Effects of photophase illuminance on locomotor activity, urine production and urinary 6-sulfatoxymelatonin in nocturnal and diurnal South African rodents

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Summary statement
Daytime light intensity affects physiological and behavioral functions of not only diurnal, but also nocturnal small mammals.

ABSTRACT
Effects of photophase illuminance (1, 10, 100 and 330 lux of white incandescent lighting) on daily rhythms of locomotor activity, urine production and 6-sulfatoxymelatonin (6-SMT; 10 lux vs. 330 lux) were studied in nocturnal Namaqua rock mice (*Micaelamys namaquensis*) and diurnal four striped field mice (*Rhabdomys pumilio*). *Micaelamys namaquensis* was consistently nocturnal (~90-94% nocturnal activity), whereas considerable individual variation marked activity profiles in *R. pumilio*, but with activity mostly pronounced around twilight (~55-66% diurnal activity). The amplitude of daily activity was distinctly affected by light intensity and this effect was greater in *M. namaquensis* than in *R. pumilio*. Only *M. namaquensis* displayed a distinctive daily rhythm of urine production which correlated with its activity rhythm. Mean daily urine production appears to be attenuated under dim photophase conditions, particularly in *R. pumilio*. The results suggest that the circadian regulation of locomotor activity and urine production each possesses separate sensitivity thresholds to photophase illuminance. *Micaelamys namaquensis* expressed a significant daily 6-SMT rhythm that peaked during the late night, but the rhythm was attenuated by the brighter photophase cycle (330 lux). *R. pumilio* appears to have expressed an ultradian 6-SMT rhythm under both lighting regimes with comparable mean daily 6-SMT values, but with different temporal patterns. It is widely known that a natural dark phase that is undisturbed by artificial light, is essential for optimal circadian function. Here we show that light intensity during the photophase also plays a key role in maintaining circadian rhythms in rodents, irrespective of their temporal activity rhythm.
INTRODUCTION
The lives of mammals are governed by a plethora of physiological and behavioural rhythms that oscillate on a daily and seasonal basis. Most of these rhythms are either directly or indirectly generated by a master biological pacemaker, which resides in the hypothalamic suprachiasmatic nucleus (SCN; Cassone et al., 1988; Ralph et al., 1990). Within neurons of the SCN, transcriptional/translational feedback loops of clock genes with near 24 h cycle lengths, form the backbone of self-sustained circadian oscillations (Reppert and Weaver, 2002). In fact, most cells throughout the body possess such genetically based circadian clocks (Yoo et al., 2004).

However, for the internal time-keeping system to have an adaptive significance, it must be attuned to the solar day-night cycle. This is achieved most notably through the photic entrainment of the SCN cells and is initiated through ocular light exposure (Lucas et al., 2001). Although photic cues for circadian entrainment are projected fundamentally from melanopsin-expressing intrinsically photosensitive ganglion cells (ipRGCs), known also as non-image forming photoreceptors (NIFP), recent studies have revealed the involvement of visual rods and cones in shaping the signal (Dkhissi-Benyahya et al., 2007; Altimus et al., 2010; van Diepen et al., 2013; Weng et al., 2013). This raises the important question of whether variations in visual photoreceptor compositions amongst different species impose distinct effects on the entrainment of the SCN.

Suprachiasmatic nucleus clock signals are transformed into downstream circadian rhythms through various output pathways. For example, one of the most commonly studied circadian rhythms, namely that of locomotor activity, is regulated by the dorsomedial nucleus that receives SCN input via the ventral subparaventricular zone (Saper et al., 2005). In another major output pathway, projections to the paraventricular nucleus extend to the sympathetic preganglionic neurons of the upper spinal column, from where the synthesis of pineal melatonin is regulated (Reiter et al., 2011). The cyclic secretion of melatonin subsequently disseminates photoperiodic information to peripheral tissues and plays a central role in the regulation of circadian adjustments and also seasonal adjustments in photoperiodic species (Reiter, 1993; Dubocovich and Markowska, 2005). Melatonin production is mostly induced by darkness, irrespective of whether a species is diurnal or nocturnal and its main metabolite, 6-sulfatoxymelatonin (6-SMT),
can easily be measured from urine to indicate the concentration of pineal secreted melatonin (Bojkowski et al., 1987; Challet, 2007). Furthermore, evidence suggests that species possess characteristic sensitivity thresholds in their circadian rhythms, such as the melatonin rhythm, to different light intensities and wavelengths. It is believed that these thresholds most likely reflect the habitat to which the species are adapted and that it may accordingly also reflect specific visual adaptations of the species (Kumar and Rani, 1999; Peichl, 2005; Zubidat et al., 2009, 2010a, 2010b).

The Namaqua rock mouse (*Micaelamys namaquensis*) and the four striped field mouse (*Rhabdomys pumilio*) are two terrestrial species that occupy contrasting temporal niches. Locomotor activity is robustly entrained by the light-dark cycle in both of these species, with *M. namaquensis* expressing a strongly nocturnal activity rhythm and *R. pumilio* a strongly diurnal activity rhythm, but with marked activity at dusk and dawn (Schumann et al., 2005; Skinner and Chimimba, 2005; van der Merwe et al., 2014). The retinal photoreceptor arrangements of these two species have previously been described as overall complementary to their temporal lifestyles. In addition, ipRGCs are observed at nearly equal densities in the two species (unpubl. data). Evidently, photic cues administered by the light-dark cycle are expected to prompt different physiological and behavioural reactions in these two species due to their contrasting temporal niches. In the present study we set out to evaluate and compare their responses across a range of different photophase illuminance levels. Locomotor activity, urine production and urinary 6-SMT concentration were selected as parameters of rhythmicity because it is well recognized that these processes are regulated by the light-dark cycle through the SCN (Negoro et al., 2012).
MATERIALS AND METHODS

Animal housing
Nine adult wild *M. namaquensis* (four males, five females; mean body mass = 34.9 g) and nine adult wild *R. pumilio* (six males, three females; mean body mass = 35.1 g) were used in this experiment. *Micaelamys namaquensis* were captured in the rocky outcrops of the Soutpansberg region at Goro Game Reserve, Limpopo Province, South Africa (22°58’S, 29°25’E). *Rhabdomys pumilio* were live-trapped at Birha farms near Birha in the Eastern Cape Province, South Africa (33°22’S, 27°19’E). All animals were kept individually in semi-transparent plastic cages (58 x 38 x 36 cm) in a controlled room with an ambient temperature of 25°C (± 1°C) and approximately 60% relative humidity. Each animal was given a small open plastic shelter and tissue paper for nesting material and had ad libitum access to food and water. Water and food (parrot seed mix; Marlton’s, Durban, South Africa) were topped up and fresh food (apple and carrot) was replaced at random times every second or third day to avoid activity entrainment to the feeding schedule. Trapping permits were acquired (001-CPM403-00014, CRO95/12CR, CRO 96/12CR) and approval of all experimentation was by the Animal Ethics Committee of the University of Pretoria, Pretoria, South Africa (EC063-11).

Experimental protocol
One incandescent lamp (white light, 100W, OSRAM, Germany) was positioned centrally above every two neighboring cages, approximately 50 cm above the cage floor level. The lights were coupled to a dimmer circuit, which permitted the manual adjustment of the light intensity. Illuminance was measured at the central cage floor level with a hand-held digital light meter (Major Tech; Johannesburg, South Africa; basic accuracy: ± 5% + 10dgt) and the dimmer adjusted to obtain the desired level of illuminance. A timer automatically switched the lights on at 06h00 (start of photophase) and off at 18h00 (start of scotophase) each day. The animals were consecutively exposed for a period of 21 days to each of four illuminance light cycles (ILCs), during which the illuminance of the photophases differed between the ILCs, but with complete darkness during the scotophase; starting with a 1 lux-ILC, followed by a 10 lux-ILC, a 100 lux-ILC and ending with a 330 lux-ILC. Locomotor activity was recorded throughout the second and third week while urine samples were collected throughout the 21’st day of each ILC. Urine
collection was used for the extrapolation of production rates across the ILCs and were also analysed for 6-SMT levels, which were compared between the 10-lux ILC (dim-photophase) and the 330 lux-ILC (bright photophase).

**Locomotor activity: Recording and data analysis**
Activity was recorded by infra-red motion captors (Quest PIR internal passive infrared detector; Elite Security Products, Electronic Lines, London, UK) that were fixed centrally above each cage and detected movement over the entire cage floor area. The collective number of locomotory movements (activity counts) for each minute was stored on a computer using VitalView software (VitalView™, Minimitter Co., Sunriver, OR, USA). The activity data were analyzed and the daily activity rhythms visually presented as double-plotted actograms using the computer program ActiView (ActiView™, Minimitter Co., Sunriver, OR, USA). Activity counts and percentages of activity were compared between the four ILCs as well as within each ILC (photophase vs. scotophase) for all of the mice within a species. The sums of the activity counts per photophase and per scotophase of each day were calculated for each individual across all four light cycles. These values were then used to estimate the mean number of activity counts (of all individuals combined) for each entire ILC as well as for the photophase and scotophase of each ILC separately. The number of activity counts during either the photophase or scotophase of each day was further expressed as a percentage against the total number of activity counts for each day and was calculated for all animals individually. The mean of these values within each of the ILCs were then presented as percentages of activity.

**Urine production rate: Collection and analysis**
During the last day of each ILC (day 21), urine samples were collected from all tested individuals at 3 h intervals for the duration of 24 h. For this, the regular cages were switched with modified cages and the animals carefully transferred to the latter. The modified cages matched the regular cages (semi-transparent, 58 x 38 x 36 cm) had a stainless steel wire mesh floors fixed approximately 2 cm above the cage bottoms. An open slit right below the stainless steel mesh floor, across the breadth of the cage, allowed the insertion of a plastic plate, which covered the cage floor and could be removed whenever the screened urine had to be collected. Urine was transferred to Eppendorf tubes using disposable glass Pasteur pipettes, weighed
immediately after collection using a Mettler digital scale (Mettler, Zurich, Switzerland) and stored at -30°C until further analysis. When calculating urine volume (sample mass divided by urine specific gravity), urine specific gravity was assumed to be 1 g/ml (Schoorlemmer et al., 2001; Tendron-Franzin et al., 2004). Urine volume was converted to indicate the hourly urine production rates (μl/h) and the collective means are presented per species for each ILC as well as for the scotophase and photophase of each ILC, respectively. In addition, the mean values for all of the animals within a species were calculated at each 3 h point to present the 24 h rhythms in urine production rate.

Measurement of urinary 6-sulfatoxymelatonin (6-SMT) concentrations
Urinary 6-SMT levels were measured in six individuals from each species using a commercial enzyme-linked immunosorbent assay kit (IBL, Hamburg, Germany, cat. no. RE54031). In brief, 50 μl of diluted urine (1:50), enzyme conjugate, and melatonin sulfate rabbit-antiserum was pipetted into microtiter wells and incubated for 2h at RT. After washing, 100 μl of TMB (tetramethylbenzidine) substrate solution was added. 100 μl stop solution was added after an incubation of 30 min. at RT and absorbance was measured at 450 nm. Samples were analysed in duplicates and the intra- and inter-assay coefficients of variation were 5.2-12.2% and 4.0-6.0%, respectively. To be able to correct 6-SMT concentrations, urinary creatinine values were additionally determined. Creatinine is a breakdown product from tissue proteins, usually formed by muscle tissues in mammals (Schmidt-Nielsen, 1997) it is excreted at a relatively constant rate and can be used to adjust concentrations of hormone in urine. In brief, 7 μl of urine were pipetted into 210 μl of freshly prepared Picric reagent (1 vol of saturated picric acid solution and alkaline triton solution (4.2 ml triton + 12.5 ml 1 N NaOH in 66 ml distilled water) in 10 vols. of distilled water) and incubated for 2 h in the dark at RT. Absorbance was measured at 492 nm.

Statistical analysis
Data and statistical analyses were performed using Microsoft Excel (Microsoft Corp., Redmond, WA, USA) and IBM SPSS Statistics version 21.0 (SPSS Inc., Chicago, IL, USA). Data were not normally distributed and hence were analyzed for statistical significance by generalized linear mixed models; the post hoc least significant difference test was used where significant differences were detected. P<0.05 were considered significantly different and all values are
expressed as means ± standard error of the mean (s.e.m.). The generalized linear mixed models (GLMM) tested for mean effects of illuminance on the different variables and for the interaction effects of illuminance with the phase of the day (photophase/scotophase), as well as the interaction effects of illuminance with the time of the day (h), on the different variables. A gamma distribution with an identity link function were selected for the statistical analyses of locomotor activity and urinary 6-SMT, whereas a gamma distribution with a log link function was selected for the analyses of urine production rate.
RESULTS

Locomotor activity

Namaqua rock mouse

*Micalemys namaquensis* displayed a robust daily locomotor activity rhythm in accordance with the light-dark cycle and all individuals were invariably nocturnal. The mean numbers of activity counts were significantly higher by night than by day (Table 1) and the percentages of nocturnal activity were similar across all ILCs (1 lux-ILC: 89.56%; 10 lux-ILC: 92.75%; 100 lux-ILC: 94.28%; 330 lux-ILC: 93.63%). The level of photophase illuminance had a significant effect on daily locomotor activity ($F_{3,985}=7.883, P<0.001$). The mean number of activity counts of the 1 lux-ILC (489.85 ± 30.34) differed significantly from that of the 10 lux-ILC (655.92 ± 40.76; $F_{3,985}=7.883, P<0.05$), the 100 lux-ILC value (584.75 ± 37.16; $F_{3,985}=7.883, P<0.05$) and the 330 lux-ILC (716.87 ± 44.68; $F_{3,985}=7.883, P<0.001$; Fig. 1). Furthermore, a significant difference was obtained between mean number of activity counts of the 100 lux-ILC and the 330 lux-ILC ($F_{3,985}=7.883, P<0.05$).

Four striped field mouse

*Rhabdomys pumilio* was overall less active compared to *M. namaquensis* and its locomotor activity rhythm appeared to be less robust. A high degree of inter-individual variation was observed in the daily activity patterns (Fig. A1). Some mice were fundamentally crepuscular, some expressed activity intermittently throughout the 24 h day either with or without peaks around twilight, and yet others were mainly active during the photophase, but without any pronounced periods of activity around twilight. Consequently, the overall percentages of nocturnal activity (1 lux-ILC: 40.41%; 10 lux-ILC: 43.90%; 100 lux-ILC: 44.00%; 330 lux-ILC: 44.71%) were nearly equivalent to the percentages of diurnal activity. The mean number of activity counts were significantly higher during photophase than scotophase under all of the ILCs (Table 1). The level of photophase illuminance had an overall significant effect on locomotor activity ($F_{3,941}=2.759, P<0.05$). The mean number of daily activity counts was lowest during exposure to the 10 lux-ILC (175.88 ± 11.83) and this value differed significantly from the highest value, which was obtained under the 330 lux-ILC (202.02 ±14.16; $F_{3,477}=2.283, P<0.05$).
Under the 1 lux-ILC and the 100 lux-ILC, values were 180.52 ± 12.24 and 196.22 ± 13.2, respectively (Fig. 1).

**Urine production rate**

*Namaqua rock mouse*

Photophase illuminance had no significant influence on the mean daily urine production rate in *M. namaquensis* ($F_{3,186}=1.335$, $P=0.264$); values were similarly low under the 1 lux-ILC (137.65 ± 47.75 μl/h) and the 10 lux-ILC (139.12 ± 47.64 μl/h), highest under the 100 lux-ILC (196.92 ± 68.35 μl/h) and in-between (171.15 ± 59.09 μl/h) under the brightest ILC (Fig. 2). Urine production rates were consistently higher during nighttime than daytime across all experimental groups, but only differ significantly between the 1 lux-ILC and 10 lux-ILC (Table 1). Daily rhythms in urine production rate for *M. namaquensis* are displayed in Fig. 3A. The overall interactive effect of photophase illuminance with the time of the day did not produce a significant effect in any of the ILCs (1 lux-ILC: $F_{7,186}=1.073$, $P=0.382$; 10 lux-ILC: $F_{7,186}=1.013$, $P=0.423$; 100 lux-ILC: $F_{7,186}=0.839$, $P=0.556$; 330 lux-ILC: $F_{7,186}=0.849$, $P=0.548$). Under the 1 lux-ILC, the peak value was obtained at 19:00 (178.71 ± 79.80 μl/h) and the lowest value at 13:00 (20.22 ± 9.03 μl/h). Under the 10 lux-ILC, the highest and lowest values were at 04:00 (116.79 ± 48.95 μl/h) and 10:00 (23.99 ± 11.20 μl/h), respectively. Under the 100 lux-ILC, the peak value was at 04:00 (168.08 ± 72.44 μl/h), and the lowest value at 13:00 (51.69 ± 22.23 μl/h). In the final experimental group, the rhythm peaked slightly earlier than during the preceding two ILCs (22:00: 137.13 ± 56.18 μl/h) but the lowest value was again at 13:00 (46.18 ± 20.61 μl/h).

*Four striped field mouse*

The effect of photophase illuminance on urine production rate was significant in *R. pumilio* ($F_{3,173}=4.104$, $P=0.008$). Exposure to the 1 lux-ILC produced the lowest total mean urine production rate (66.18 ± 15.31 μl/h), exposure to the 10 lux-ILC produced a rate of 90.22 ± 20.12 μl/h, the highest rate was obtained under the 100 lux-ILC (138.90 ± 31.15 μl/h) and under the 330 lux-ILC the rate was 121.87 ± 28.43 (Fig. 2). Mean urine production rates were significantly different between the 1 lux-ILC and the 100 lux-ILC ($F_{3,173}=4.104$, $P=0.001$), the 1 lux-ILC and the 330 lux-ILC ($F_{3,173}=4.104$, $P=0.007$) and between the 10 lux-ILC and the 100 lux-ILC.
Urine production rate was higher during the photophase than scotophase under the 1 lux-ILC and significantly higher during the photophase under the 10 lux-ILC (Table 1). Under the 100 lux-ILC, photophase and scotophase values were comparable and under the 330 lux-ILC, the rate was slightly higher during the scotophase (Table 1). The daily rhythms in urine production rate for *R. pumilio* are illustrated in Fig. 3B. The interactive effect of photophase illuminance with the time of the day did not produce significant effects in any of the ILCs (1 lux-ILC: $F_{7,173}=1.189$, $P=0.311$; 10 lux-ILC: $F_{7,173}=1.317$, $P=0.245$; 100 lux-ILC: $F_{7,173}=0.464$, $P=0.860$; 330 lux-ILC: $F_{7,173}=0.660$, $P=0.705$). The daily urine production rhythms peaked consistently around dawn in all of the ILCs (Fig. 3B). During the 1 lux-ILC and the 10 lux-ILC, peak values were at 07h00 (1 lux-ILC: $60.81 \pm 20.70 \mu l/h$; 10 lux-ILC: $83.60 \pm 27.29 \mu l/h$). Under the 100 lux-ILC, the peak was slightly earlier (04h00: $102.22 \pm 45.34 \mu l/h$), but during the 330 lux-ILC it once again peaked at 07h00 ($93.97 \pm 32.00 \mu l/h$).

**Urinary 6-sulfatoxymelatonin (6-SMT) concentrations**

*Namaqua rock mouse*

Urinary 6-SMT level were higher under the 10 lux-ILC ($181.49 \pm 22.82 \text{ ng/mg}$) than the 330 lux-ILC ($146.41 \pm 18.03 \text{ ng/mg}$; Fig. 4), though the effect was not significant ($F_{1,54}=2.317$, $P=0.134$). The interactive effect of photophase illuminance with the phase (light/dark) of the day did not show any significant effects under either of the two ILCs but, scotophase values were consistently higher than photophase values (Fig. 4). The 24h 6-SMT profile for *M. namaquensis* is illustrated in Fig. 5. The interactive effect of photophase illuminance with the time of day significantly affected the urinary 6-SMT level during both ILCs (10 lux-ILC: $F_{7,54}=6.505$, $P<0.001$; 330 lux-ILC: $F_{7,54}=6.039$, $P<0.001$). During the 10 lux-ILC, the lowest value of the rhythm was at 22h00 (28.80 $\pm$ 7.68 ng/mg), whereas the peak of the rhythm was at 04h00 (262.41 $\pm$ 55.28 ng/mg; Fig. 5). Exposure to the 330 lux-ILC yielded a similar 24h urinary 6-SMT rhythm, whereby the lowest value was again at 22h00 (27.91 $\pm$ 7.85 ng/mg) and the highest value also at 04h00 (152.17 $\pm$ 32.59 ng/mg; Fig. 5).

*Four striped field mouse*

A non-significant effect ($F_{1,39}=0.008$, $P=0.930$; Fig. 4) was observed when comparing The response in urinary 6-SMT production under a low (10 lux-ILC: $113.52 \pm 20.58 \text{ ng/mg}$) and a
high (330 lux-ILC: 116.42 ± 25.54 ng/mg) photophase illuminance level. The interactive effect of photophase illuminance with the phase (light/dark) of the day was also not significant in both ILCs, but nighttime 6-SMT values were consistently higher than daytime values (Fig. 4). The 24h 6-SMT profiles for *R. pumilio* are illustrated in Fig. 5. The interactive effect of photophase illuminance with the time of day had a non-significant influence on the level of urinary 6-SMT under the 10 lux-ILC (*F*₇,₃⁹=1.887, *P*=0.098) and also a non-significant influence under the 330 lux-ILC (*F*₇,₃⁹=1.030, *P*=0.426). During subjection to the 10 lux-ILC, there was a distinct increase in the urinary 6-SMT level at dusk (between 16h00 and 19h00) that was followed by a further increase towards the first major peak (22h00: 105.43 ± 46.91 ng/mg). A second, and largest peak, was observed at dawn (07h00: 121.23 ± 48.25 ng/mg), whereas the lowest value was observed at 13h00 (15.98 ± 7.11 ng/mg). Exposure to the 330 lux-ILC yielded a different 24h 6-SMT profile in which the peak was around dusk (19h00: 110.51 ± 56.78 ng/mg) and the lowest value at 10h00 (17.51 ± 15.58 ng/mg).
DISCUSSION

*Micaelamys namaquensis* is fundamentally nocturnal while *R. pumilio* is essentially diurnal, consequently the species’ active phases coincide with different photoenvironments in their natural habitats. The present results suggest that the level of photophase illuminance plays a key role in regulating locomotor activity, urine production and melatonin expression in *M. namaquensis* and *R. pumilio*.

**Daily rhythms in locomotor activity**

Since the availability of environmental resources change in concurrence with changing levels of illumination and spectral dominant wavelength across the day, the temporal layout of locomotor activity of a species is vital. Several studies suggest that species hold unique thresholds to different qualities of lighting and that these reflect the environments they are adapted to (Tast et al., 2001; Zubidat et al., 2009, 2010a, 2010b). The advantage of distinguishing between light intensities within the boundaries of specific thresholds is best demonstrated in species that adjust their activity patterns according to the level of moonlight. For example, many nocturnal animals either avoid open areas or reduce their locomotor activity altogether in an attempt to decrease predation risks under ranges of lighting that are equivalent to increasing moonlight (Alkon and Saltz, 1988; Julien-Laferrière, 1997; Topping et al., 1999).

The present study indicate that *M. namaquensis* and *R. pumilio* possess distinct locomotor activity responses to varying photophase illuminance levels, which appear to be associated with their temporal niches. Under the range of lighting conditions used here, all individuals of *M. namaquensis* and most individuals of *R. pumilio* had well defined daily locomotor activity rhythms. As expected, *M. namaquensis* consistently exhibited robust nocturnal activity, whereas a considerable amount of inter-individual variation marked the temporal expression of activity in *R. pumilio*. However, each individual retained its own characteristic locomotor activity rhythm across the range of ILCs. Under natural conditions, ambient illuminance levels vary considerably throughout the day and could reach approximately 110 000 lux during the brightest part of the day. Most small diurnal mammals refrain from being active during the middle of the day and often make use of pathways that run underneath vegetation cover, perhaps not only to escape the
heat but also the bright levels of lighting. Although the photophase illuminance did not influence the timing of daily activity expression, it determined the overall amount thereof. This effect also appears to be more pronounced in *M. namaquensis* than in *R. pumilio*. During its active phase and in its natural environment, *R. pumilio* faces greater fluctuations in the photoenvironment than *M. namaquensis*. Not only do the lighting conditions change radically at dawn and dusk, when *R. pumilio* exerts most of its activity, but also at times such as when the mice move between their darker underground burrows or nests and the outside field throughout the day. Therefore, *R. pumilio*’s expressed capacity to tolerate a wide range of illuminances is an extension of its more diurnal lifestyle and serves to preserve the stability and thus the adaptive capacity of its photically entrained circadian rhythm of locomotor activity. In contrast, under natural circumstances *M. namaquensis* is exposed to a photoenvironment with smaller fluctuations while it is active by night; for example, the illuminance level during full moon nights only reaches approximately 2 lux (Vásquez, 1994). This likely explains the greater sensitivity of *M. namaquensis* to the range of photophase illuminances used in the present study. It also highlights that light exposure during the species’ sleep phase could play an important role in regulating the extent to which animals are active at night. In humans, even low levels of light can be transmitted through closed eyelids. The spectral transmittance of light through the human eyelid is attenuated at short-wavelengths, i.e. the spectral region to which the circadian system is most sensitive (Bierman et al., 2011) and it is reasonable to assume that the same is true in the studied species. Interestingly, a relatively small increase from 1 lux to 10 lux, also a relatively dim ILC, yielded an amount of daily locomotor activity comparable to that obtained under the 330 lux-ILC. Therefore, the species was able to distinguish day from night under 1 lux of photophase lighting, but at this level of illuminance stimulation of the pathway resulting in the expression of activity was attenuated. A different correlation has previously been reported in another nocturnal species, the social vole (*Microtus socialis*), whereby the mean daily energy expenditure rates, which is an index of locomotor activity, decreased with increased photophase intensity (Bennett and Ruben, 1979; Zubidat et al., 2010a). In Macaque monkeys, distinct differences in activity onset times were observed in bright compared to dim light (Takasu et al., 2002). However, the bright light presented was an order of magnitude higher than in the current study (3500 lx) and the mice in the current study may also require higher light intensities for clear-cut differences to be observed. However, in some cases changes as small as 1 or 2 lux can
induce changes in locomotor activity patterns (Kramer and Birney, 2001). Whether differences in the densities and distributions of visual and non-visual retinal photoreceptors could be attributed to these species-related differences remains to be elucidated.

**Urine production rate**

Urine production fluctuates across the day not as a mere function of the daily eating and drinking patterns of an organism, but as a function of the circadian timing system (Negoro et al., 2012). Several processes that are tied to urine production clearly exhibit daily or circadian variations and are essential to the well-being of organisms (Kamperis et al., 2004; Noh et al., 2011). For example, the circadian timing of a bladder gap junction protein (connexin43), which regulates changes in bladder capacity, is fundamental for preventing micturition during sleep and thus improves sleep quality (Negoro et al., 2012). Another example is that of the relationship between atypical circadian rhythms of arginine vasopressin, a hormone renowned for regulating renal water excretion in mammals, and nocturnal polyuria syndrome (Asplund, 1995; Rittig et al., 2008). The endogenous timing of urine production is thus expected to ensure that the highest rates coincide with the wake period rather than the sleep period. Evidence also suggests an effect of photophase intensity on the timing of urine production, which might be species specific. In social voles (*Microtis socialis*) for instance, significant daily rhythms of urine excretion were observed at higher as opposed to dimmer photophase light intensities whereas, in blind mole rats (*Spalax ehrenbergi*), the rhythms were significant under both dim and bright photophase intensities (Zubidat et al., 2009).

In the present study, the 24 h urine production rhythms in *M. namaquensis* largely correlated to the species’ nocturnal activity rhythm. On the other hand, *R. pumilo* lacked an obvious 24 h urine production rhythm yet the highest rates consistently coincided with the start of the species’ active phase. Interestingly, similar trends in the response of mean daily urine production rates across the varying ILCs were observed in the two species but, significant differences were only observed in *R. pumilio*. In both species, brighter photophase illuminances appear to have a stimulating effect on urine production, yet values peaked under the 100 lux-ILC as opposed to the 330 lux-ILC. This might suggest similar sensitivity thresholds in these two species. It is interesting to note that mean daily urine production and mean daily locomotor activity responses
were uncorrelated. This could indicate that the circadian regulation of locomotor activity and of urine production each possesses their own sensitivity threshold to the level of photophase illuminance. Knowing that daily urine production rhythms result from the integrative workings of several hormones (e.g. arginine vasopressin, aldosterone, plasma renin and natriuretic peptide), which are also regulated by the circadian timing system (Kamperis et al., 2004; Noh et al., 2011), it is possible that the regulatory pathways of each of these hormones respond differently to varying qualities of lighting and that this caused the dissociation between locomotor activity and urine production, quantitatively.

**Urinary 6-sulfatoxymelatonin concentration**

In rats, light exposure of as low as 0.2 lux during the normal dark phase has been shown to reduce melatonin by 87%, compared to the 94% reduction by constant lighting (Dauchy et al., 1997). The present results indicate that the photophase illuminance level is an important component in the regulation of melatonin expression in both of the studied species. Only *M. namaquensis* showed significant interactive effects between the photophase illuminance and the time of the day under both ILCs (10 lux and 330 lux). In *M. namaquensis* 6-SMT values peaked at around two hours before the lights went on. It is likely that the period between pineal melatonin production and catabolism until 6-SMT detection in the urine caused a slight (1-2h) delay (Nowak et al., 1987). The peak in melatonin might thus have occurred slightly earlier and this could imply that the contrast between daytime and nighttime melatonin values is slightly larger in *M. namaquensis* than revealed here. Similar peaks in the melatonin content during the late night have also been observed in different strains of mice and in hamsters (Panke et al., 1978; Goto et al., 1989). In *M. namaquensis*, brighter photophase lighting actually had a slight attenuating effect on the melatonin rhythm. A similar effect has been reported in the strictly fossorial ‘blind’ mole rat (*Spalax ehrenbergi*), whereby the melatonin rhythm was weakened by brighter photophase lighting conditions, presumably as a result of an increased stress response in the animals under bright lighting (Zubidat et al., 2009). Although *M. namaquensis* is not fossorial like *S. ehrenbergi*, it still sleeps and rests in protected spaces such as between rock crevices that are sheltered from intense daytime illumination. For this reason, the attenuated melatonin levels under bright lighting conditions may be stress related. Nevertheless, the significant interactive effect between the photophase illuminance and the time of the day under
the 330 lux-ILC suggests that the photoreception system of the mice was not yet saturated at this intensity. Also in contrast to what was observed in *M. namaquensis*, Afrotropical stonechats (*Saxicola torquata axillaris*) show higher melatonin levels (with activity peaking in the early and late night) when exposed to bright light, but the light presented to the birds was in orders of magnitude higher than in the mice (Kumar et al., 2007).

In *R. pumilio*, the intensity of the photophase lighting did not affect the overall amount of melatonin produced daily. Interestingly, two relatively different daily rhythms in 6-SMT were observed, but both rhythms appeared to be ultradian. Ultradian patterns of melatonin secretion have previously been observed in humans and in mice and it has been suggested that two separate oscillators are foundational to these patterns (Wehr et al., 1995; Nakahara et al., 2003). Evidence also suggests that daytime and nighttime melatonin is regulated by different processes. At night, neurological signals from the superior cervical ganglia stimulates the rise in pineal melatonin production via sympathetic nerves (Moore, 1996; Reiter et al., 2011). In CBA mice, application of tetrodotoxin, a Na\(^+\) channel blocker, effectively suppresses neurological stimulation of pineal melatonin production by night, but not by day (Nakahara et al., 2003). It is therefore suggested that daytime melatonin regulation primarily results from endogenous activation inside the pinealocyte (Li et al., 2000). The present results strongly indicate that daytime and nighttime melatonin production is also regulated separately in *R. pumilio*; this is best demonstrated by the prominent rise in daytime 6-SMT levels under the bright ILC, but not the dim ILC. It also reveals that the daytime illuminance level plays an essential role in regulating melatonin production. Furthermore, the relatively high levels of 6-SMT during the day while subjected to the bright ILC was not unusual since a similar pattern has been observed in B6D2F\(_1\) mice (Li et al., 2000). Tast et al. (2001) determined that photophase light intensity has a relatively low threshold light intensity (40 lux) in diurnal domestic pigs, whereafter further increases in light intensity does not affect melatonin levels any longer.
CONCLUSION
Light plays a vital role in the regulation of the circadian timing system. However, when it intrudes into the natural dark phase, it may lead to the disruption of circadian organization and desynchronization in timing of the different biological rhythms, which is associated with various health risks and the disturbance of ecological systems (Bird et al., 2004; Navara and Nelson, 2007; Haim and Portnov, 2013). Although the effects of nighttime light exposure on circadian organization is widespread, few studies have investigated the effects of varying intensities of daytime lighting on photoentrainment. Here we show that the level of photophase illuminance proves to be an essential element in the process of photic entrainment of physiology and behavior, irrespective of whether the species is active by day or by night. The present results indicate that *M. namaquensis* and *R. pumilio* adjust their daily patterns in locomotor activity, urine production and melatonin excretion according to the light-dark cycle. *Micealamys namaquensis* and *R. pumilio* respond differently to varying photophase light intensities, most likely because they are adapted to different temporal niches in their natural environments where they are exposed to very different photoenvironments. The level of photophase illuminance proves to be an essential element in the process of photic entrainment of physiology and behavior, irrespective of whether the species is active by day or by night. Apart from light intensity varying across the day, the dominant wavelength within the photophase also changes across the day and this should be studied in the future.
LIST OF SYMBOLS AND ABBREVIATIONS

6-SMT  6-sulfatoxymelatonin
ipRGC  Intrinsically photosensitive retinal ganglion cell
SCN    Suprachiasmatic nucleus
ILC    Illuminance light cycle

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COMPETING INTERESTS
The authors declare no competing financial interests.

AUTHOR CONTRIBUTIONS
Conception and design - IvdM, MKO, AH, NCB
Data collection, analysis and/or interpretation - IvdM, MKO, AG
Drafting the article - IvdM
Revision of the article - MKO, AH, AG, NCB

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REFERENCES


Table 1. Mean ± s.e.m. values of locomotor activity (activity counts) and of urine production rate (µl/h) per photophase and per scotophase in *Micaelamys namaquensis* and *Rhabdomys pumilio* under different illuminance light cycles (ILCs).

<table>
<thead>
<tr>
<th></th>
<th>1 lux-ILC</th>
<th>10 lux-ILC</th>
<th>100 lux-ILC</th>
<th>330 lux-ILC</th>
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<tr>
<td><strong>M. namaquensis</strong></td>
<td></td>
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<tr>
<td>Photophase</td>
<td>51.07 ± 6.03</td>
<td>47.53 ± 5.92</td>
<td>33.47 ± 5.58</td>
<td>45.65 ± 5.91</td>
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<tr>
<td>Scotophase</td>
<td>438.00 ± 28.79</td>
<td>608.39 ± 39.63</td>
<td>551.29 ± 35.97</td>
<td>671.22 ± 43.66</td>
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<td>GLMM</td>
<td>F$_{1,985}$; <em>P</em></td>
<td>185.550; <strong>0.00</strong></td>
<td>202.871; <strong>0.00</strong></td>
<td>211.124; <strong>0.00</strong></td>
</tr>
<tr>
<td><strong>R. pumilio</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Photophase</td>
<td>108.95 ± 8.37</td>
<td>99.00 ± 7.54</td>
<td>109.88 ± 8.36</td>
<td>110.72 ± 8.88</td>
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<tr>
<td>Scotophase</td>
<td>73.90 ± 5.74</td>
<td>77.32 ± 5.89</td>
<td>86.35 ± 6.57</td>
<td>89.54 ± 7.01</td>
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<tr>
<td>GLMM</td>
<td>F$_{1,957}$; <em>P</em></td>
<td>21.275; <strong>0.00</strong></td>
<td>9.673; <strong>0.00</strong></td>
<td>9.217; <strong>0.00</strong></td>
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</table>

<table>
<thead>
<tr>
<th></th>
<th>1 lux-ILC</th>
<th>10 lux-ILC</th>
<th>100 lux-ILC</th>
<th>330 lux-ILC</th>
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<tr>
<td><strong>M. namaquensis</strong></td>
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<tr>
<td>Photophase</td>
<td>37.68 ± 14.07</td>
<td>45.45 ± 16.33</td>
<td>77.48 ± 28.85</td>
<td>65.15 ± 24.03</td>
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<td>Scotophase</td>
<td>125.72 ± 44.59</td>
<td>106.46 ± 37.54</td>
<td>125.12 ± 44.68</td>
<td>112.42 ± 39.75</td>
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<td>GLMM</td>
<td>F$_{1,186}$; <em>P</em></td>
<td>6.67; <strong>0.011</strong></td>
<td>5.889; <strong>0.016</strong></td>
<td>3.079; 0.081</td>
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<tr>
<td><strong>R. pumilio</strong></td>
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<tr>
<td>Photophase</td>
<td>37.95 ± 9.94</td>
<td>58.80 ± 14.29</td>
<td>68.91 ± 17.05</td>
<td>52.55 ± 13.86</td>
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<tr>
<td>Scotophase</td>
<td>28.85 ± 7.51</td>
<td>34.61 ± 8.76</td>
<td>69.99 ± 17.70</td>
<td>70.66 ± 18.92</td>
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<td>GLMM</td>
<td>F$_{1,173}$; <em>P</em></td>
<td>1.18; 0.279</td>
<td>4.622; <strong>0.033</strong></td>
<td>0.005; 0.944</td>
</tr>
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</table>

GLMM, generalized linear mixed model; *P*-value for the rejection of the null hypothesis when *P*<0.05, significant values are in bold.
Fig. 1.
Mean ± s.e.m. values of daily activity counts in *N*=9 *Micaelamys namaquensis* and in *N*=9 *Rhabdomys pumilio* following exposure to four successive ILCs. The photophase of each ILCs had a different illuminance level (i.e. 1 lux, 10 lux, 100 lux and 330 lux), whereas all scotophases consisted of complete darkness (*=P<0.05; **=P<0.001).
Mean ± s.e.m. values of daily urine production rate in \( N=9 \) *Micaelamys namaquensis* and in \( N=9 \) *Rhabdomys pumilio* following exposure to four successive ILCs. The photophase of each ILCs had a different illuminance level (i.e. 1 lux, 10 lux, 100 lux and 330 lux), whereas all scotophases consisted of complete darkness (*\( P<0.05 \); **\( P<0.001 \)).
Fig. 3.
The 24h rhythms of urine production rate (µl/h) under four ILCs in (A) *Micaelamys namaquensis* (N=9) and (B), *Rhabdomys pumilio* (N=9). The black and white bars at the bottom of each graph indicate the scotophase (18h00-06h00) and photophase (06h00-18h00) of each ILC, separately.
Fig. 4.
Mean ± s.e.m. urinary 6-sulfatoxymelatonin values (6-SMT; ng/mg) in $N=6$ *Micaelamys namaquensis* and in $N=6$ *Rhabdomys pumilio*. Levels of 6-SMT were measured under a dim ILC (10 lux-ILC) and under a bright ILC (330 lux-ILC).
Fig. 5.
The 24h rhythms of urinary 6-sulfatoxymelatonin (6-SMT; ng/mg) in \(N=6\) *Micaelamys namaquensis* and in \(N=6\) *Rhabdomys pumilio* under a dim illuminance light cycle (10 lux-ILC) and a bright illuminance light cycle (330 lux-ILC). Values are means ± s.e.m. and the black and white bars at the bottom of each graph indicate the scotophase (18h00-06h00) and photophase (06h00-18h00) of each ILC, separately.
**Fig. A1.**

Representative actograms for *Rhabdomys pumilio*. The present actograms were recorded under the 1 lux-ILC; in mouse number 2 (A), activity was concentrated around dusk and dawn whereas in mouse number 7 (B), activity was expressed intermittently throughout the day but with activity elevated around dusk and during the period between midnight and dawn.