Physiological rhythms are influenced by photophase wavelength in a nocturnal and a diurnal rodent species from South Africa

Ingrid van der Merwe[1], Nigel C. Bennett[1,2], Abraham Haim[3,†] Andre Ganswindt[2] and Maria K. Oosthuizen[1,2]

[1]Department of Zoology and Entomology, University of Pretoria, Private Bag X20, Hatfield, 0028, South Africa.

[2]Mammal Research Institute, University of Pretoria, Hatfield, 0028, South Africa.

[3]Israeli Center for Interdisciplinary Studies in Chronobiology, University of Haifa, Haifa 31905, Israel.

Corresponding author:

M.K. Oosthuizen

Department of Zoology and Entomology, University of Pretoria, Private Bag X20, Hatfield, 0028, South Africa.

Tel: +27 82 483 2529

Fax: +27 12 3625242

E-mail: moosthuizen@zoology.up.ac.za).

Running title: Effects of light wavelength on physiological rhythms of a diurnal and a nocturnal rodent

ABSTRACT

The quality and quantity of light changes significantly over the course of the day. The effect of light intensity on physiological and behavioural responses of animals has been well documented, particularly during the scotophase, but the effect of the wavelength of light, particularly during the photophase, less so. We assessed the daily responses in urine production, urinary 6-sulfatoxymelatonin (6-SMT) and glucocorticoid metabolite (uGCM) concentrations in the nocturnal Namaqua rock mouse (*Micaelamys namaquensis*) and diurnal four striped field mouse (*Rhabdomys pumilio*) under varying wavelengths of near monochromatic photophase (daytime) lighting. Animals were exposed to a short-wavelength light cycle (SWLC; ~465–470 nm), a medium-wavelength light cycle (MWLC; ~515–520 nm) and a long-wavelength light cycle (LWLC; ~625–630 nm). The SWLC significantly attenuated mean daily urine production rates and the mean daily levels of urinary 6-SMT and of uGCM were inversely correlated with wavelength in both species. The presence of the SWLC greatly augmented overall daily 6-SMT levels, and simultaneously led to the highest uGCM concentrations in both species. In *M. namaquensis*, the urine production rate and urinary 6-SMT concentrations were significantly higher during the scotophase compared to the photophase under the SWLC and MWLC, whereas the uGCM concentrations were significantly higher during the scotophase under all WLCs. In *R. pumilio*, the urine production rate and uGCM were significantly higher during the scotophase of the SWLC, not the MWLC and LWLC. Our results illustrate that wavelength in the photophase plays a central role in the entrainment of rhythms in diurnal and nocturnal African rodent species.

Key words: daily rhythms, glucocorticoid metabolite, photophase wavelength, urinary 6-sulfatoxymelatonin, urine production.

1. INTRODUCTION

Most mammals respond and adjust cellular, physiological and behavioural functions to the light-dark cycle by means of an endogenous time-keeping system that is coordinated by a master clock, the suprachiasmatic nucleus (SCN) (Doyle and Menaker, 2007). For the circadian clock to be effective, it must be synchronized with the external environmental rhythms by daily resetting of the clock (Rea, 1998). In mammals, photic signals reach the SCN via the retinohypothalamic-tract, after initially being absorbed in the retina by classical photoreceptors (rods and cones) and by melanopsin-expressing intrinsically photosensitive retinal ganglion cells (ipRGCs) (Aggelopoulos and Meissl, 2000; Hattar et al., 2006). Although the ipRGCs are independently light sensitive (Lucas et al., 1999; Lucas et al., 2001), evidence now suggests that rods and cones also contribute to non-visual SCN-related processes (Dkhissi-Benyahya et al., 2007; Altimus et al., 2010; Gooley et al., 2010; Van Diepen et al., 2013; Weng et al., 2013). In the SCN, the circadian clock is reset by the alteration of clock gene expression, which in turn modulates the downstream physiological and behavioural rhythms (Golombek and Rosenstein, 2010). Entrainment is an adaptive feature, and the general process thereof appears to be similar for diurnal and nocturnal animals, with differences in temporal organization occurring further downstream (Challet, 2007).

The circadian timing system is differentially affected by a number of qualities of the light input, these include the intensity, spectral wavelength, duration and timing of the light exposure (Gorman et al., 2003; Duffy and Wright, 2005; Aral et al., 2006; Zubidat et al., 2009; Zubidat et al., 2010b). Moreover, mammalian species exhibit specific sensitivity thresholds in their circadian or daily rhythms to different qualities of lighting, that most likely reflects the ecological niches to which species are adapted to (Kumar and Rani, 1999; Peichl, 2005; Zubidat et al., 2009; Zubidat et al., 2010b; Zubidat et al., 2010a). Accordingly, animals may exhibit stress responses when they are subjected to certain qualities of lighting that differ substantially from their natural photo-

environments. It is well known that artificial light at night (ALAN) is one of the most effective ways to disrupt coherence within the circadian timing system and to induce stress, and evidence suggests that certain qualities of light during the photophase can also act as a stressor (Zubidat et al., 2007; Zubidat et al., 2009).

As a result of adaptation, the ratio of cones to rods in rodent retinas typically corresponds to the temporal activity rhythm of a species and thus tends to be considerably higher in diurnal- than in nocturnal species (Jacobs, 1993a). This raises the possibility that different photoreceptor compositions between species may, at least in part, explain different circadian responses to photic stimuli between diurnal and nocturnal species. From the SCN, circadian cues reach the pineal through efferent connections and modulate the synthesis or suppression of melatonin (Moore, 1996; Reiter et al., 2011). Melatonin production is induced by the circadian clock at night (or subjective night), but can be suppressed by light. The circulating melatonin plays a central role in regulating daily and seasonal adjustments in photoperiodic species (Reiter, 1993; Challet, 2007). Exposure to light of shorter wavelengths at night (~420-500nm, blue light) is deemed to be the most potent spectra for suppressing melatonin in both humans and animals (Thapan et al., 2001; Brainard et al., 2008; Rahman et al., 2008). However, light of longer wavelengths is also capable of suppressing nighttime melatonin signals either quickly (at high intensities; Hanifin et al., 2006) or over the long-term (at low intensities; Dauchy et al., 2015).

We studied two rodent species native to Africa, the Namaqua rock mouse (*Micaelamys namaquensis*) and the four striped field mouse (*Rhabdomys pumilio*). The rock mouse is strictly nocturnal in the laboratory (Van der Merwe et al., 2014; Ackermann et al., 2020), whereas the four striped field mouse is active during the day with peaks at dawn and dusk, both in the field and in the laboratory (Schumann et al., 2005; Ackermann et al., 2020). The photoreceptors of both species appear to be complementary to their temporal niches. As expected for a nocturnal species,

M. namaquensis possesses both short wavelength-sensitive cones (S-cones) as well as medium- to long wavelength-sensitive cones (M/L-cones) in low quantities; whereas the average M/L-cone density value was over five times higher in the diurnal *R. pumilio* (Van der Merwe et al., 2018). The peak sensitivity of M/L cones in *R. pumilio* is around 500 nm whereas that of S-cones reaches quite far into the ultraviolet range (estimated at around 360 nm; Allen et al., 2020). Although the spectral sensitivities for cones in *M. namaquensis* have not yet been quantified, in many nocturnal rodents it seems comparable to that of *R. pumilio* (Veilleux and Cummings, 2012). In both species, ipRGC's have been identified, quantities were slightly higher in *R. pumilio* compared to *M. namaquensis* (Van der Merwe et al., 2018). Melanopsin (IpRGC photopigment) peak spectral sensitivity is around 460-485 nm in mammals (Lucas et al., 2013).

The locomotor activity of the two species under investigation is affected by the wavelength of light. When exposed to the same lighting conditions, the level of activity in the diurnal species displayed the highest levels of activity under short wavelength light, whereas the opposite was true in the nocturnal species. Both species displayed more diurnal activity and less nocturnal activity under long wavelength light (Van der Merwe et al., 2019). Here we looked to see if behavioural differences occur in response to light of different wavelengths which in turn may be reflected in the physiological functions of the animals.

We evaluated the daily rhythms in urine production, urinary 6-sulfatoxymelatonin (6-SMT) and glucocorticoid metabolite (uGCM) concentrations in the two species in response to three different photophase wavelengths. We anticipated a dampened effect on the amplitude of the rhythms under long wavelength light and an amplified effect on the rhythms under short wavelength light, as is reflected in the locomotor activity under similar conditions (Van der Merwe et al., 2019).

2. MATERIALS AND METHODS

2.1 Animal collection and housing

The experiments were carried out on nine wild trapped *M. namaquensis* males (mean body mass = 41.1 g) and nine wild trapped *R. pumilio* males (mean body mass = 38 g). *M. namaquensis* were captured at Goro Game Reserve in the Soutpansberg region (Limpopo Province, South Africa, 22°58'S, 29°25'E), whereas *R. pumilio* were collected from Birha farms near Birha (Eastern Cape Province, South Africa, 33°22'S, 27°19'E). Animals were housed individually in semi-transparent plastic cages (58 x 38 x 36 cm) in a temperature-controlled room (25 °C ± 1°C and ~60% relative humidity). Animals were provided with a small open plastic shelter and tissue paper as nesting material. The mice had *ad libitum* access to water and food, comprising of parrot seed mix (Marlton's, Durban, South Africa), apple and carrot. Seeds were topped up and fresh food replaced at random times every second or third day to avoid activity entrainment to the feeding schedule. All experimentation was approved by the Animal Ethics Committee of the University of Pretoria, Pretoria, South Africa (EC063-11) and the required trapping permits were acquired (001-CPM403-00014, CRO95/12CR, CRO 96/12CR).

2.2 Experimental protocol

Double strands of Light Emitting Diodes (LED) strip lights (RGB, DC12V, 14.4 W/m, IP55, SMD 5050, 30 LED/m) were installed approximately 50 cm above cage floor level, with irradiance levels of 0.2-0.3 $W.m^{-2}$ for all light colours (Table S1). Photophase was between 06h00 and 18h00 and scotophase between 18h00 and 06h00. The animals were exposed to three wavelength light cycles (WLCs), a short-wavelength light cycle (SWLC; blue: 465 – 470 nm), followed by a medium-wavelength light cycle (MWLC; green: 515 - 520 nm) and ending with a long-wavelength light cycle (LWLC; red: 625 – 630 nm). The light cycles were presented in the same order for both species. The blue light (SWLC) was expected to overlap with the spectral sensitivity range of the

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melanopsin expressing ipRGC's and the green light (MWLC) with the M/L-cones, while the red light (LWLC) was probably not in the range of any of the photoreceptors.

Animals were exposed to each cycle for three weeks, one week to acclimatize to the light conditions and locomotor activity was recorded for the last two weeks (Van der Merwe et al., 2019). Urine samples were collected on day 21 of each WLC, while still maintaining the light conditions, and were used to evaluate the rate of urine production and for determining urinary 6-SMT as well as uGCM levels.

2.3 Urine production rate: Collection and analysis

Urine samples were collected at 3h intervals over a 24h period on the last day of each WLC. Animals were transferred to modified urine collection cages with stainless steel wire mesh floors 2 cm above the cage bottoms. At the short end of the cage, an open slit allowed the insertion of a plastic sheet between the wire mesh floor and the cage bottom, which could be removed whenever the screened urine had to be collected. Urine was collected using disposable glass Pasteur pipettes and stored in 1.5 ml Eppendorf tubes. The samples were immediately weighed with a Mettler digital scale (Mettler, Zurich, Switzerland) and stored at -20ºC until analyses. Urine specific gravity was assumed to be 1 g/ml (Schoorlemmer et al., 2001; Tendron-Franzin et al., 2004) when the urine volume (sample mass divided by urine specific gravity) was calculated. These values were then transformed to indicate hourly urine production rates (μl/h). The summed means were calculated for each WLC as well as for the respective photophases and scotophases per species. Furthermore, the mean values for all of the animals within a species were calculated at each 3h point to present the 24h daily rhythms in urine production rate.

2.4 Quantification of urinary 6-sulfatoxymelatonin (6-SMT)

The main melatonin metabolite, 6-sulphatoxymelatonin (6-SMT), is quantifiable in urine and accurately represents the concentration of secreted melatonin (Bojkowski et al., 1987). Urinary 6-

SMT concentrations were determined using a commercial enzyme immunoassay (EIA, IBL, Hamburg, Germany, cat. no. RE54031). Prior to analysis, 10 μl urine was diluted with 500 μl Assay Buffer. Subsequently 50 μl of the diluted sample have been analyzed in duplicate using 96-well microtiter plates by adding 50 μl of enzyme labelled antigen and Melatonin Sulfate rabbit-antiserum. After an incubation for 2h at room temperature and a subsequent washing step, 100 μl of a pre-prepared tetramethylbenzidine solution was added. Following incubation for 30 min. at RT, the reaction was terminated by adding 100 μl H_2SO_4 (1M) and absorbance was measured at 450 nm. The intra- and inter-assay coefficients of variation were 5.2-12.2% and 4.0-6.0%, respectively.

2.5 Quantification of urinary glucocorticoid metabolite levels

In mammals, one of the principal effectors of the autonomic nervous system, for the preservation of homeostasis, is the activation of the hypothalamic pituitary adrenal axis, resulting, amongst others in a temporal increase in glucocorticoid output (Möstl and Palme, 2002; Palme et al., 2005). Therefore, glucocorticoids or their metabolites are commonly quantified in rodents as a biological stress-related indicator. Urinary glucocorticoid metabolite (uGCM) concentrations were determined using an EIA utilizing antibodies against corticosterone-3-CMO. Further details of the EIA, including cross-reactivities were previously described (Palme and Möstl, 1997). In brief, 50 μl aliquots of standards, quality controls and diluted urine were pipetted in duplicate into the microtiter plate wells. After adding 50 μl of biotinylated label and antibody the plates were incubated overnight at 4°C. After washing, 150 μl of streptavidin-peroxidase was added. After incubation for 45 min., the plates were washed again before 150 μl peroxidase substrate solution was added. After another incubation for 30-60 min., the reaction was terminated by adding 50 μl of 4N H_2SO_4 and the absorbance subsequently measured at 450nm. Serial dilutions of sample material gave displacement curves that were parallel to the respective standard curve (yielding a

slope of <1%). The intra assay coefficient of variation of high- and low-concentration controls was 6.4% and 7.1%, respectively, and the inter assay coefficient of variation was 14.2% and16.4%, respectively. All hormone analyses were conducted at the Endocrine Research Laboratory, University of Pretoria, South Africa.

2.6 Creatinine

Urinary creatinine levels were obtained to correct the determined urinary 6-SMT and uGCM values for urine concentration. The urine samples were analyzed in duplicate. A standard curve was constructed from eight standard samples of known creatinine concentration. 7 μl urine samples were incubated for 2h in the dark in 210 μl of freshly prepared Picric reagent, which comprised of 1 volume saturated picric acid solution, 10 volumes in distilled- water and 1 volume alkaline triton solution (4.2 ml triton and 12.5 ml NaOH 1N dissolved in 66.0 ml distilled water). The spectral absorbency of the samples was then determined with a universal plate reader (at 492 nm) and the creatinine concentration values extrapolated from the standard curve.

2.7 Statistical analysis

Microsoft Excel (Microsoft Corp., Redmond, WA, USA) and IBM SPSS Statistics version 21.0 (SPSS Inc., Chicago, IL, USA) were used for descriptive and analytical statistical analyses. Data were not normally distributed, therefore a generalized linear mixed effect model with a gamma distribution and an identity link function was used to test the mean effects of spectral wavelength on the range of variables (daily rhythms in urine production, 6-SMT and uGCM concentrations). Consecutive measurements over the 24 day were used as repeated measures with spectral wavelength and the phase of the day (photophase or scotophase) were used as fixed factors. All interactions were also considered. Least significant difference *post hoc* tests were performed when needed. Statistical significance was set at $P<0.05$ and values are expressed as mean ± standard error of the mean (s.e.m).

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3. RESULTS

3.1 Urine production rate

The spectral exposure during photophase had an overall significant effect on urine production rate in *M. namaquensis* ($F_{2,139}$=6.488, P=0.002). The SWLC yielded the lowest mean daily urine production rate and differed significantly from that of the MWLC (P=0.004) and the LWLC (P=0.004; Fig. 1A). Daily urine production rates showed significant day/night differences, with increased levels at night under SWLC and MWLC conditions (Table 1). Daily rhythms of urine production rate are depicted in Fig. 1B.

In *R. pumilio,* photophase wavelength had an overall significant effect on urine production rate ($F_{2,104}$=3.931, P=0.023). The mean daily urine production values showed a similar trend across the three WLCs comparable to that for *M. namaquensis* (Fig. 1C). Mean daily urine production rate was lowest under the SWLC and differed significantly from that obtained under the MWLC- (P=0.024) and LWLC-conditions (P=0.014; Fig. 1C). Daytime urine production rates were significantly higher than nighttime rates only under the SWLC (Table 2) and no significant time-related variations were detected under any of the experimental conditions. See Fig. 1D for daily urine production rates in *R. pumilio*.

3.2 Urinary 6-sulfatoxymelatonin (6-SMT) concentrations

In *M. namaquensis,* significant effects of the photophase wavelengths on urinary 6-SMT levels ($F_{2,66}$=8.489, P=0.001) were revealed. An increase in photophase wavelength induced a progressive decrease in the mean daily 6-SMT levels from SWLC to MWLC to LWLC (Fig. 2A). The latter value differed significantly from that of the SWLC ($F_{2,66}$=8.489, P=0.001) and from the MWLC ($F_{2,66}$=8.489, P=0.005). Under the SWLC, 6-SMT levels were significantly higher during

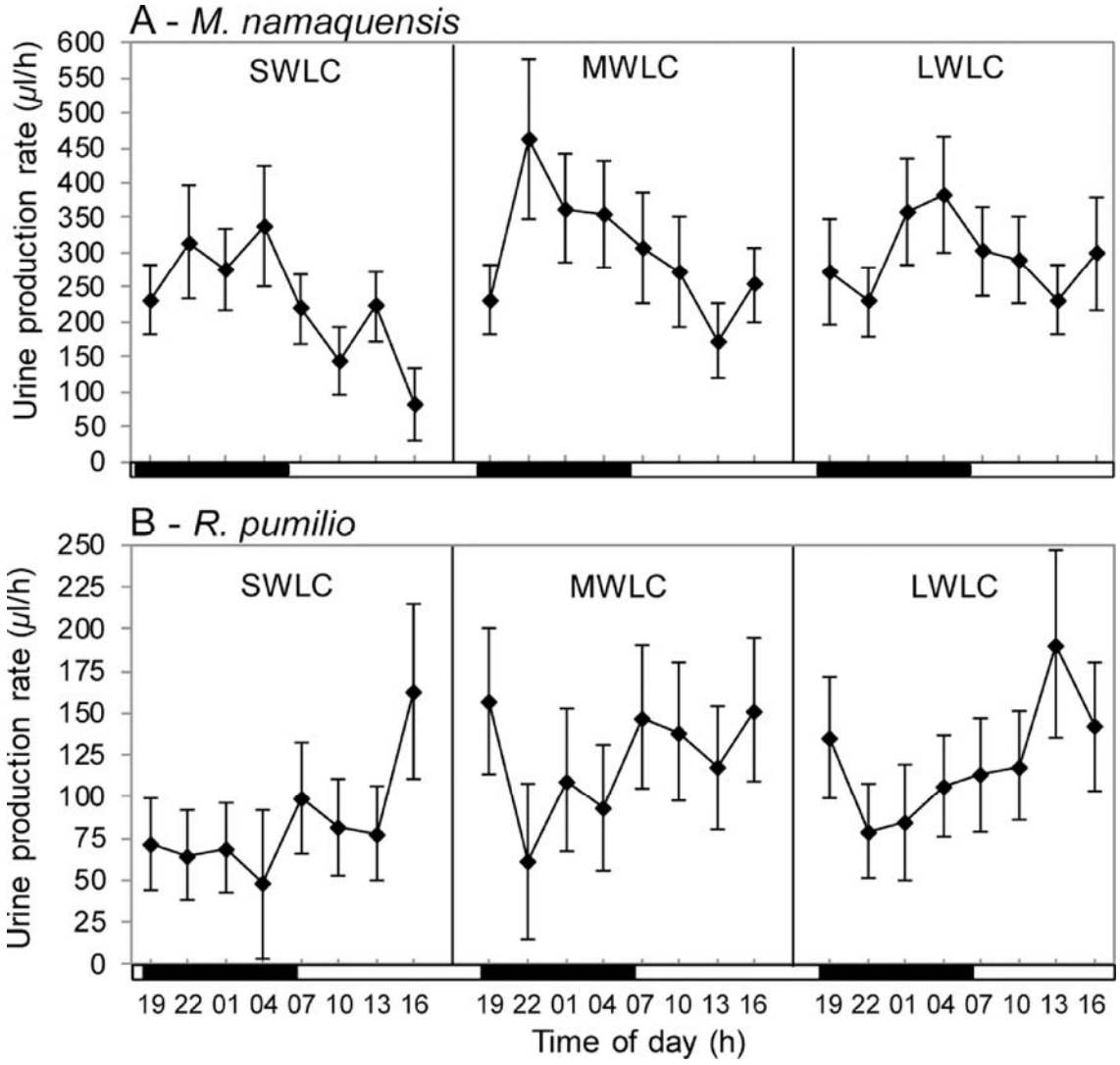

Fig. 1. Daily rhythms of urine production rate (µl/h) in (A) *M. namaquensis* and (B) *R. pumilio* after exposure to three WLCs. The photophases of the respective WLCs were illuminated using near-monochromatic short-, medium-, or long-wavelength lighting while the scotophases were completely dark. Black and white bars indicate the scotophases and photophases, separately. (Data are means ± s.e.m. *=$P < 0.05$).

Table 1. Urine production rate, urinary 6-sulfatoxymelatonin and urinary glucocorticoid metabolite conncentration during photophase and scotophase in *Micaelamys namaquensis* under different wavelength light cycles (WLCs). Data are means±s.e.m. GLMM, generalized linear mixed model. Significant P-values ($P < 0.05$) are in bold.

M. namaquensis	SWLC	MWLC	LWLC
Urine production rate (μl)			
Photophase	166.98 ± 47.31	251.65 ± 52.49	280.22 ± 51.66
Scotophase	290.10 ± 53.48	353.41 ± 57.36	310.75 ± 54.05
GLMM ($F_{1,139}$; P)	19.20; **0.000**	5.74; **0.018**	0.74; 0.391
Urinary 6-SMT (ng/mg)			
Photophase	72.28 ± 17.52	88.93 ± 18.60	39.54 ± 13.77
Scotophase	145.09 ± 30.36	74.68 ± 17.10	42.82 ± 14.80
GLMM ($F_{1,66}$; P)	4.88; **0.031**	0.41; 0.524	0.048; 0.827
Urinary GCM (μg/mg)			
Photophase	2.77 ± 0.73	3.86 ± 0.86	1.72 ± 0.53
Scotophase	9.85 ± 1.70	3.79 ± 0.70	2.81 ± 0.63
GLMM ($F_{1,86}$; P)	17.03; **0.000**	0.01; 0.934	5.98; **0.017**

Table 2. Urine production rate, urinary 6-sulfatoxymelatonin and urinary glucocorticoid metabolite concentration per photophase and per scotophase in *Rhabdomys pumilio* under different wavelength light cycles (WLCs). Data are means±s.e.m. GLMM, generalized linear mixed model. Significant *P*-values ($P < 0.05$) are in bold.

R. pumilio	SWLC	MWLC	LWLC
Urine production rate (μl)			
Photophase	105.42 ± 28.46	138.62 ± 29.38	141.02 ± 29.45
Scotophase	63.33 ± 26.61	105.23 ± 29.29	101.47 ± 26.61
GLMM ($F_{1,104}$; P)	6.16; **0.015**	1.71; 0.194	3.82; 0.053
Urinary 6-SMT (ng/mg)			
Photophase	45.70 ± 8.20	25.82 ± 5.89	16.25 ± 3.96
Scotophase	93.49 ± 26.76	37.07 ± 8.59	16.21 ± 3.10
GLMM ($F_{1,73}$; P)	2.93; 0.091	1.20; 0.277	0.00; 0.994
Urinary GCM (μg/mg)			
Photophase	14.84 ± 2.34	7.75 ± 1.29	6.18 ± 0.99
Scotophase	17.10 ± 3.29	6.90 ± 1.16	4.44 ± 0.67
GLMM ($F_{1,65}$; P)	0.32; 0.577	0.26; 0.615	2.31; 0.134

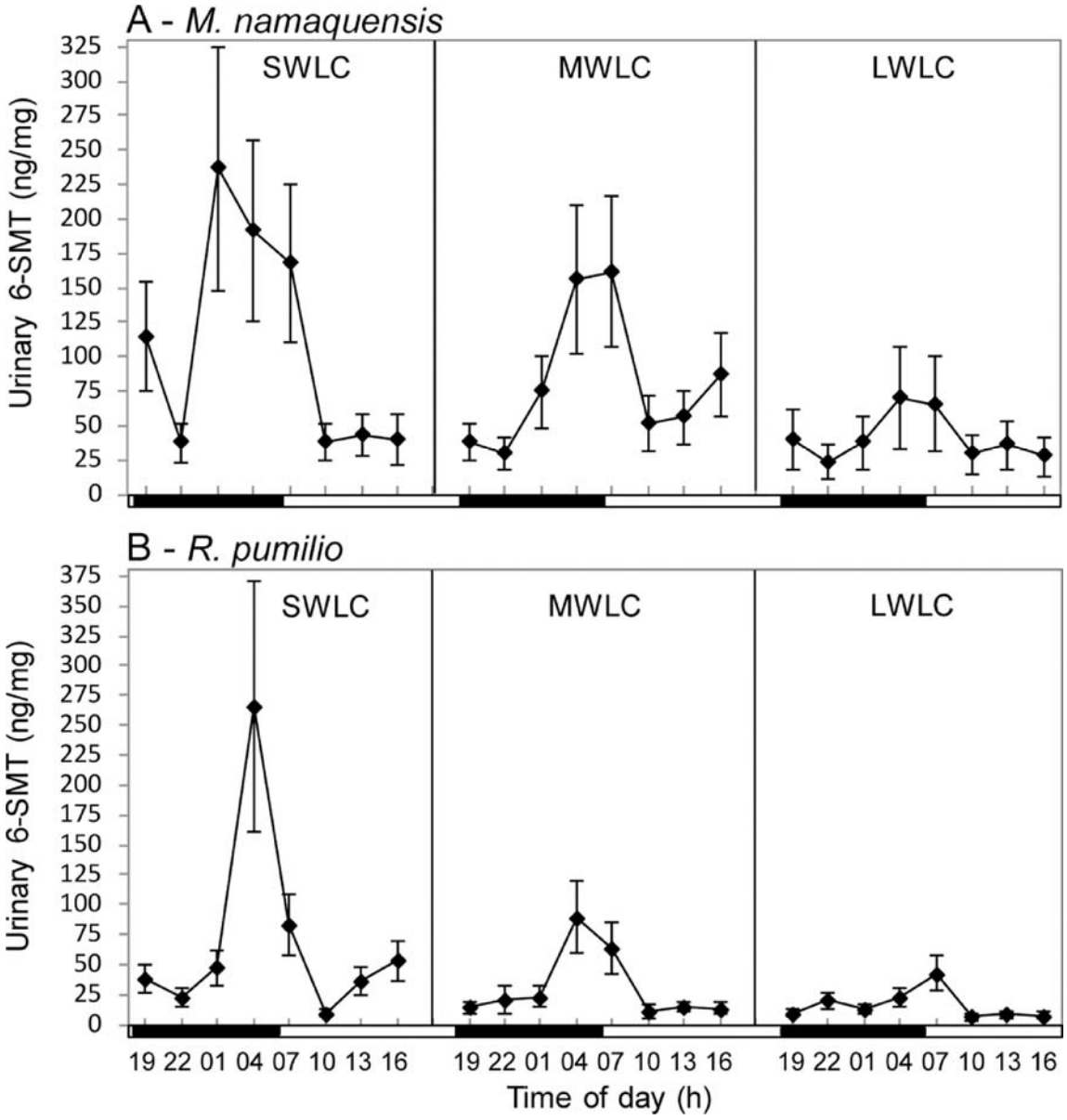

Fig. 2. Daily rhythms of urinary 6-SMT (ng/mg) in (A) *M. namaquensis* and (B) *R. pumilio* after exposure to three successive WLCs. The photophases of the respective WLCs were illuminated using near-monochromatic short-, medium-, or long-wavelength lighting while the scotophases were completely dark. Black and white bars indicate the scotophases and photophases, separately. (Data are means ± s.e.m. *=$P < 0.05$).

scotophase than during photophase ($F_{2,66}$=4.882, P=0.031; Table 1). The 24h urinary 6-SMT rhythm for *M. namaquensis* is presented in Fig. 2B. The interactive effect of photophase wavelength with the time of the day significantly affected 6-SMT levels under the SWLC ($F_{7,66}$=2.691, P=0.016) and MWLC ($F_{7,66}$=2.460, P=0.026), but not the LWLC ($F_{7,66}$=0.549, P=0.794).

In *R. pumilio*, significant effects of photophase wavelength on urinary 6-SMT levels were detected ($F_{2,73}$=10.064, P<0.001). As in *M. namaquensis*, an increase of the photophase wavelength led to a progressive significant decrease in the mean daily 6-SMT levels from the SWLC to the MWLC ($F_{2,73}$=10.064, P=0.012) and from the MWLC to the LWLC ($F_{2,66}$=8.489, P=0.009). The LWLC value was also significantly different from the SWLC value ($F_{2,73}$=10.064, P<0.001; Fig. 2C). Nighttime 6-SMT values were 34.34%, and 17.88% higher (yet not significantly) than daytime values under the SWLC and MWLC conditions, respectively. Under LWLC conditions, this difference was 0.12% (Table 2). The 24h urinary 6-SMT rhythm in *R. pumilio* is presented in Fig. 2D. Under the SWLC, the interactive effect between the photophase wavelength and the time of the day had a significant effect on 6-SMT levels ($F_{7,73}$=5.260, P<0.001), under the MWLC this was not significant ($F_{7,73}$=1.828, P=0.095) while under the LWLC there was a stronger yet non-significant effect ($F_{7,73}$=2.110, P=0.053).

3.3 Urinary glucocorticoid metabolite (uGCM) concentrations

A significant inverse effect of photophase wavelength on uGCM levels was found in *M. namaquensis* ($F_{2,86}$=13.984, P<0.001). Mean daily uGCM levels decreased significantly from the SWLC to the MWLC (P=0.012) and further from the MWLC to the LWLC (P=0.002), SWLC and LWLC also differed significantly (P<0.001; Fig. 3A). Furthermore, nighttime uGCM concentrations were significantly higher than daytime concentrations under MWLC- and LWLC-conditions, while day and night values were similar under the MWLC (See Table 1). Fig. 3B shows

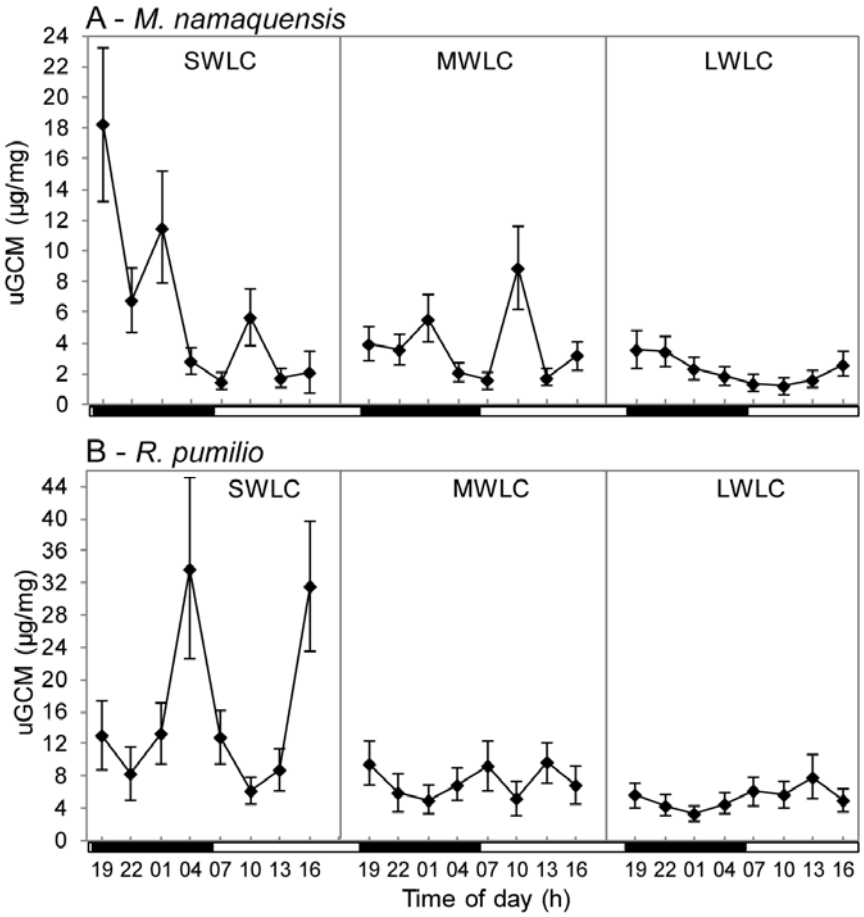

Fig. 3. Daily rhythms of urinary uGCM (*µ*g/mg) in (A) *M. namaquensis* and (B) *R. pumilio* after exposure to three successive wavelength light cycles WLCs. The photophases of the respective WLCs were illuminated using near-monochromatic short-, medium-, or long-wavelength lighting while the scotophases were completely dark. Black and white bars the scotophases and photophases, separately. (Data are means ± s.e.m. *=$P < 0.05$).

the 24h rhythms of uGCM concentrations for *M. namaquensis* under the various experimental conditions. The interactive effects of time of day with photophase wavelength yielded significant results under all three WLCs (SWLC: $F_{7,86}$=4.677, P<0.001; MWLC: $F_{7,86}$=3.558, P=0.002; LWLC: $F_{7,86}$=2.922, P=0.009).

A significant effect of photophase spectra on uGCM levels was observed in *R. pumilio* ($F_{2,65}$=13.666, P<0.001). Similar to *M. namaquensis*, an increase in the spectral wavelength during the photophase was related to lower uGCM levels in *R. pumilio* (Fig. 3C). Mean daily uGCM values decreased significantly from the SWLC to the MWLC (P<0.001) and displayed a further non-significant decrease to the LWLC (P=0.054). Values obtained under SWLC and LWLC exposures also differed significantly (P<0.001; Fig. 3C). There was no significant phase-related variation in uGCM concentrations for any of the spectral groups (Table 2). The 24h-trends in uGCM levels for *R. pumilio* are illustrated in Fig. 3D. Significant effects for the time of day on uGCM concentration were detected only under the SWLC ($F_{7,65}$=2.745, P=0.015).

4. DISCUSSION

The timing of activity and other physiological factors is an important adaptation for the survival of animals (Hut et al., 2012). Among the murids, most species occupy nocturnal niches, with fewer being diurnal or crepuscular. The temporal niches of animals are frequently shaped by environmental conditions (Halle and Stenseth, 2000). Over time, species show anatomical and physiological adaptations to their specific niches in features such as photoreceptor complements in the retina and sensitivity to light (Jacobs, 1993b) that may lead to differential responses to the same light conditions. We investigated a diurnal and a nocturnal rodent species, aiming to evaluate the daily rhythms in urine production, urinary 6-sulfatoxymelatonin (6-SMT) and glucocorticoid

metabolite (uGCM) concentrations in response to three different photophase wavelengths. In a previous study, we showed that changes in light intensity of the photophase did not affect locomotor activity, urine production or urinary 6-SMT in the same manner in *M. namaquensis* and *R. pumilio* (Van der Merwe et al., 2017). These variations could stem from differences in the SCN response to light of different intensities and wavelengths (Bonmati-Carrion et al., 2017; Dkhissi-Benyahya et al., 2000; Oosthuizen et al., 2010), and most likely reflected adaptations to the vastly distinct photo-environments of the temporal niches to which these two species are confined in their natural habitats.

Urine production is regulated by a combination of factors, most of which are strongly tied to the circadian timing system (Negoro et al., 2012). For example, the bladder gap junction protein (connexin43) which is involved in the regulation of bladder capacity, also expresses distinct daily variations (Kamperis et al., 2004; Noh et al., 2011). As a consequence, urine production exhibits daily rhythmicity whereby uresis is attenuated during the sleep/rest phase in healthy humans and animals in order to improve sleep quality (Asplund, 1995; Rittig et al., 2008; Negoro et al., 2012). Evidence suggest that melatonin is involved in the circadian regulation of at least some of these factors. Antidiuretic hormone (ADH), a major regulator of water homeostasis, stimulates water reabsorption in the kidneys, causing an increase in urine osmolality and a decrease in urine volume in healthy subjects. Melatonin has been shown to have an inhibitory effect on neurohypophyseal ADH secretion (Richardson et al., 1992; Bojanowska and Forsling, 1997; Juszczak and Stempniak, 2003).

The results of the present study revealed significant 24h rhythms of urine production in *M. namaquensis* in all but the LWLC. This agrees with previous data in another nocturnal species (*M. socialis*) where a distinct rhythm of urine production was observed under short-wavelength photophase lighting, but not long-wavelength photophase lighting (Zubidat et al., 2010b). The

daily rhythm of urine production largely correlated with the nocturnal activity rhythm of *M. namaquensis*. Our results suggest that melatonin could be involved in the regulation of the urine production rhythm in *M. namaquensis*. Urine production levels and melatonin levels were simultaneously and significantly pronounced at nighttime under SWLC conditions. However, the contrast between day and night levels were diminished under LWLC conditions, while MWLC conditions led to pronounced nighttime urine production levels, but not melatonin concentrations. Although *R. pumilio* did not display distinct 24h urine production rhythms, urine production was higher during the daytime across all three WLC's, corresponding to the species' diurnal activity rhythm. Nevertheless, a significant contrast between day and night urine volumes only occurred under SWLC conditions. The effect of the changing light spectra on the overall daily urine production in *R. pumilio* was similar to that of *M. namaquensis.* Therefore, irrespective of the temporal niche of the species, SWLC conditions had an attenuating effect on urine production compared to MWLC and LWLC conditions. This suggests the involvement of the short-wavelength light-sensitive ipRGCs and confirms the notion that urine production is physiologically timed by the circadian system, which in turn is regulated by the quantity and quality of light (Negoro et al., 2012). In the diurnal *R. pumilio*, urine production was significantly higher during photophase compared to scotophase despite the nighttime melatonin concentrations being double that of the daytime concentrations under SWLC conditions, indicating that melatonin may have an inhibitory effect on the urine production.

The urine production rhythm in both of our study species is less well defined compared to that of the other variables measured. A potential explanation may be the influence of non-photic cues since the peripheral clock of the kidney is also strongly influenced by non-photic cues such as food intake and glucocorticoids (Damiola 2000; Son et al., 2018). Depending on the interaction between

photic and non-photic cues, it may have been better to measure the urine output over several days to obtain a more representative rhythm profile.

Several qualities of light including intensity, spectral wavelength, duration and timing of the light exposure, affect the circadian rhythm of melatonin synthesis (Gorman et al., 2003; Duffy and Wright, 2005; Aral et al., 2006; Zubidat et al., 2009; Zubidat et al., 2010b). There is also a large variation in the capacity of light to suppress melatonin rhythms among different species. Although numerous studies have tested the effects of light exposure during the night on melatonin production, few studies have looked at the effects of different qualities of photophase lighting on melatonin production. In the present study, robust daily rhythms in urinary 6-SMT production were only detected under SWLC and MWLC conditions in *M. namaquensis*. The shape of the 6-SMT rhythms of *M. namaquensis* reflects that of other rodents (Panke et al., 1978; Goto et al., 1989). The melatonin rhythm peaks slightly earlier under SWLC conditions than under MWLC and LWLC and the mean daily melatonin concentrations showed a significant inverse relationship with the wavelength of the photophase lighting. Zubidat et al. (2010b) obtained a similar trend in *M. socialis* where mean daily melatonin values were lower under long- as opposed to short-wavelength photophase lighting. Our results confirm that in a nocturnal mammal, the quality of daytime lighting, in this case light spectra, clearly influences nighttime melatonin production and that it is not solely dependent on the quality of darkness during scotophase (Griffith and Minton, 1992; Hashimoto et al., 1997; Park and Tokura, 1999). Short wavelength light (~420nm-500nm, blue light) exposure during the dark phase has been shown to be most effective at suppressing melatonin in both humans and animals (Thapan et al., 2001; Brainard et al., 2008; Rahman et al., 2008). In our study, daytime exposure to LWLC conditions proved to be a weak stimulus of the nocturnal melatonin rhythm in *M. namaquensis*. Interestingly, Zubidat et al (2010b) also reported that long-wavelength light exposure amplified daily melatonin output in the blind mole rat (*Spalax*

ehrenbergi), but that this was most likely due to the species being adapted to a subterranean environment where spectral power is weak.

Short wavelength light during photophase was shown to play a key role in the regulation of the daily melatonin production in *R. pumilio*. Significant day-night differences in urinary 6-SMT levels were only detected under the SWLC, where nighttime melatonin levels were double that of the daytime levels. The mean daily melatonin was similar across the various WLC's in the nocturnal *M. namaquensis* and the diurnal *R. pumilio,* despite differences in their retinal photoreceptor compositions. The ipRGCs in the two species have similar densities (*R. pumilio*: total number=1012 ipRGCs/mm^2; *M. namaquensis*: total number=862 ipRGCs) but are distributed differently (Van der Merwe et al., 2018). Blue light that overlaps with the spectral range of the ipRGC's, renders the largest response in both the nocturnal and diurnal species, and the response reduces as the wavelength of the light becomes longer. This confirms the fundamental involvement of the ipRGC's in conveying photoperiodic information for melatonin regulation (Thapan et al., 2001). The profile of melatonin production differs in the nocturnal and diurnal species, the nocturnal *M. namaquensis* appears to have a longer peak in melatonin whereas the melatonin peak of the diurnal *R. pumilio* is defined more sharply at the end of the scotophase. This may be mediated by the classic photoreceptors, although it is not clear how different distributional patterns of photoreceptors facilitate vision and photic entrainment. It is however apparent that photophase spectral wavelength is an important component in the regulation of melatonin production in both the nocturnal and the diurnal species.

The adrenocortical glucocorticoid corticosterone exhibits distinct daily rhythms, and it is well established that the phase of the peripheral adrenal oscillator is orchestrated by light (Ishida et al., 2005; Oster et al., 2006; Thorpe et al., 2012). The adrenal glands receive photic signals through the autonomic nervous system by way of the SCN pacemaker and accordingly the activation of

the adrenal clock gene (*Per1*) and corticosterone expression by light exposure exhibits circadian phase dependency (Ishida et al., 2005). A more recent study revealed a novel pathway for the photic regulation of the peripheral adrenal clock via the retinohypothalamic tract during the subjective day (Kiessling et al., 2014). Despite its pivotal role in regulating corticosterone rhythmicity, light may also act as a stressor. In Golden spiny mice (*Acomys russatus*) light interference at night caused an increase in heat shock protein HSP70 (Ashkenazi and Haim, 2012). This is congruent with previous studies demonstrating increases in stress related hormones in animals not only by ALAN, but also the light quality during the photophase (Ishida et al., 2005; Zubidat et al., 2007; Zubidat et al., 2010a; Zubidat et al., 2010b).

The results of the present study highlight the importance of the photophase wavelength in the regulation of glucocorticoid expression in both study species. However, our results do not reflect the difference between standard broad spectrum laboratory lighting and the monochrome wavelengths used in our setup but demonstrate the general fluctuation in uGCM concentration in both species, that differ under different wavelengths of monochrome light and also result in different profiles for the nocturnal and diurnal species. *M. namaquensis* exhibited clear rhythmicity in uGCM concentration under all three WLCs with mean daily uGCM levels being significantly and inversely related to the wavelength of the photophase lighting. With the exception of the daytime peaks observed during SWLC and MWLC, the rhythms generally resemble those of other nocturnal rodents, which peak approximately around the onset of the dark phase (Atkinson and Waddell, 1997). In mice, peak concentrations of glucocorticoid metabolites are visible in the urine within 2 hours following intraperitoneal corticosterone injection (Touma et al., 2002). The daytime peaks observed under the SWLC and MWLC in *M. namaquensis* therefore suggests the activation of a stress response slightly earlier than represented by our results. Likewise, the dramatic peak at the onset of the dark phase is probably reflective of an hour or two earlier.

The overall daily uGCM levels of *R. pumilio* were also inversely correlated to the photophase wavelength. Blind mole-rats (*S. ehrenbergi*) exhibit a similar relationship between the photophase wavelength and stress response, showing elevated levels of stress hormones under short wavelength light, whereas the nocturnal *M. socialis* showed the opposite response with long wavelength photophase light eliciting higher stress hormone levels (Zubidat et al., 2010b). Furthermore, in *R. pumilio*, a distinct 24h rhythm in uGCM concentrations, with a bimodal appearance, was only observed under the SWLC. A similar bimodal daily corticosterone rhythm was previously observed in another diurnal rodent species, the Sudanian grass rat (*Arvicanthis ansorgei*) (Verhagen et al., 2004).

Both the nocturnal and diurnal species display rhythmicity of uGCM concentrations, however the diurnal species in particular, some variability is obvious. These measures could therefore be in the normal range for the species, and the dampened rhythms under the longer wavelengths may result from a lower response to these wavelengths in the SCN.

Locomotor activity rhythms of these two species under the same experimental wavelength conditions appear to be more robust than the physiological rhythms in both species (Van der Merwe et al., 2019). Rhythms under short and medium wavelength light have a similar profile compared to activity rhythms under broad spectrum light, whereas long wavelength light rendered higher activity levels during the day in both species (Schumann et al., 2005; Van der Merwe et al., 2014, 2019). The physiological rhythms assessed in our study seems to require short light wavelengths to efficiently maintain rhythms.

5. CONCLUSION

Our results indicate that exposure to photophase wavelength has a significant effect on the daily physiological rhythms in both the diurnal *R. pumilio* and the nocturnal *M. namaquensis*. Both of

the species we investigated are most sensitive to the short wavelength light and are relatively insensitive to long wavelength light. The increased sensitivity to short wavelength lighting that was observed indicates the involvement of the ipRGCs in mediating physiological rhythms in both species. Both classic photoreceptors (rods and cones) and ipRGC's contribute to circadian responses, and therefore species differences in the response to light of different wavelengths might be attributed to variation in these photoreceptor proportions. It should also be noted that light is not the only factor that can influence rhythmicity, several non-photic cues such as feeding schedule, locomotor activity and ambient temperature can reset peripheral clocks independent of the central pacemaker (Astiz et al., 2019). However, these cues are usually integrally linked to light and both photic and non-photic cues are used, and reinforce each other, in the establishment of entrainment. Indeed, the innate activity habits of the species may increase or decrease exposure to photophase light, and invertedly change physiological responses thereto. The possibility of masking can also not be excluded, the nocturnal *M. namaquensis* is known to show a strong masking response to light, and its locomotor activity increased during the photophase LWLC (Van der Merwe et al., 2014, 2019). The behaviour and physiological attributes displayed by an animal likely results from a combination of the above. In natural conditions, the quality of the light changes both in terms of intensity and wavelength. It appears that both these factors affect and are vital for the entrainment of rhythms in animals.

LIST OF ABBREVIATIONS

6-SMT	6-sulfatoxymelatonin
ipRGC	intrinsically photosensitive retinal ganglion cell
LWLC	long wavelength light cycle
MWLC	medium wavelength light cycle

SCN	suprachiasmatic nucleus
SWLC	short wavelength light cycle
uGCM	urinary glucocorticoid metabolite
WLC	Wavelength light cycle

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AUTHOR CONTRIBUTIONS

Conception and design - IvdM, NCB, AH, MKO

Data collection, analysis and/or interpretation - IvdM, AG, MKO

Drafting the article - IvdM

Revision of the article - NCB, AH, AG, MKO

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