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Original Research Article

Causal link of total locomotor activity, melatonin and rectal temperature daily rhythm in small ruminants

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ABSTRACT

To improve the knowledge in chronophysiology we investigated the causal link between the most important physiological variable studied until now; ten Sarda ewes and ten Sarda goats, pluriparus not pregnant and no lactating, were used. Animals were housed under natural environmental conditions in a common stall, alfalfa hay and water were available ad libitum. Each animal was equipped with an Actiwatch-Mini® for recording total activity. Blood samples were collected every 4 h over a 48 h period for the assessment of melatonin concentration. Rectal temperature was recorded with a digital thermometer immediately before the blood sampling at each data point. Single cosinor method showed a daily rhythm of studied variables. Higher MESOR and amplitude values of melatonin and rectal temperature were observed in sheep than in goats. The diurnal acrophase of locomotor activity was statistically different from the nocturnal acrophase of melatonin and rectal temperature, with no differences between the two species. Robustness was statistically lower in total locomotor activity in comparison with the others two variables, with a differences due to species in melatonin daily rhythm. In conclusion, in small ruminants, melatonin and rectal temperature daily rhythms are strictly correlated, and are not associated with the locomotor activity rhythm.

Introduction

In mammals, information about the environmental photoperiod is relayed from the retina to the suprachiasmatic nuclei (SCN) in the anterior hypothalamus. This is a basic structure where many physiological functions are controlled. Environmental conditions, especially light information, are mediate from the eye to hypothalamic structure. The mechanism of adaptation to these variations is seen to have an influence on

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behavior such as locomotor activity and feeding, and in physiological functions, such as body temperature and hormonal secretion (Alila-Johansson et al., 2004). The light information received by the SCN is transduced into neural and hormonal output signals that affect various rhythms of the animal. Via the sympathetic nervous system the SCN sends impulse to the pineal gland where it influences the secretion of melatonin. The hormone melatonin is a chemical messenger that is synthesized during darkness; it is itself a messenger of the light/dark alternation in environment and is thought to serve as synchronizer for circadian rhythms. Its circadian periodicity may be understood as a coordinating signal for other biological rhythmicity, or as an endogenous synchronizer (Corbalan-Tutau et al., 2014). Daily oscillation in blood levels of melatonin has been documented in various species of birds and mammals (Hasegawa and Ebihara, 1992; Brandstätter et al., 2000; Zawilska et al., 2006). In sheep and goats, melatonin daily rhythm has been widely studied; it is endogenously generated and entrained by the light/dark cycle. In the study of biological rhythm the simultaneous investigation of many physiological variables plays an important role in consideration that there is a reciprocal relationship between the robustness of the endogenous circadian timing system and its dependency on regularly timed synchronizers (Berger, 2008). Although, daily rhythmicity is more robust in some physiological variables than in others, and more robust in some organisms than in other (Refinetti, 2006). As indicator of the rhythmicity of the biological clock the rhythmicity of body temperature has been widely used; because of the relative ease of monitoring, and because of the robustness of its rhythm (Piccione and Refinetti, 2003). Similarly it believed that locomotor activity ensures an optimal functioning of the biological system (Piccione et al., 2010); monitoring behavioral changes in farm animals can improve welfare by providing information on an individual health (Muller and Schrader, 2003). Previous research has shown that melatonin plays an important role in the modulation of the circadian rhythms of activity and body temperature (Aguzzi et al., 2006).

In the last years many studies have been conducted on the morpho-fucntional characteristics of the circadian system. All physiological variables that display a prominent circadian rhythm should be measured as a marker rhythm with an application in chronotherapy protocols and in the monitoring of welfare. Daily synchronization of physiological process contributes to the wellness of the organism; in order to investigate the potential causal link between activity, melatonin and body temperature we simultaneously investigated the rhythm of locomotor activity, melatonin serum levels and rectal temperature values in sheep and goats housed under environmental conditions.

Material and methods

Animals and housing

Ten Sarda breed pluriparus ewes, four years old, with a mean body weight 40.5 ± 1.8 kg, and ten Sarda breed pluriparus female goats, two years old, with a mean body weight 41.3 ± 2.8 kg, were enrolled in our study. All animals were clinically healthy, free from internal and external parasites, not pregnant and in dry period. All animals were kept in a common stall and had free access to water and to good-quality alfalfa hay. They were subjected to a natural photoperiod (Sunrise 07:00; Sunset 17:30). The environmental temperature and relative humidity ranges were 13–20 °C and 54–100%, respectively. All treatments, housing and care were carried out in accordance with the standards recommended by the EU Directive 2010/63/EU for animal experiments.

Data collection

Each animal was equipped with an Actiwatch-Mini® (Cambridge Neurotechnology Ltd., UK), actigraphy-based data loggers that record a digitally integrated measure of motor activity by means of collars that was accepted without any obvious disturbance. Total activity of each animal was recorded as the result of all movements, which includes different behaviors such as feeding, drinking, walking, grooming, and small movements during sleep, independent of the animal's position such as lying or standing, for 48 h with a sampling interval of 5 min, the values recorded every 5 min were the mean values of the sum of 32 recordings per second.

Also, all animals were cannulated the day before the start of the study and the cannula remained patent for the duration of sample collection. Blood samples were collected in heparinized tubes though jugular intravenous catheters (FEP G18 × 45 mm) secured in place with suture (Vicryl, Ethicon, Somerville, NJ) every 4 h over a 48 h period starting from 00:00 of day 1 and ending at 00:00 on day 3.

Rectal temperature was recorded with a digital thermometer (HI-92740, Hanna Instruments, Bedfordshire, UK) whose probe was insert 8 cm into the rectum immediately before the blood sampling at each data point. All animals tolerate rectal probes very well and show no sign of stress-induced hyperthermia (Piccione et al., 2002).

During the night, all data recording were performed using a dim-red light (<3 lux, 15 W Safelight lamp filter 1A, Kodak Spa) avoiding any direct lighting of the eyes in order not to influence melatonin secretion.

Melatonin assessment

Blood sample tubes were centrifuge at 2500 × *g* per 15 min and the obtained plasma were used in order to assess melatonin concentration. Plasma melatonin concentrations were determined with the aid of direct radioimmunoassay, adapted from that described by Fraser et al. (1983). The plasma sample (200 μl) was incubated with a specific antiserum to melatonin raised in the sheep (against *N*-acetyl-5-methoxy tryptophan/bovine thyroglobulin; Guildhay Antisera Ltd.) with a final dilution of 1:6000 and trace amounts of 3*H*-melatonin (*o*-methyl-3Hmelatonin, Amersham) were then added. The standard curve (Sigma melatonin) was constructed using charcoal-stripped pooled plasma from at least two ewes which had been kept in natural daylight for at least 4 h before sampling. The free and antibodybound fractions of melatonin were then separated using a dextran-coated charcoal solution. The free melatonin fraction was precipitated with charcoal by centrifugation and supernatant liquid counted in a scintillation-counter. The major

cross-reactions of the antibody were: *N*-acetyl tryptamine, 0.91%, 6-hydroxy melatonin, 0.33% and *N*-acetyl tryptophan, 0.22%. The sensitivity (90% B/B0) was 2.5 pg/tube. All samples from a ewe in all experimental conditions were run in the same assay.

Statistical analysis

Data were normally distributed (Kolmogorov–Smirnov test). Using cosinor rhythmometry (Nelson et al., 1979), four rhythmic parameters were determined: MESOR (mean level), amplitude (half the range of oscillation), acrophase (time of peak), and robustness (strength of rhythmicity).

Before the start of analyses the homoscedasticity of data was verify by the application of a Barlett's test at the significance level 2alpha 0.05. Whereas MESOR and amplitude of different rhythms cannot be compared because they refer to distinct physical quantitates; the analysis of the temporal relationship of physiological processes considers the comparison of acrophase and robustness of rhythm.

Two-way analysis of variance (ANOVA) was applied to study the effect of species and day on MESOR and amplitude for each parameter studied. General linear model-multivariate analysis of variance for repeated measures (GLM-RM-MANOVA) was applied to assess the differences among the parameters studied and the effect of species and day on acrophase and robustness of rhythm. Bonferroni's test was applied for post hoc comparison. At the significance level 2alpha 0.05 was considered statistically significant, with an alpha level of 95%. The data were analyzed with Statistica 7 (StatSofts, Inc., USA).

Result

A daily rhythm of total locomotor activity, melatonin and rectal temperature was observed in all animals, in both days of monitoring. Table 1 reports the mean ± standard deviation (SD) of circadian parameters observed in all studied physiological variables. The application of two-way ANOVA showed no effect of species and day on MESOR and amplitude of locomotor activity, and an effect of species on MESOR and amplitude of melatonin ($F_{(1,18)} = 482.83$; $p < 0.0001$) and rectal temperature ($F_{(1,18)} = 83.00$; $p < 0.0001$; $F_{(1,18)} = 64.65$; $p < 0.0001$); with highest values in sheep than in goats in both day of monitoring. The application of general linear model showed statistically differences of acrophase and robustness among the physiological variables studied ($F_{(2,36)} = 648.96$; $p < 0.0001$; $F_{(2,36)} = 1097.20$; $p < 0.0001$), an effect of species only on robustness of rhythm ($F_{(1,18)} = 18.83$; $p < 0.0001$) and any effect of day of monitoring on both circadian parameters. In particular, Bonferroni's post hoc comparison showed statistically significant differences among the diurnal acrophase of locomotor activity and the nocturnal acrophase of melatonin serum concentration and rectal temperature, in both day of monitoring; and a lower robust rhythm of locomotor activity in comparison with melatonin and rectal temperature. No differences in acrophase and robustness were observed between melatonin and rectal temperature. A difference of robustness of melatonin daily rhythm due to species has been observed in both day of monitoring.

Fig. 1 shows the mean values of locomotor activity recorded every 5 min with its acrophasogram, and the mean patterns of melatonin serum concentration and rectal temperature recorded every 4 h, for 48 h of monitoring, in the both studied species.

Discussion

Our results confirm numerous previous observations of daily rhythmicity of locomotor activity, melatonin and rectal temperature in sheep and goats housed under natural light/dark cycle (Carcangiu et al., 2015; Piccione et al., 2005, 2008a). It is supposed that the peripheral tissue clock can manifest independence from the so called master clock (Schibler and Sassone-Corsi, 2002); such as, the circadian oscillation of many physiological behavioral process and physiological parameters are paralleled (Panda et al., 2002). By the comparison of the rhythmic parameters of each physiological studied variable it has been observed that all rhythms studied remained constant in the two days of monitoring. Locomotor activity daily rhythm showed the same characteristic in both species. No differences were observed in MESOR values, amplitude, acrophase and

Table 1 – Circadian parameters of variables studied during the 2 days of monitoring.

SHEEP	Locomotor activity (counts)		Melatonin (pg/ml)		Rectal temperature (°C)	
	Day 1	Day 2	Day 1	Day 2	Day 1	Day 2
Circadian parameters						
MESOR	410.22 ± 121.14	416.30 ± 118.39	42.37 ± 1.86	41.43 ± 1.42	39.29 ± 0.06	39.29 ± 0.07
Amplitude	326.49 ± 96.00	326.19 ± 80.30	29.26 ± 3.02	28.91 ± 1.88	0.25 ± 0.04	0.28 ± 0.06
Acrophase (hour)	13:12 ± 45 min	13:19 ± 45 min	23:00 ± 10 min	23:10 ± 5 min	22:45 ± 1 h 39 min	22:22 ± 1 h 5 min
Robustness (percentage)	15.28 ± 6.94	14.15 ± 7.47	94.19 ± 5.58	94.06 ± 5.49	79.00 ± 10.89	83.26 ± 9.37
Goats						
MESOR	414.90 ± 46.00	430.39 ± 53.02	37.26 ± 2.30	37.58 ± 1.12	38.89 ± 0.22	38.97 ± 0.05
Amplitude	380.67 ± 125.00	381.73 ± 99.44	8.95 ± 2.25	9.37 ± 2.56	0.16 ± 0.02	0.14 ± 0.03
Acrophase (hour)	13:10 ± 12 min	13:25 ± 48 min	00:15 ± 1 h 03 min	01:50 ± 1 h 10 min	21:08 ± 2 h 58 min	23:50 ± 3 h 48 min
Robustness (percentage)	16.84 ± 6.70	17.63 ± 4.29	76.18 ± 8.02	79.17 ± 10.02	77.26 ± 8.66	74.15 ± 8.54

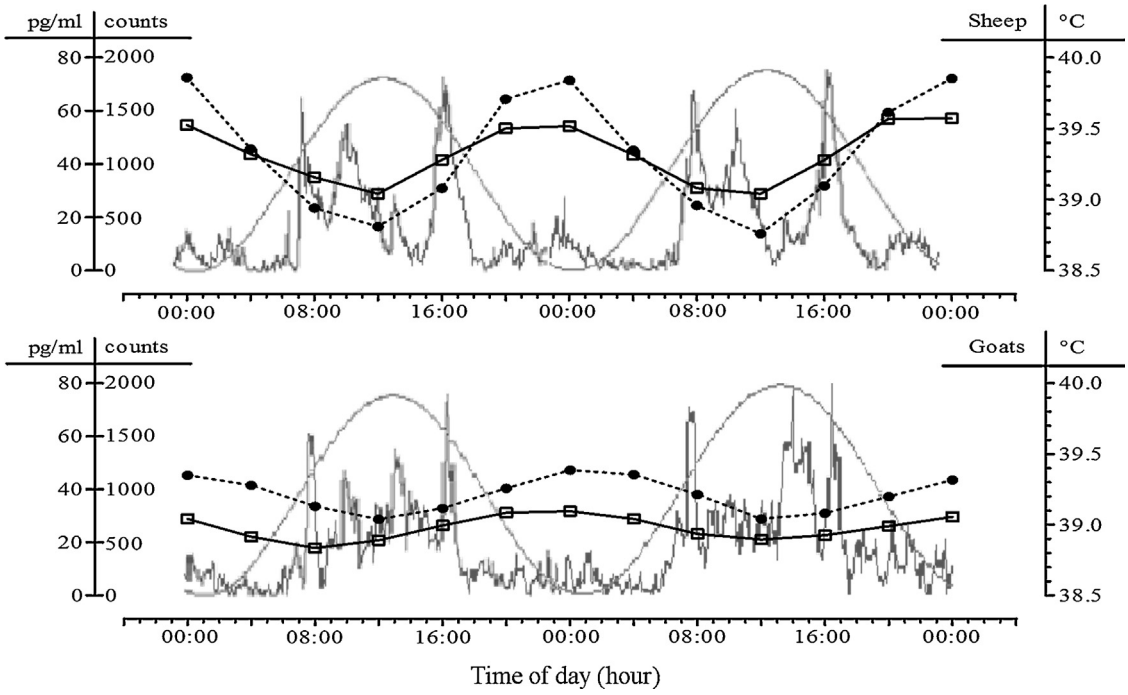

Fig. 1 – Mean of locomotor activity recorded every 5 min, with its acrophasogram (gray graph), and the mean pattern of melatonin serum concentration (•) and rectal temperature (□) recorded every 4 h, during the 48 h of data collection, in sheep and goats.

robustness, but differences were observed between locomotor activity and the others two studied variables. In particular, locomotor activity showed a diurnal acrophase that was statistically different from the nocturnal acrophase observed in melatonin concentration and rectal temperature daily rhythms. Also the robustness of locomotor activity daily rhythm was statistically lower than the robustness of the others two studied variables. Reiter (1991) reported that melatonin production is always circumscribed to the night, regardless the behavioral distribution of activity and rest of diurnal, nocturnal and crepuscular species. Aguzzi et al. (2006) suggested that in rats the circadian pacemaker driving melatonin synthesis is rather independent from the circadian pacemaker driving the locomotor activity. In most researches where body temperature and locomotor activity were measured simultaneously, the two variables had very similar circadian rhythms with the high phase of body temperature circadian rhythm occurring during the active phase of locomotor activity circadian rhythm (Refinetti and Menaker, 1992). The high phases of melatonin and rectal temperature were postponed of about 5 h from the active phase of locomotor activity that started at about 07:00 and ended at about 17:00 in both studied species. Studies in humans subjected to constant best rest and correlation studies in animals have shown that the body temperature rhythm is autonomous, even if affected by the activity rhythm (Giannetto et al., 2016). In rabbits and horses (Piccione et al., 2011a, 2013) has been reported that locomotor activity for the issue of causation, is not able to influence the rhythm of rectal temperature, even though it not imply that the rectal temperature daily rhythm cannot be enhanced or masked by changes in total locomotor activity.

By the comparison of melatonin concentration and rectal temperature differences due to species were observed in MESOR and amplitude values, that were statistically higher in sheep in comparison to goats; however no differences in acrophase were observed. In rats has been shown that the regulation of melatonin daily rhythm is independent from the regulation not only of activity but also of rectal temperature. Alteration in some circadian parameters of the melatonin rhythm had no effect on MESOR and amplitude to the rhythm of locomotor activity and rectal temperature. On the contrary, our results showed that rectal temperature plays an important role in the regulation of the circadian timing system as well as melatonin (Aguzzi et al., 2006). Differently from that observed in horses (Piccione et al., 2005, 2013) in which body temperature rhythm was slight less robust than the melatonin rhythm. No differences in robustness of rhythm were observed between melatonin and rectal temperature in sheep and goats. Even thought, melatonin daily rhythm was more robust in sheep than in goats. Also, our results were inconsistently with finding in a previous study where activity, melatonin and temperature exhibited robust rhythmicity (Piccione et al., 2005). These differences could be attributed to a different housing condition used in the two studies, that, as observed in other mammals species (Piccione et al., 2008b, 2011b), can influence locomotor activity.

Conclusion

In conclusion, we can claim that the daily rhythm of melatonin and rectal temperature are consistently different from the rhythm of locomotor activity in sheep and goats. The differences in acrophase and robustness are probably due to the fact that peripheral tissue clock can manifest independence from the master clock. The differences in MESOR and amplitude, within the same variable studied, are due to intrinsic differences of species. The mechanism that regulates the close temporal association between the daily rhythm of melatonin and rectal temperature in sheep and goats is unknown and need of further investigation.

Conflict of interest

The authors declare no conflicts of interest to disclose.

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