of correlation between plasma melatonin levels and increasing temperature, a linear regression was performed. The annual plasma melatonin rhythm was determined using Cosinor analysis.

RESULTS

Plasma melatonin varied during the year, influenced by both photoperiod and seasonal water temperature changes. Statistically significant differences in MD melatonin levels were found between different months (ANOVA, $P< 0.001$) (Fig. 1). From January to March, the average plasma melatonin concentration at night was 91 ± 4 pg/ml while the water temperature ranged from 11.5 to 13.6ºC. In April, the water temperature started to increase, and the mean value for nocturnal plasma melatonin from April to June increased to 142 ± 15 pg/ml, the water temperature ranging between 16.7 and 20.5ºC. In August we registered the highest water temperature (25ºC) and nocturnal plasma melatonin concentrations (203 ± 44 pg/ml). From September to November the temperature started to fall, as did plasma melatonin levels at night (127 ± 23 pg/ml). In July and October fish were not sampled to avoid the outbreak of any pathology. Finally, in December both water temperature and nocturnal plasma melatonin levels dropped to 14.5ºC and 103 ± 46 pg/ml, respectively. As regards ML, curiously, plasma melatonin remained between 11-28 pg/ml throughout the year, except in

November. Plasma melatonin levels at MD were plotted against water temperature, and linear regression confirmed the existence of a positive correlation between higher plasma melatonin and increasing water temperature. Note that only MD melatonin correlated with water temperature, while ML melatonin did not (Fig. 2).

The Cosinor analysis revealed the existence of a significant sinusoidal rhythm of annual plasma melatonin in sole ($P<0.001$), with mesor (130 pg/ml), amplitude (44 pg/ml) and acrophase (July). When the Cosinor analysis was performed for the annual temperature rhythm, the acrophase was also mid July, indicating that melatonin rhythm and temperature cycle were in phase. However, the photophase cycle was out of phase since its acrophase (summer solstice) occurred one month earlier.

A total of 6 spawning events were observed, from mid May to mid June, when temperature ranged from 20 to 21 ºC. The total volume of eggs recovered during the spawning period was 415 cc. In all cases, embryonic development stopped 24 h after spawning (Table I).

Daily melatonin peaks were nocturnal in all cases, although some differences in the daily profile were detected during the year. At spring equinox, plasma melatonin peaked in the last third of the scotophase (120 ± 24 pg/ml), the mean value during the dark phase being 88 ± 10 pg/ml. One hour before daybreak, plasma melatonin levels started to fall, reaching their lowest levels during the photophase (around 22-44 pg/ml). Statistically significant differences among sampling times were found (ANOVA, $P < 0.001$) (Fig. 3A).

At the summer solstice, plasma melatonin peaked at the beginning of the night (172 ± 34 pg/ml) and after it decreased slightly, dropping once again one hour before the lights came on. The average nocturnal melatonin value was 138 ± 19 pg/ml. In this case, the nocturnal elevation of plasma melatonin was shorter than at the spring equinox, since the night itself

was shorter. Statistically significant differences among sampling points were found (ANOVA, $P < 0.001$) (Fig. 3B).

At the autumn equinox, plasma melatonin peaked one hour after night fell reaching a concentration of 268 ± 64 pg/ml. However, after this sampling point melatonin dropped, so that the mean melatonin value during the night was 149 ± 49 pg/ml. Indeed, it was observed that two hours before day break plasma melatonin descended (68 ± 31 pg/ml), anticipating the light. Statistically significant differences among sampling points were found (ANOVA, $P < 0.001$) (Fig. 3C).

At the winter solstice, plasma melatonin showed an atypical daily profile. One hour after night arrived, melatonin peaked at 304 ± 9 pg/ml, however after which it dropped sharply to remain at 79-155 pg/ml. Statistically significant differences were found between the first sampling point during the scotophase and the rest of sampling points (ANOVA, $P < 0.001$) (Fig 3D).

Concerning the influence of constant water temperature on plasma melatonin, we observed that differences on plasma melatonin levels between group I and II depended on the season. In winter, animals from group I were kept at 17 ºC, while those from group II were kept at 13.2 ºC, resulting in plasma melatonin levels at MD of 136 ± 38 pg/ml and 88 ± 25 pg/ml, respectively. As regards ML, plasma melatonin levels were higher in group I (40 ± 13 pg/ml) than in group II (11 ± 2 pg/ml) (ANOVA, $P<0.001$).

In spring, however, when differences in water temperature were smaller (19 ºC in controlled conditions vs 16.7 ºC in sea water) plasma melatonin levels in both groups were around 120 pg/ml at MD and 20-40 pg/ml at ML. In summer, differences in water temperature increased again (20.0ºC for group I and 25.0ºC for group II), resulting in plasma melatonin levels at MD of 103 ± 31 pg/ml (group I) and 203 ± 44 pg/ml (group II), the

difference being statistically significant (ANOVA, $P < 0.001$). At ML, however, melatonin concentrations remained at around 20 pg/ml in both cases.

Finally, in autumn differences in water temperature were small (18.4 ºC in controlled conditions vs 17.2 ºC in environmental conditions) and plasma melatonin levels at ML and MD were similar in both groups, around 85 and 104 pg/ml respectively. At ML, we observed unusually high melatonin levels and no statistically significant differences between groups or between ML and MD were found (Fig. 4).

In so far as reproductive rhythms in sole are concerned, those kept in controlled temperature showed no spawning events despite the fact that females were seen to be sexually mature.

DISCUSSION

For the first time the existence of an annual rhythm of plasma melatonin at MD was demonstrated in Senegal sole, appearing a positive correlation between increasing temperature and melatonin concentration, as well as seasonal differences, with mean nocturnal levels in summer and autumn being higher than those in winter and spring. Moreover, when Senegal sole were kept all year round at controlled temperature significant differences were obtained in the plasma melatonin levels. Finally, the spawning time began at the end of spring in sole kept under environmental temperature, whereas no spawning events were registered in sole maintained at controlled temperature.

The parallelism between higher plasma melatonin levels of sole (MD) and increasing water temperature supports previous findings in the pineal of other fish cultured *in vitro*. For instance, in the lamprey *Lampetra japonica* a typical melatonin daily rhythm was observed under light-dark (LD) conditions at 20 ºC, but at 10 ºC and 7 ºC, the melatonin rhythm disappeared [13]. In pike pineals cultured at 10 ºC, the nocturnal melatonin elevation

increased when a 20 ºC temperature pulse was applied during the dark phase. Indeed, under constant darkness (DD), temperature cycles synchronized the melatonin rhythm, which peaked with the highest temperature [14]. It is of note that sole are ectotherm animals and that temperature influences biochemical reactions such as the synthesis of melatonin in which the arylalkylamine *N*-acetyltransferase (AANAT) converts serotonin into *N*-acetylserotonin [15]. For example, in northern pike *Esox lucius* pineal AANAT activity increased with increasing temperatures (4 ºC to 56 ºC), optimal values occurring between 17 ºC and 20 ºC, after which it gradually decreased [16]. Furthermore, temperature influences molecular and cellular processes such as membrane properties, ion homeostasis, calcium influx, and signal cascades (cAMP, cGMP, and protein kinases A and C) which may affect protein phosphorylation processes of the circadian clock mechanism [17].

The fact that in our experiment ML plasma melatonin levels during the year did not differ with increasing temperature could be explained by the light inhibition of melatonin production in the pineal organ. The LD cycle could act as the main factor influencing melatonin synthesis by the pineal, whereas temperature could modulate this production. This may not always be the case, since temperature increased melatonin synthesis during darkness and increased the sensitivity of the response to light in the trout pineal [18].

Concerning daily rhythms of plasma melatonin, we observed that the shape of the nocturnal elevation differed between seasons. At the summer solstice and autumn equinox a melatonin peak was observed in the first hours of the night, which is in accordance with previous results obtained in this species [10]. However, at the spring equinox we observed a plasma melatonin peak at the end of the night, as observed in Atlantic cod [19]. Other seasonal differences were found for the amplitude and length of the nocturnal elevation. On the one hand, we observed the highest values of nocturnal melatonin at the autumn equinox and summer solstice, when the temperature was 19.5 ºC and 21.0 ºC, respectively. On the

other hand, the nocturnal elevation was shorter at the summer solstice, when night was also shorter. We expected to find the longest nocturnal elevation at the winter solstice, but in fact, an atypical plasma melatonin profile was recorded, probably due to the altered light intensity during the sampling day, when it was a raining.

Our results concerning seasonal variations of melatonin production are consistent with those described for other species. For example, in Atlantic salmon *Salmo salar* it was observed that the nocturnal increase of plasma melatonin reflected the duration of the dark phase. Indeed, this increase was higher in samples taken in June and August, when water temperature ranged from 13 to 19 ºC, than in other months when water temperature was between 1.75 and 8.25 ºC [20]. The same conclusion has been drawn from results obtained in several fish species: photoperiod determines the length of the nocturnal melatonin elevation, while water temperature controls its amplitude [5, 21]. The differences observed in plasma melatonin levels in sole kept at controlled temperature *versus* sole maintained under natural temperature oscillations support this idea.

The pineal organ transduces both photoperiod and temperature into melatonin production, which strongly influence reproduction rhythms in Senegal sole [11]. In sea bass *Dicentrarchus labrax*, it was suggested that melatonin is involved in the LH elevation during the dark period [7]. In catfish *Heteropneustes fossilis* maintained during prespawning and spawning periods in LD 12:12, melatonin injections inhibited ovarian weight, pituitary gonadotropic hormone (GTH) cells and oocyte maturation [22]. However, in Senegal sole further studies are needed to reveal the specific influence of melatonin on reproductive cycles as well as the neuroendocrine pathways involved in such control.

Spawning at the end of spring (when water temperature was 20-21 ºC) was observed in sole maintained in fluctuating environmental water temperatures. This finding is in accordance with previous results obtained in this species when located in the Gulf of Cadiz

(south of Spain), which showed that spring is the main spawning period with some occasional spawning during autumn and winter [11]. However the spawning period was shorter, probably because the sole used for our study did not come from the wild but were obtained in captivity from a previous stock. This may explain the low spawning rates and survival of eggs observed in our experiments.

In sole kept at a controlled temperature, no spawning events were observed at all, which lends weight to the theory that natural temperature variations are required to induce spawning in this species [11], as in southern flounder [23]. The importance of temperature controlling reproductive maturation of striped bass (*Morone saxatilis*) has been observed in females exposed to seasonally-changing water temperature, but invariable photoperiod, which showed profiles of circulating sex steroids and vitellogenin similar to females kept under natural photothermal conditions. However, under constant temperature, but with seasonal changes of photoperiod, females and males showed abnormal endocrine profiles and patterns of oogenesis and spermiation [24].

To conclude, our results revealed the existence of both an annual plasma melatonin rhythm at MD, and seasonal differences in the daily plasma melatonin rhythm of Senegal sole. We also found differences in plasma melatonin at MD between fish kept in naturally fluctuating temperature and fish kept in controlled water temperature, which failed to spawn. It seems that some sort of natural variations rather than constant levels are required for normal reproduction, pineal and melatonin rhythms transducing such variations and triggering spawning.

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FIGURE LEGENDS

Fig. 1. Annual rhythm of plasma melatonin levels at ML (white bars) and MD (black bars). The upper continuous and dotted lines represents the water temperature and photoperiod, respectively. Months are indicated at the bottom. Different letters indicate statistically significant differences (ANOVA, $P< 0.001$).

Fig. 2. Correlation between plasma melatonin levels and water temperature at ML (white circles) and MD (black squares). The figure shows the positive correlation between plasma melatonin at MD and increasing temperature.

Fig. 3. Daily plasma melatonin rhythm during the spring equinox (A), summer solstice (B), autumn equinox (C) and winter solstice (D). Black and white bars at the top represent the night and day, respectively. Water temperature during the sampling day is indicated at the bottom. Different letters indicate statistically significant differences (ANOVA, $P<0.001$).

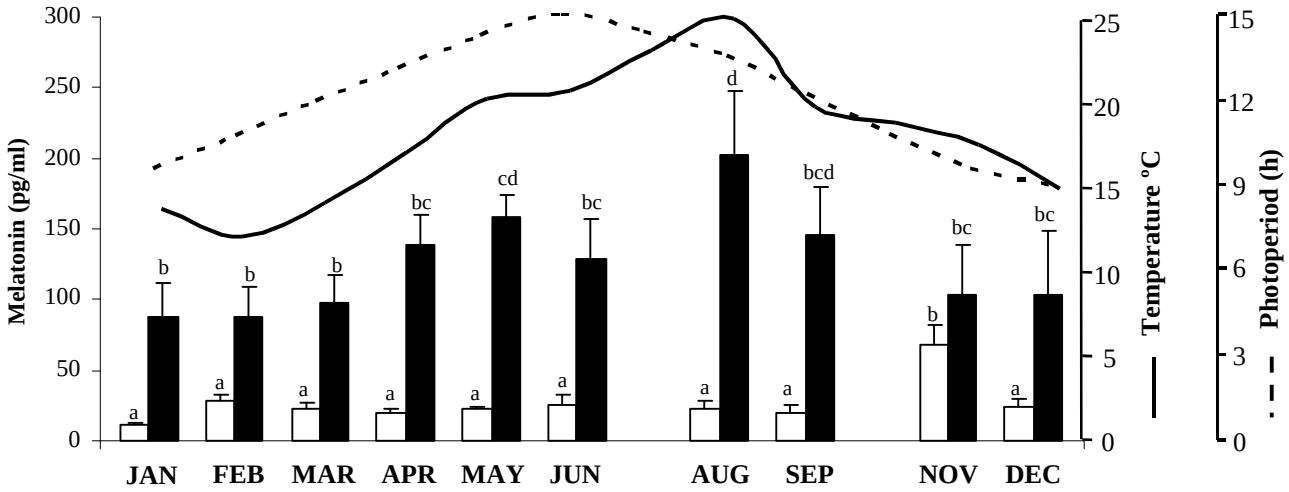
Fig. 4. Comparison between plasma melatonin levels from fish submitted to controlled temperature (ML: dotted white bars, MD: dotted black bars) and reared under the environmental temperature cycle (ML: white bars, MD: black bars). The upper continuous and dotted lines represents the water temperature of fish reared under natural and controlled temperature, respectively. Different letters indicate statistically significant differences (ANOVA, $P<0.001$).

Table I.

SPAWNING EVENTS

Spawning date	Total volume of eggs (cc)	Volume of floating eggs (cc)	Viability (%)
12th May	30	0	0
21st May	25	0	0
24th May	70	30	42
26th May	150	90	60
30th May	70	0	0
9th June	70	10	14

Figure 1


Melatonin (pg/ml)
300
250
200
150
100
50
0
25
20
15
10
5
0
15
12
9
6
3
0
Temperature °C
Photoperiod (h)
a
b
a
b
a
b
a
bc
a
cd
a
bc
a
d
a
bcd
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a
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JAN
FEB
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JUN
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SEP
NOV
DEC

Figure 2

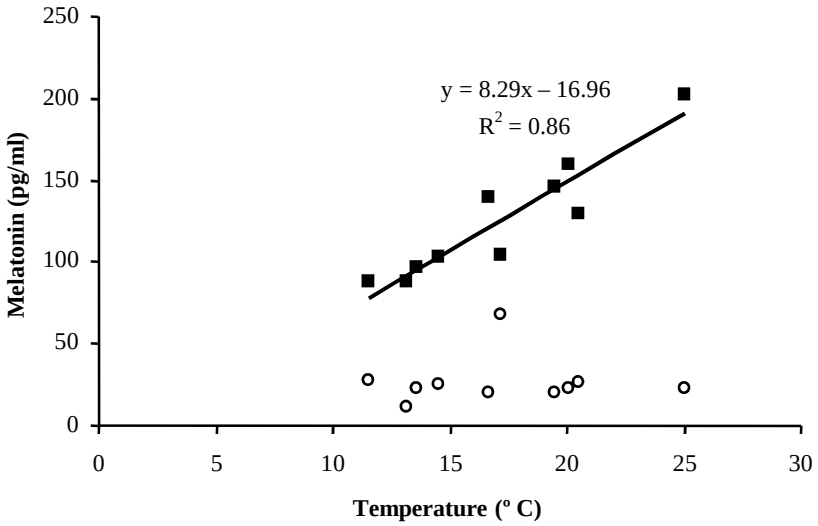

y = 8.29x – 16.96
R2 = 0.86
Melatonin (pg/ml)
Temperature (º C)
0
50
100
150
200
250
0
5
10
15
20
25
30

Figure 3

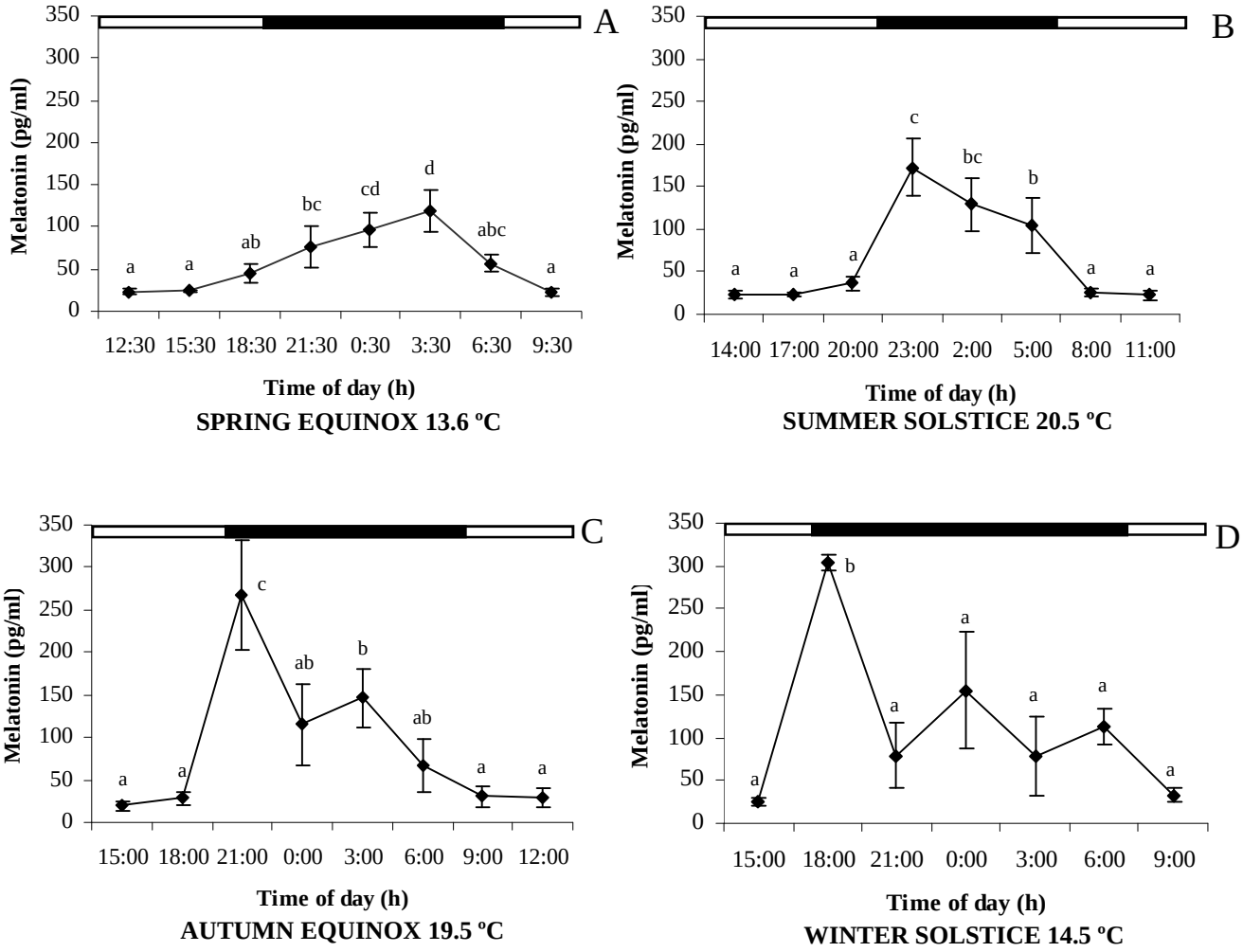
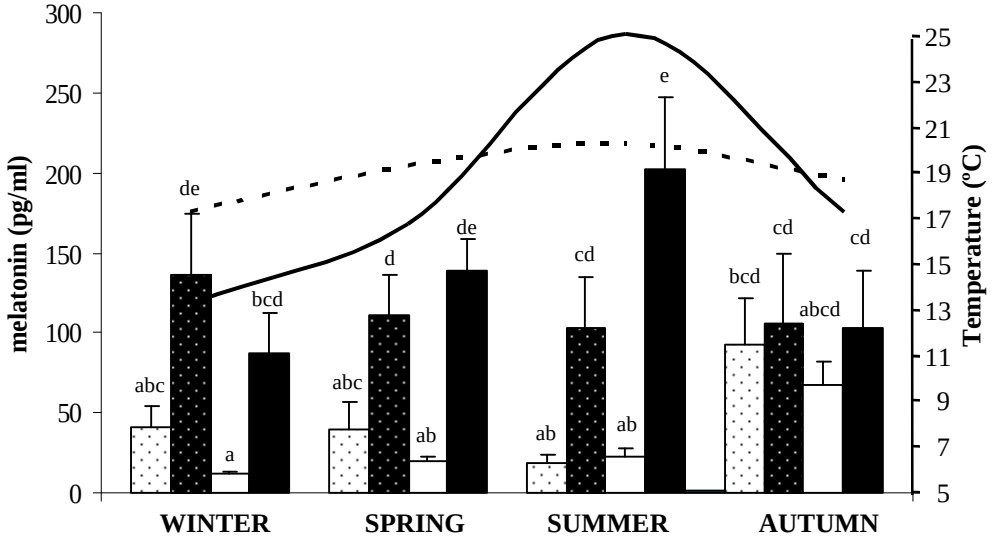

A
Melatonin (pg/ml)
350
300
250
200
150
100
50
0
a a ab bc cd d abc a
12:30 15:30 18:30 21:30 0:30 3:30 6:30 9:30
Time of day (h)
SPRING EQUINOX 13.6 ºC
B
Melatonin (pg/ml)
a a a c bc b a a
14:00 17:00 20:00 23:00 2:00 5:00 8:00 11:00
Time of day (h)
SUMMER SOLSTICE 20.5 ºC
C
Melatonin (pg/ml)
a a c ab b ab a a
15:00 18:00 21:00 0:00 3:00 6:00 9:00 12:00
Time of day (h)
AUTUMN EQUINOX 19.5 ºC
D
Melatonin (pg/ml)
a b a a a a a
15:00 18:00 21:00 0:00 3:00 6:00 9:00
Time of day (h)
WINTER SOLSTICE 14.5 ºC

Figure 4


melatonin (pg/ml)
Temperature (°C)
300
250
200
150
100
50
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abc
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WINTER
SPRING
SUMMER
AUTUMN