Twenty-four-hour rhythm patterns of plasma melatonin in short-day and long-day breeders maintained under natural environmental conditions

ABSTRACT

Photoperiodic treatments have been of practical interest in controlling seasonal reproduction in sheep, goats and horses. Melatonin is the principal mediator of the environmental photoperiodic message. To investigate the intra- and inter-subject variability of melatonin 24 h rhythm, ten female Italian Saddle horses (8–10 yrs old, mean body weight 525 ± 30 kg), ten female Sarda breed sheep (2–3 yrs old, mean body weight 40.5 ± 2.8 kg) and ten female Sarda breed goats (3–4 yrs old, mean body weight 38.9 ± 4.1 kg), housed individually in a 4 × 4 m soundproof box equipped with 50 × 100 cm opening windows, were subjected to a natural photoperiod of the vernal equinox (sunrise 06:00 h; sunset 18:00 h). Blood samples were collected from each animal, every 3 h over a 48 h period starting at 00:00 h of day 1 and ending at 00:00 h of day 3. Plasma melatonin concentrations were determined by direct radioimmunoassay (MelatoninDirect RIA, Labor Diagnostika Nord GmbH, Nordhorn, Germany). The application of single cosinor method substantiated a circadian rhythm of melatonin with a nocturnal peak in all studied species. The application of two-way ANOVA on the rhythmic parameters indicated statistically significant differences between the three species in all of the cosinor analysis-derived parameters of MESOR, amplitude, acrophase and robustness of rhythm. Analyses of intra- and inter-subject variability indicate that organization of the melatonin 24 h rhythm is characterized by great accuracy of control within and between the individuals of a breed. In conclusion, features of the 24 h rhythm of melatonin among species; however, the 24 h rhythmicity of melatonin each species showed high stability within the various subjects and within the same subject. These findings must be taken into consideration when applying photoperiod and melatonin treatments for breeding purposes.

KEYWORDS

Circadian rhythm; melatonin; species differences; horses; sheep and goats

Introduction

Melatonin is a hormone synthesized mainly in the pineal gland and secreted into the general circulation. Melatonin has received great attention from circadian physiologists because of its central role in photoperiodism (Refinetti 2006). It is involved in the organization of the circadian system and plays a central role in the control of photoperiodic response (Gorman et al. 2001). The hormone, which is a messenger of the environmental light/ dark cycle, is synthesized by the pineal gland almost exclusively during darkness. Melatonin circadian periodicity may be understood as a coordinating signal for other biological rhythmicities, i.e. as an endogenous synchronizer (Cordalan-Tutau et al. 2014). The effects of photoperiod on the hormonal control of reproductive seasonality are mediated by pineal melatonin, which transforms the photoperiodic message into a chemical message (Coelho et al. 2006). Encoding the duration of darkness and reflecting the change in the length of day and night, it provides a neuroendocrine signal that conveys seasonal timing and regulates reproduction in seasonal breeding animals (Bartness and Goldman 1989; Morgan and Mercer 1994). Studies conducted on Syrian hamsters and rats revealed that exposure to short days or daily administration of melatonin changes the function of the pituitary-gonadal axis, and these and other such findings convinced the scientific community of the legitimacy of the pineal gland as an organ of internal secretion involved in the regulation of reproductive function, particularly in seasonal breeders (Reiter et al. 2009). On the basis of the length of the gestation period, photoperiodic species can be classified as short-day or long-day breeders. In both long-day and short-day breeders there is a critical amount of light that determines whether an animal becomes and/or remains reproductively competent or whether it becomes anestrus (Moore-Ede and Moline 1985). Investigations of both long-day and short-day breeders have obviously contributed significantly to the understanding of the mechanisms whereby day length and melatonin govern seasonal reproduction (Coelho et al. 2006). Studies on farm animals have contributed significantly to the understanding of the basic melatonin-related physiological mechanism, as well as to the regulation of reproduction. In sheep and goats, many aspects of the physiological and biochemical parameters, including glucose, locomotor activity and rectal temperature are correlated with features of the melatonin 24 h rhythm (Carcangiu et al. 2018; Giannetto et al. 2016). However, in horses the 24 h pattern in melatonin is not endogenously but photoperiod-generated (Piccione et al. 2013).

Farm animals express seasonal variations in their production traits, thus inducing changing availability of fresh animal products (meat, milk and cheese) or performance (horses). The importance of circadian rhythmicity for the health and welfare of livestock is becoming increasingly recognized and forms the basis for melatonin treatment, for example, control seasonal function, in particular reproduction. On this basis, the aim of this study was to investigate the 24 h rhythm of melatonin in short- (sheep and goats) and long-day (horses) breeders, as to improve the knowledge on the melatonin rhythmicity in species nowadays extensively subjected to artificial photoperiodic and/or melatonin treatment to adjust their breeding season and improve their productivity.

Materials and methods

Animals

Ten female horses (Italian Saddle, 8–10 yrs old, mean body weight 525 ± 30 kg), ten female sheep (Sarda breed, 2–3 yrs old, mean body weight 40.5 ± 2.8 kg) and ten female goats (Sarda breed, 3–4 yrs old, mean body weight 38.9 ± 4.1 kg) were enrolled in the study after a clinical exam verifying their health status, including the absence of internal and external parasites. Animals were subjected to a natural photoperiod (sunrise 06:00 h; sunset 18:00 h) during the vernal equinox and housed individually in a 4 × 4 m soundproof box equipped with 50 × 100 cm opening windows that allowed natural ventilation. The vernal equinox was chosen to guarantee equal distribution of light stimulus so as to not favor any species. The animals were placed into the experimental box 30 d before the commencement of the study to

avoid changes in their behavior and physiology due to the induction of fear by isolation (Carbonaro et al. 1992).

Thermal and hygrometric records were collected inside the box throughout the study by means of a data logger (Gemini, UK). Minimal and maximal temperatures during the experimental period were 12°C and 18° C, and mean humidity was 60–70%, which are normal autumn season in the locality. All animals had free access to water from a commercial waterer and goodquality hay placed on the floor in abundance three times a day (06:00, 12:00 and 18:00 h).

All treatments, housing and animal care conformed to the standards recommended by the Guide for the care and use of animals (D.L. 27/1/1992, n 116) and UE (Directive 86/609/CEE), and also Journal standards (Portaluppi et al. 2010).

Blood sample collection

All animals were cannulated the day before the start of the study, and the cannula, secured in place with sutures, remained patent for the duration of the study. Blood samples were collected every 3 h for 48 h commencing 00:00 h of day 1 and ending 00:00 h of day 3. Samples were drawn into heparinized tubes (Terumo Corporation, Japan), centrifuged at 2000 g × 10 min for horses and at 2500 g × 15 min for sheep and goats; and frozen at -20°C until later analyzed. Plasma melatonin concentrations were determined by direct radioimmunoassay (MelatoninDirect RIA, Labor Diagnostika Nord GmbH, Nordhorn, Germany), with a resolution of 1.5 pg/mL and intra coefficients of variation of 13% for horses; and sensitivity of 2.5 pg/tube with intra-assay coefficient of variation <5% for sheep and goats. Nighttime blood samples were collected using a dimred light (<3 lux, 15 W Safelight lamp filter 1A, Kodak Spa) to avoid any direct lighting of the eyes in order to not influence melatonin secretion.

Statistical analysis

All results are presented as means ± SD. Data were normally distributed ($P < .05$, Kolmogorov–Smirnov test). Using cosinor rhythmometry (Nelson et al. 1979), four rhythmic parameters were determined by the best approximating cosine waveform by the least squares method: MESOR (mean level, a time series 24 h mean), amplitude (half the range of oscillation), acrophase (time of peak) and robustness (strength of rhythmicity, i.e. quotient of the variance accounted for by the approximation of the cosine waveform relative to the variance accounted by a straight-line approximation to the time series data) (Refinetti 2004).

For each group (n = 10 animals), intra- and intersubject variabilities in the rhythmic parameters of melatonin daily rhythm were computed as the standard deviations of the means. The standard deviations of the individual mean of the ten subjects of each group across the 2 days of monitoring were used as the measure of intra-subject variability. Likewise, the standard deviations of the means for the 2 days of monitoring across the ten subjects were used as the measure of inter-subject variability. Analysis of variance (ANOVA) was used to determine the effects of species, time-of-day and day-ofmonitoring on melatonin; to determine the effect of species and days of monitoring on rhythmic parameters and to identified statistically significant effects of species and type of variability (intra-subject *versus* inter-subject). Bonferroni's test was applied for post hoc comparison. Data were analyzed using the software STATISTICA 7 (StatSoft Inc.). P values <.05 were considered statistically significant.

Results

Application of multivariate ANOVA showed a significant effect of the breed ($F(2,528) = 905.16$; $p < .0001$) and time-of -day ($F(8,528) = 114.07$; $p < .0001$) on melatonin level, but no statistically significant differences in melatonin level between day 1 and day 2 of monitoring. Daily fluctuation of plasma melatonin in the 10 subjects for each group is reported in Figure 1. The single cosinor method substantiated the 24 h rhythm of melatonin in all species for each day (day1 and day 2) of monitoring. Application of two-way ANOVA on the rhythmic parameters revealed significant effect of species on the circadian MESOR ($F(2,27) = 3285.00$; $p < .0001$), amplitude ($F(2,27) = 2281.00$; $p < .0001$) and acrophase ($F(2,27) = 128.30$; $p < .0001$), and also robustness of the melatonin circadian rhythm ($F(2,27) = 15.14$; $p < .0001$). In particular, Bonferroni post hoc comparison showed the lowest MESOR and amplitude values in horses, followed by goats and sheep. In horses, the acrophase was advanced in a statistically significant way by about 2 h relative to that of sheep and goats. The

robustness of rhythm was greater in sheep and goats than horses (Figure 2). Intrasubject variability refers to the extent of variability of the melatonin 24 h rhythm pattern of an individual from day to day; inter-subject variability refers to the extent of variability in the melatonin 24 h rhythm patterns between different individuals (Table 1). ANOVA conducted for each of the four rhythm parameters indicated the absence of significant main effects for species and condition (intra-subject versus inter-subject), except for amplitude of rhythm, in particular, a lower intra-subject variability was observed in horses than sheep. In no case in any species was intra-subject variability significantly different from inter-subject variability.

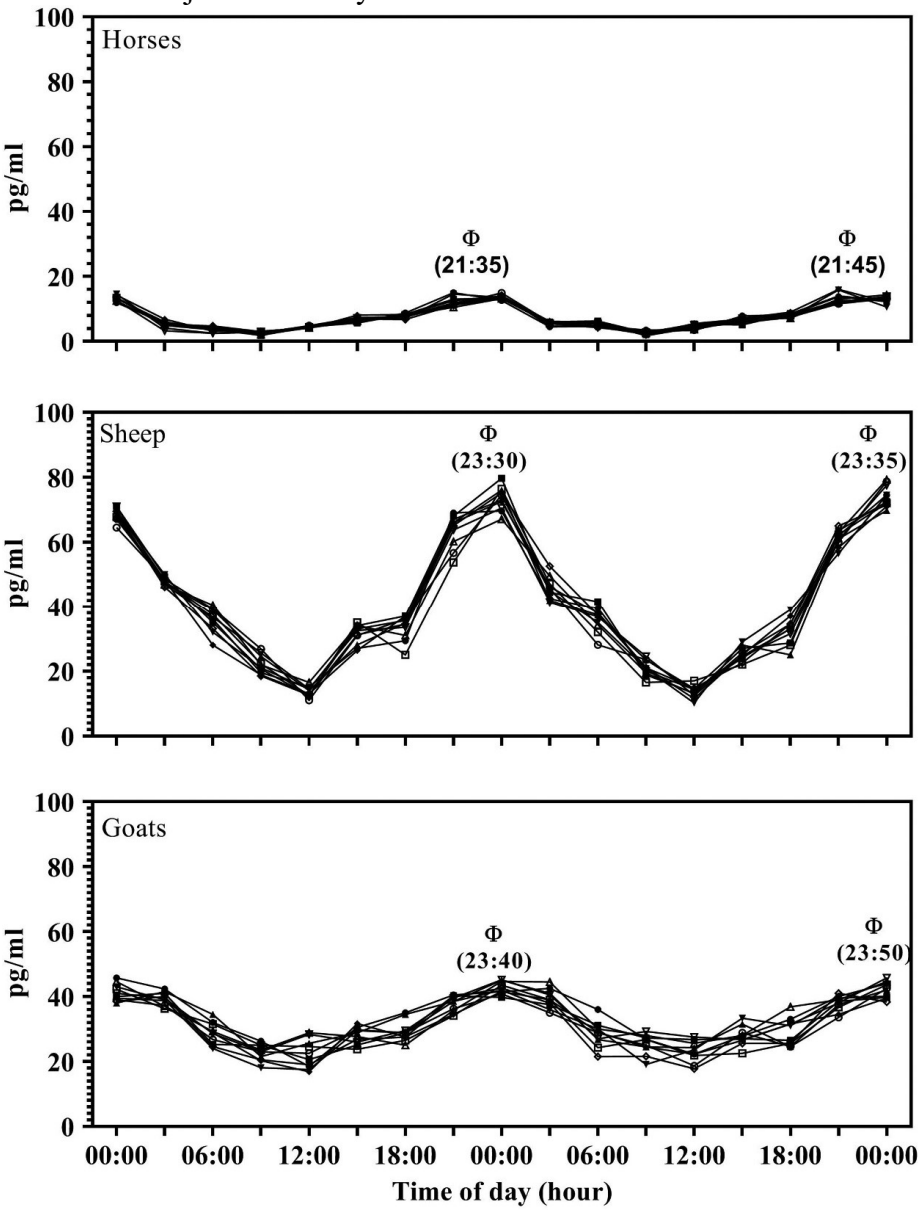

Figure 1. Daily fluctuation of melatonin recorded in the 10 subjects of each species. ϕ indicates the acrophase (peak time) of rhythm.

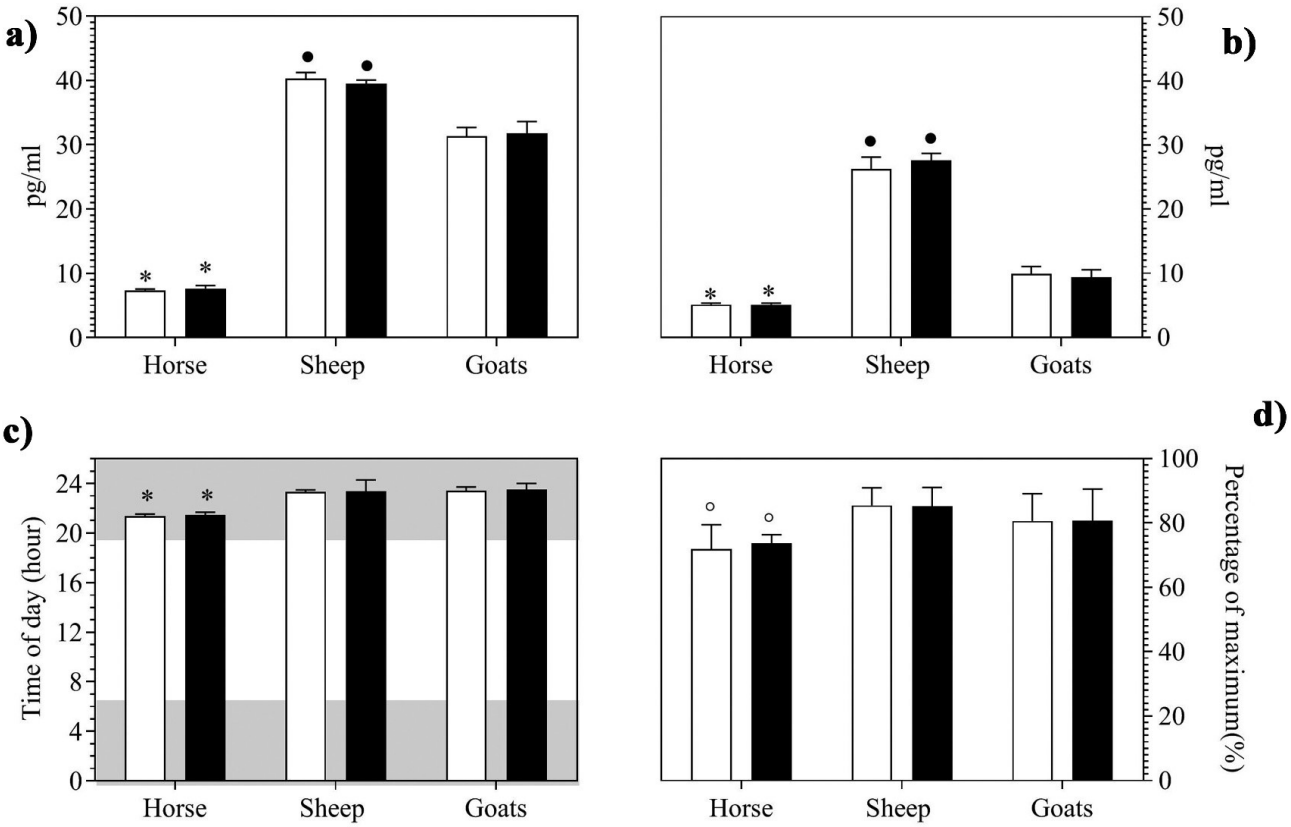

Figure 2. Mean (±SD) of circadian parameters (a: Mesor, b: Amplitude, c: Acrophase, d: Robustness) expressed in their conventional units, for the melatonin time series data of day 1 (□) and day 2 (■) in the 3 species studied. Statistical significance: *$p < .001$ versus sheep and goats; ●$p < .001$ versus goats; °$p < .05$ versus sheep and goats.

Table 1. Mean (±SD) of intra-subject and inter-subject variabilities of circadian parameters of the three species studied, with their coefficient of variation (CV).

	Variability	Mesor (pg/ml)		Amplitude (pg/ml)		Acrophase (min)		Robustness (%)	
			cv		cv		cv		cv
Horses	Intra	0.28±0.24	0.86	0.18±0.14*	0.80	17±8	0.14	3.53±2.13	0.60
	Inter	0.36±0.14	0.38	0.25±0.01	0.06	19±2	0.46	5.08±3.38	0.66
Sheep	Intra	0.80±0.50	0.62	1.39±1.29	0.92	12±5	0.48	3.19±2.95	0.92
	Inter	0.77±0.20	0.27	1.45±0.51	0.35	14±5	0.47	5.62±0.12	0.02
Goats	Intra	0.83±0.49	0.59	0.80±0.70	0.87	21±12	0.57	7.70±5.70	0.74
	Inter	1.59±0.29	0.18	1.19±0.02	0.02	33±4	0.12	9.15±0.85	0.09

Discussion

It is well established that a variety of species utilize daylight as an environmental cue for restricting reproduction to a particular time of the year. That this confers a selective advantage is suggested by the differences in
breeding seasons both within species, when populations are geographically separated, and between species, when gestation lengths differ. By responding to a photoperiod, animals optimize the time of birth to maximize the chances of survival of their young (Bittman 1985). Our results confirm the statistically significant difference in the amount of circulating plasma melatonin among species for the condition of our study, i.e. vernal equinox, and a statistically significant effect of time-of-day attributable to its 24 h rhythmicity. Previous studies have showed that melatonin is not stored in the pineal gland and thus its plasma concentration reflects its synthesis and secretion, which is high at night and low during the day (Carcangiu et al. 2014; Cordalan-Tutau et al. 2014). Annual rhythms

are distinct in two types. Type I annual rhythms are dependent upon the environment, they are generally photoperiodic driven because they require transduction of seasonal changes in day length. Type II annual rhythms are dependent upon an endogenous biological clock; they free run in constant conditions (Conn 2003). It is hypothesized that the involvement of melatonin in photoperiodic time measurement is similar for Type I and Type II mammals. In a given species, long-duration nocturnal melatonin elevations are associated with responses of short days, and short-duration nocturnal elevations of melatonin are associated with responses of long days. Thus, the quantitative relation between hours of elevated melatonin and day length varies somewhat among species (Dunlap et al. 2004). We found the peak time of the 24 h rhythm of plasma melatonin was clearly nocturnal both in the long- and short-day breeders. Comparing the circadian rhythm parameters of the melatonin 24 h rhythm revealed differences between the short- and long-day breeders. The differences involved not only MESOR values but also the amplitude of rhythm, which were lower in long- than short-day breeders. Moreover, the acrophase occurred 2 h earlier in the long- than short-day breeders. Both in long- and shortday breeders the rhythmicity of plasma melatonin accounted for a high percentage of the total variance, although it was greater in short- than in long-day breeders. Within the short-day breeders, differences were observed in MESOR and amplitude, but no differences were observed in acrophase and robustness of rhythm. Except for amplitude that was statistically significantly different between sheep and horses, inter-subject variability and intra-subject variability was found to be similar in the three studied species for all other circadian parameters investigated. This means that in the 3 species of our study, there was low day-to-day variability in the 24 h rhythmicity of plasma melatonin, as well as the variability between the 24 hy rhythmicity of melatonin of different individuals. In other words, there was great consistency in the 24 h rhythmicity of melatonin within individuals and between individuals. As shown in Table 1, some data reported a “high” standard deviation. Their coefficient of variation was lower than 1, indicating the distribution of data can be considered to be of lowvariance, confirming the great accuracy of the circadian rhythm organization in the control of the 24 h rhythm melatonin secretion within and between the individuals of a species. Comparisons of intra- and inter-subject variabilities in circadian rhythms have rarely been conducted; previous studies of the variability in the freerunning periods of various species have found smaller intra-subject variability than inter-subject variability (Refinetti et al. 2016). Distinguishing the two types of variability has important implications for the
management of individual needs, such as in the clinical practice of veterinary or human medicine. If intersubject variability is greater than intra-subject variability, then treatments designed for an “average” patient may turn out to be either too weak or too strong for a patient who is not average. On the other hand, if intrasubject variability is greater than inter-subject variability, then the notion of an average patient may be useful, but the administration of the treatment to patients may have to be adjusted on a daily or weekly basis. Our finding that inter-subject variability is similar to the intra-subject variability indicates that the concept of an average patient should not be applied in all the studied species. Also, the absence of statistically significant differences in any of the species between intra- and intervariability of MESOR, amplitude, acrophase, and robustness of rhythm confirms these circadian parameters are very dependable for the analysis and characterization of circadian rhythms. In conclusion, the results of this study indicate differences in the 24 h rhythmicity of plasma melatonin among species must be taken into consideration, and in light of the high stability within the various subjects and within the same subject, any time photoperiod and melatonin treatments are performed in animal breeding programs.

Disclosure statement

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