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Current Biology 20, 533–537, March 23, 2010 ©2010 Elsevier Ltd All rights reserved DOI 10.1016/j.cub.2010.01.042

Report

# A Circadian Clock Is Not Required in an Arctic Mammal

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## Summary

**Seasonally breeding mammals use the annual change in the photoperiod cycle to drive rhythmic nocturnal melatonin signals from the pineal gland, providing a critical cue to time seasonal reproduction [1]. Paradoxically, species resident at high latitudes achieve tight regulation of the temporal pattern of growth and reproduction despite the absence of photoperiodic information for most of the year [2]. In this study, we show that the melatonin rhythm of reindeer (*Rangifer tarandus*) is acutely responsive to the light/dark cycle but not to circadian phase, and also that two key clock genes monitored in reindeer fibroblast cells display little, if any, circadian rhythmicity. The molecular clockwork that normally drives cellular circadian rhythms is evidently weak or even absent in this species, and instead, melatonin-mediated seasonal timing may be driven directly by photic information received at a limited time of year specific to the equinoxes.**

## Results and Discussion

### Melatonin Rhythmicity

It has been suggested that the pineal production of melatonin in reindeer may be largely independent of circadian regulation and may be driven instead passively by the light/dark (LD) cycle [3]. To test this, we measured melatonin concentrations in reindeer exposed to artificial LD cycles of 2.5 hr light followed by 2.5 hr dark over two cycles, administered during the photophase of a natural LD cycle. By selecting a period of 2.5 hr, we were able to avoid the possibility of frequency demultiplication to modulo 24 hr.

Concentrations of melatonin remained at or below the level of detection of the radioimmunoassay (5 pg/ml) when the animals were exposed to daylight. Entrance into the dark phase of the experimental cycle induced an immediate ($\leq$15 min) rise in the melatonin titer in all animals. Levels of the hormone remained high until the onset of the light phase, when they fell precipitously, in every case returning to baseline levels within 2 sample points ($\leq$30 min). The melatonin titer rose again abruptly at the onset of the next dark phase. The pattern of the increase was individually consistent across the two cycles (Figure 1). Our previous studies have also reported a rapid rise of melatonin to peak levels within 30 min on exposure to continuous darkness, but no subsequent circadian oscillations [3].

The biosynthesis of melatonin in the mammalian pineal gland is subject to dual control involving endogenous (circadian pacemakers) and exogenous (LD cycle) components. The rate-limiting enzyme *N*-acetyltransferase (NAT) is tightly gated by transcriptional (rodents) or posttranslational (ungulates) control [4]. It is stimulated by autonomic neurons leading from the paraventricular nuclei (PVN) according to a temporal pattern that is modulated by both photic and circadian information. Thus, the conventional model is that the daily LD cycle entrains circadian oscillators in the suprachiasmatic nuclei (SCN) of the hypothalamus that rhythmically inhibit the output of the PVN [5, 6]. Consequently, the production of melatonin is intimately associated with the dark phase of the LD cycle, and the rhythmic pattern of its secretion persists when animals are exposed to continuous darkness, with levels of the hormone rising spontaneously during each subjective night [7–10]. The melatonin pattern is sculpted to the LD cycle because light acutely inhibits sympathetic outflow from the PVN to the pineal gland and suppresses NAT activity, such that the period of secretion closely matches the duration of the dark phase of the LD cycle [11]. Hence, continuous lighting, which most mammals experience only as an artificial experimental treatment but which is a characteristic feature of the polar summer, abolishes rhythmicity, and blood levels of melatonin generally remain permanently below the level of detection [12, 13].

The regulation of melatonin observed in our study diverges strongly from the standard model in which secretion of the hormone is tightly gated by the circadian clock. The duration of the daily melatonin signal in reindeer seems instead to be controlled by a strict "hourglass"-like mechanism and does not involve gating by the circadian axis. This is a novel finding in mammals and has previously been reported only in trout (*Oncorhynchus mykiss*) [14] and lizard (*Dipsosaurus dorsalis*) [15]. The data reported here are also consistent with our earlier observations that reindeer maintained under natural photoperiodic conditions at 70°N fail to exhibit robust melatonin rhythms in midwinter [16]. In the earlier study, peak nocturnal concentrations of melatonin exhibited marked seasonal variation, with maximal concentrations coincident with the equinoxes [16], the time of year also used in the present study.

### Fibroblast Clock Gene Rhythmicity

Fibroblast cells in species including laboratory rodents and man [17–19] display robust rhythmic regulation of circadian clock genes and therefore provide a convenient model in which to examine the basis of the observed absence of circadian input. To measure rhythmic activity in clock genes, we generated "insulated" *Bmal1* or *Per2* murine promoter constructs, which earlier studies have shown to exhibit robust cell-autonomous oscillations for up to 7 days in fibroblast cells [20]. These were packaged into lentiviral vectors to allow transduction of primary cells. The validity of the approach was tested by infecting mouse fibroblast cells derived from skin and lung tissue. All transduced primary mouse fibroblast

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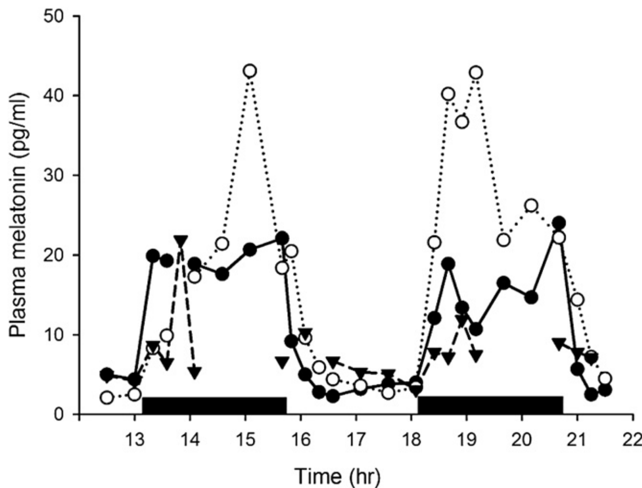


Figure 1. Melatonin Rhythms in Reindeer

Plasma melatonin concentrations in reindeer (n = 3) subjected to two cycles of 2.5 hr light/2.5 hr dark during the photophase in September.

cultures displayed robust and persistent rhythms (period for *Bmal1*::luc, 23.18 + 0.12 hr; period for *Per2*::luc, 22.74 + 0.33 hr; n = 6) (Figure 2A). The oscillations of these two promoters were in antiphase to each other when cultures were synchronized at the same time, as predicted from the known function of these genes [21]. Similar results were obtained in Syrian hamster skin-derived fibroblasts (data not shown).

Next, we tested the same constructs in reindeer fibroblast cells derived from three different animals. After transduction, cells were synchronized with forskolin before bioluminescence recording. Transduction efficiency was high, as judged by the production of a strong luciferase signal. However, in contrast to mice, reindeer fibroblasts transduced with these reporters failed to exhibit overt circadian oscillations. Using normalization against a 24 hr moving average [20], we checked to see whether we could identify any low-amplitude residual rhythmicity. In some instances, a normalized rhythm was detectable, but only for two to three cycles after synchronization (Figure 2B), after which the signal degenerated and became arrhythmic. In these cases, we did observe an initial antiphase oscillation of *Bmal1* and *Per2*, but in contrast to the mouse data, there was a wide range of periods, from 19 to 31 hr. Furthermore, our reindeer samples showed that only a low percentage of the variance of the normalized data could be explained by rhythmic oscillations, in marked contrast to the mouse data (11%–59% versus 79%–80% in mice; see Table S1 available online).

In order to assess whether the synchronization protocol that we used above (forskolin) was in some way less effective in reindeer fibroblasts, we used a glucocorticoid treatment (100 nM dexamethasone, Dex). Here, Dex induced a sustained rise in luciferase signal but, again, no overt circadian rhythmicity. Following normalization, we were able to detect low-amplitude oscillations over a few cycles in some cultures (with a wide range of periods), but most showed no significant oscillations. In contrast, the same procedure applied to a fibroblast cell line revealed strong sustained oscillations with a tight range of periods around 22.5 hr. Thus, we conclude from two different synchronization protocols that reindeer fibroblasts do not exhibit clear circadian rhythms (Figure S1).

### Seasonal Rhythms in Reindeer

Measurement of photoperiodic change is normally intimately dependent on circadian mechanisms [22], and the resulting signal is coded as a 24 hr melatonin cycle that is interpreted and translated by “calendar cells” in structures such as the pituitary pars tuberalis [23, 24]. Dual control of pineal melatonin production, involving both endogenous and exogenous components, provides the organism with a robust, precise, and flexible biological timer [1]. The regulation of melatonin production in reindeer appears to be fundamentally different in that it lacks circadian input. Our results trace this novel observation to the molecular clocks that normally drive cellular circadian rhythms throughout the body, including in the SCN.

It is generally believed that circadian rhythmicity has a significant survival value to the organism [25]. However, there can be little selective advantage to an animal being driven through subjective 24 hr cycles in an environment that, in effect, lacks 24 hr rhythmicity. Indeed, strong circadian mechanisms constrain opportunistic behavior and are therefore likely to be selected against under such circumstances [2, 26]. The absence of robust circadian clock mechanisms in reindeer (see also [2, 26]) may be an adaptive evolutionary consequence for life in the extreme photic environment to which these animals are naturally exposed (Figure 3). At high latitudes, there are long periods with continuous light in summer (“polar day”) and darkness in winter (“polar night”), whereas LD cycles occur for only a few weeks around the spring and autumn equinoxes. Our previous studies of telemetered free-ranging reindeer have shown that the mainland subspecies (used here) only exhibits clear daily (24 hr) patterns of behavior for part of the year, coincident with the equinoxes and likely entrained to the prevailing equinoctial LD cycle [26]. To date, no studies have been undertaken to determine whether behavioral cycles in mainland reindeer can free run in the absence of external entrainment (i.e., are truly circadian). Intriguingly, in the northern subspecies inhabiting the archipelago of Svalbard, no clear patterns of daily activity can be detected at any time of the year [26]. Although differences in behavior of these two subspecies may be attributable to local prevailing photoperiods, an underlying genetic contribution cannot be ruled out, an option which could be tested by examining responses of fibroblast cells from the two populations.

Synchronization of seasonal cycles in mammals is a prominent feature of physiological adaptation in northern temperate and Arctic species (see [26, 27]). Studies of seasonal sheep reveal that melatonin signals need only be present for a few weeks of the year to entrain an annual reproductive cycle [28]. It is attractive to speculate that in reindeer, informative melatonin signals associated with equinoxes directly entrain a “circannual clock” [24, 29] that, at least in reindeer, may not involve circadian mechanisms (Figure 3).

## Experimental Procedures

### Blood Sampling for Melatonin Assay

The experiment was conducted at the University of Tromsø (69°46′N) with three castrated reindeer (*Rangifer tarandus tarandus*) 15 months of age. At this age, intact reindeer are reproductively competent. The animals had free access to pelleted feed and water and were kept indoors, subject to LD cycles closely matching outdoors conditions (September; LD 17:7, lights on at 04:10). Cannulation of the exterior jugular vein allowed repeated collection of heparinized blood samples (4 ml) from each animal during two periods of darkness (each lasting 2.5 hr) separated by 2.5 hr with lights on, beginning 9 hr after morning lights on. Blood samples were collected twice at 30 min intervals prior to lights off. During darkness, four samples

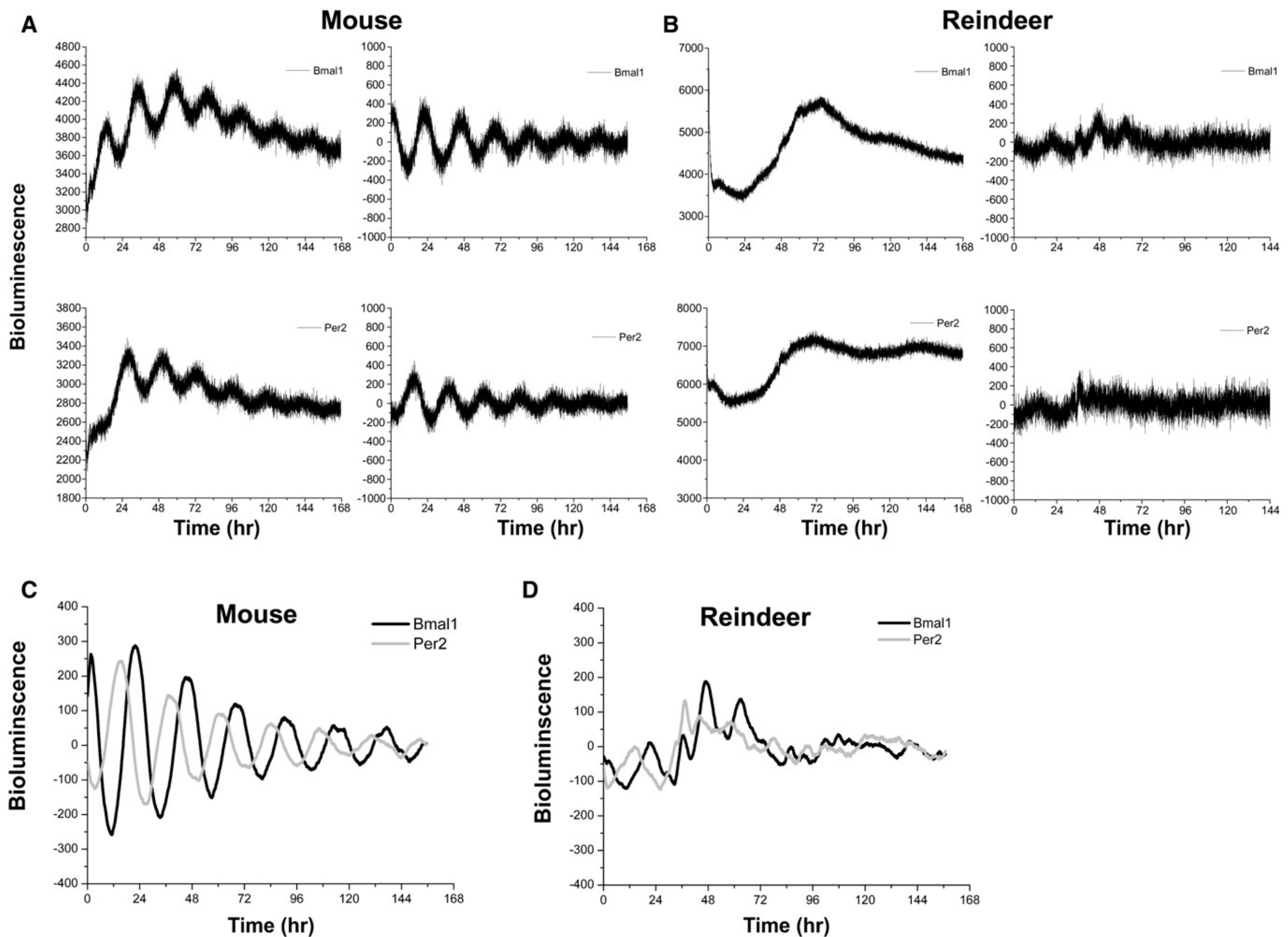


Figure 2. Molecular Circadian Rhythms in Mouse and Reindeer Primary Fibroblasts

Representative bioluminescence recordings from fibroblasts of mice and reindeer are shown. Each panel represents raw data (left) or normalized data (right).

(A) Mouse fibroblasts transduced with *Bmal1*::luc or *Per2*::luc reporters.

(B) Reindeer fibroblasts transduced with *Bmal1*::luc or *Per2*::luc reporters.

(C) Mouse fibroblast data (same as in A) replotted as spline fit (MATLAB).

(D) Reindeer fibroblast data (same as in B) replotted as spline fit (MATLAB). See also Table S1 and Figure S1.

were initially taken at 15 min intervals followed by three at 30 min intervals. This sampling regime was repeated in the intervening light period and during the second dark period. Three samples at 15 min intervals were taken after the final transfer to light. Immediately after each sampling, plasma was separated by centrifugation and stored at −20°C until assay.

A red light yielding <0.1 lux at the animals' heads remained on during darkness. The mean intensity of white light was 300 lux at the animals' heads, measured by a handheld sensor (EC1, Hagner AB). Permission to conduct the experiment on reindeer was granted by the National Animal Research Authority of Norway (NARA).

#### Radioimmunoassay

Plasma melatonin was assayed via a radioimmunoassay procedure described previously [30]. Reindeer plasma was spiked with 25 or 50 pg/ml melatonin. Levels measured in two assays were 5.2 and 5.9 pg/ml in untreated plasma, 28.6 and 30.5 pg/ml for 25 pg spike, and 46.0 and 44.4 pg/ml for 50 pg spike. Intra-assay coefficients of variation were 13.8%, 9.6%, and 7.3% at 13.6, 40.6, and 82.8 pg/ml, respectively.

#### Primary Fibroblast Culture from Mice and Reindeer

Primary mouse fibroblasts were generated from lung tissue by a standard enzymatic digestion procedure [19, 20]. Cells were then dissociated by collagenase digestion, filtered through a sterile nylon mesh, washed twice, and centrifuged in chilled Hank's solution. Pellets were resuspended in culture medium and plated on a T25 cell culture flask. Cultures were maintained at 37°C (5% $CO_2$) for 2–3 days until confluent and ready for splitting. Mouse fibroblasts were then cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum and antibiotics (100 U/ml penicillin and 100 μg/ml streptomycin).

Primary mouse and reindeer skin fibroblasts cultures were established via a tissue explant adherent method. Briefly, skin samples were cut into pieces of 2 mm size and put into culture flasks for an initial 15 min. After the tissue pieces attached to the flasks, culture medium (Amniomax II, GIBCO Invitrogen) was added. Typical spindle-like cells organized in "whirls" indicate the healthy growth of the fibroblast population.

#### Generation of Reporter Constructs

Lentiviral transfer construct pLV and packaging plasmids pMD2-VSV-G, pMDLg/pRRE, and pRSV-REV were a gift from O.T. Jones (University of Manchester, UK). To generate a plasmid mediating transduction of m*Bmal1* and m*Per2* by lentivirus (pLV-*Bmal1* or pLV-*Per2*), we digested pGL4-*Bmal1*-luc and pGL4-*Per2*-luc [20] with the restriction enzymes NdeI and SalI. The resulting fragments were then cloned into the corresponding sites of the lentiviral transfer vector. The promoter constructs had been engineered with chicken *beta-globin* insulator sequences to avoid inappropriate gene silencing or positioning effects of chromatin [20]. The identity of all constructs was confirmed by DNA sequencing (University of Manchester, Core Facility).

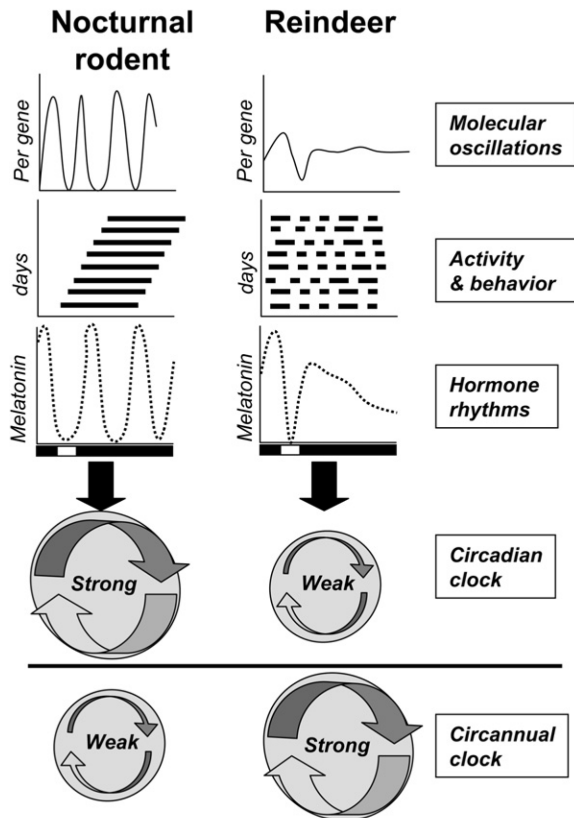


Figure 3. Schematic Model for Circadian and Circannual Clocks in Rodents and Reindeer

In a short-lived nocturnal rodent, strong clock gene circadian oscillations have been reported for multiple tissues, including primary fibroblasts. Furthermore, activity cycles are robustly circadian under constant conditions and are entrained to the prevailing light/dark cycle, allowing the animal to "light sample" and constrain activity to the hours of darkness [36]. Hormone rhythms are also strongly rhythmic and, in the case of melatonin, track the prevailing photoperiod cycle. In seasonal rodents (e.g., Syrian and Siberian hamsters), melatonin-regulated reproductive cycles are activated by photoperiod change, but there is no evidence in such short-lived animals for the occurrence of spontaneous "circannual" reproductive rhythms when exposed to constant photoperiods. In reindeer, strong molecular oscillations of clock genes are not detected, and this is associated with lack of free-running activity cycles under constant photic conditions in the wild [26]. Melatonin is acutely responsive to photoperiod but is not regulated by a circadian clock ([3], present study). In contrast to short-lived nocturnal rodents, long-lived seasonal mammals exhibit robust circannual cycles, which we speculate in reindeer may be entrained by a short period of melatonin information provided at the two equinoxes, which act as a seasonal "zeitgeber."

### Viral Packaging and Transduction

Recombinant lentiviral particles were produced by transient cotransfection of HEK293FT cells (Invitrogen) via the calcium phosphate method [31]. After 2 days, supernatants from transfected cells were collected, concentrated with Vivaspin 20 centrifugal concentrators (Sartorius Ltd.), and used immediately. Lentiviral transduction of mouse and reindeer fibroblast cells was performed by adding 100 μl of the viral preparation (see above) to cell culture medium (50% confluence) in 35 mm dishes. After 48 hr, cells were subjected to real-time bioluminescence recording. All steps were performed under level II biosafety conditions.

### Real-Time Bioluminescence Recording

Confluent cells in 35 mm dishes were synchronized by treatment with forskolin (10 μM) for 1 hr. The medium was changed to non-phenol red DMEM supplemented with 0.1 mM luciferin substrate [32]. Each 35 mm dish was sealed with vacuum grease and placed in a light-tight and temperature-controlled environment at 37°C. Light emission (bioluminescence) was measured continuously with a photomultiplier tube (H6240 MOD1, Hamamatsu Photonics). Data are presented as photon counts per minute. Baseline correction was calculated by using a 24 hr moving average and then subjected to spline fit (MATLAB). In some instances, a second corticosterone treatment (200nM, 500 nM, or 1 μM concentration) or 0.1% dimethyl sulfoxide (vehicle control) was administered, and samples were monitored continuously for at least 6 days. Periods were analyzed by RAP software [33].

Bioluminescence signals from reindeer cells were assessed with Clockwise curve fitting software (developed in-house by T. Brown, University of Manchester) [34, 35] to determine the period and significance of circadian variation. Briefly, data were normalized such that they spanned a range of values between 100 and −100. Normalized data were fit with the equation $Y = A \sin[B(x + C)]$ via the Newton-Raphson iterative method, where A equaled the amplitude of the rhythm, B equaled the period in radians per hour, and C determined the phase. Initial values of A, B, and C were estimated from the best-fitting curve of a series of >3000 standard curves with periodicities between 3 and 34 hr and a range of different amplitudes and phasing. Significant rhythmic variation in the data was assessed by repeating the curve fitting procedure 1000 times with the same data set, but with the order of observations randomized with respect to time.

### Supplemental Information

Supplemental Information includes one table and one figure and can be found with this article online at doi:10.1016/j.cub.2010.01.042.

### Acknowledgments

We would like to thank M. Nystad for preparing reindeer fibroblast cells and T. Brown for statistical advice. This work was supported by research grant BB/E022553/1 from the UK Biotechnology and Biological Sciences Research Council.

Received: December 1, 2009
Revised: January 14, 2010
Accepted: January 15, 2010
Published online: March 11, 2010

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