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RESEARCH ARTICLE

Spectral and duration sensitivity to light-at-night in 'blind' and sighted rodent species

Abed E. Zubidat[1,*], Randy J. Nelson[2] and Abraham Haim[1,3]

[1]Department of Evolution and Environmental Biology, University of Haifa, Mount Carmel, Haifa 31905, Israel, [2]Departments of Neuroscience and Psychology, Ohio State University, Columbus, OH 43210, USA and [3]The Israeli Center for Interdisciplinary Research in Chronobiology, University of Haifa, Mount Carmel, Haifa 31905, Israel

*Author for correspondence (zubidat3@013.net)

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SUMMARY

Light-at-night (LAN) has become a defining feature of human and animal ecosystems and may possibly compromise human and animal physiology and health. Spectral and acclimation duration (AD) sensitivity were compared between social voles (*Microtus socialis*) and 'blind' mole rats (*Spalax ehrenbergi*) in four increasing ADs (0, 1, 7 and 21 days) to LAN (1×30 min, 293 μW cm^{-2}) of three different monochromatic lights [blue (479 nm), yellow (586 nm) and red (697 nm)]. Animals were sampled for urine and oxygen consumption (V_{O_2}) promptly after each LAN-AD. Urine samples were analyzed for production rate, urinary 6-sulfatoxymelatonin and urinary metabolites of adrenalin and cortisol. Overall, the blue light elicited the greatest effects on the biological markers of *M. socialis*, whereas similar effects were detected for *S. ehrenbergi* in response to red light. The increasing LAN-AD resulted in a dose-dependent decrement of all markers tested, except of stress hormones, which showed a direct positive correlation with LAN-AD. Our results suggest that: (1) photoperiod is an important cue for entraining physiological functions in the 'blind' *S. ehrenbergi*, which is essentially characterized by red-shifted sensitivity compared with the blue-shifted sensitivity detected for the sighted counterpart species, and (2) there is a strong association between LAN of the appropriate wavelength and adrenal endocrine responses, suggesting that LAN is a potential environmental stressor.

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Key words: cosinor analysis, daily energy expenditure, daily rhythm, melatonin suppression, percentage change in body mass, retinal photoreceptor.

INTRODUCTION

For the past approximately 130 years since the invention of electric lights, artificial light-at-night (LAN) has increasingly invaded our night. Several lines of experimental evidence from rodent species have demonstrated unequivocal LAN-induced disruption across virtually all biological domains, including gene transcription (Ben-Shlomo and Kyriacou, 2010), immune responses (Bedrosian et al., 2011), endocrine responses (Devarajan and Rusak, 2004), metabolism (Haim et al., 2005; Dauchy et al., 2010), temperature and activity (Song and Rusak, 2000), body mass (Fonken et al., 2010) and reproduction (Hoffmann, 1979).

LAN-induced circadian disruptions are likely mediated by disrupting the nocturnal transmission of the pineal melatonin (MLT) message. In mammals, the circadian rhythm of MLT is controlled by the hypothalamic suprachiasmatic nucleus (SCN), which basically translates day length (photoperiod) by tracking night length through special retinal photoreceptors (Dibner et al., 2010; Nakagawa and Okumura, 2010). In mammals, two arrays of retinal photoreceptors are recognized: (1) image-forming photoreceptors (IFPRs) located in the outer retina and (2) non-image-forming photoreceptors (NIFPRs) distributed in the inner retina. The melanopsin-containing NIFPRs form a few distinctive and intrinsically photosensitive retinal ganglion cells (mRGCs), which play an important role in circadian entrainment, and little if any role in visual imaging (Bailes and Lucas, 2010; Pickard and Sollars, 2010).

Social voles, *Microtus socialis* (Pallas 1773), and 'blind' mole rats, *Spalax ehrenbergi* (Nehring 1898), are important species among the pest rodents of Israel. As a nocturnal species, *M. socialis* forage above ground during the dark period and take refuge from the surface challenges during the day in a complex underground system. Diurnal activity is also reported for *M. socialis*, but the shifting takes place only during winter under the cover of clouds (Harrison and Bates, 1991; Benjamini, 1989). *Spalax ehrenbergi* presents a contrasting adaptive strategy with respect to the *M. socialis* ecology and ocular system. It is a solitary, strictly subterranean species that has practically lost visual competence (Haim et al., 1983) during millions of years of evolution (Nevo, 1999); the animal's eyes are severely degenerated and the evolutionary retinal remnants are completely concealed by solid layers of fur and skin (Sanyal et al., 1990). Remarkably, the vestigial retina of the 'blind' species remains sensitive to ambient light, but mainly for circadian responses rather than image-forming responses (Pevet et al., 1984; David-Gray et al., 1998).

Despite the contrasting life history strategies, the two species display comparable levels of photoperiodic responses in biological functions. For *M. socialis*, photoperiodic changes are an important environmental cue for seasonal acclimatization (Banin et al., 1994). Similarly, various photoperiodic responses in physiology (Haim et al., 1983), gene expression (Avivi et al., 2004) and behavior (Goldman et al., 1997) were reported for *S. ehrenbergi*. Photoperiod-adjusted daily rhythms in urine production rates, 6-sulfatoxymelatonin [6-SMT; the major metabolite of MLT in urine (Arendt, 2006)], urinary metabolites of adrenalin and cortisol, and oxygen consumption are well established for *M. socialis* and *S.*

ehrenbergi as well (Zubidat et al., 2010a; Zubidat et al., 2010b). These recent studies have clearly demonstrated that the two species respond to changes in light characteristics during the light period (photophase), with effective responses under the blue and red ranges of the visual spectrum for *M. socialis* and *S. ehrenbergi*, respectively. Most electrophysiological evidence indicates that sighted rodent species such as mice, rats, hamsters and, inferentially, *M. socialis* have retinal photoperiodic spectral sensitivity that ranges from 450 to 530 nm (Reiter and Richardson, 1992; Bullough et al., 2006). The NIFPRs in *S. ehrenbergi* have dual spectral sensitivity with green-shifted (λ_{max}=497 nm) and red-shifted (λ_{max}=534 nm) wavelengths (Janssen et al., 2000; Janssen et al., 2003).

Results of field experiments performed in semi-natural conditions during winter revealed that 15 min of LAN hourly or up to once in 4 h significantly decreased activity (no active holes were evident) in *M. socialis*, whereas the control animals did not show a similar tendency (Haim et al., 2004; Haim et al., 2007). Furthermore, under laboratory conditions, LAN treatment adversely affects several aspects of physiology in short-day-acclimated *M. socialis* (Haim et al., 2001; Haim et al., 2005). More recently, it has been revealed that LAN can also modulate adrenal endocrine responses in *M. socialis*; for example, urinary adrenalin and serum cortisol levels were boosted in response to LAN exposures (Zubidat et al., 2007). Subsequently, LAN was suggested as a novel tool for rodent pest control that should be integrated in the spectrum of traditional approaches such as biological control by raptors (Haim et al., 2007).

The main objective of our research was to compare the effect of LAN on photoentrainment responses in *M. socialis* and *S. ehrenbergi* as two natural models for contrasting vision and ecology. To meet this ultimate objective, the two species were exposed to a single nightly 30 min LAN exposure in the form of blue, yellow or red monochromatic wavelengths at increasing acclimation duration (AD). Daily rhythms of urine production rates, urinary 6-SMT, urinary metabolites of stress hormones and oxygen consumption were used as physiological markers to measure the photoentrainment sensitivity of the two species to LAN exposure.

MATERIALS AND METHODS

All individuals used in our study were mature males of *M. socialis* and *S. ehrenbergi*. Twenty-four second- and third-generation individuals of wild-type *M. socialis* (~4 months old, 56±2 g), reared in our rodent breeding colony (Oranim, University of Haifa, Israel), were used in total for this study. Mole rats (*N*=24, 215±9 g) representing the 2*N*=60 chromosomal superspecies of *S. ehrenbergi* (Nevo, 1985) were caught in their natural underground tunnels in fields around the Rehovot area (31°53′33.98″N, 34°48′40.58″E). All individuals were kept in standard laboratory rodent cages (43×23×26 cm) and had free access to commercial Purina rodent pellets (Koffolk Ltd, Tel Aviv, Israel; 21% crude protein, 4% crude fat, 4% cellulose, 13% moisture, 7% ash, 18.7 kJ g^{-1} gross energy) and carrots as a water source. Cages were placed in an environmentally controlled light-tight room at an ambient temperature (T_a) of 25±2°C and a relative humidity of 60% with an 8 h:16 h light:dark photoperiod rotation [lights on from 08:00 to 16:00 h; short day (SD)]. Light fixtures (*N*=8, 40 W; OSRAM, Molesheim, France), were installed 50 cm from the rodent cage floor and 60 cm apart. Light measurements were completed with a hand-held fiber optic spectrometer (AvaSpec-2048-FT-SDU, Avantes, Eerbeek, The Netherlands) directly from the animal cage floor level. All animal procedures were performed according to a protocol (no. 111/2008) approved by the Ethics and Animal Care Committee of the University of Haifa.

Experimental protocols

Individuals of either species were arbitrarily assigned to one of three groups (*N*=8) for studying their photoentrainment responses to LAN exposure at three different spectral compositions: blue (479 nm), yellow (586 nm) and red (679 nm) wavelengths. The experimental groups were exposed to LAN (1×30 min per scotophase occurring at 24:00 h) of the appropriate wavelength at the maximal effective photophase intensity (MEPI; 293 μW cm^{-2}) for photoperiodic entrainment in *M. socialis* and *S. ehrenbergi* (Zubidat et al., 2009; Zubidat et al., 2010a). Photoentrainment responses were assessed in four LAN-AD repeated subgroups of each of the three spectra designated above: (1) control conditions, without LAN; (2) acute conditions, 1 day; (3) mild conditions, 1 week; and (4) chronic conditions, 3 weeks.

Photoentrainment responses were evaluated physiologically by constructing daily rhythms of urine production rates (UPRs), 6-SMT, urinary metabolites of adrenalin (UMAdr) and cortisol (UMCort), and total daily energy expenditure (DEE) enumerated by monitoring oxygen consumption (V_{O_2}).

UPR, 6-SMT, UMAdr and UMCort daily rhythms

Urine collection

Urine sampling was conducted according to the non-invasive method described previously (Zubidat et al., 2010a). Urine samples were collected regularly over a period of 24 h at 4 h intervals using a single animal method for rodent species (Kurien et al., 2004). Urine volume was assessed gravimetrically by assuming a specific gravity of 1 g ml^{-1} (Schoorlemmer et al., 2001; Tendron-Franzin et al., 2004).

Hormone analyses

Urine samples were subjected in duplicate to solid phase enzyme-linked immunosorbent assay (IBL, Hamburg, Germany) to determine the immunoreactivity of 6-SMT, UMAdr and UMCort as described previously (Zubidat et al., 2010b). The intra- and inter-assay coefficients were 5.8–204 ng ml^{-1} (5.2–12.2%) and 12.4–220 ng ml^{-1} (5.1–14.9%) for 6-SMT, 5.4 and 12.8% for UMAdr and 3.5 and 6.9% for UMCort, respectively.

Total DEE assessed from V_{O_2} daily rhythms

Total DEE levels were calculated as the product of V_{O_2} assuming a conversion coefficient of 20.92 kJ per liter of O_2 utilized (Speakman, 2000). The levels of V_{O_2} were monitored using an electrochemistry micro oxygen sensor analyzer (Servomex 4100) in an open gas respirometric system as previously described (Zubidat et al., 2009). The respirometric metabolic chambers were placed in a LAB-Line EnvironETTE® environmental incubator (Dubuque, IA, USA) at T_a=25±2°C, 60% relative humidity and SD conditions. V_{O_2} levels were monitored only under control and chronic conditions (3 weeks).

Statistical analysis

Our results are tabulated as means ± s.e.m. except for rhythm estimates, which are constructed as means ± 95% confidence intervals. Statistical effects of the independent factors were first completed by three-way repeated-measures ANOVA (3RM-ANOVA) and then by two-way or one-way repeated-measures ANOVA (2RM-ANOVA or 1RM-ANOVA, respectively) if mean differences and relevant first-order interaction effects were significant. The ANOVA models were followed by a Bonferroni correction test for repeated-measures data or a Student–Newman–Keuls (SNK) test for mean effect factor where appropriate. The Pearson correlation coefficient (*r*) was used to

evaluate the statistical relationship between the four levels of the LAN-AD (control, acute, mild and chronic) and mean levels of a known physiological variable. Additionally, pairwise comparisons between mean levels were realized by Student's *t*-tests as appropriate.

These standard statistical analyses were further integrated with the population cosinor method for spectral and rhythmicity analyses for population time series (Nelson et al., 1979; Refinetti et al., 2007). The cosinor method is an effective nonlinear procedure for data fitting using the function:

$$Y(t) = Mesor + Amplitude \times \cos\left(\frac{2\pi \bullet (t + Acrophase)}{\tau}\right), \quad (1)$$

where Y is the level of a certain variable at a specific time t. The solved rhythm estimates include the rhythm-adjusted arithmetic mean (mesor); the difference between the culmination of the rhythm and the estimated mesor (amplitude); the phase angle of the culmination of the cosine curve approximated to the entire data (acrophase) with 360 deg=24 h in reference to 24:00 h; and the period (τ), which describes the approximated interval of a complete cycle. The statistical significance of the rhythm was evaluated using an F-test on the variances of the linear distribution (amplitude=0) and the trigonometric distribution generated by the best-fitted cosine curve to the entire data, with $P<0.05$ indicating statistical significance (i.e. unequal variance). The general significance for rejecting the null hypothesis that mean effects are equal was $P\leq0.05$; when multiple pairwise comparisons (n) were conducted the threshold was adjusted accordingly ($0.05/n$).

RESULTS

UPR daily rhythms

Daily variations in mean UPR levels of *M. socialis* under different spectral compositions and increasing LAN-AD are shown in supplementary material Fig. S1. A 3RM-ANOVA revealed significant effects of wavelength ($F_{2,21}$=10.29, P=0.001), sampling time ($F_{6,126}$=3.00, P=0.001) and LAN-AD ($F_{3,63}$=7.49, P=0.0001), and non-significant interaction effects among these variables. The mean UPR calculated for the blue spectral group was 0.62 ± 0.03 ml 100 g^{-1} h^{-1}, representing a 48 and 130% increase compared with the yellow and red spectral groups, respectively. Overall, significant 24 h rhythms were estimated by the population cosinor analysis for the blue and yellow spectral groups, but not for the red spectral group (see supplementary material Table S1).

Mean UPR did not differ across the LAN-AD within each of the spectral groups of *S. ehrenbergi* (supplementary material Fig. S2). A 3RM-ANOVA detected significant effects of sampling time ($F_{2,126}$=6.62, P=0.0001) and wavelength ($F_{2,21}$=34.72, P=0.0001), as well as their interaction ($F_{36,378}$=4.26, P=0.001). Mean UPR for blue-treated *S. ehrenbergi* reached 1.17 ± 0.07 ml 100 g^{-1} h^{-1} and increased significantly (SNK, $P<0.05$) from the yellow- and red-treated groups by 29 and 64%, respectively. The spectral analysis generally revealed significant rhythms of 24 h or ultradian periods ($\tau<24$ h), particularly within the blue spectral group (see supplementary material Table S2).

6-SMT daily rhythms

Mean urinary 6-SMT levels were greater in yellow-treated *M. socialis* (49.75 ± 4.51 pg ml^{-1} g^{-1}) than in the other spectral groups (34.40 ± 2.33 and 27.93 ± 2.44 pg ml^{-1} g^{-1} for blue and red spectral groups, respectively), suggesting a greater MLT inhibitory potential compared with the former group (SNK, $P<0.05$; Fig. 1). Urinary 6-SMT levels significantly differed with sampling time ($F_{6,108}$=26.13, P=0.0001), LAN-AD ($F_{3,54}$=18.08, P=0.0001), and interaction effects between each of the two variables with different wavelengths ($F_{36,324}$=3.62, P=0.0001). A 2RM-ANOVA detected significant LAN exposure effects on mean urinary 6-SMT in both the blue and yellow spectral groups ($F_{3,18}$=18.08 and 22.24, P=0.0001 and 0.04, respectively). LAN exposure had no effect on the red spectral group ($F_{3,18}$=3.00, $P>0.05$). A strong and negative correlation (r=–0.70, P=0.0001, N=28) was detected between mean urinary 6-SMT levels and LAN-AD in blue-treated *M. socialis*, but not in yellow- and red-treated *M. socialis* counterpart spectral groups. The cosinor analysis approximated significant 24 h rhythms for the blue and yellow spectral groups, but not for the red spectral group, at all LAN-AD levels except for controls. Mesor and amplitude values increased with decreasing LAN-AD, and acrophases were generally delayed with increasing LAN-AD (see supplementary material Table S3).

Increasing LAN-AD resulted in significant differences in mean urinary 6-SMT of *S. ehrenbergi* between the different wavelengths (Fig. 2). A 3RM-ANOVA showed significant effects of sampling time ($F_{6,108}$=20.53, P=0.0001), LAN-AD ($F_{3,54}$=4.29, P=0.0001), wavelength ($F_{2,18}$=13.34, P=0.0001) and the interactions among the three variables ($F_{36,324}$=5.11, P=0.0001). 6-SMT mean levels (34.92 ± 2.42 pg ml^{-1} g^{-1}) were markedly lower in red-treated *S. ehrenbergi* than either blue- (59.55 ± 3.65 pg ml^{-1} g^{-1}) or yellow-treated (82.43 ± 11.37 pg ml^{-1} g^{-1}) groups (SNK, $P<0.05$), indicating a higher sensitivity to LAN of red wavelength. The correlation analysis indicated negative relationships between LAN-AD and mean 6-SMT levels for the blue (r=–0.70, P=0.0001, N=28) and red (r=–0.74, P=0.0001, N=28) spectral groups, whereas a positive correlation was detected in the yellow spectral group (r=0.42, P=0.03, N=28). All rhythms were of significant amplitudes that cycled with $\tau\leq24$ h. In the blue- and red-treated groups, mesors and amplitudes decreased across all LAN-AD levels. Phase-delayed acrophases were observed with increasing LAN-AD in the red-treated groups; however, no potent direction of effect was revealed for the other wavelengths (see supplementary material Table S4).

Urinary UMAdr and UMCort daily rhythms

UMAdr daily rhythms

Responses in UMAdr daily rhythms to increasing LAN-AD and different wavelengths in *M. socialis* are shown in supplementary material Fig. S3. A 3RM-ANOVA showed significant effects of wavelength ($F_{2,18}$=11.02, P=0.001), LAN-AD ($F_{3,54}$=11.10, P=0.0001) and sampling time ($F_{6,108}$=2.68, P=0.02), but no significant interaction effects between the three variables ($F_{36,324}$=1.11, $P>0.05$). Pearson's correlation analysis indicated a significant positive association between increasing LAN-AD and UMAdr excretion levels in blue- and yellow-treated groups (r=0.41 and 0.67, P=0.03 and 0.0001, N=28; respectively), but not in the red-treated group (r=–0.17, $P>0.05$, N=28). Significant rhythms were detected by the cosinor analysis under both control and acute (1 day) subgroups in the blue and red spectral groups (control, τ=~15 h; acute, τ=24 h; see supplementary material Table S5).

Supplementary material Fig. S4 presents UMAdr daily rhythm responses to increasing LAN-AD under different wavelengths in *S. ehrenbergi*. A 3RM-ANOVA detected significant effects of sampling time ($F_{6,108}$=5.70, P=0.001), LAN-AD ($F_{3,54}$=31.44, P=0.001) and wavelength ($F_{2,18}$=28.04, P=0.001), as well as significant interaction effects between these three variables ($F_{36,324}$=2.78, P=0.01) on UMAdr levels. Mean UMAdr levels differed significantly (SNK; $P<0.05$) across the three wavelengths, with the highest levels in the

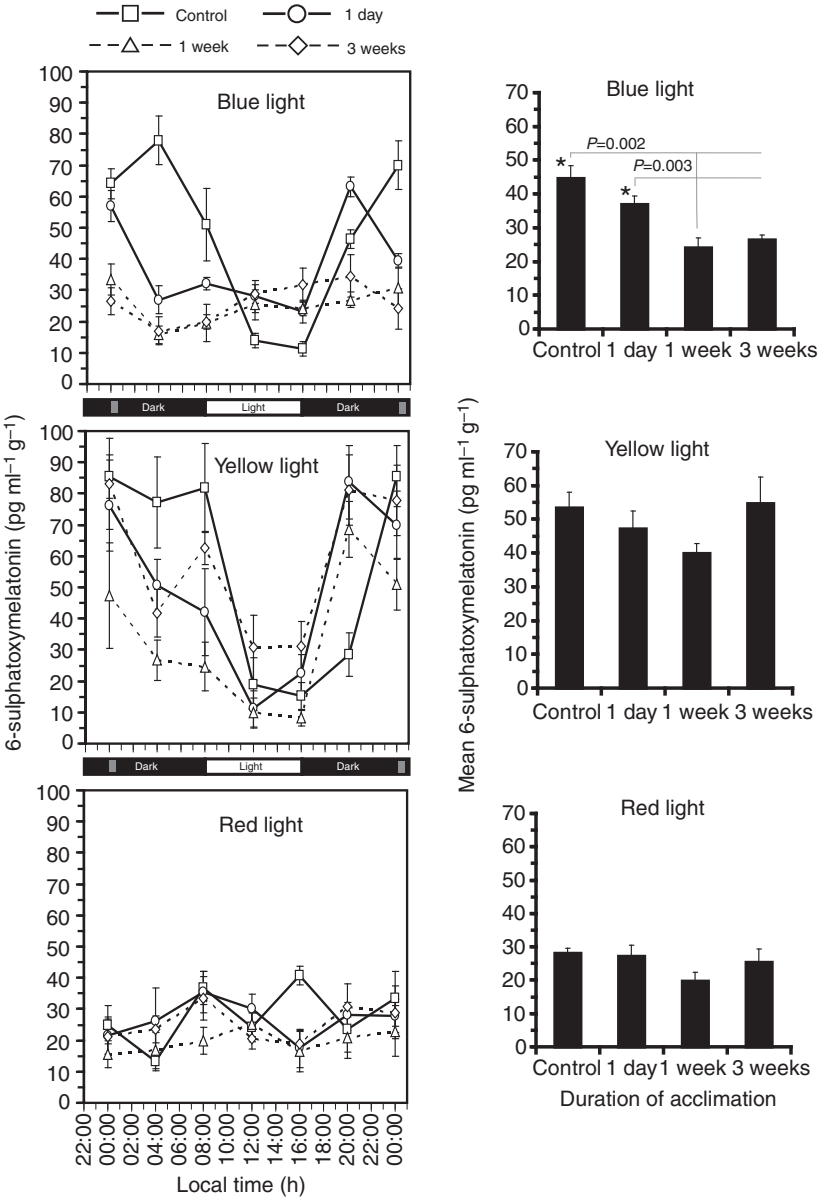

Fig. 1. Daily rhythms (left) and mean levels (right) of urinary 6-sulphatoxymelaton (6-SMT) concentrations of *Microtus socialis* exposed to 30 min of light-at-night (LAN, $1\times293\,\mu W\,cm^{-2}$) at three different wavelengths at increasing durations of acclimation (1 day, 1 week and 3 weeks) starting from control levels. Individuals were acclimated to short-day photoperiod (8 h:16 h light:dark cycles, lights on from 08:00 to 16:00 h). Values are means ± s.e.m. of *N*=7 individuals per subgroup. Black and white horizontal bars represent dark and light periods, respectively, and small gray segments at 00:00 h represent the 30 min of LAN exposure every dark period. *P*-values for significant differences between subgroup mean levels under a given wavelength are presented (Bonferroni *post hoc*).

blue-treated group ($231\pm08\,pg\,ml^{-1}\,g^{-1}$) and the lowest levels in the red-treated group ($63.83\pm7.23\,pg\,ml^{-1}\,g^{-1}$). The cosinor analysis generally detected significant daily rhythms in UMAdr excretion levels across all of the spectral groups. Rhythms were mainly of 24 h, but shorter (14 and 8 h) cycling rhythms were also detected (see supplementary material Table S6).

UMCort daily rhythms

A 3RM-ANOVA showed a significant effect of LAN-AD on *M. socialis* UMCort daily rhythms ($F_{3,54}$=35.46, *P*=0.001), but non-significant effects of sampling time ($F_{6,108}$=2.05, *P*>0.05) and wavelength ($F_{3,18}$=2.26, *P*>0.05) and no significant interaction effects among the three variables ($F_{36,324}$=1.24, *P*>0.05). Pearson's correlation coefficient revealed significant direct associations between LAN-AD and UMCort levels for wavelength groups (Fig. 3), with higher correlation coefficients for both blue (*r*=0.74, *P*=0.0001, *N*=28) and yellow (*r*=0.52, *P*=0.01, *N*=28) spectral groups compared with the red spectral group (*r*=0.39, *P*=0.04, *N*=28). Cosinor and spectral analyses detected significant 24 h rhythms for all blue-treated subgroups (i.e. control, acute, mild and chronic), and control and acute yellow-treated subgroups, but no significant rhythms were detected for the remaining experimental subgroups, especially in the red spectral group (see supplementary material Table S7).

Daily rhythms in UMCort excretion for *S. ehrenbergi* are described in Fig. 4. A 3RM-ANOVA detected significant effects of LAN-AD ($F_{3,548}$=10.24, *P*=0.0001) and wavelength ($F_{2,18}$=10.09, *P*=0.001) but no effect of sampling time ($F_{6,108}$=1.64, *P*>0.05); however, mean UMCort levels were significantly affected by the interaction between these variables ($F_{36,324}$=1.70, *P*=0.01). Correlation analysis detected a direct association between LAN-AD and mean UMCort levels for all spectral groups, but a significant relationship was only detected for the red spectral group (*r*=0.41, *P*=0.03, *N*=28). The cosinor analysis detected sporadic significant rhythms ($\tau\leq24$ h) across all experimental groups, without any clear pattern with respect to the different LAN-AD subgroups and wavelengths (see supplementary material Table S8).

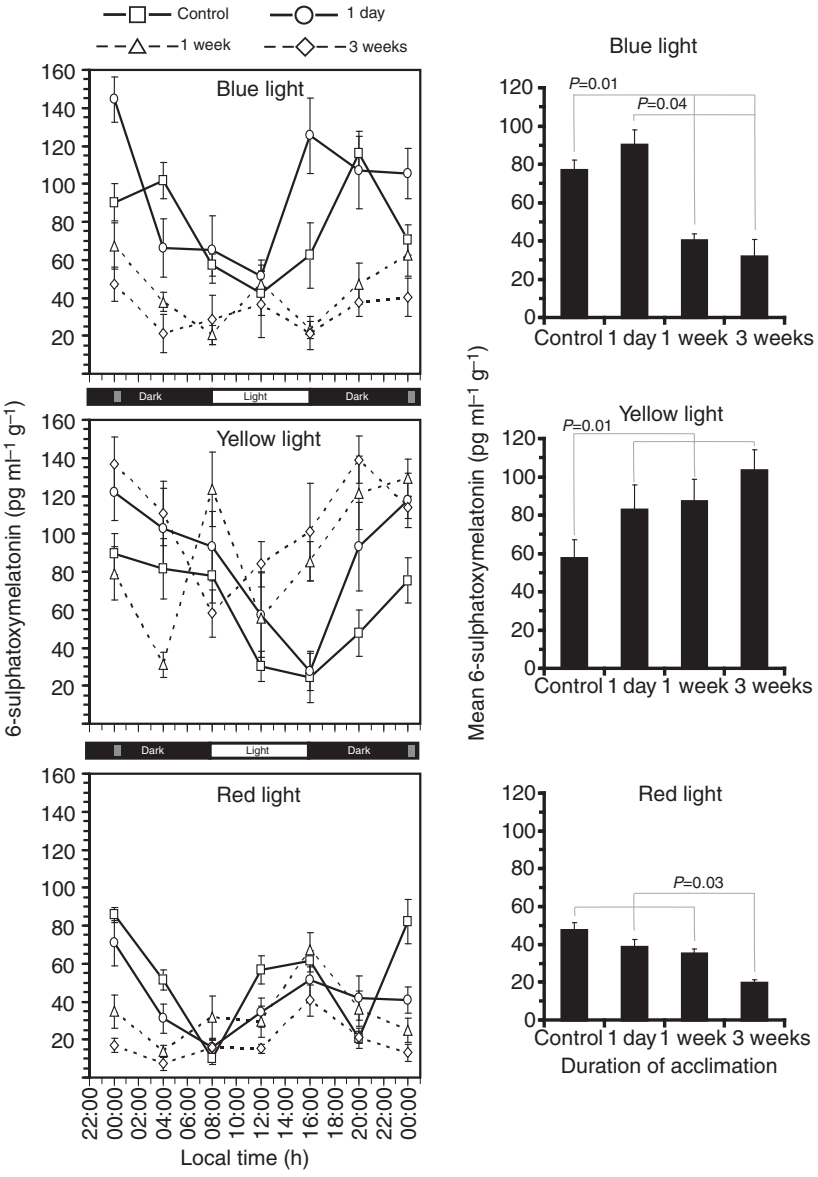

Fig. 2. Daily rhythms (left) and mean levels (right) of urinary 6-SMT concentrations of *Spalax ehrenbergi* exposed to 30 min of LAN (1×293 μW cm^{-2}) at three different wavelengths at increasing durations of acclimation (1 day, 1 week and 3 weeks) starting from control levels. Individuals were acclimated to short-day photoperiod. Values are means ± s.e.m. of *N*=7 individuals per subgroup. Black and white horizontal bars represent dark and light periods, respectively, and small gray segments at 00:00 h represent the 30 min of LAN exposure every dark period. *P*-values for significant differences between subgroup mean levels under a given wavelength are presented (Bonferroni *post hoc*).

Changes in body mass and DEE daily rhythms

Body mass

Fig. 5A shows the percentage change of body mass (M_b) in the *M. socialis* and *S. ehrenbergi* experimental subgroups. In *M. socialis*, ANOVA revealed significant effects of wavelength ($F_{4,21}$=4.87, P=0.02) and the LAN-AD × wavelength interaction ($F_{4,42}$=4.87, P=0.01). Significant wavelength ($F_{4,21}$=24.20, P=0.04) and LAN-AD × wavelength interaction effects ($F_{4,42}$=2.93, P=0.03) were also revealed for *S. ehrenbergi* experimental subgroups. The M_b of blue-treated *M. socialis* decreased significantly (Bonferroni, P<0.05) after 1 week (8.13%) and 3 weeks (11.06%) of LAN-AD from control levels, indicating a dose-dependent response to the increasing LAN-AD. The M_b of *M. socialis* in the yellow-treated mild subgroup decreased by 4.3% from controls. The M_b of *S. ehrenbergi* also progressively decreased with increasing LAN-AD in the blue- and red-treated groups, but significant effects were only detected for blue-treated animals ($F_{2,14}$=5.47, P=0.02). The mean M_b of animals in the blue-treated chronic group was 185±5 g, representing a significant decrease of 8% from control levels.

DEE daily rhythms

The daily rhythms of V_{O_2} in the *M. socialis* experimental subgroups are presented in Fig. 6. A 3RM-ANOVA indicated significant effects of LAN-AD ($F_{1,21}$=21.38, P=0.0001), sampling time ($F_{48,1008}$=3.83, P=0.0001) and wavelength ($F_{2,21}$=26.81, P=0.0001), as well as the sampling time × LAN interaction ($F_{96,1008}$=2.82, P=0.0001). Bonferroni analysis indicated that animals subjected to 3 weeks of blue LAN treatment exhibited significantly (P=0.04) decreased mean V_{O_2} levels of 1.51±0.10 ml O_2 100 g^{-1} h^{-1}, demonstrating a 19% change from control levels. Similarly, in the red-treated spectral group, V_{O_2} levels after 3 weeks of LAN exposure decreased by 29% in comparison with controls (2.72±0.12 ml O_2 100 g^{-1} h^{-1}; Bonferroni, P=0.002). Likewise, in the blue-treated spectral group, mean total DEE (Fig. 5B) was 0.77±0.06 and 0.96±0.05 kJ g^{-1} day^{-1} (paired t-test, t=–2.48, d.f.=7, P=0.04) in 3 weeks of LAN and controls, respectively, whereas in the red-treated group, DEE levels after 3 weeks of LAN (0.98±0.06 kJ g^{-1} day^{-1}) decreased significantly (paired t-test, t=4.81, d.f.=7, P=0.002) compared with control levels (1.39±0.06 kJ g^{-1} day^{-1}). The cosinor analysis estimated significant

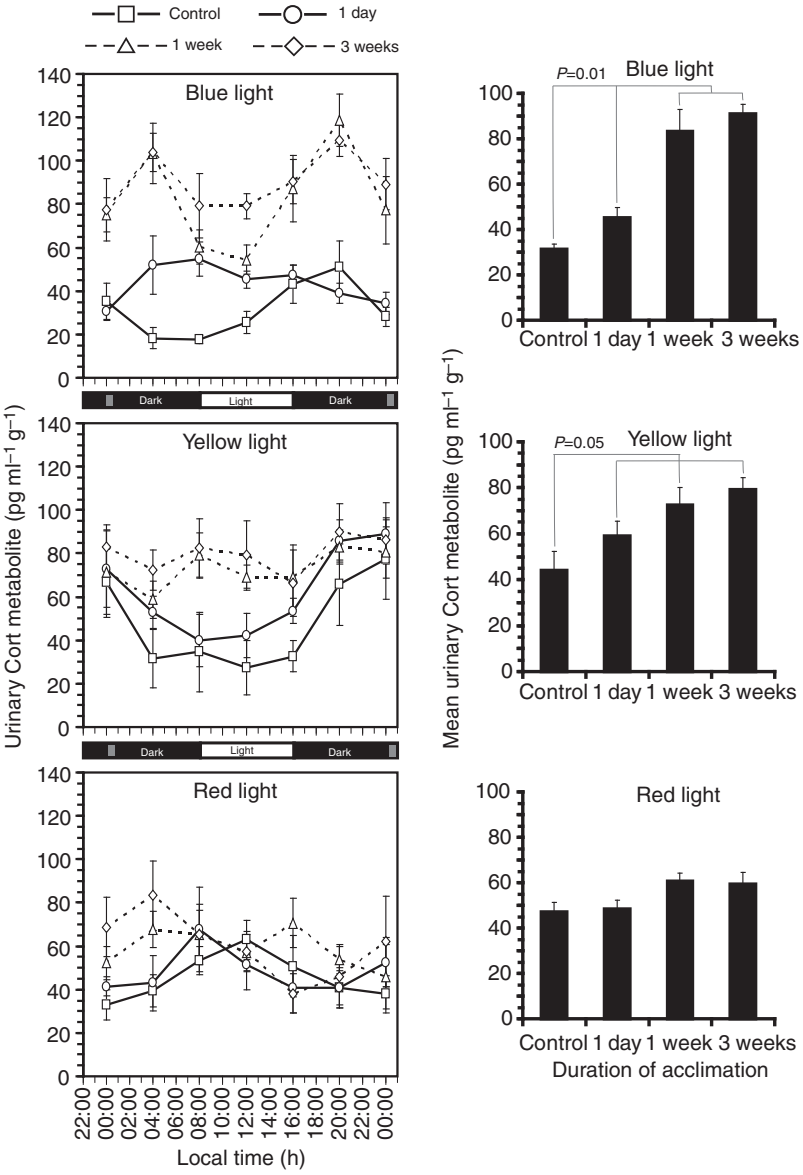

Fig. 3. Daily rhythms (left) and mean levels (right) of urinary metabolites of cortisol (Cort) concentrations of *M. socialis* exposed to 30 min of LAN (1×293 μW cm^{-2}) at three different wavelengths at increasing durations of acclimation (1 day, 1 week and 3 weeks) starting from control levels. Individuals were acclimated to short-day photoperiod. Values are means ± s.e.m. of *N*=7 individuals per subgroup. Black and white horizontal bars represent dark and light periods, respectively, and small gray segments at 00:00 h represent the 30 min of LAN exposure every dark period. *P*-values for significant differences between subgroup mean levels under a given wavelength are presented (Bonferroni *post hoc*).

rhythms across all experimental subgroups, with τ=24 h for controls and compound rhythms of two harmonics for the blue (τ=24 and 12 h) and red spectral groups (τ=12 and 7 h). In the yellow LAN subgroups, a significant rhythm was also detected but with a much shorter period (τ=12 h) compared with controls (see supplementary material Table S9).

Results of 48 h V_{O_2} monitoring for *S. ehrenbergi* are presented in Fig. 7. A 3RM-ANOVA showed that subgroups differed significantly with respect to LAN-AD ($F_{1,21}$=9.81, *P*=0.03), sampling time ($F_{48,1008}$=5.00, *P*=0.0001), wavelength ($F_{2,21}$=24.19, *P*=0.0001) and their interactions ($F_{96,1008}$=2.96, *P*=0.0001). LAN exposure had opposite effects across wavelengths, as it increased V_{O_2} in the blue-treated group and decreased V_{O_2} in the red-treated group compared with equivalent controls; no significant difference was found between treatment and control in response to yellow LAN exposure. The mean level of total DEE (Fig. 5B) in the blue-treated group (1.20±0.08 kJ g^{-1} day^{-1}) was markedly higher (paired *t*-test, *t*=–3.17, d.f.=7, *P*=0.01) than that calculated for controls (0.82±0.10 kJ g^{-1} day^{-1}), whereas the mean value in the red-treated group (0.37±0.01 kJ g^{-1} day^{-1}) was significantly (paired *t*-test, *t*=–6.61, d.f.=7, *P*=0.0001) lower compared with controls (0.79±0.01 kJ g^{-1} day^{-1}). The cosinor analysis retrieved significant rhythms of 24 h in all groups during control and chronic LAN exposure; no significant rhythm was detected for the red-treated group. Furthermore, 12 h ultradian rhythms were also revealed for the blue- and yellow-treated groups under control conditions, signifying the existence of compound rhythms under these conditions (see supplementary material Table S10).

DISCUSSION

UPR and 6-SMT daily rhythms

Exposure to a single 30 min exposure of LAN at the MEPI (293 μW cm^{-2}) per scotophase at midnight comparably reduced UPR and urinary 6-SMT excretion in a dose-dependent pattern in *M. socialis* and *S. ehrenbergi*, principally when applied as 479 nm monochromatic light (blue). Similarly, the decrement in 6-SMT was also observed for *M. socialis* in response to LAN at 586 nm (yellow) and for *S. ehrenbergi* at 679 nm (red). Our data suggest that the differential responses in daily rhythms of UPR and 6-SMT of the two species are related to wavelength, with greater sensitivity

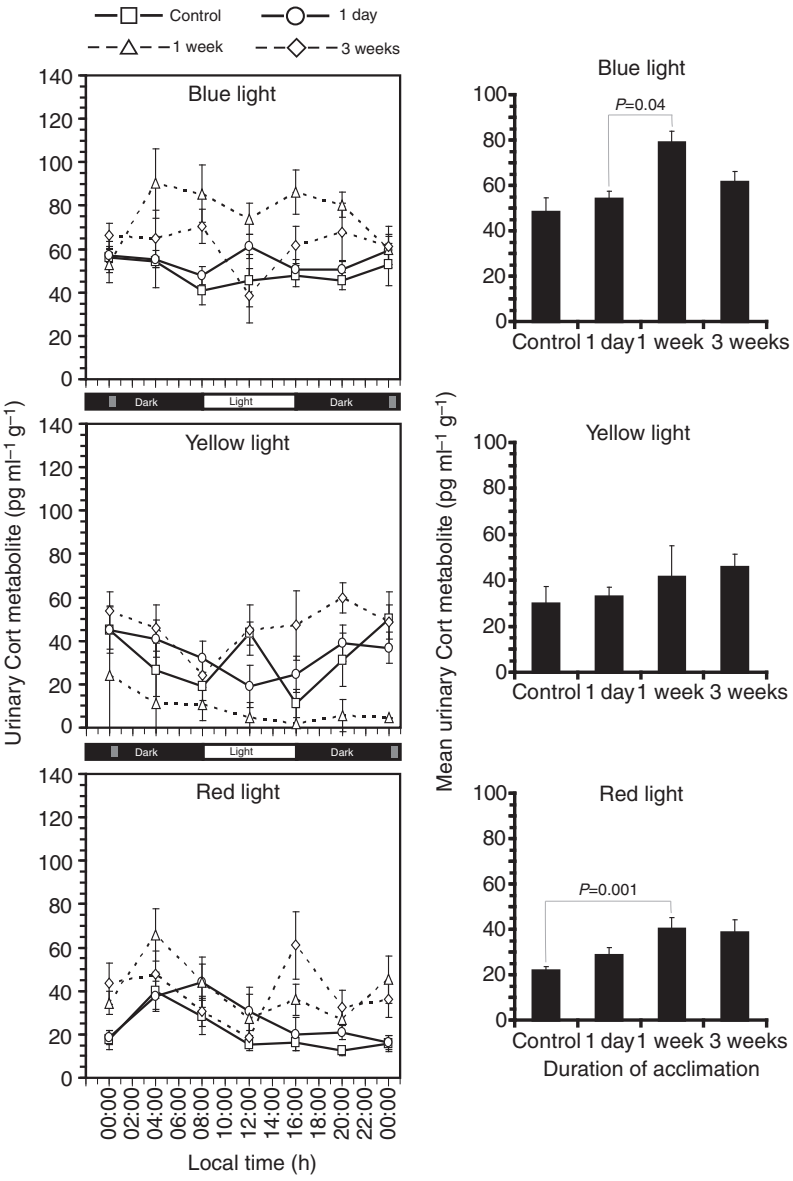

Fig. 4. Daily rhythms (left) and mean levels (right) of urinary metabolites of cortisol (Cort) concentrations of *S. ehrenbergi* exposed to 30 min of LAN ($1\times293\,\mu W\,cm^{-2}$) at three different wavelengths at increasing durations of acclimation (1 day, 1 week and 3 weeks) starting from control levels. Individuals were acclimated to short-day photoperiod. Values are means ± s.e.m. of $N=7$ individuals per subgroup. Black and white horizontal bars represent dark and light periods, respectively, and small gray segments at 00:00 h represent the 30 min of LAN exposure every dark period. *P*-values for significant differences between subgroup mean levels under a given wavelength are presented (Bonferroni *post hoc*).

of the sighted species at high spectral frequencies and of the 'blind' species at both high and low frequencies.

The spectral sensitivity observed in our study for the two species is in agreement with that reported previously for other mammals. In human males, exposure to 2 h of short-wavelength (460 nm) LAN before midnight suppressed MLT production, whereas exposure to a longer wavelength (550 nm) at the same irradiance level and for the same duration did not affect MLT (Cajochen et al., 2005). Overall, visually competent mammals, including nocturnal small rodents such as *M. socialis*, express significant suppression and phase shifting of MLT rhythms in the blue (~430 nm) to yellow (~530 nm) range of the visible spectrum (Peichl, 2005; Bullough et al., 2006; Rea et al., 2002). The vestigial retina of *S. ehrenbergi* has been demonstrated to express a red-shifted cone opsin with peak sensitivity at ~530 nm, which incontrovertibly contributes to circadian photoentrainment modulations in this species (David-Gray et al., 1999; Janssen et al., 2000). Additionally, a green-shifted rod-like opsin with peak sensitivity at approximately 497 nm was also characterized for functional remnants of the buried retina in the 'blind' species (Janssen et al., 2003).

Although the spectral sensitivity for MLT suppression in *M. socialis* is undoubtedly blue-shifted, the yellow LAN at the MEPI inhibited pineal MLT, as reflected by reduced urinary 6-SMT levels in response to 30 min of LAN 8 h after lights were extinguished. However, the effects of the two wavelengths at the same irradiance were not comparable, given that the inhibitory effect of the blue wavelength on pineal MLT synthesis was approximately twofold greater than that elicited by the yellow light. These results suggest that the retinal photoreceptors mediating the effect of visual light on MLT levels in *M. socialis* are governed by the blue-shifted wavelength, but the influence of other wavelengths of lower frequency (up to 586 nm) cannot be eliminated.

At long wavelengths, previously published data are often contradictory regarding the inhibitory effects of LAN in some rodent species (Vanecek and Illnerova, 1982; Poeggeler et al., 1995; Hanifin et al., 2006), and in some data there is a lack of a well-defined pattern of effects (Cardinali et al., 1972; Brainard et al., 1984; Benshoff et al., 1987). Our results demonstrated no clear sensitivity in MLT levels of *M. socialis* to LAN at 679 nm (red), indicating a marginal role for red-shifted retinal photoreceptors, such as NIFPRs,

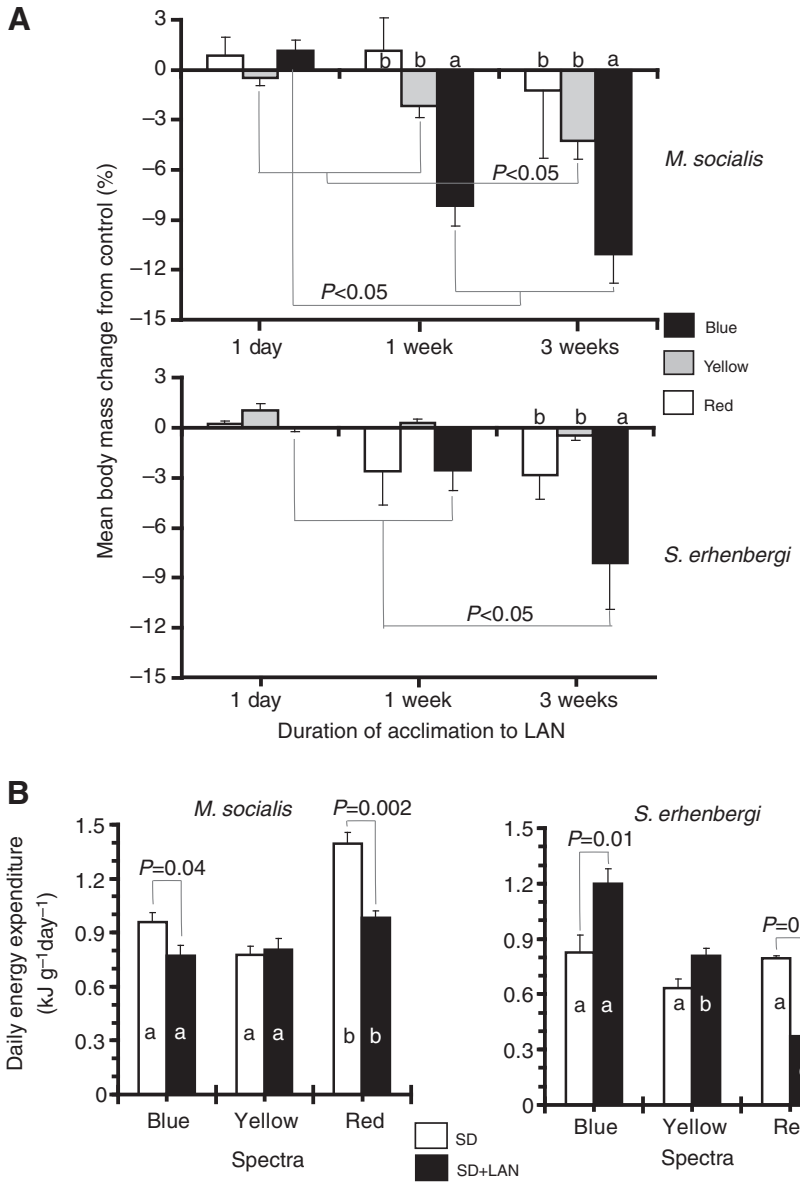

Fig. 5. Effect of 30 min of LAN ($1\times293\,\mu W\,cm^{-2}$) at three different wavelengths on percentage change of body mass (A) and mean daily energy expenditure (DEE) levels (B) in *M. socialis* and *S. ehrenbergi*. Percentage change of body mass was calculated for animals exposed to the different wavelengths with increasing duration of exposure (1 day, 1 week and 3 weeks) starting from control levels. DEE was calculated for individuals acclimated for 3 weeks to either short-day (SD) photoperiod or SD+LAN. Different letters indicate significant wavelength group mean differences in DEE (one-way ANOVA) and significant mean differences in DEE between SD and SD+LAN (paired *t*-test). The significant effect of duration of exposure to LAN is also shown for percentage change in body mass (Bonferroni *post hoc*).

in controlling pineal MLT circadian rhythms. Together, these results suggest species-specific wavelength-dependent sensitivity of MLT suppression in sighted mammals at the retinal level or higher. In the 'blind' *S. ehrenbergi*, the effects of blue and red lights on damping urinary 6-SMT were comparable in terms of magnitude and dose-dependent suppression LAN-AD. Our results are consistent with the observed dual spectral sensitivity (green- and red-shifted photoreceptors) of the retinal NIFPRs in *S. ehrenbergi* (David-Gray et al., 1999; Janssen et al., 2003). These studies have demonstrated that the molecular basis of the spectral tuning of these photoreceptors is chloride-dependent and thus is different than that in sighted mammals. The shift in phototransduction photoreceptors in favor of melanopsin in blind animals such as *S. ehrenbergi* is suggested to have a significant adaptive value in keeping circadian photoreception functioning under the subterranean long-wavelength conditions (David-Gray et al., 1998; Janssen et al., 2000).

The species-specific spectral differential effects in our study were also reflected in the results of the cosinor population analysis. In *M. socialis*, rhythm estimates were most affected by the short-wavelength LAN exposure and were much less affected by the long-wavelength interruption. In contrast to the sighted species, the temporal analysis showed maximal effective change in the rhythmic characteristics of 6-SMT in *S. ehrenbergi* at the red monochromatic light. Our results suggest that pineal MLT circadian modulations in the two species are wavelength dependent.

Urinary metabolites of UMAdr and UMCort daily rhythms

The results presented here show an acute impact of a single 30 min LAN exposure on the sympathetic adrenomedullary (SAM) system and the hypothalamus–pituitary–adrenal (HPA) axis of both *M. socialis* and *S. ehrenbergi*, represented by the quantitative increase in UMAdr and UMCort compared with control levels. Furthermore, the adrenal endocrine responses of the two species were directly dose dependent with increasing LAN-AD and varied with respect to wavelength, stress system and species. In *M. socialis*, elevated levels of UMAdr and UMCort were detected under the blue and yellow wavelengths, with defined, AD-induced, aggravated endocrine responses under the blue light. Our results for *S. ehrenbergi* showed significant elevation of UMAdr in response to LAN of either blue or red wavelength and of UMCort in response

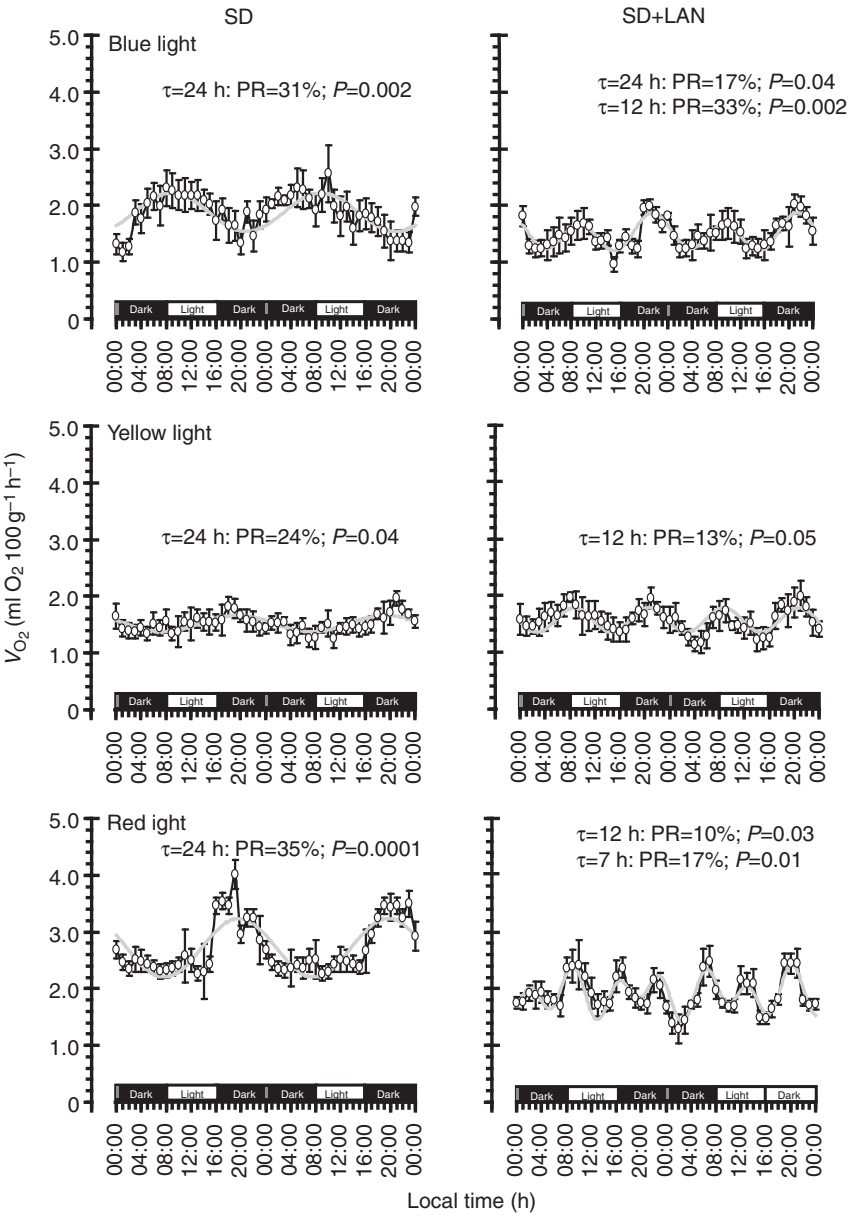

Fig. 6. Daily rhythms of oxygen consumption rates (V_{O_2}) in *M. socialis* exposed to 30 min of LAN ($1\times293\,\mu W\,cm^{-2}$) at three different wavelengths. Individuals were acclimated for 3 weeks to either SD photoperiod or SD+LAN. Experimental data are presented as means ± s.e.m. of *N*=8 individuals per subgroup and population cosinor best-fitted curves are plotted as gray wavy lines. Period length (τ), percentage rhythm (PR) and *P*-values of the *F*-statistic for rejection of the amplitude=0 hypothesis at $P<0.05$ are indicated for every curve approximated. Black and white horizontal bars represent dark and light periods, respectively, and small gray segments at 00:00 h represent the 30 min LAN exposure every dark period.

to exposure to long-wavelength (red) only. Additionally, the magnitude of the response in the SAM system and the HPA axis was greater after the exposure to long-wavelength compared with short-wavelength LAN. One possible explanation for the increased adrenal endocrine responses is that changes in the animal photic environment are likely to promote stress responses, at least in our rodent models.

Overall, our results suggest that the SAM system and HPA axis of *M. socialis* are more sensitive to short-wavelength interruption by LAN, whereas the sensitivity of the 'blind' species is essentially shifted towards the long wavelength of the visible spectrum. The results presented here are consistent with those of a previous study conducted at our laboratory indicating short-wavelength-shifted sensitivity in *M. socialis* and long-wavelength-shifted sensitivity in *S. ehrenbergi* (Zubidat et al., 2010b). *Microtus socialis*, as a visually competent and diurnal (primarily during winter) species, is expected to be exposed to light of greater frequency compared with the visually challenged subterranean species *S. ehrenbergi*. Consequently, the LAN-induced adrenal endocrine responses in *M. socialis* are most likely mediated through the cone's IFPRs, which are responsible for color vision detection; responses in *S. ehrenbergi* are likely to be mediated *via* the cone's red-shifted NIFPRs, which contain the well-defined photopigment melanopsin, as previously described (David-Gray et al., 1999; Janssen et al., 2003). The species-specific wavelength-dependent sensitivity could reflect an adaptive ecological spectral selectivity, designed to modulate the circadian photoentrainment system to match the challenging external environment, by which survival is maximized (Emerson et al., 2008; Yerushalmi and Green, 2009).

The population cosinor analysis detected robust UMAdr and UMCort rhythms in the two species mainly oscillating at τ=24 h, and shorter τ-values (e.g. 17, 15, 12 and 8 h) were also detected for few rhythms particularly among red-treated subgroups. Fundamental temporal oscillations in catecholamines and corticosteroids have been recently reviewed (Haus, 2007; Dickmeis, 2009). Several chronobiological and stress studies have detected substantial circadian and ultradian variations in stress hormones in mice (Weinert et al., 1994), rats (Atkinson and Waddel, 1997; Ahlers et

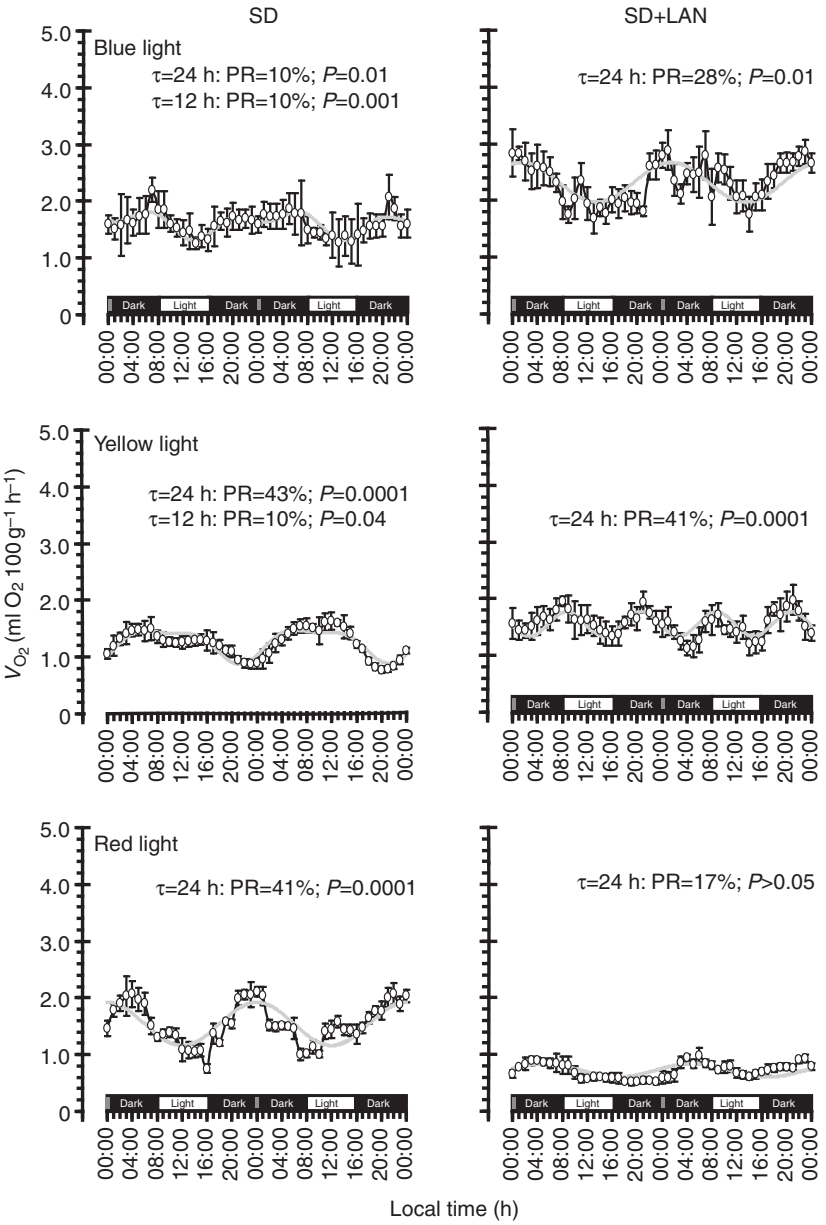

Fig. 7. Daily rhythms of V_{O_2} in *S. ehrenbergi* exposed to 30 min of LAN (1×293 μW cm^{-2}) at three different wavelengths. Individuals were acclimated for 3 weeks to either SD photoperiod or SD+LAN. Experimental data are presented as means ± s.e.m. of *N*=8 individuals per subgroup and population cosinor best-fitted curves are plotted as gray wavy lines. Period length (τ), percentage rhythm (PR) and *P*-values of the *F*-statistic for rejection of the amplitude=0 hypothesis at $P<0.05$ are indicated for every curve approximated. Black and white horizontal bars represent dark and light periods, respectively, and small gray segments at 00:00 h represent the 30 min LAN exposure every dark period.

al., 1999) and voles (Krame and Sothern, 2001). Overall, the rhythmicity of these stress hormones responds strongly to phase shifting of the day–night cycle (Sudo and Miki, 1995), is promptly refurbished upon stress relief (Droste et al., 2009) and is likely regulated directly by the SCN (Oster et al., 2006). Recently, we have reported potent daily variation in urinary UMAdr and UMCort for both *M. socialis* and *S. ehrenbergi* that was irradiance and wavelength dependent (Zubidat et al., 2010a; Zubidat et al., 2010b).

Changes in body mass and DEE

Our results showed that 30 min of LAN applied once at midnight decreased the M_b of *M. socialis* and *S. ehrenbergi* in an AD- and wavelength-dependent manner. The blue wavelength elicited comparable M_b loss in the two species, with maximal percentage change after 3 weeks of LAN-AD. A similar effect was detected in yellow-LAN treated *M. socialis*, but this wavelength had no clear effect on *S. ehrenbergi*. The M_b of the two species changed after 3 weeks of red-LAN treatment but without any clear pattern. Concurrent with a previous report from our laboratory (Zubidat et al., 2007), these results provide further support for the hypothesis that LAN exposure can disrupt M_b balance and engender mass loss in non-laboratory rodents. Similarly, LAN-induced mass loss was also documented in laboratory rats after 9 weeks of exposure to 30 min (1×300 lx) incandescent light at the middle of the scotophase (Cos et al., 2006).

Generally, changes in M_b are a consequence of a balance between caloric energy intake and DEE and are shaped by behavioral and physiological factors, where physical activity is the most potent behavioral factor and body temperature and food-related hormones (i.e. leptin, insulin, thyroxin and stress hormones) are the major physiological contributors (Dokken and Tsao, 2007; Keesey and Powley, 2008). The M_b loss observed here for the two species is consistent with previous data from our laboratory from LAN-designed experiments, which have shown that 15 min of white light (3×450 lx) exposure notably decreased energy intake in *M. socialis* (Haim et al., 2004). Thus, the LAN-induced energy intake decrement could, at least partially, account for the M_b loss measured in *M. socialis* and *S. ehrenbergi*.

The results of our study also revealed that *M. socialis* responded equally to blue and red LAN exposure at the same irradiance level, by dramatically reducing total DEE (calculated from V_{O_2}). Total DEE levels of *S. ehrenbergi* were also altered by the blue and red LAN exposure, but the two wavelengths elicited opposite effects, decreasing total DEE under the short-wavelength and increasing it under the long-wavelength light exposure. The reduced DEE responses have also been reported in SD-acclimated *M. socialis* 3 weeks after LAN acclimation (3×15 min) with polychromatic fluorescent light at 450 lx (Zubidat et al., 2007). The significant decline in total DEE could have also contributed to the decrease in M_b of the two species in the present study.

Several lines of evidence in both human and non-human mammals suggest that energy intake, energy expenditure and body mass exhibit circadian and seasonal rhythms that are SCN-controlled at the molecular, physiological and behavioral levels (Jéquier and Tappy, 1999; Morgan et al., 2003; Turek et al., 2005; Williams and Schwartz, 2005). The mechanism underlining the LAN-induced M_b loss observed here for the two species is not clear, though the alteration of metabolic-related circadian rhythms *via* the retinal photoreceptors is a promising candidate. The role of the SCN in circadian-induced metabolic responses at the cellular and systemic levels has been recently reviewed (Kalsbeek et al., 2007; Li and Lin, 2009). Overall, in mammals, the SCN-originated signals modulate activity of nuclear receptors that co-regulate expression of clock and adjacent metabolic genes. At the systemic level, these genes coordinate complex and diverse satiety mechanisms involved in the establishment and maintenance of metabolism and energy balance. We suggest that the LAN-induced M_b loss reported here is SCN-mediated by pineal MLT rhythms that were significantly altered in the two species (represented by urinary 6-SMT) in response to LAN with either short-wavelength or long-wavelength exposures.

CONCLUSIONS

Here we provide strong experimental support that LAN exposure (1×30 min) of appropriate irradiance and wavelength equally challenges the circadian photoentrainment of sighted and 'blind' rodent species. The fully sighted *M. socialis* is sensitive to light of greater frequency (shorter wavelength) compared with the 'blind' species. LAN at the appropriate wavelength changed physiological variables of the two species in a dose-dependent manner with respect to AD. Taking into account the fact that glucocorticoids have been postulated to be involved in the adjusting machinery of the mammalian circadian clock to the light:dark cycle (Dickmeis, 2009), it is reasonable to suggest that the enhanced glucocorticoid responses reported here play a role in mediating the circadian LAN-induced urinary and metabolic responses.

These adverse effects of LAN are expected to be mediated by retinal photoreceptors, whose LAN-induced circadian signals disrupt pineal MLT synthesis and release. The comparable responses in the two species suggest similar mechanisms of action at the central level of the circadian photoentrainment system, whereas the different spectral sensitivity represents an adaptive life history strategy at the retinal level. Finally, the circadian relationships between the IFPRs and NIFPRs in sighted species warrant additional research to determine to what extent each contributes to the mechanism mediating the LAN signal to the mammalian circadian clock.

LIST OF SYMBOLS AND ABBREVIATIONS

6-SMT	6-sulphatoxymelatonin
AD	acclimation duration
DEE	daily energy expenditure
IFPR	image-forming photoreceptor
LAN	light-at-night
M_b	body mass
MEPI	maximal effective photophase irradiance
MLT	melatonin
mRGC	melanopsin-containing retinal ganglion cell
NIFPR	non-image-forming photoreceptor
RGC	retinal ganglion cell
SCN	suprachiasmatic nucleus
SD	short day
UMAdr	urinary metabolite of adrenaline
UMCort	urinary metabolite of cortisol
UPR	urine production rate
V_{O_2}	oxygen consumption
τ	period

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