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Circadian rhythms of melatonin and behaviour in juvenile sheep in field conditions: Effects of photoperiod, environment and weaning

Wyse C.A.[a,b,*], Zhang X.[a], McLaughlin M.[d], Biello S.M.[c], Hough D.[a], Bellingham M.[a], Curtis A.M.[b], Robinson J.E.[a], Evans N.P.[a]

[a] Institute of Biodiversity, Animal Health and Comparative Medicine, University of Glasgow, Glasgow G61 1QH, United Kingdom
[b] Department of Molecular and Cellular Therapeutics, Royal College of Surgeons in, Ireland, 123, St Stephens Green, Dublin
[c] School of Psychology, 58 Hillhead Street, Glasgow G12 8QB, United Kingdom
[d] School of Veterinary Medicine, University of Glasgow, G61 1QH, United Kingdom

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ABSTRACT

Entrainment of circadian rhythms (CR) to the light dark cycle has been well described under controlled, experimental conditions. However, studies in rodents have reported that rhythms in the laboratory are not always reproduced under field conditions. The aim of this study was to characterise the CR of sheep maintained under conditions of standard UK farm animal husbandry and to investigate the effects of environmental challenges presented by season, weaning and changes in housing on CR. Male sheep (n = 9) were kept at pasture, or group housed in barns, under natural photoperiod for one year. CR in locomotor activity were monitored using accelerometry, and 24 h patterns in plasma cortisol and melatonin were measured every 4 h by ELISA. CR was measured before and after weaning, in summer and winter, and at pasture and by barn housing. Cosinor analysis revealed high amplitude, diurnal rhythms in locomotor activity that were disrupted by weaning and by barn housing. Rhythms in winter showed an interrupted night time activity pattern, but only when the sheep were kept at pasture. Cortisol and melatonin secretion followed typical circadian patterns in winter and summer. The CR of the sheep under the field conditions of this study were strikingly robust under basal conditions, but easily disrupted by environmental challenges. Interrupted patterns of activity during the long nights of wintertime, not previously reported for sheep kept in experimental conditions were recorded. Based on these findings, we propose that animals require exposure to more complex environments than the laboratory in order to exhibit their true circadian phenotype.

1. Introduction

Photoperiodic animals, such as the sheep, synchronise their physiology and behaviour to the seasons at northern latitudes principally by entrainment to variations in daylength [1] [2, 3]. This photic signal is transduced through multi-synaptic pathways from the suprachiasmatic nucleus that innervate the pineal gland and suppress secretion of melatonin during daylight. In this way, the circadian pattern of melatonin secretion provides a seasonal timing cue that serves to regulate annual changes in physiology and behaviour in photoperiodic animals [4].

Decades of literature describe a preponderant role of the light dark cycle in the regulation of seasonal and circadian biology, based largely on the capacity of light to entrain rhythmicity in laboratory rodents. These animals are often housed alone, with constant temperature and humidity, and *ad libitum* food and water access. Such conditions would support robust entrainment of CR to the light dark cycle photoperiod, as it is the most variable factor in the laboratory environment. Biological rhythmicity in the laboratory cannot truly represent the chronobiology of wild animals that are entrained to the CR of predators and conspecifics, in addition to the complex cycles in temperature, feeding opportunities, noise and other challenges that they encounter in their environment. In this regard, the CR and nocturnal preference of the two classical chronobiological laboratory animals, the mouse and the hamster, are strikingly different when measured under field and laboratory conditions [5, 6]. Similarly, the fruit fly (*Drosophila*), another seemingly well understood animal model of circadian rhythmicity, is nocturnal in the laboratory but expresses a diurnal preference in natural conditions [7]. Furthermore, the effects of lesions of the clock are also profoundly different in laboratory and field conditions. Mice with a homozygous mutation of a core clock gene, *Per2* show multiple phenotypic abnormalities and premature senescence under laboratory

* Corresponding author at: Institute of Biodiversity, Animal Health and Comparative Medicine, University of Glasgow, Glasgow G61 1QH, United Kingdom.
E-mail address: cathywyse@rcsi.com (C.A. Wyse).

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conditions, yet their survival in the field was unaffected [5]. In contrast, surgical lesion of the SCN induces metabolic and behavioural arrhythmicity but only mild phenotypic effects in the laboratory [8], yet lesioned animals are spectacularly unsuccessful in field conditions [9]. This evidence suggests that animals require exposure to the full repertoire of environmental challenges to exhibit their true chronobiological phenotype and assumptions based on laboratory studies now require re-investigation in more complex environments.

The chronobiology of the sheep has been well-described due to their seasonal physiology and diurnal behaviour patterns. There have been many studies reporting photoperiodic regulation of rhythmicity in sheep kept in controlled, experimental conditions, [10] but little is known about the rhythmicity of sheep under field conditions. The aim of this study was to characterise the circadian rhythmicity of young male sheep maintained under conditions of standard UK farm animal husbandry and to investigate the effects of environmental challenges presented by season, weaning and changes in housing on CR in melatonin, cortisol and locomotor activity.

2. Methods

2.1. Animals, housing and lighting regimes

The animals in this study were March-born male sheep (n = 9) (*Ovis aries*; Texel cross, Scottish mules) aged 3–9 months over the experimental period. The animals were kept at pasture with minimal human contact and no supplemental food, or group housed in barns, under the natural photoperiod of the West of Scotland (55°N). Mean daytime environmental light intensity was 28,118 ± 34,146 lx in summer and 4571 ± 5518 lx in winter. Night time light intensity ranged between 0 and 0.8 lx outside the barns, and 0–0.4 lx inside the barns. The pasture where the sheep were housed was darker than the barns, with values ranging between 0 and 0.1 lx. Barn housed animals were bedded on straw with *ad libitum* access to hay and water, and twice daily (8 am and 4 pm) received commercial sheep concentrate as per normal husbandry practice. The sheep were kept with their dams until weaned at approximately 6 months of age, at which time the ewes were removed and the lambs kept at pasture until they were 9 months old, when they were transferred to barn housing. These studies were carried out in accordance with the Animals (Scientific Procedures) Act, 1986 under project licence 60/4422.

2.2. Locomotor activity

Patterns of locomotor activity were measured continuously while the animals were at pasture, over 14 day study periods in summer (June–August) and winter (November–December). Locomotor activity rhythms were also monitored for 14 days before and after weaning, and for 14 days before and after the animals were moved from pasture to the barn. CR in locomotor activity were monitored by fitting an accelerometer with data recording capacities (Actiwatch Mini, Camntech, UK) to a collar fastened around the sheep's neck. Data were acquired every minute, and then downloaded and analysed using Sleep Analysis 7 software (Camntech, UK).

2.3. Plasma melatonin and cortisol measurement

Patterns of melatonin and cortisol secretion were measured in 4 hourly jugular blood samples collected over 24 h on two occasions; August 12th, and November 11th under conditions of natural photoperiod. Sheep were barn housed for the duration of the blood sampling experiments. Blood was collected into heparinised tubes and plasma recovered after centrifugation for 20 min at 3000 rpm. Plasma cortisol concentrations were measured using an ELISA kit (Stratech, UK) designed for application in multiple species, with mean recovery of cortisol standard in sheep plasma recorded at 92%. Intra and inter assay coefficients of variation were reported by the manufacturer as 5.4% and 9.3%, and the sensitivity (mean minimum detectable dose of cortisol) was reported to be 0.071 ng/ml. Plasma melatonin was measured using an ELISA kit (IBL International, Germany) previously applied in sheep [11], following extraction on C18 reversed phase columns, and elution with methanol. Intra and inter assay coefficients of variation were reported by the manufacturer to range between 3 and 11% and 6–19% respectively, and sensitivity (limit of detection) were 1.6 pg/ml (IBL International, Germany).

2.4. Data analysis

Disruption to the circadian rhythmicity of the sheep was quantified using parameters described for assessment of fragmentation of human rhythmicity [12]. The interdaily stability index (IS) was calculated as the ratio between the variance of the average 24-hour pattern around the mean and the overall variance, and reflects the predictability of the diurnal pattern over sequential days. IS was defined as:

$$IS = \frac{n \sum_{h-1}^{p} (\bar{x}_h - \bar{x})^2}{p \sum_{i-1}^{n} (\bar{x}_i - \bar{x})^2}$$

where n is the total number of data, p is the number of data per day, $\bar{x}_h$ are the hourly means, $\bar{x}$ is the mean of all data, and x_i are the data points. The intradaily variability index (IV) was measured as an indicator of the fragmentation of the rhythm, with high values indicative of multiple transitions between periods of rest and activity. IV was defined as the ratio of the mean squares of the difference between successive hours and the total mean squares:

$$IV = \frac{n \sum_{i-2}^{n} (\bar{x}_i - \bar{x}_{i-1})^2}{(n-1) \sum_{i-1}^{n} (\bar{x}_i - \bar{x})^2}$$

The relative amplitude (RA) of the activity rhythm was calculated as the difference between the mean of the most active 10-hour period and the least active 5-hour period over a mean 24 h period.

Clocklab software (Actimetrics, US) was used to derive the time of onset and offset of activity by calculating the timepoint at which the activity level was less than or greater than a threshold of 20% of all non-zero counts for 6 h. The derived values for onset and offset were adjusted by visual inspection of the actigrams, where necessary. The phase angle of entrainment (ψ) was defined as the difference in minutes between the average time of activity onset and the time of civil dawn, (where positive values represent activity onset in advance of civil dawn). The time of civil dawn was taken from the values released by the US Department of Commerce National Oceanic and Atmospheric Administration (http://www.esrl.noaa.gov/gmd/grad/solcalc/index.html). Circadian rhythmicity was assessed by plotting a periodogram using the cosinor procedure described by Nelson et al. [13] to calculate the following parameters: midline estimating statistic of rhythm (Mesor, middle value of fitted curve), acrophase (timepoint of peak value of fitted curve), robustness (goodness of fit of the cosine function, F statistic) and amplitude (difference between maximum and mesor of the fitted cosine function. The dominant periods of the time series activity data were detected by plotting Lomb-Scargle periodograms [14] using software provided by Refinetti et al. [15] to detect significant power of rhythms with frequencies between 2 and 25 h. Central tendency and dispersion of data were compared between test conditions by calculating the mean and the standard error of the mean. Where data conformed to the parameters of the normal distribution, differences were assessed using Student's *t*-test for paired samples or a generalised linear model where more than one factor was included. Statistical significance was accepted at $p < 0.05$ and all analyses were performed

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