SHORT COMMUNICATION

Waiting for the Sun: the circannual programme of reindeer is delayed by the recurrence of rhythmical melatonin secretion after the arctic night

David Hazlerigg[1,*], Arnoldus Schytte Blix[1,2] and Karl-Arne Stokkan[1,*]

ABSTRACT

At temperate latitudes, the annual cycle of day length synchronizes circannual rhythms, and, in mammals, this is mediated via nocturnal production of the pineal hormone melatonin, proportional to the length of the night. Here, we studied circannual synchronization in an arctic species, the reindeer (*Rangifer tarandus tarandus*), which ceases to produce a rhythmic melatonin signal when it is exposed to extended periods of continuous midwinter darkness and continuous midsummer light. Using food intake, antler growth and moult as endpoints, we demonstrate that when animals living at 70°N are transferred from natural photoperiods in late autumn to either continuous light or continuous darkness, they undergo a conspicuous acceleration of the circannual programme. We conclude that rhythmical melatonin secretion, recommencing when the Sun reappears late in January, is required for proper timing of spring physiological responses, through a delaying effect on the circannual programme set in motion during the preceding autumn.

KEY WORDS: *Rangifer*, Photoperiod, Pars tuberalis, Pineal gland, Circadian rhythms

INTRODUCTION

Seasonal rhythms in physiological responses depend upon innate long-term (circannual) timers, the phase of which is determined by photoperiodic synchronization. Circannual timers are synchronized to the annual environmental cycle through a process known as photoperiodic entrainment. This involves measurement of day length, which, in mammals, is transduced into a nocturnal rhythm of melatonin secretion by the pineal gland. Without the pineal gland, mammals either cease to express circannual rhythms or express disorganized rhythms that are no longer entrained to the solar year (Hazlerigg and Simonneaux, 2014).

In rodents and in sheep, the mechanisms through which changes in melatonin signal duration are transduced rely upon a circadian-based 'coincidence timer' residing in the pars tuberalis (PT) of the anterior pituitary (Dardente et al., 2010; Matsumoto et al., 2010). This timer converts changes in nightly duration of melatonin exposure into changes in the amplitude of expression of a transcription coactivator and developmental switch, Eya3. Eya3 promotes the expression of differentiated thyrotrophs in the PT, in turn governing hypothalamic function through thyroid hormone-dependent mechanisms (Dardente et al., 2014). According to the current model (Dardente et al., 2010; Matsumoto et al., 2010; Dardente et al., 2014), shorter melatonin signals generate large amplitude daily oscillations of Eya3 RNA expression in the PT, while long-duration signals suppress the oscillation via a direct suppressive effect of melatonin in a critical time window occurring approximately 12 h after dark onset. Hence, induction of a physiological programme typical of spring, such as antler growth and moult in reindeer (*Rangifer tarandus*), is seen as a consequence of an increasing amplitude of a melatonin-dependent circadian rhythm of PT gene expression, induced by exposure to long days. The importance of the nightly rhythm of melatonin secretion is supported by experiments in pinealectomized rodents and sheep, in which the absence of melatonin or melatonin replacement as a continuous infusion failed to synchronize or maintain circannual rhythms in reproduction, body mass or prolactin secretion (Bartness and Goldman, 1989; Bartness et al., 1993; Lincoln et al., 2006).

The capacity of arctic species to maintain photoperiodic synchronization of circannual rhythms challenges this model, because for long periods of the year surrounding the summer and winter solstices, the rhythmicity of melatonin secretion disappears (Stokkan et al., 1994). This suggests either that circannual entrainment relies upon narrow time windows around the equinoxes or that in species adapted to high latitudes, periods of continuous presence or absence of melatonin can act as circannual synchronizers. Experiments to distinguish between these possibilities have not been undertaken.

If autumnal day length exposure is sufficient to set the phase of the circannual programme that runs during the winter, then subsequent exposure to continuous darkness or continuous light should not affect the timing of the spring programme of physiological change. Here, we tested this prediction in reindeer raised under semi-natural conditions at 70°N. Our data demonstrate that rhythmical melatonin secretion, which reappears with the return of the Sun in late January (Stokkan et al., 1994), is an essential requirement for proper phasing of the circannual rhythm.

MATERIALS AND METHODS
Animals

A total of 15 semi-domesticated male Eurasian reindeer, *Rangifer tarandus* (Linnaeus 1758), aged 17 months were used for the described photoperiod treatments. Experimental procedures underwent local ethical review before the study commenced.

Protocol

Space limitations permitted up to two indoor photoperiod treatments to be conducted at any one time.

[1]Department of Arctic and Marine Biology, UiT – The Arctic University of Norway, Tromsø NO-9037, Norway. [2]St Catharine's College, Cambridge CB2 1RL, UK.

*Authors for correspondence (david.hazlerigg@uit.no; kst002@post.uit.no)

D.H., 0000-0003-4884-8409

Effect of transfer from outdoor natural photoperiod to continuous darkness

In late January, at the time of year when rhythmical melatonin secretion begins again after the winter solstice (Stokkan et al., 1994), a group of six reindeer were moved indoors from an outdoor paddock in Tromsø, Norway (70°N, 19°E), where they had been exposed to natural photoperiod (NP). They were kept in a 50 m² room where they could move freely and where the temperature was kept constant at 11°C. The room was isolated from natural light. One dim red light bulb gave a constant ca. 0.1 lx at head level to facilitate animal care; this treatment is referred to hereafter as constant darkness (DD). The animals had *ad libitum* access to pelleted reindeer food (FK Reinfôr, Felleskjøpet, Norway) and water throughout. Food was provided in troughs, and the daily food intake of the whole group was recorded by weighing the added and remaining food. Throughout the study, the behaviour of the animals and the status of their fur and antler condition were routinely assessed. The experiment ended in mid-June. The response to this DD exposure was compared with previously published reference data from animals in the outdoor enclosure that the DD group were taken from (Tyler et al., 1999).

Comparison of exposure to simulated NP and continuous light

In early October, a total of nine reindeer were taken indoors from the same Tromsø outdoor enclosure, and were held in the same housing as used for the DD manipulation. Here, they were exposed to artificial white lighting provided by fluorescent tubes, giving a mean light intensity of approximately 1000 lx at head level, measured by a hand-held sensor (EC1; Hagner AB, Solna, Sweden).

From mid-October, the day length was reduced weekly, mimicking the natural photoperiod decline in a stepwise fashion and reaching 4 h of light per day in mid-November. The photoperiod was kept at this level until 1 December, mimicking the period of approximately constant photoperiod at the winter solstice. From this point forward, four reindeer were transferred to constant light (LL) until the end of the study, while five reindeer were exposed to a progressively increasing, simulated natural photoperiod (SNP) mimicking the outdoor increase in day length. Data on voluntary food intake (VFI), pelage and antler growth were collected as described for the DD animals.

Moulting

Moulting of winter fur is a slow process and can be difficult to assess (Mesteig et al., 2000). We used the appearance of a dark ring of new hairs around the eye as the first sign of shedding of winter fur, and a general assessment of no remaining winter fur as indicative of a completed moult.

Antler growth

Visual inspections were made at regular intervals to assess the growth of new antlers. In DD, the length of the antlers and the degree of branching were assessed weekly by visual inspection. For the animals in natural light and on LL, photos were taken to document the growth process.

Statistical analysis

All dates were converted to Julian days (Julian day 1=1 January) to allow regression analyses to be performed.

The data on VFI in DD were compared with previously published data from animals held in the Tromsø outdoor enclosure (Tyler et al., 1999) by fitting linear regression lines from the date on which recording of VFI in DD animals commenced (Julian day 30).

For the SNP versus LL comparison, changes in VFI over time were modelled as a segmental regression, effectively fitting one regression line to the period of the winter nadir in VFI and a second line from

when the upturn in VFI occurs in the spring. As well as the slopes and intercepts for the two regression lines, this approach gives an estimate of the breakpoint (X_0) between the two lines, and this constitutes an estimate of when the spring increase in VFI commences. Regressions were performed in GraphPad Prism version 6, using the non-linear curve fitting function, and setting no constraint on parameter values. Only fits giving a *P*-value less than 0.05 were accepted, and all fits were unambiguous solutions obtained within 1000 rounds of iteration. For significant fits, parameter values (slope and intercepts of two lines, and best estimate of X_0) were compared by an extra sum of squares *F*-test.

RESULTS AND DISCUSSION

Effect of photoperiod conditions on VFI

Previous work (Tyler et al., 1999) showed that in reindeer raised outdoors under semi-natural conditions, VFI gradually increases from a midwinter nadir during the first half of the year. Data from this earlier work are replotted in Fig. 1 for reference (grey shaded area).

Compared with outdoor animals, animals transferred indoors into DD showed a much steeper increase in VFI (regression slope coefficients, mean±s.e.m.: DD 0.018±0.001; outdoors 0.005± 0.001, *P*<0.001) with no evidence of any delay in the upturn in VFI following transfer to DD (comparison of linear regression versus segmental regression fits, *P*=0.57).

We next compared VFI in reindeer housed indoors under SNP and under LL (Fig. 1). In both cases, a segmental regression fit to the data was significantly better than a simple straight-line fit (*P*<0.001). In SNP control animals, daily VFI remained low until about the end of March, after which point a shift to progressively increasing daily intake was observed. By segmental regression analysis, the best estimate of the break point (X_0) between stable low VFI and increasing springtime VFI occurred on Julian day 108±37. In LL animals, food intake was stable at about 2 kg animal⁻¹ day⁻¹ until the end of February, when a conspicuous increase began, reaching a peak in mid-May at 4.7 kg animal⁻¹ day⁻¹ (Fig. 1). The timing of the breakpoint (X_0) occurred some 70 days earlier than in
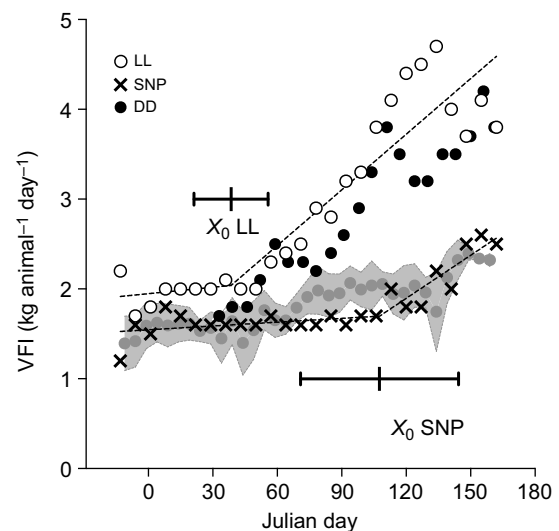

Fig. 1. Food intake in reindeer under different indoor photoperiod conditions. SNP, simulated natural photoperiod (crosses, *n*=5 males); DD, constant darkness (black circles, *n*=6 males); LL, constant light (white circles, *n*=4 males). Grey circles and shading show mean±s.e.m. voluntary food intake (VFI) in reindeer held in the Tromsø outdoor enclosure (*n*=6 females and *n*=3 males). X_0, breakpoint between the regression lines. Adapted from Tyler et al., 1999.

the corresponding SNP controls (Julian day 39±17; $P<0.001$ for comparison of best-fit segmental regressions).

Moulting and antler growth

Under SNP, moulting began in early June and was completed in early August. The antlers of the animals were photographed at intervals (Fig. 2), and their growth began in April, whereas cleaning (velvet shedding) began in August. In animals placed in LL, moulting of winter fur began in early March, and was completed in late April/early May. Growth of new antlers began in early March (Fig. 2). Cleaning of the antlers began in early June and rutting behaviour began in late June. In DD animals, moulting of winter fur began in late April and was completed in early June. Growth of antlers began in late February/early March (Fig. 2). Cleaning of the antlers had not started when the study ended in mid-June and no aggressive/rutting behaviour had been observed up to that time.

Conclusions

We have shown that, compared with reindeer held outdoors on NP or indoors on SNP, reindeer transferred to LL or maintained in DD from

Fig. 2. Summary of antler growth, moult and rutting in reindeer exposed to different photoperiods. Top: natural changes in daylight (SNP, simulated natural photoperiod). Middle: continuous light (LL). Bottom: continuous darkness (DD). Photographs of reindeer in SNP and LL were taken at times indicated by arrows. Coloured bars show the observed ranges of the time windows for different stages in antler and pelage condition and for the timing of the rut.

mid-winter onwards show conspicuous accelerated increases in food intake, antler growth and pelage moult. This result implies an escape from a winter state maintained by exposure to short photoperiods. Previous studies in temperate species have shown that responses to continuous melatonin implants are often similar to those seen for pinealectomy, suggesting that both manipulations carry no day length information (Bartness et al., 1993; Lincoln et al., 2006). As reindeer have a remarkably weak circadian clock (van Oort et al., 2005; Lu et al., 2010), we suspect a similar basis for the responses to LL and DD seen in the present study. Indeed, a year-round analysis of melatonin secretion in reindeer held in Tromsø revealed that the rhythmical production of melatonin disappears for approximately 2 months around the winter and summer solstices (Stokkan et al., 1994), being replaced by continuous production of melatonin, differing only in the level of tonic secretion. Hence, we consider LL and DD to be equally neutral as circannual synchronizers.

Given that, even under NP, no rhythmic melatonin production occurs from November to January, the observed effects of the LL and DD treatments must be due to their impact on processes going on after the return of the Sun and the re-establishment of the melatonin rhythm. Accordingly, we surmise that for a period around the winter solstice, reactivation of spring physiology is not possible, presumably because necessary preparative processes are not complete. Subsequently, from February onwards, reactivation is possible, but is held back by short day-type patterns of rhythmical melatonin secretion. Animals exposed to LL or DD at this time escape this braking effect of rhythmical melatonin production, giving the premature reactivation we observe. Accordingly, the prediction is that under NP, the braking effect is released sometime around the spring equinox when rhythmical photoperiods of sufficient duration are encountered. Whether such 'long photoperiods' applied mid-winter might further accelerate the upturn in food intake, beyond the effect seen with LL/DD exposure, remains to be determined.

Previously, Karsch and colleagues found that the breeding season of pinealectomized sheep could be synchronized through infusion of melatonin to simulate endogenous melatonin production at specific times of year (Woodfill et al., 1994). This work emphasized the importance of summer photoperiods to set the annual programme in motion, and suggested that winter day lengths are weak synchronizing signals. In actual fact, a re-examination of their data reveals that autumn breeding happens prematurely in ewes exposed only to a melatonin signal mimicking the summer period, also implying an important braking effect of the photoperiod cycle on the circannual rhythm.

We propose that these different observations can be reconciled by a model in which photoperiod has dual effects on the expression of circannual rhythms (Fig. 3): periods of exposure to long days set in motion circannual cycles of change, the precise phasing of which is further modulated by patterns of short day exposure. According to this model, if long day-type melatonin signals are removed (as is the case in pinealectomized animals), short-day melatonin signals are insufficient to maintain a circannual rhythm (this is the case for the 'winter melatonin infusion' of Woodfill et al., 1994). However, if short day-type melatonin signals are removed, as is the case in the present study, a robust but phase-advanced circannual rhythm ensues. Hence, appropriate circannual phasing depends upon important braking effects of photoperiod at multiple phases of the year.

The concept that spring day lengths are of major importance for photoperiodic entrainment shapes thinking regarding molecular time-keeping mechanisms. In current working models for photoperiodic entrainment, as the duration of the nocturnal melatonin signal declines with increasing day length, it triggers a spring response by enhancing the amplitude of circadian rhythms of key transcriptional
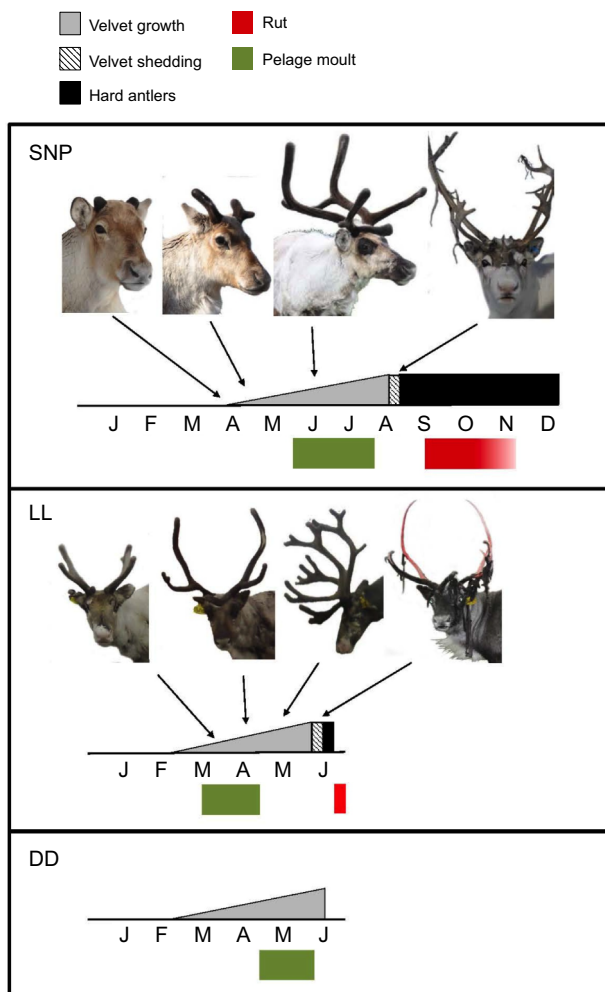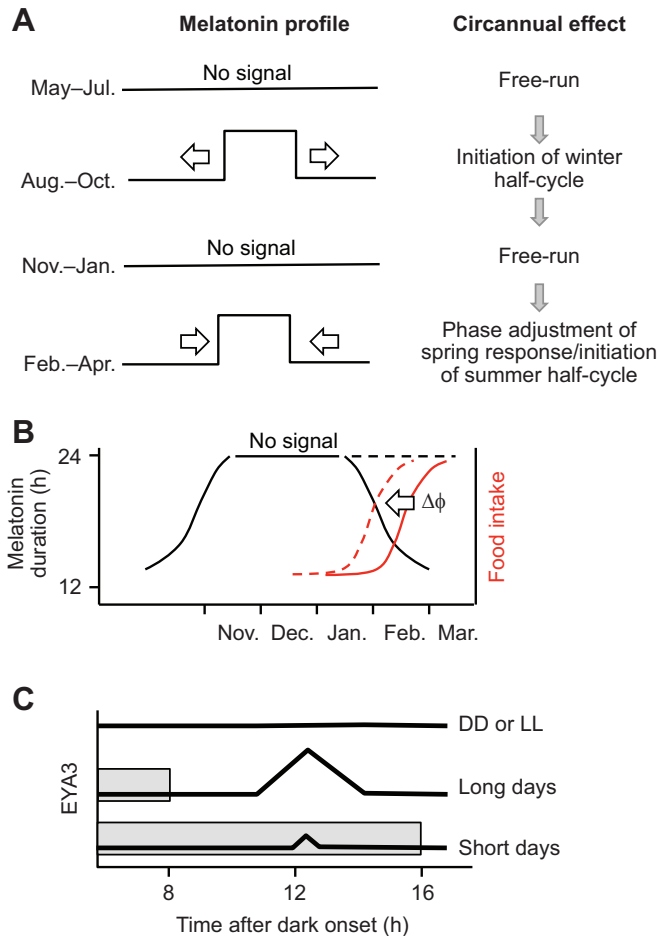
Fig. 3. Phasing of seasonal physiological cycles through a decelerated circannual rhythm. (A) Cartoon of melatonin signal waveforms in arctic reindeer based on data collected under outdoor conditions (Stokkan et al., 1994). From the end of the arctic summer until the onset of the arctic night, the nocturnal melatonin signal increases in duration and this autumn signal initiates a circannual timer for the expression of the winter phenotype. During the arctic night, when the melatonin rhythm breaks down, this circannual timer free-runs. From the end of the arctic night to the onset of the arctic summer, rhythmical melatonin secretion returns, with a nocturnal signal that becomes progressively shorter, offering a spring synchronizing signal. With the onset of the arctic summer, the nocturnal melatonin signal disappears, and a further bout of free running occurs. (B) The observed phase-advancing (Δφ) effect of continuing artificial exposure to LL or DD after the onset of the arctic night. Solid black lines indicate the nocturnal duration of the melatonin signal, becoming continuous in late November. Dashed black line indicates the consequence of transfer to LL or DD after this point, i.e. a continuation of the 'no signal' state for melatonin. Solid and dashed red lines indicate the observed food intake profiles under natural conditions and following LL/DD exposure, respectively. The graph indicates that in the absence of early spring photoperiodic signals, the circannual rhythm runs fast, leading to a premature reactivation of spring metabolic physiology. (C) Implications of this result for our understanding of melatonin-based 'coincidence timing' in the pars tuberalis, based on Dardente et al. (2010) and Matsumoto et al. (2010). EYA3 is a developmental switch driving increased expression of thyroid-stimulating hormone, and this is the signal for activation of spring physiological state by the hypothalamus. When there is a rhythmical melatonin signal, EYA3 expression peaks about 12 h after dark onset. Because melatonin suppresses EYA3 expression, this leads to low levels of EYA3 under short days, but a strong peak under long days. The response to LL/DD observed in the present study implies that short-duration nocturnal melatonin signals are not required for elevation of EYA3 expression.

activators in the PT (Dardente et al., 2010; Matsumoto et al., 2010; Dardente et al., 2014). The present study challenges the assumption that circadian rhythmicity is important because both LL and DD block circadian rhythmicity in reindeer (Stokkan et al., 1994; Van Oort et al., 2005). Moreover, constant low or high levels of melatonin are likely to abolish all rhythmicity in the PT – as has been shown previously in pinealectomized hamsters or mice genetically deficient in melatonin synthesis (Messager et al., 2000; van Gall et al., 2002). If this is indeed the case, then research should focus on why rhythmical patterns of PT gene expression on short days hold back the spring programme, rather than on the importance of circadian waveforms induced by exposure to longs days in spring.

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