Photoperiodic modulation of adrenal gland function in the rhesus macaque: effect on 24-h plasma cortisol and dehydroepiandrosterone sulfate rhythms and adrenal gland gene expression

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Abstract

In temperate zones, day length changes markedly across the year, and in many mammals these photoperiodic variations are associated with physiological adaptations. However, the influence of this environmental variable on human behavior and physiology is less clear, and the potential underlying mechanisms are unknown. To address this issue, we examined the effect of changing photoperiods on adrenal gland function in ovariectomized female rhesus macaques (*Macaca mulatta*), both in terms of steroid hormone output and in terms of gene expression. The animals were sequentially exposed to the following lighting regimens, which were designed to simulate photoperiods associated with winter, spring/autumn and summer respectively: 8 h light:16 h darkness (short days), 12 h light:12 h darkness and 16 h light:8 h darkness (long days). Remote 24-h serial blood sampling failed to disclose

any effect of photoperiod on mean or peak plasma levels of cortisol or dehydroepiandrosterone sulfate. However, there was a marked phase-advancement of both hormonal rhythms in short days, which was reflected as a similar phase-advancement of the daily motor activity rhythm. Gene microarray analysis of the adrenal gland transcriptome revealed photoperiod-induced differences in the expression of genes associated with homeostatic functions, including: development, lipid synthesis and metabolism, and immune function. Taken together, the results indicate that in primates, both circadian adrenal physiology and gene expression are influenced by seasonal changes in day length, which may have implications for adrenal-regulated physiology and behavior.

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Introduction

In mammals, many aspects of physiology and behavior are temporally regulated, showing circadian as well as circannual rhythms. Whereas, circadian rhythms reflect the daily organization of body functions, circannual rhythms represent an adaptation to the seasonal variations that occur in a natural environment throughout the year. It is well established that circadian rhythms are intrinsic to a wide range of body functions, including the sleep–wake cycle, metabolism, immune response, and reproduction (Hastings *et al.* 2007). On the other hand, circannual rhythms have been reported in metabolism, reproduction, and immune function in a number of mammalian species (Bilbo *et al.* 2002, Nakao *et al.* 2008). In humans, seasonal variations have been reported for blood pressure, immune response, birth rate, and sleep duration, as well as for behavioral traits associated with seasonal affective disorders, bulimia nervosa, anorexia, and suicide (Bronson 2004).

Although the underlying mechanism that regulates circannual rhythms is unclear, there is evidence that seasonal neuroendocrine changes are among the main causal agents.

In particular, circannual oscillations in one of the major neuroendocrine structures, the hypothalamus–pituitary–adrenal (HPA) axis has been regarded as a potential mediator of seasonal changes, a view supported by the major role played by adrenal steroids in physiology and behavior. This hypothesis stems from the fact that the HPA axis is temporally regulated by the circadian pacemaker located in the suprachiasmatic nuclei (SCN) of the hypothalamus, which drives the rhythmic secretion of two major adrenal steroids, cortisol, and dehydroepiandrosterone sulfate (DHEAS; Hastings 1991, Urbanski *et al.* 2004, Downs *et al.* 2008). Furthermore, light itself exerts a remarkable effect on adrenal physiology; in mice, light exposure at night induces both gene expression and the secretion of corticosterone, through a pathway that involves the SCN and the sympathetic nervous system (Ishida *et al.* 2005).

Although circannual variations have also been reported for adrenal corticoids, the available data are largely inconclusive. In the case of cortisol, some human studies have reported seasonal differences (Van Cauter *et al.* 1981, Levine *et al.* 1994, Walker *et al.* 1997, King *et al.* 2000, Hansen *et al.* 2001), whereas others have failed to disclose such variations

(Agrimonti *et al.* 1982, Wehr *et al.* 1993, Van Dongen *et al.* 1998, Lac & Chamoux 2006). Similarly, some studies have reported seasonal differences in DHEAS levels (Deslypere *et al.* 1983, Nicolau *et al.* 1984, Garde *et al.* 2000), whereas one study found no seasonal difference (Bjornerem *et al.* 2006).

In the present study, we used the rhesus macaque animal model to examine if circannual changes in day length can significantly influence adrenal gland function. Specifically, our first aim was to examine the effect of different photoperiods on plasma corticosteroid rhythms, both in terms of magnitude and in relation to motor activity rhythms. Our second aim was to explore potential photoperiod-induced gene expression changes within the adrenal gland using gene microarray. The results show that day length affects specific parameters of the 24-h plasma cortisol and DHEAS rhythms as well as the daily activity–rest cycle. Moreover, seasonal-like changes in day length influence the expression of genes involved in development, lipid synthesis and metabolism, and immune response.

Materials and Methods

Animals

To avoid the confounding influence of changing sex-steroid concentrations across the menstrual cycle and across different photoperiods, the study used three long-term (>3 months) ovariectomized adult female rhesus macaques (*Macaca mulatta*; age range: 8.5–12 years old). Each animal was fitted with an indwelling sub-clavian vein catheter, as previously described (Downs *et al.* 2008), which remained implanted for the duration of the study. The animals were caged singly in a temperature-controlled environment, with fixed light cycles that comprised 12 h of light per day (i.e. 12 h light:12 h darkness), and were cared for by the Oregon National Primate Research Center (ONPRC) Division of Animal Resources, in accordance with the National Research Council's Guide for the Care and Use of Laboratory Animals.

As part of a longitudinal experimental design, the animals were then sequentially exposed to the following three photoperiods: 8 h light:16 h darkness; 12 h light:12 h darkness and 16 h light:8 h darkness, for 10 weeks in each case. These three photoperiods were selected because they represent the winter, spring equinox, and summer respectively in Oregon. Note, in order to further facilitate the comparison of phases between the photoperiods, the time of lights on was fixed at 0700 h for each photoperiod. Primate chow (Purina Mills Inc., St Louis, MO, USA) was made available to the animals twice daily, at 0800 h and again at 1500 h. This diet was supplemented with fresh fruit and vegetables, which were provided at the time of the afternoon meal; drinking water was available at all times.

For the gene microarray analysis, nine ovariectomized adult (age range: 8.5–12 years old) female rhesus macaques

were used. The animals (three per light regimen) were maintained under the photoperiods 12 h light:12 h darkness, 8 h light:16 h darkness and 16 h light:8 h darkness for 10 weeks. At the end of that period, the animals were anesthetized with ketamine (15–25 mg/kg i.m.) followed by pentobarbital sodium (25–30 mg/kg i.v.) and exsanguinated. This method of euthanasia is consistent with the recommendations of the American Veterinary Medical Association's Panel on Euthanasia. In all cases, necropsies were performed within a narrow window of time (1000–1300 h). Various postmortem tissues were collected and made available to other investigators through the ONPRC Tissue Distribution Program. This research was approved by the Institutional Animal Care and Use Committee.

Activity recording and analysis

The activity–rest cycles of individual animals were continuously monitored using Actiwatch activity recorders (Mini Mitter Company Inc., Bend, OR, USA). Measure of gross motor activity by the accelerometers was digitally integrated into activity bouts of 5 min duration. Analysis of the activity bouts was performed using Sleepwatch software (Mini Mitter Company Inc). Total daily activity was estimated by calculating the average number of bouts per day, per animal, over time. Total photophase and scotophase activity were calculated by measuring the average number of bouts per animal, during photophase and scotophase respectively. Cosine correlation was used to estimate the phase of motor activity rhythm in each photoperiod.

Remote 24-h blood sampling

After ~10 weeks of exposure to each of the three photoperiods, blood samples were collected remotely from an adjacent room, via the indwelling vascular catheter and a swivel/tether-based sampling system (Downs *et al.* 2008). Beginning at 0700 h, hourly blood samples were collected from the undisturbed animals for an entire day, including their sleep period. The blood was immediately transferred into EDTA-coated borosilicate glass tubes, centrifuged at 4 °C, and the plasma supernatant was stored at –20 °C until assay.

Hormone assays

Plasma concentrations of cortisol and DHEAS were determined at the ONPRC Endocrine Services Laboratory as previously described (Downs *et al.* 2008). Briefly, cortisol levels were measured by electrochemiluminescence using an Elecsys 2010 Platform (Roche Diagnostics). DHEAS levels were determined by RIA, using an antibody raised against DHEAS-17-(O-carboxymethyl)oxime-BSA, and [³H]DHEAS (22 Ci/mmol). Intra-assay and inter-assay coefficients of variation were <10% for each assay. Assay detection limits were 3 ng/ml for both the steroids.

Terminology and statistical analysis

The terms photophase, and scotophase refer to the illuminated and the dark segment of a light–darkness cycle respectively. Rhythmic parameters, including amplitude and mesor were determined by Cosinor analysis. The acrophase measures the timing of a rhythm relative to a reference time point defined by the investigator, and is used for data which fits a mathematical model; for example, in a cosine curve fit the acrophase represents the rhythm's peak. The mesor represents the adjusted mean of a cosine function fitted to the data. Statistical comparisons for motor activity, as well as hormone measurements, were performed using repeated measures ANOVA followed by the Bonferroni test.

RNA extraction and gene microarray

During necropsy, the adrenal glands were quickly frozen in liquid N₂ and total RNA was extracted using RNeasy columns (Qiagen); the final concentration and purity were determined by spectrophotometry, and the integrity was assessed using an Agilent 2100 Bioanalyzer (Agilent Technology, Palo Alto, CA, USA). Complementary cDNA synthesis, cRNA synthesis, hybridization, and array scanning were performed by the OHSU Affymetrix Microarray Core, as described in the Affymetrix GeneChip Expression Analysis Technical Manual. As in previous studies (Wang *et al.* 2004, Dillman & Phillips 2005), the Affymetrix human HG_U133A gene microarray platform was used. RNA samples from individual adrenal glands were hybridized separately on their own GeneChip arrays.

Raw scanner image files were analyzed using the Affymetrix MAS 5.0 absolute expression analysis comprised in the GeneSifter software (Greospiza Inc, Seattle, WA, USA). The parameters α_1 and α_2 , which set the point at which a probe set is called present (P), marginal (M) or undetectable (A) were set to 0.1 and 0.15 respectively. Comparisons were performed using global scaling, with the target intensity value set to an average intensity of 325; this allowed for the direct comparison of hybridization values from the different arrays. Pair-wise statistical comparisons between photoperiods were made using Student's *t*-test, with photoperiod 12 h light:12 h darkness as the control group.

Taqman quantitative reverse-transcription PCR

Random-primed reverse transcription was performed to prepare the cDNA samples, using 200 ng RNA and the Omniscript kit (Qiagen). The reverse transcription reaction was diluted 1:100 for PCR analysis. The PCR reactions mixtures contained 5 μ l Taqman Universal PCR Master Mix, 300 nM specific target gene primers, 50 nM human β -actin primers, 250 nM specific probes, and 2 μ l cDNA, as previously described (Lemos *et al.* 2006). Standard curves were used to convert the critical threshold values into relative RNA concentrations for each sample, and the results

were expressed relative to β -actin, a housekeeping gene. The primers and probes were designed based on human sequences, using PrimerExpress software (Applied Biosystems), and purchased from Invitrogen and Sigma Genosys respectively.

The sequences were: NCKAP1-F, CATTGGCACAA-GAAGCACTTAGAG; NCKAP1-R, GGTAATCCG GCA GCTGATGA, NCKAP1 probe, 5'-6FAM-CCACATTCC TTTTCTTGTAAGTTCAA-TAMRA-3'; FADS1-F, CCC CTGGTGCAGTCCTTGT; FADS1-R, GGCAGGGTGG CTGTTGTTAT; FADS1 probe, 5'-6FAM-TCCAAGCCC CTGCTGTCAGC-TAMRA-3'; IL11-F, GGCTGCACCT-GACACTTGACT; IL11-R, TGTTCGCCCCCAGT ACTG; IL11- probe, 5'-6FAM-TGCTGCTGAAGACT CGGCTGTGA CC-TAMRA-3'; ACSL1-F, CAAGCCTC-CAGCACCTGAAG; ACSL1-R, TGGGCAAGGATTGA CTG TATTCT, ACSL1 probe, 5'-6FAM-CAAAAGCT-GAACAATCGCTCACT-TAMRA-3'; β -actin-F, CGTGG ACATCCGCAAAGAC; β -actin-R, GGGCGGTGATCTC CTTCTG; β -actin probe, 5'-VIC-TGCTGTCTGGCGG CACCACC-TAMRA-3'.

Results

Effect of photoperiod on plasma cortisol and DHEAS rhythms in the rhesus macaque

Mean 24-h plasma cortisol rhythms, obtained under each of the three photoperiods are depicted in Fig. 1A. No significant differences were detected in either the mesor or amplitude (Table 1). The acrophases under both the 12 h light:12 h darkness and the 16 h light:8 h darkness photoperiods were attained around the time when lights came on (i.e. at 0700 h), but a significant phase advancement was disclosed under 8 h light:16 h darkness ($P < 0.05$ vs 12 h light:12 h darkness; $P < 0.01$ vs 8 h light:16 h darkness); notably, plasma cortisol levels reached a maximum while the animals were still in the darkness phase of their daily photoperiodic cycle (Fig. 1A, Table 1). Although the ascending phase of the 24-h plasma cortisol rhythm appears to be shorter in 8 h light:16 h darkness, this change was not statistically significant (Table 1). Mean 24-h plasma DHEAS rhythms, obtained under each of the three photoperiods are depicted in Fig. 1B. No significant differences were detected in either the mesor or amplitude, although both parameters showed a tendency to decrease under the 8 h light:16 h darkness photoperiod (Table 2). Under both the 12 h light:12 h darkness and the 8 h light:16 h darkness photoperiods the acrophase of the DHEAS rhythm occurred ~ 3 –4 h after the lights came on in the morning; by contrast, under 8 h light:16 h darkness the acrophase occurred significantly earlier ($P < 0.05$ vs 12 h light:12 h darkness and 8 h light:16 h darkness), within an hour of the beginning of the light phase (Fig. 1B, Table 2). As with cortisol, the ascending phase of the 24-h plasma DHEAS rhythm appeared to be shorter in 8 h light:16 h darkness, but this change was not statistically significant (Table 2).

Figure 1 Effect of photoperiod on the 24-h circulating (A) cortisol and (B) DHEAS rhythms in female rhesus macaques. Plasma samples were collected from the same animals after 10 weeks of exposure to each of the three photoperiods. Values are expressed as means $\pm$ S.E.M. ($N=3$). Note, the data are double plotted to aid visualization of circadian changes. Vertical dashed lines within each panel indicate the acrophases. The vertical dashed line across all panels indicates the beginning of the light phase. The horizontal white and black bars represent day and night respectively.

Effect of photoperiod on motor activity

Activity–rest profiles from a representative animal, obtained under each of the three photoperiodic regimens are depicted in Fig. 2. One-way ANOVA analysis of total activity spectra from

the three animals showed that there was no significant difference in average total activity, or average light-phase activity between the different photoperiods (Fig. 2B). Interestingly, the hourly activity during the light phase, although not significantly different, tended to be higher under 8 h light:16 h darkness than

Table 1 Effect of photoperiod on plasma cortisol in rhesus macaques

	Mesor (mean $\pm$ S.E.M.)	Amplitude (mean $\pm$ S.E.M.)	Acrophase (h, min)	Rising period (h)
Photo period				
8 h light:16 h darkness	79.4 $\pm$ 3.9	64.9 $\pm$ 10.5	0448 $\pm$ 0013	1100 $\pm$ 0109
12 h light:12 h darkness	96.9 $\pm$ 8.9	73.5 $\pm$ 5.1	0724 $\pm$ 0012*	1118 $\pm$ 0118
16 h light:8 h darkness	76.1 $\pm$ 11.9	61.0 $\pm$ 5.9	0757 $\pm$ 0014†	0918 $\pm$ 0150

Statistical comparisons were made by repeated measures ANOVA, followed by Bonferroni test (* $P<0.05$, † $P<0.01$, vs 8 h light:16 h darkness). No significant differences were found for mesor, amplitude or rising period ($P>0.05$).

Table 2 Effect of photoperiod on plasma dehydroepiandrosterone sulfate (DHEAS) in rhesus macaques

	Mesor (mean ± S.E.M.)	Amplitude (mean ± S.E.M.)	Acrophase (h, min)	Rising period (h)
Photoperiod				
8 h light:16 h darkness	105.4 ± 24.0	55.8 ± 5.2	0744 ± 0020	1018 ± 0112
12 h light:12 h darkness	125.6 ± 32.8	63.7 ± 12.4	1012 ± 0012*	1000 ± 0034
16 h light:8 h darkness	91.2 ± 21.8	36.6 ± 5.0	1011 ± 0004*	0840 ± 0140

Statistical comparisons were made by repeated measures of ANOVA, followed by Bonferroni test (**P* < 0.05, vs 8 h light:16 h darkness). No significant differences were found for mesor, amplitude or rising period (*P* > 0.05).

Figure 2 Effect of photoperiod on motor activity in ovariectomized female rhesus macaques. (A) Left panels: representative actograms from an individual animal that was exposed sequentially for 10 weeks to 8 h light:16 h darkness, 12 h light:12 h darkness and 16 h light:8 h darkness lighting regimens. Note, the activity data are double plotted to aid visualization of circadian changes. Right panels: representative mean activity during the 10-week periods. The arrow indicates the advancement of activity onset, and the vertical dashed line indicates the beginning of the diurnal phase. The horizontal white and black bars indicate day and night respectively. (B) Cosinor analysis was used to assess motor activity variables over each 10-week period. Comparisons were made using repeated-measures ANOVA followed by Bonferroni test. Values represent the means ± S.E.M. from all three animals. **P* < 0.05, ***P* < 0.01.

12 h light:12 h darkness or 8 h light:16 h darkness (data not shown). On the other hand, average darkness-phase activity was clearly higher under 8 h light:16 h darkness than 12 h light:12 h darkness or 16 h light:8 h darkness ($P < 0.05$ vs 12 h light:12 h darkness; $P < 0.01$ vs 16 h light:8 h darkness; Fig. 2B). In addition, exposure to the 8 h light:16 h darkness photoperiod was associated with earlier activity onset (Fig. 2A), which occurred during the dark phase, while the lights were still off, as well as a significant advancement of the activity acrophase, compared with the 16 h light:8 h darkness photoperiod ($P < 0.05$, Fig. 2B).

Effect of photoperiod on adrenal gland gene expression

Rhesus macaque gene microarrays (Affymetrix, Santa Clara, CA, USA) were used to study the effect of photoperiod on the adrenal transcriptome. Gene expression profiles were obtained from long-term ovariectomized animals that had been maintained for 10 weeks in either 8 h light:16 h darkness, 12 h light:12 h darkness or 16 h light:8 h darkness photoperiods, with three animals per group. Comparisons were made between 12 h light:12 h darkness versus 8 h light:16 h darkness, and 12 h light:12 h darkness versus 16 h light:8 h darkness, with fold changes higher than 1.8 or lower than -1.8 being considered significant. A gene annotation tool was used to obtain gene abbreviations and descriptions. The data analysis revealed three main functional clusters: 1) development, 2) lipid synthesis and metabolism, and 3) immune function.

Genes identified as being differentially expressed after exposure to short photoperiods

Genes that were differentially regulated in 8 h light:16 h darkness compared with 12 h light:12 h darkness are depicted in Fig. 3. The set of genes associated with development included genes that play functional roles in morphogenesis (*HOXD11*, *ALX3*, *WNT2*), cytoskeletal regulation (*NCKAP1*), axonal growth (*SEMA6B*, *ROBO1*), and neural cell development (*NPTX1*). The set of genes associated with lipid synthesis and metabolism included genes that play functional roles in the activation of long-chain fatty acids (*ACSL1*) and in the synthesis of glycosylphosphatidylinositol (*PIGO*) anchor in the endoplasmic reticulum. Finally, the set of genes associated with immune response included genes that play functional roles in acute inflammatory response (*CSF1*), recognition of antigen particles bound to major histocompatibility complex (*TRAα*), interferon-inducible response to double stranded RNA (*PRKR*), early lymphoid development (*CD7* antigen), and proliferation of hematopoietic stem cells (*IL11*). To validate the GeneChip analysis, we used quantitative real-time polymerase chain reaction (qRT-PCR) to corroborate differences in the expression of three selected genes (*NCKAP1*, *IL-11*, and *ACSL1*) between the short (8 h light:16 h darkness) and medium (12 h light:12 h darkness) photoperiods (Fig. 5).

Genes identified as being differentially expressed after exposure to long photoperiods

Genes that were differentially regulated in 16 h light:8 h darkness compared with 12 h light:12 h darkness are depicted in Fig. 4. The set of genes associated with development included genes that play functional roles in nervous tissue differentiation (*BMP7*, *DSP*, *WNT2*), cell proliferation (*MAC30*), and cell-cell contact (*PCDHB13*). Interestingly, some of the developmental genes that showed differential regulation by short days were also regulated by long days (i.e. *NCKAP1*, *MYBPC3*, *ROBO1*, *NPTX1*, *SEMA6*, *CASP10*, *SOCS1*, *HOXD11*, *CTF1*, and *LIMS1*). The set of genes associated with lipid synthesis and metabolism included several genes that encode enzymes involved in lipid synthesis, modification, and degradation (*FADS1*, *CDS2*, *UGCG*, *PLCB3*). The set of genes associated with immune response included genes that encode chemokine ligands (*XCL2*, *CXCL14*), and genes that are involved in peripheral inflammation (*TACR1*), virus response (*IRF4*, *IFNA10*), and early activation of T cells (*CD69*), among others. Differences in the expression of *NCKAP1*, *IL-11*, and *FADS1* between the long (16 h light:8 h darkness) and medium (12 h light:12 h darkness) photoperiods were corroborated using (qRT-PCR; Fig. 5).

Discussion

Day/night cycles can exert a profound effect on physiology and behavior, as well as gene expression (Hastings *et al.* 2003). On the other hand, it is unclear how seasonal changes in environmental conditions can influence these parameters, especially in humans and non-human primates. To address this issue, we examined the impact of photoperiodic manipulation on circadian functions and adrenal gene expression in a diurnal non-human primate, the rhesus macaque.

In their native habitat, in Northern India and China, these monkeys restrict their breeding activity to autumn and winter. This biological adaptation ensures that their offspring are born when environmental conditions are more favorable for survival, in the late spring and summer. The well-defined breeding season of rhesus macaques is especially evident at more extreme latitudes (e.g., Oregon at $\sim 45^\circ$ North), where the most likely proximate factor (Baker 1938) synchronizing it to the environment is the annual change in photoperiod (Urbanski 1995). Although less obvious, a seasonal reproductive rhythm also occurs in humans, with presumed conception rates peaking around the time of the vernal equinox (Roenneberg & Aschoff 1990a,b, Bronson 1995, 2004). Consequently, both rhesus macaques and humans may show some seasonal fluctuation in circulating sex-steroid concentrations, which would be partially masked by the marked concentration changes that occur across the menstrual cycle. In female rhesus macaques and women, the menstrual

Figure 3 Effect of short days on adrenal gland gene expression. The gene microarrays were analyzed using the algorithm MAS 5.0. (A) Histogram depicting functional clustering of genes differentially regulated after exposure to short photoperiod. (B) Table of genes involved in development, lipid synthesis and metabolism, and immune response, differentially expressed in 8 h light:16 h darkness versus 12 h light:12 h darkness ($P < 0.05$). Statistical comparisons were made by Student's *t*-test.

cycle is remarkably similar, with a peak of circulating estradiol concentrations occurring during the late follicular phase and a peak of progesterone concentrations occurring during the mid-luteal phase (Urbanski 1995, Downs & Urbanski 2006). It was unclear to us, whether seasonal and monthly fluctuating sex-steroid concentrations could affect adrenal gland function (Fonseca *et al.* 2001, Stavisky *et al.* 2003), and so as to avoid this potentially confounding issue, we performed our study using long-term ovariectomized rhesus macaques, exclusively.

The animals were chronically maintained under each of the three photoperiods, which were selected because they resemble the natural photoperiods that occur in temperate

zones during the winter, spring/autumn, and summer. Also, the use of a moderately long 16 h light:8 h darkness photoperiod to mimic summer was expected to induce a larger effect on clock-mediated gene expression, than the use of a very long photoperiod (> 20 h of light per day; Wagner *et al.* 2007). Although natural photoperiods increase and decrease gradually across the seasons, we chose to use more abrupt photoperiodic transitions. This ensured that the animals had a full 10 weeks to stabilize their physiological rhythms to each new photoperiod. Equally important, by limiting the exposure to only 10 weeks, we reduced the chances of developing photorefractoriness. This condition is characterized by a spontaneous reversion in physiology to that

Figure 4 Effect of long days on adrenal gland gene expression. The gene microarrays were analyzed using the algorithm MAS 5.0. (A) Histogram depicting functional clustering of genes differentially regulated after exposure to short photoperiod. (B) Table of genes involved in development, lipid synthesis and metabolism, and immune response, differentially expressed in 16 h light:8 h darkness versus 12 h light:12 h darkness ($P<0.05$). Statistical comparisons were made by Students t -test.

of the previous photoperiodic condition, and has been observed in other species, such as sheep, when they are exposed to constant photoperiods for extended periods (Lincoln *et al.* 2005). At the end of each photoperiodic treatment, the serial collection of blood samples at multiple time points across the day and night, enabled changes in plasma hormone rhythms to be determined more precisely. The results indicate that exposing the animals longitudinally to the different photoperiods had no obvious impact on either plasma cortisol mean levels or amplitude. On the other hand, these photoperiodic manipulations did affect

some circadian aspects of the hormonal rhythm, especially the acrophase and rising kinetics. Also, the nocturnal periods of rising cortisol levels changed in response to the duration of the scotoperiod. These findings are in agreement with the clinical results of Wehr *et al.* (1993), which showed adaptive changes in the rhythm of cortisol when human subjects were chronically maintained under controlled experimental conditions. They also support the idea that the rhythm-generating component of the cortisol secretion mechanism undergoes adjustments in response to length of day, or duration of scotophase. In the present study, a significant

Figure 5 Expression levels for *NCKAP1*, *FADS1*, *IL-11*, and *ACSL1*, determined by Taqman qRT-PCR. Values are expressed as means $\pm$ S.E.M. ($N=3$). Statistical comparisons were made by one way ANOVA, followed by Bonferroni test * $P<0.05$, ** $P<0.01$.

phase-advancement of ~ 2.5 h was evident for the cortisol rhythm under short photoperiods, reaching the acrophase in the dark phase.

In addition, we observed that the phase of the motor activity rhythm adjusts to photoperiodic changes. An advance in both the onset and phase of the activity rhythm were observed under short days, resulting in higher levels of activity during the scotophase. Since the phase-advancement in cortisol rhythm parallels that of the activity rhythm a causal relationship may exist between the two rhythms. It could be hypothesized that, in view of its physiological functions, an earlier peak of cortisol helps an individual to achieve a state of arousal earlier in the day, thus facilitating an earlier awakening relative to dawn. In natural environments, short photoperiods are generally associated with the onset of unfavorably low temperatures and a scarce food supply. In this context, the advancement of the activity rhythm and other physiological functions in the winter would help to optimize the use of a shorter light phase. Another way of interpreting these data is that the animals centralize their daily activity around the middle of the day, regardless of photoperiod. This means that in short winter photoperiods, their daily activity onset occurs several hours before dawn. Note that in our study, we kept dawn fixed for each of the photoperiods, at 0700 h. Thus, under 8 h light:16 h darkness the animals woke up at ~ 0500 h, 6 h before the middle of the day (i.e. at 1100 h). Similarly, under 12 h light:12 h darkness they woke up at ~ 0700 h, again 6 h before the middle of the day (i.e. at 1300 h). Under 16 h light:8 h darkness, however, they woke up at ~ 0700 h, which was 8 h before the middle of the day (i.e. at 1500 h). When viewed from this alternative perspective (i.e. relative to mid-day rather than dawn), our monkey activity data are in complete agreement with those of

Honma *et al.* (1992), which showed that humans wake-up earlier in summer than in winter.

Also in agreement with previous human studies (Wehr *et al.* 1993), we did not observe changes in daily average plasma cortisol levels or amplitude, in response to different day lengths. Consequently, if such variations do occur seasonally, they are unlikely to be triggered by changes in the photoperiod *per se*. A potential effect of other external factors such as temperature, diet or social habits, which show seasonal variations in increased latitudes, cannot be excluded.

Analysis of the DHEAS rhythm did not reveal an effect of day length on either plasma DHEAS mean levels or amplitude, although both values tended to decrease slightly during exposure to a long photoperiod; more conclusive analysis of these parameters would necessitate a larger number of animals, to reduce the impact of high variability between individuals. Nevertheless, the lack of an obvious photoperiodic impact on the magnitude of plasma DHEAS levels contrasts with the marked attenuation of circulating DHEAS concentrations that have been observed during aging (Downs *et al.* 2008, Perret & Aujard 2005). Similar to cortisol, a significant phase advancement of ~ 2.5 h was observed for DHEAS under short photoperiods. Considering the reported effects of DHEAS within the central nervous system, this circadian alteration may have important behavioral consequences. Although no receptor has been reported for DHEAS to date, this steroid acts as an excitatory neuromodulator with proconvulsant activity (Carette & Poulin 1984, Demigoren *et al.* 1991). It has been reported that DHEAS acts both as a negative allosteric modulator on GABA_A receptors and as a positive modulator on glutamate NMDA receptor activity. In support of this view is the finding that DHEAS increases the excitability of CA3 neurons in the hippocampus (Bergeron *et al.* 1996), suggesting that DHEAS helps to promote a general arousal state. In agreement with the phase-shifts observed for the plasma cortisol and activity rhythms, the phase-advancement in DHEAS rhythm may be interpreted as another component in the adaptation to short winter days, allowing for an earlier increase in the alert/arousal state.

The gene microarray and real-time PCR data demonstrate that photoperiod can influence gene expression in the primate adrenal gland, upregulating the expression of certain genes while downregulating the expression of others. The microarray analysis focused on changes in gene expression that are likely to occur during the annual cycle, especially after the transition from a winter photoperiod to a spring/autumn photoperiod, and from the latter to a summer photoperiod. The three main sets of genes identified (i.e. development, lipid synthesis and metabolism, and immune response) suggest that the adrenal gland undergoes both structural and functional changes as an adaptive response to long-term exposure to both short and long photoperiods. This hypothesis is supported by our observation that homeobox regulators are differentially expressed in both environmental conditions.

The adrenal gland is a dynamic organ that undergoes structural changes as part of its adaptive role in stress

response. Ultrastructural changes in this gland have been reported in response to environmental stressors such as noise (Soldani *et al.* 1999) and heat (Koko *et al.* 2004). More importantly, from the perspective of the present study, the effect of light on gene expression in the adrenal gland has previously been examined in rodents. For example, a brief light stimulus (400 lux, 30 min) was shown to affect gene expression in the adrenal glands of mice kept under constant darkness, through a pathway that involves the SCN and the autonomic nervous system (Ishida *et al.* 2005). As demonstrated by Kalsbeek *et al.* (2007), signals from the SCN are transmitted to peripheral organs, including the adrenal glands, through a pathway that involves vasopressin secretion activating preautonomic neurons of the paraventricular nucleus, and autonomic neurons from the intermediolateral column. Furthermore, exposure of male rats to either constant light or constant darkness was found to upregulate the expression of genes that encode catecholamine biosynthetic enzymes in the adrenal gland (Gallara *et al.* 2004). In keeping with these previous reports, our results demonstrate that some genes can be activated by exposure to extreme photoperiods (i.e. either short days or long days), and suggest that common pathways may be involved. In the natural environment, photoperiodic extremes are separated by the vernal and autumnal equinoxes (i.e. 12 h light:12 h darkness), which could allow resetting of these genes to a more basal level twice per year.

The gene expression data also suggest that the primate adrenal gland may be capable of responding to changes in photoperiod by undergoing tissue remodeling. These structural changes do not seem to affect the endocrine functions of the adrenal cortex; instead, they likely constitute cellular adaptations that allow the tissue to maintain normal function under environmental conditions associated with long and short photoperiods (e.g., high and low temperatures respectively, and changing food availability). A potential candidate that could underlie the observed changes in gene expression is the pineal hormone melatonin. In mammals, melatonin secretion conveys photoperiodic information to the rest of the body, in part by modulating the secretion of other hormones. In addition, melatonin receptor activation regulates gene expression through a pathway that involves protein kinase C and the extracellular signal-regulated kinases 1 and 2 (Sainz *et al.* 1999, Roy & Belsham 2002). Expression of MT1 melatonin receptor has been reported in the primate adrenal gland, and it has been suggested that melatonin may play a role in regulating adrenal clock-gene expression (Valenzuela *et al.* 2008). Therefore, although the autonomic nervous system is known to regulate adrenal gland function, it is plausible that a melatonin-mediated pathway plays a key role in the seasonal modulation of adrenal gland gene expression.

In summary, the present study represents the first multi-level analysis of photoperiod-induced responses in a non-human primate, involving 24-h endocrine profiling, whole animal behavioral observations, as well as gene expression

analysis. Although the responses of rhesus macaques to photoperiodic manipulations may be more pronounced than those of humans, the similar organization and endocrine function of the rhesus and human adrenal glands (Conley *et al.* 2004, Abbott & Bird 2008), emphasize the translational importance of our data. Together, they provide a new perspective on the effects of seasonal variations in photoperiod on primate behavior, physiology, and gene expression, and may have clinical value in the development of therapies for seasonal human pathophysiology, such as seasonal affective disorders (Wirz-Justice *et al.* 1984).

Declaration of interest

The authors declare that there was no conflict of interest that would prejudice the impartiality of the research reported.

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References

- Abbott DH & Bird IM 2008 Nonhuman primates as models for human adrenal androgen production: function and dysfunction. *Reviews in Endocrine and Metabolic Disorders* (DOI 10.1007/s11154-008-9099-8).
- Agrimonti F, Angeli A, Frairia R, Fazzari A, Tamagnone C, Fornaro D & Ceresa F 1982 Circannual rhythmicities of cortisol levels in the peripheral plasma of healthy subjects. *Chronobiologia* **9** 107–114.
- Baker JR 1938 The evolution of breeding season. In *Evolution (Essays presented to ES Goodrich)*, pp 161–177. Ed. GR de Beer. London: Oxford University Press.
- Bergeron R, de Montigny C & Debonnel G 1996 Potentiation of neuronal NMDA response induced by dehydroepiandrosterone and its suppression by progesterone: effects mediated via sigma receptors. *Journal of Neuroscience* **16** 1193–1202.
- Bilbo SD, Dhabhar FS, Viswanathan K, Saul A, Yellon SM & Nelson RJ 2002 Short day lengths augment stress-induced leukocyte trafficking and stress-induced enhancement of skin immune function. *PNAS* **99** 4067–4072.
- Bjornerem A, Straume B, Oian P & Berntsen GK 2006 Seasonal variation of estradiol, follicle stimulating hormone, and dehydroepiandrosterone sulfate in women and men. *Journal of Clinical Endocrinology and Metabolism* **91** 3798–3802.
- Bronson FH 1995 Seasonal variation in human reproduction: environmental factors. *Quarterly Review of Biology* **70** 141–164.
- Bronson FH 2004 Are humans seasonally photoperiodic? *Journal of Biological Rhythms* **19** 180–192.
- Carette B & Poulain P 1984 Excitatory effect of dehydroepiandrosterone, its sulphate ester and pregnenolone sulphate, applied by iontophoresis and pressure, on single neurones in the septo-preoptic area of the guinea pig. *Neuroscience Letters* **45** 205–210.

- Van Cauter EW, Virasoro E, Leclercq R & Copinschi G 1981 Seasonal, circadian and episodic variations of human immunoreactive beta-MSH, ACTH and cortisol. *International Journal of Peptide and Protein Research* **17** 3–13.
- Conley AJ, Pattison JC & Bird IM 2004 Variations in adrenal androgen production among (nonhuman) primates. *Seminars in Reproductive Medicine* **22** 311–326.
- Demiregoren S, Majewska MD, Spivak CE & London ED 1991 Receptor binding and electrophysiological effects of dehydroepiandrosterone sulfate, an antagonist of the GABAA receptor. *Neuroscience* **45** 127–135.
- Deslypere JP, de Biscop G & Vermeulen A 1983 Seasonal variation of plasma dehydroepiandrosterone sulphate and urinary androgen excretion in post-menopausal women. *Clinical Endocrinology* **18** 25–30.
- Dillman JF & Phillips CS 2005 Comparison of non-human primate and human whole blood tissue gene expression profiles. *Toxicological Science* **87** 306–314.
- Van Dongen HP, Kerkhof GA & Souterijn JH 1998 Absence of seasonal variation in the phase of the endogenous circadian rhythm in humans. *Chronobiology International* **15** 623–632.
- Downs JL & Urbanski HF 2006 Neuroendocrine changes in the aging reproductive axis of female rhesus macaques (*Macaca mulatta*). *Biology of Reproduction* **75** 539–546.
- Downs JL, Mattison JA, Ingram DK & Urbanski HF 2008 Effect of age and caloric restriction on circadian adrenal steroid rhythms in rhesus macaques. *Neurobiology of Aging* **29** 1412–1422.
- Fonseca E, Basurto L, Velázquez S & Zárate A 2001 Hormone replacement therapy increases ACTH/dehydroepiandrosterone sulfate in menopause. *Maturitas* **39** 57–62.
- Gallara RV, Bellavia SL, Serova LL & Sabban EL 2004 Environmental light conditions alter gene expression of rat catecholamine biosynthetic enzymes and Neuropeptide Y: differential effect in superior cervical ganglia and adrenal gland. *Brain Research. Molecular Brain Research* **124** 152–158.
- Garde AH, Hansen AM, Skovgaard LT & Christensen JM 2000 Seasonal and biological variation of blood concentrations of total cholesterol, dehydroepiandrosterone sulfate, hemoglobin A(1c), IgA, prolactin, and free testosterone in healthy women. *Clinical Chemistry* **47** 1877.
- Hansen AM, Garde AH, Skovgaard LT & Christensen JM 2001 Seasonal and biological variation of urinary epinephrine, norepinephrine, and cortisol in healthy women. *Clinica Chimica Acta* **309** 25–35.
- Hastings MH 1991 Neuroendocrine rhythms. *Pharmacology and Therapeutics* **50** 35–71.
- Hastings MH, Reddy AB & Maywood ES 2003 A clockwork web: circadian timing in brain and periphery, in health and disease. *Nature Reviews. Neuroscience* **4** 649–661.
- Hastings M, O'Neill JS & Maywood ES 2007 Circadian clocks: regulators of endocrine and metabolic rhythms. *Journal of Endocrinology* **195** 187–198.
- Honma K, Honma S, Kosaka M & Fukuda N 1992 Seasonal changes of human circadian rhythms in Antarctica. *American Journal of Physiology. Regulatory, Integrative and Comparative Physiology* **262** 885–891.
- Ishida A, Mutoh T, Ueyama T, Bando H, Masubuchi S, Nakahara D, Tsujimoto G & Okamura H 2005 Light activates the adrenal gland: timing of gene expression and glucocorticoid release. *Cell Metabolism* **2** 297–307.
- Kalsbeek A, Kreier F, Fliers E, Sauerwein HP, Romijn JA & Buijs RM 2007 Minireview: Circadian control of metabolism by the suprachiasmatic nuclei. *Endocrinology* **148** 5635–5639.
- King JA, Rosal MC, Ma Y, Reed G, Kelly TA, Stanek EJ III & Ockene IS 2000 Sequence and seasonal effects of salivary cortisol. *Behavioral Medicine* **26** 67–73.
- Koko V, Djordjevic J, Cvijic G & Davidovic V 2004 Effect of acute heat stress on rat adrenal glands: a morphological and stereological study. *Journal of Experimental Biology* **207** 4225–4230.
- Lac G & Chamoux A 2006 Do circannual rhythm of cortisol and testosterone interfere with variations induced by other events? *Annales d'Endocrinologie* **67** 60–63.
- Lemos DR, Downs JL & Urbanski HF 2006 Twenty-four-hour rhythmic gene expression in the rhesus macaque adrenal gland. *Molecular Endocrinology* **20** 1164–1176.
- Levine ME, Milliron AN & Duffy LK 1994 Diurnal and seasonal rhythms of melatonin, cortisol and testosterone in interior Alaska. *Arctic Medical Research* **53** 25–34.
- Lincoln GA, Johnston JD, Andersson H, Wagner G & Hazlerigg DG 2005 Photorefractoriness in mammals: dissociating a seasonal timer from the circadian-based photoperiod response. *Endocrinology* **146** 3782–3790.
- Nakao N, Ono H, Yamamura T, Anraku T, Takagi T, Higashi K, Yasuo S, Katou Y, Kageyama S, Uno Y *et al.* 2008 Thyrotrophin in the pars tuberalis triggers photoperiodic response. *Nature* **20** 317–322.
- Nicolau GY, Lakata D, Sackett-Lundeen L & Haus E 1984 Circadian and circannual rhythms of hormonal variables in elderly men and women. *Chronobiology International* **1** 301–319.
- Perret M & Aujard F 2005 Aging and season affect plasma dehydroepiandrosterone sulfate (DHEA-S) levels in a primate. *Experimental Gerontology* **40** 582–587.
- Roenneberg T & Aschoff J 1990a Annual rhythm of human reproduction: I. biology, sociology, or both? *Journal of Biological Rhythms* **5** 195–216.
- Roenneberg T & Aschoff J 1990b Annual rhythm of human reproduction: II. Environmental correlations. *Journal of Biological Rhythms* **5** 217–239.
- Roy D & Belsham DD 2002 Melatonin receptor activation regulates GnRH gene expression and secretion in GT1-7 GnRH neurons. Signal transduction mechanisms. *Journal of Biological Chemistry* **277** 251–258.
- Sainz RM, Mayo JC, Reiter RJ, Antolin I, Esteban MM & Rodriguez C 1999 Melatonin regulates glucocorticoid receptor: an answer to its antiapoptotic action in thymus. *FASEB Journal* **13** 1547–1556.
- Soldani P, Gesi M, Lenzi P, Natale G, Fornai F, Pellegrini A, Ricciardi MP & Paparelli A 1999 Long-term exposure to noise modifies rat adrenal cortex ultrastructure and corticosterone plasma levels. *Journal of Submicroscopic Cytology and Pathology* **31** 441–448.
- Stavisky RC, Watson SL, Anthony MS, Manuck SB, Adams MR & Kaplan JR 2003 Influence of estradiol on cortisol secretion in ovariectomized cynomolgus macaques (*Macaca fascicularis*). *American Journal of Primatology* **60** 17–22.
- Urbanski HF 1995 Excitatory amino acids and the control of seasonal breeding. In *Excitatory Amino Acids: their Role in Neuroendocrine Function*, pp 253–279. Eds DW Brann & VB Mahesh. Boca Raton: CRC Press ch 9.
- Urbanski HF, Downs JL, Garyfallou VT, Mattison JA, Lane MA, Roth GS & Ingram DK 2004 Effect of caloric restriction on the 24-hour plasma DHEAS and cortisol profiles of young and old male rhesus macaques. *Annals of the New York Academy of Sciences* **1019** 443–447.
- Valenzuela FJ, Torres-Farfan C, Richter HG, Mendez N, Campino C, Torrealba F, Valenzuela GJ & Serón-Ferré M 2008 Clock gene expression in adult primate suprachiasmatic nuclei and adrenal: is the adrenal a peripheral clock responsive to melatonin? *Endocrinology* **149** 1454–1461.
- Wagner GC, Johnston JD, Clarke JJ, Lincoln GA & Hazlerigg DG 2007 Redefining the limits of day length responsiveness in a seasonal mammal. *Endocrinology* **149** 32–39.
- Walker BR, Best R, Noon JP, Watt GC & Webb DJ 1997 Seasonal variation in glucocorticoid activity in healthy men. *Journal of Clinical Endocrinology and Metabolism* **82** 4015–4019.
- Wang ZN, Lewis MG, Nau ME, Arnold A & Vahey MT 2004 Identification and utilization of inter-species conserved (ISC) probesets on Affymetrix human GeneChip platforms for the optimization of the assessment of expression patterns in nonhuman primate (NHP) samples. *BMC Bioinformatics* **5** 165.
- Wehr TA, Moul DE, Barbato G, Giesen HA, Seidel JA, Barker C & Bender C 1993 Conservation of photoperiod-responsive mechanisms in humans. *American Journal of Physiology* **265** 846–857.
- Wirz-Justice A, Wever RA & Aschoff J 1984 Seasonality in freerunning circadian rhythms in man. *Naturwissenschaften* **71** 316–319.

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