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Early chronotype with advanced activity rhythms and dim light melatonin onset in a rural population

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Abstract

Studying communities at different stages of urbanisation and industrialisation can teach us how timing and intensity of light affect the circadian clock under real-life conditions. We have previously described a strong tendency towards morningness in the Baependi Heart Study, located in a small rural town in Brazil. Here, we tested the hypothesis that this morningness tendency is associated with early circadian phase based on objective measurements (as determined by dim light melatonin onset, DLMO, and activity) and light exposure. We also analysed how well the previously collected chronotype questionnaire data were able to predict these DLMO values. The average DLMO observed in 73 participants (40 female) was $20:03 \pm 01:21$, SD, with an earlier average onset in men ($19:38 \pm 01:16$) than in women ($20:24 \pm 01:21$; $P \le .01$). However, men presented larger phase angle between DLMO and sleep onset time as measured by actigraphy (4.11 hours vs 3.16 hours; $P \le .01$). Correlational analysis indicated associations between light exposure, activity rhythms and DLMO, such that early DLMO was observed in participants with higher exposure to light, higher activity and earlier light exposure. The strongest significant predictor of DLMO was morningness-eveningness questionnaire (MEQ) (beta=-0.35, $P \le .05$), followed by age (beta = -0.47, $P \le .01$). Sex, light exposure and variables derived from the Munich chronotype questionnaire were not significant predictors. Our observations demonstrate that both early sleep patterns and earlier circadian phase have been retained in this small rural town in spite of availability of electrification, in contrast to metropolitan postindustrial areas.

KEYWORDS

actigraphy, circadian rhythms, neuroendocrinology, phase angle, sleep-wake rhythm

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Ruiz and Beijamini contributed equally to this work.

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1 | INTRODUCTION

Circadian rhythms are observed as physical, mental and behavioural daily oscillations. The circadian pacemaker is differentially sensitive to the resetting effects of retinal light exposure depending upon the circadian phase at which the light exposure occurs. Light exposure during biological evening delays the phase of the human circadian pacemaker, whilst light exposure in the early hours of the morning advances it.[1,2]

Dim light melatonin onset (DLMO) is considered the most reliable measure of circadian phase in humans[3,4] and is determined as the point in time where melatonin levels rise above a set threshold, which typically occurs 2-3 hours before the habitual onset of nocturnal sleep.[5] DLMO assessment is typically performed in small numbers of individuals, and most studies to date have been performed in higher-income countries in laboratory settings. Obtaining this gold standard measure of circadian timing from a variety of populations can help us better understand variability in circadian timing, and ultimately the multitude of ways in which it relates to health.

Until recently, human activity was largely constrained by the natural photoperiod. The generally accepted view is that this was radically changed by industrialisation, which enabled both production and leisure during the hours of natural darkness under increasingly more affordable artificial light. Although the process was not studied in real time, there is a general consensus that this development has delayed bedtimes and circadian phase, although it is still controversial whether it has also resulted in a reduction of total sleep time.[6] The importance of studying communities at different stages of the path towards urbanisation and industrialisation is becoming increasingly recognised, with a number of key recent publications providing important data points.[6-12]

Studying a cohort in a rural town in south-eastern Brazil, the Baependi Heart Study Cohort, and comparing it to metropolitan populations, we have previously described a strong tendency towards early chronotype (morningness).[13] Baependi is a small rural town (population: 19 148 habitants[14]) in the state of Minas Gerais in Brazil (21.95° S, 44.88° W). Duration of daylight in Baependi ranges from 10:47 (sunrise 06:37 and sunset 17:25) to 13:27 hours (sunrise 06:18 and sunset 19:46 during daylight saving time, which has subsequently been abolished in Brazil but was still in operation during the study period). Baependi has followed a stable upward trajectory in Municipal Human Development Index—MHDI (a measure that assesses the same dimensions as the global HDI—health, education and income—on a scale between 0 and 1), which has increased from 0.430 to 0.681 between 1991 and 2010. The lack of knowledge about how the quantity of daylight, including mixed day/electric light conditions, impacts circadian entrainment and whether

this effect is dependent on the moment of the day has recently been identified as a key research priority[15]. In spite of universal access to electricity in Baependi, both diurnal preference/chronotype[13] and sleep timing[16] are still significantly earlier than in metropolitan areas. We hypothesised that these observations were associated with an earlier circadian phase and high exposure to natural light/dark cycle. Testing this hypothesis required the assessment of the circadian phase under habitual sleep/wake conditions (*i.e*. not sleeping in a laboratory). The main purpose of this study was to investigate circadian phase of healthy adults who were following their habitual sleep-wake schedule. We investigated the associations between DLMO, and chronotype, sleep/wake cycle, activity and light exposure, by objective means of actigraphy recording.

2 | METHODS

2.1 | Participants

We collected saliva samples from 76 participants (42 female; ages 19-60 years). The study protocol conformed to the tenets of the Declaration of Helsinki and was approved by the Ethics Committee of the Hospital das Clínicas, University of São Paulo, Brazil. All procedures were conducted with informed and written consent of the participants. The methodology for recruitment has been described previously.[17,18] Briefly, all extended family members aged above 18 years of probands who had initially been randomly selected from 11 out of 12 census districts in Baependi were initially invited to participate in the full study.

2.2 | Protocol

Sampling for DLMO was scheduled for Tuesdays, Wednesdays and Thursdays, between 26 August 2016 and 28 September 2016 (before the switch to daylight savings time). Participants were invited to participate through a telephone interview, and those who reported being prescribed beta-blockers or antidepressants as well as those reporting shift work or sleep disorders were excluded. All participants were asked to maintain their habitual sleep-wake pattern for a minimum of 7 days before the DLMO assessment and were instructed to wear an actigraph device for a fourteen-day period, starting from the day of the saliva collection. Saliva sampling took place in the research station, located in an easily accessible sector of the town, where all questionnaire-based assessments were also conducted. The sunrise times during the months of August and September ranged from 05:42 to 06:14h, while the sunset time ranged from 17:48 to 17:57 hours, resulting in a day length range of 11:34 to 12:14 hours.

2.3 | Measurements

2.3.1 | Chronotype questionnaires

The following questionnaires were administered to the participants between March and November 2016:

1. The morningness-eveningness questionnaire (MEQ),[19] consists of 19 items which measure a participant's preference for the timing of daily activities. Lower scores indicate evening preference and higher scores morning preference.
2. The Munich chronotype questionnaire (MCTQ)[20], which focuses primarily on sleep timing, has 14 questions that assess the regularity of the participant's sleep timing on workdays and work-free days. Chronotype is estimated as the midpoint of sleep on work-free days minus half of the difference between sleep duration on work-free days and average sleep duration of the week to control for sleep debt (midpoint of sleep on work-free days, sleep corrected, MSF_{sc}). The following measurements from the MCTQ were also analysed, SOw, sleep onset workdays; SOf, sleep onset work-free days; SEw, local time of getting out of bed workdays; SEf, local time of getting out of bed work-free days; SDw, sleep duration workdays; SD, sleep duration work-free days; MSW, mid-sleep phase during working days; and MSF, mid-sleep phase during free days.

2.3.2 | Dim light melatonin onset (DLMO) assessment

Participants were invited to the research station for one evening, arriving at 17:30 hours. The saliva sampling started 4.5 hours before the mean bedtime of the population, as described previously.[16] During the phase assessment, participants remained awake from 18:00 to 23:00 hours in dim light (<10 lux), as verified at the level of the participant's neck with an actigraphy monitor (ActTrust AT0503, Condor Instruments, São Paulo, Brazil). Participants were seated in comfortable chairs and had access to toilet facilities (also < 10 lux). They spent the sampling period watching a dimmed TV (<10 lux) and talking to each other and to supervising staff. A research assistant was always present to make sure that participants were not falling asleep. Participants provided a 2-mL saliva sample every 60 minutes (1800, 1900, 2000, 2100, 2200 and 2300h, respectively) directly into a centrifuge tube. Snacks and water were provided after each sampling, and participants rinsed their mouths with water 10 minutes before the collection of each sample. Samples were immediately frozen at −20°C after the collection and shipped on dry ice to the UK to be assayed for melatonin. All samples from an individual participant were assayed in the

same batch. DLMO was defined as the point in time (as determined with linear interpolation) when the melatonin concentration exceeded the threshold of 3 pg/mL. DLMO was used as the marker of circadian phase in each participant.[21] Three out of the 76 participants were excluded because their salivary melatonin levels did not exceed the threshold.

2.3.3 | Actigraphy protocol

Participants were instructed to wear an actimetry device that contained an accelerometer and also light and temperature sensors (ActTrust AT0503) on their nondominant wrist on the outside of any long-sleeved clothing for a fourteen-day period. The proportional integration mode (PIM) algorithm was used to derive a measure of user activity from acceleration readings. The PIM data with epochs of 60 s were integrated every hour to generate 24 epochs of 3600 s each day. Participants were instructed to use the event button to report any time they removed the actigraph and the times they went to bed and woke up. All data files were visually screened for sufficient wear time and then processed for analysis. A day was considered valid if it was recorded with no more than 1 hour of nonvalid signal (ie nonwear periods). Out of the total of 76 participants with DLMO, 67 presented valid actigraphy recordings, with full 14-day records available from a majority of participants (n = 53). For eight participants, 10 to 13 days of valid recordings were available, for a further five, six days, and for one, five days.

2.3.4 | Sleep variables

For each day, we manually identified a main rest interval as the primary sleep period in agreement with standard procedures in sleep actigraphy.[22,23] All valid days of recording were averaged to produce the variables related to sleep. The actigraph software, ActStudio, provided an estimation of the sleep duration (SD), sleep onset time (SO) and sleep offset time (SE) based on a modified version of the Cole-Kripke algorithm (Condor Instruments).

2.3.5 | Nonparametric variables

We applied a nonparametric approach to describe the 24-hour rest-activity profile of the participants, as daily rest-activity pattern did not perfectly follow the sinusoidal waveform assumed by the cosinor parametric method. We selected 5 consecutive valid days, starting at midnight of the first day of recording, in order to have a consistent number of days across all participants. The following nonparametric variables were derived from the data: M10 (a measure of total activity in

most active 10-hour period, indicating diurnal motor activity), L5 (a measure of total motor activity in the least active 5-hour period, indicating nocturnal motor activity), IV (intradaily variability), IS (interdaily stability), F5 (beginning of L5, representing a measure of the beginning of rest phase), F10 (beginning of M10, representing a measure of the beginning of active phase), RA (relative amplitude), M16 (a measure of total light exposure in the 16-hour period with maximum illumination, indicating diurnal light exposure) and M3i (total light exposure within 3 hours after sunrise).[24] Phase angle was calculated as the difference between DLMO and sleep onset, or the difference between DLMO and F5, or difference between DLMO and F10.

2.4 | Data analysis

Data are presented as mean ± SD unless otherwise specified. Normal distribution was confirmed by the Kolmogorov-Smirnov test for all variables of interest. Data were log10 transformed prior to statistical analyses in the case of non-normal distribution (M3i and SEw, L5, IV and IS). A general linear model (GLM) was used for comparisons of DLMO, MEQ, MCTQ and actigraphy parameters between sexes, controlling by age. The partial eta-squared (η^2_p) was used as estimates of effect size. For all participants, initial Pearson's correlations were calculated to determine associations between DLMO, chronotype (MEQ and mid-sleep),

sleep parameters, light exposure (M3i) and activity (M10). As a second step, a multiple linear regression analysis was performed with MEQ, MSw, MSf, SOw, SOf, M10, M3i, age and sex together to assess the accuracy of these variables in predicting DLMO, using the enter method. The Durbin-Watson statistic was applied to test for autocorrelation in the residuals. A P-value of ≤ 0.05 was considered significant. Statistical analysis was performed using SPSS (version 25) (IBM, Armonk, NY), and graphs were obtained with RStudio (version 1.1.463) using the ggplot2 package.[25]

3 | RESULTS

Demographic information (including official urban/rural residential zone classification),[13,26] circadian parameters and results derived from the chronotype questionnaires are provided in Table 1. The 73 participants (aged from 19 to 60 years) included 33 men (aged 38 ± 10) and 40 women (aged 36 ± 12).

3.1 | Dim light melatonin onset profiles, chronotype and questionnaire-derived sleep characteristics

Mean DLMO time (h ± min) was 20:03 ± 01:21 (Table 1). The GLM analysis adjusted by age revealed a sex effect

TABLE 1 Demographics, DLMO and questionnaire-derived sleep parameters

Demographics	Total (n = 73)	Female (n = 40)	Male (n = 33)
Age, years	36.4 ± 10.9	35.5 ± 10.9	37.6 ± 10.1
Body mass index	24.7 ± 5.8	24.4 ± 7.1	25.1 ± 4.0
Residential zone (Urban/ Rural)	63/10	37/3	26/7
Employed, n (%)	59 (80.8%)	32 (80.0%)	27 (81.8%)
DLMO (hh:min)	20:03 ± 01:21	20:24 ± 01:21	19:38 ± 01:16**
MEQ Score (n = 56; 25 Male)	59.1 ± 9.2	57.9 ± 9.6	60.6 ± 8.7
MCTQ (n = 64; 29 Male)			
SOw (hh:min)	23:15 ± 01:20	23:29 ± 01:12	23:00 ± 01:26
SOf (hh:min)	23:40 ± 01:15	23:53 ± 01:17	23:24 ± 01:11
SEw (hh:min)	06:13 ± 00:47	06:21 ± 00:46	06:03 ± 00:48
SEf (hh:min)	07:43 ± 01:31	07:53 ± 01:33	7:32 ± 01:28
SDw (hh:min)	06:57 ± 01:15	06:52 ± 01:17	07:03 ± 01:14
SDf (hh:min)	08:03 ± 01:29	08:00 ± 01:44	8:07 ± 01:09
MSFsc (hh:min)	03:16 ± 01:05	03:25 ± 01:07	03:05 ± 01:02

Note: Data are presented as mean ± SD. GLM p-values: **$P < .01$.

Abbreviations: MEQ, morningness-eveningness questionnaire score; MCTQ, Munich chronotype questionnaire score; SOw, sleep onset workdays; SOf, sleep onset work-free days; SEw, sleep offset workdays; SEf, sleep offset work-free days; SDw, sleep duration workdays; SDf, sleep duration work-free days; MSFsc, mid-sleep on free days corrected for sleep debts on workdays.

$[F(1,72)=6.82; P = .01;$ observed power $= 0.73]$. The effect size, as indexed by partial eta-squared (η^2_p), was 0.10. DLMO time was significantly earlier in the male participants ($19:38 \pm 01:16$ vs $20:24 \pm 01:21$, $P = .01$); 95%CI [19:00, 20:04] vs [20:00, 20:49]. To explore our hypothesis that the relatively greater morningness in the Baependi population is associated with a correspondingly early circadian phase, we present in Figure 1 a comparison between DLMO time from our data set and previously published studies performed in nonclinical populations of similar age and an unrestricted sleep schedule.

Two different assessments for chronotype were considered for this analysis, MEQ score and the phase of entrainment (the time of MSFsc) derived from the MCTQ. No significant sex difference was observed for MEQ score or MSFsc (Table 1). Furthermore, sleep timing assessed by the MCTQ was similar between females and males.

The GLM repeated measure analysis adjusted by age revealed differences in sleep end $[F(1,63) = 16.3; P = .01;$ observed power $= 0.98, \eta^2_p = 0.22]$ and sleep duration $[F(1,63) = 14.2; P = .01;$ observed power $= 0.95, \eta^2_p = 0.20]$. In general, participants reported waking up earlier on work days ($06:13 \pm 00:47$ vs $7:47 \pm 01:31$, $P = .01$) compared to work-free days; 95%CI [6:07, 7:36] vs [8:01, 10:38]. In addition, participants slept longer on work-free days ($8:06 \pm 1:29$) compared to work days ($6:57 \pm 1:15$); 95%CI [8:15, 10:49] vs [6:04, 8:18] The relationship between DLMO time and MCTQ sleep onset and offset variables is presented in Figure 2A.

To test for associations between DLMO and chronotype and sleep timing, correlational analysis was performed. We found that DLMO significantly correlated with MEQ ($r = -0.30$, $P = .02$), the higher the MEQ scores, the earlier the DLMO, as shown in Figure 3A. MSW and MSF also correlated with DLMO ($r = 0.25$, $P = .04$ and $r = 0.27$, $P = .03$), but not MSFsc ($r = 0.25$, $P = .13$), the later the DLMO, the later the mid-sleep phase, as shown in Figure 3B-D. Similar results were found for SOf, where later DLMO time was associated with later sleep onset on work-free days ($r = 0.27$, $P = .03$), as shown in Figure 3E-F.

3.2 | Actigraphy-derived variables and Dim Light Melatonin Onset

Table 2 summarises the actigraphy-derived variables. Sex differences were not observed for sleep onset time, measured through actigraphy. Females presented longer total sleep time in comparison with males ($7:11 \pm 0:44$ vs $6:39 \pm 0:57$; 95%CI [6:53, 7:27] vs [6:22, 6:59] $[F(1,66) = 5.48$; $P = .02.$; observed power $= 0.64, \eta^2_p = 0.08]$. Figure 2B-D shows circular histograms to illustrate the relationship between DLMO and sleep onset and offset obtained by actigraphy. GLM analysis revealed a sex effect on sleep onset phase angle (ie the timing of sleep onset relative to DLMO) $[F(1,66)=7.26; P = .01;$ observed power $= 0.75, \eta^2_p = 0.10]$. The phase angle to sleep onset observed with actigraphy was larger in men than in women ($4:11 \pm 1:31$ vs $3:16 \pm 1:53$,
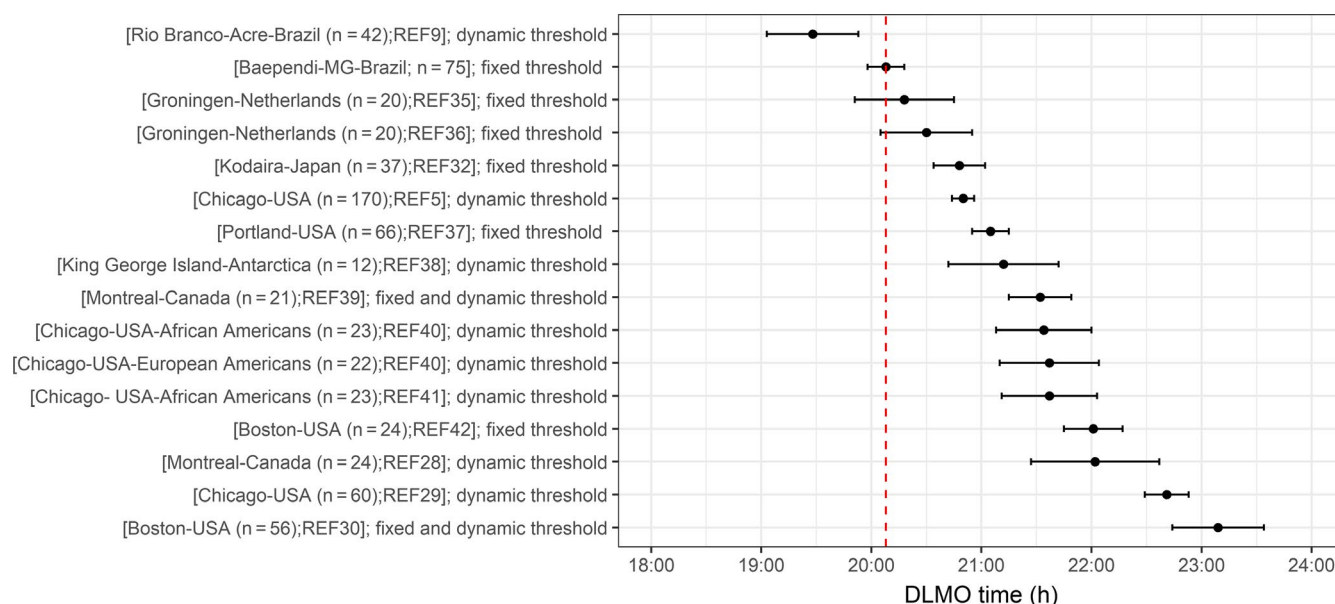

FIGURE 1 DLMO for the Baependi population (mean is marked with red dotted line) compared with published DLMO data from other adult populations.[5]9,28-30,32,35-42 Data are presented as mean ± SEM. Different methodologies for determining DLMO are shown as follows: dynamic threshold refers to defining DLMO based on two standard deviations above the mean of three or more prerise values or from parameters describing a fitted curve to the time series of melatonin samples. Fixed threshold refers to analysing DLMO based on a time of attaining a 1 or 3 pg/mL level for saliva or a comparable 10 pg/mL for plasma. The threshold used in the present study was 3 pg/mL
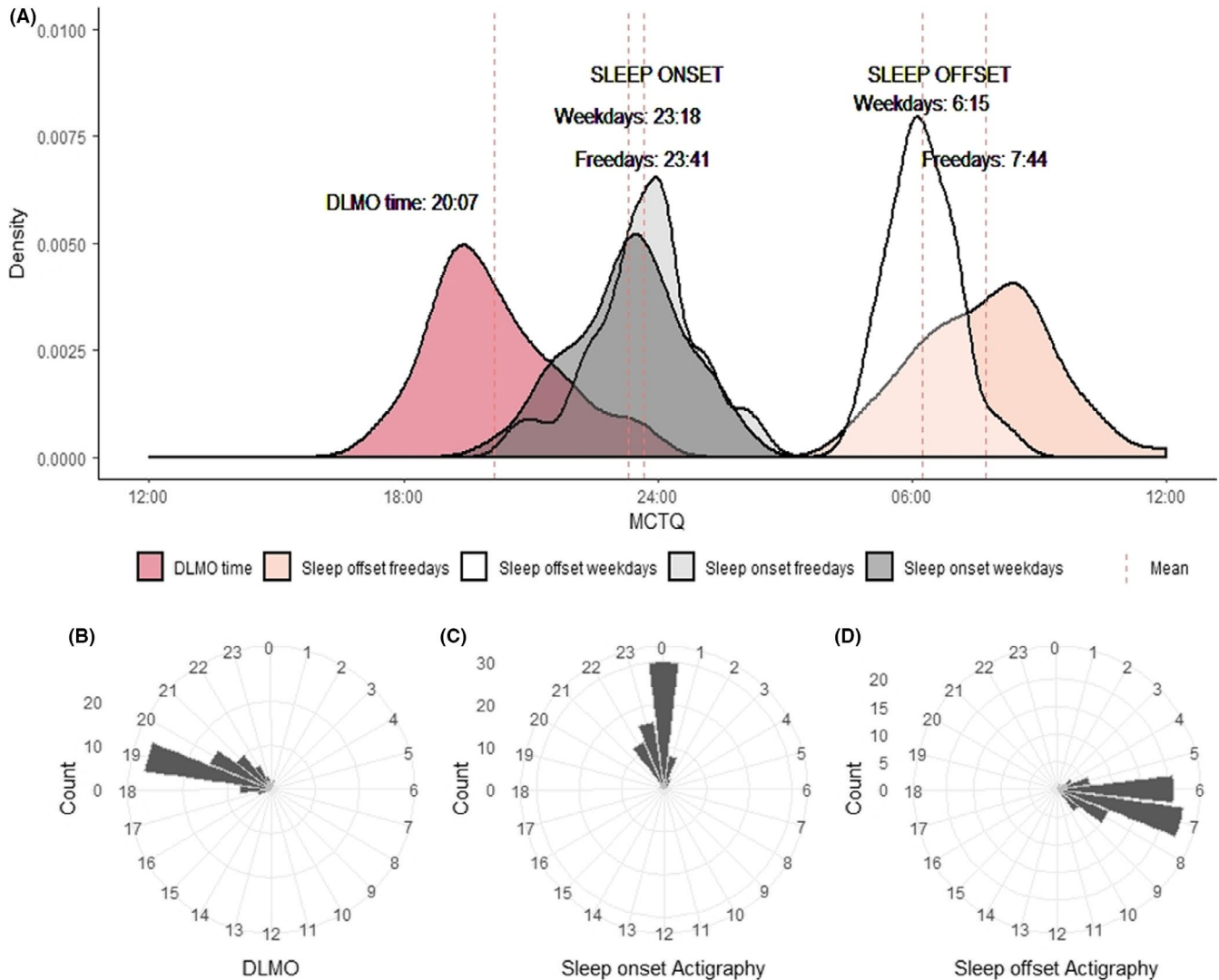
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FIGURE 2 Relationship between DLMO time and sleep schedules obtained from MCTQ and actigraphy. (A) Density plots representing DLMO time compared with reported sleep habits at work days and work-free days from MCTQ, (B) daily hour circular plots representing DLMO time, (C) sleep onset from actigraphy and (D) sleep offset from actigraphy. Bar heights are proportional to the number of individuals with DLMO, sleep onset and offset time from actigraphy within each hour

$P = .009$; 95%CI [3:42, 4:52] vs [2:37, 3:44]). Figure 4 depicts the individual differences in DLMO and phase relation with nighttime sleep. Figure 5A presents the mean profile of the activity pattern comparing both sexes. The GLM analysis revealed a sex effect on diurnal motor activity (M10) [$F(1,62) = 21.55$; $P = .001$; observed power = 0.99, $\eta^2_p = 0.26$]. Figure 5B represents the mean profile of light exposure. GLM analysis also revealed a sex effect on diurnal light exposure (M16lu) [$F(1,57)=11.13$; $P = .002$; observed power = 0.90, $\eta^2_p = 0.17$] and on light exposure within the first 3 hours after sunrise (M3i) [$F(1,61)=10.95$; $P = .002$; observed power = 0.90, $\eta^2_p = 0.16$]. In summary, male participants were significantly more active ($4.7 \times 10^6 \pm 1.3 \times 10^6$ vs $3.3 \times 10^6 \pm 0.9 \times 10^6$, $P = .001$; 95%CI[4.2×10^6, 5.1×10^6] vs [2.9×10^6, 3.7×10^6]) and were exposed to higher amounts of light (3808 vs 3195, $P = .002$; 95%CI

[3545, 4066] vs [2941, 3453]), mainly during the first 3 hours after sunrise (877 vs 359, $P = .002$; 95%CI [6471, 1085] vs [163, 574]). Correlational analysis indicated association between DLMO and higher light exposure ($r = -0.25$, $P = .04$) and activity ($r = -0.33$, $P = .008$; Figure 3G-H). Furthermore, we also observed associations between DLMO and the rest onset phase angle (ie the timing of the beginning of rest phase relative to DLMO; $r = -0.74$, $P = .01$), and the active onset phase angle (ie the timing of the beginning of the active phase relative to DLMO; $r = 0.61$, $P = .01$).

Light exposure and activity patterns in the population were also associated with earlier sleep. We found that light exposure within the first 3 hours after sunrise (M3i) correlated with objective sleep onset ($r = -0.53$; $P = .001$), MSF ($r = -0.39$; $P = .01$) and MSFsc ($r = -0.36$; $P = .01$), and RA ($r = 0.52$; $P = .01$) as shown in Figure S1A-D. Similarly,

FIGURE 3 Associations between DLMO and (A) morningness-eveningness questionnaire score (MEQ), (B) mid-sleep on free days corrected for sleep debts on workdays (MSFsc), (C) mid-sleep on workdays (MSW), (D) mid-sleep on work-free days (MSF), (E) sleep onset weekdays (SOw), (F) sleep onset on work-free days (SOf), (G) total light exposure within 3 h after sunrise (M3i) and (H) diurnal motor activity (M10). The blue line represents the linear association between each pair of variables. Pearson's correlation coefficients and p-values for each analysis are shown in the corresponding graph
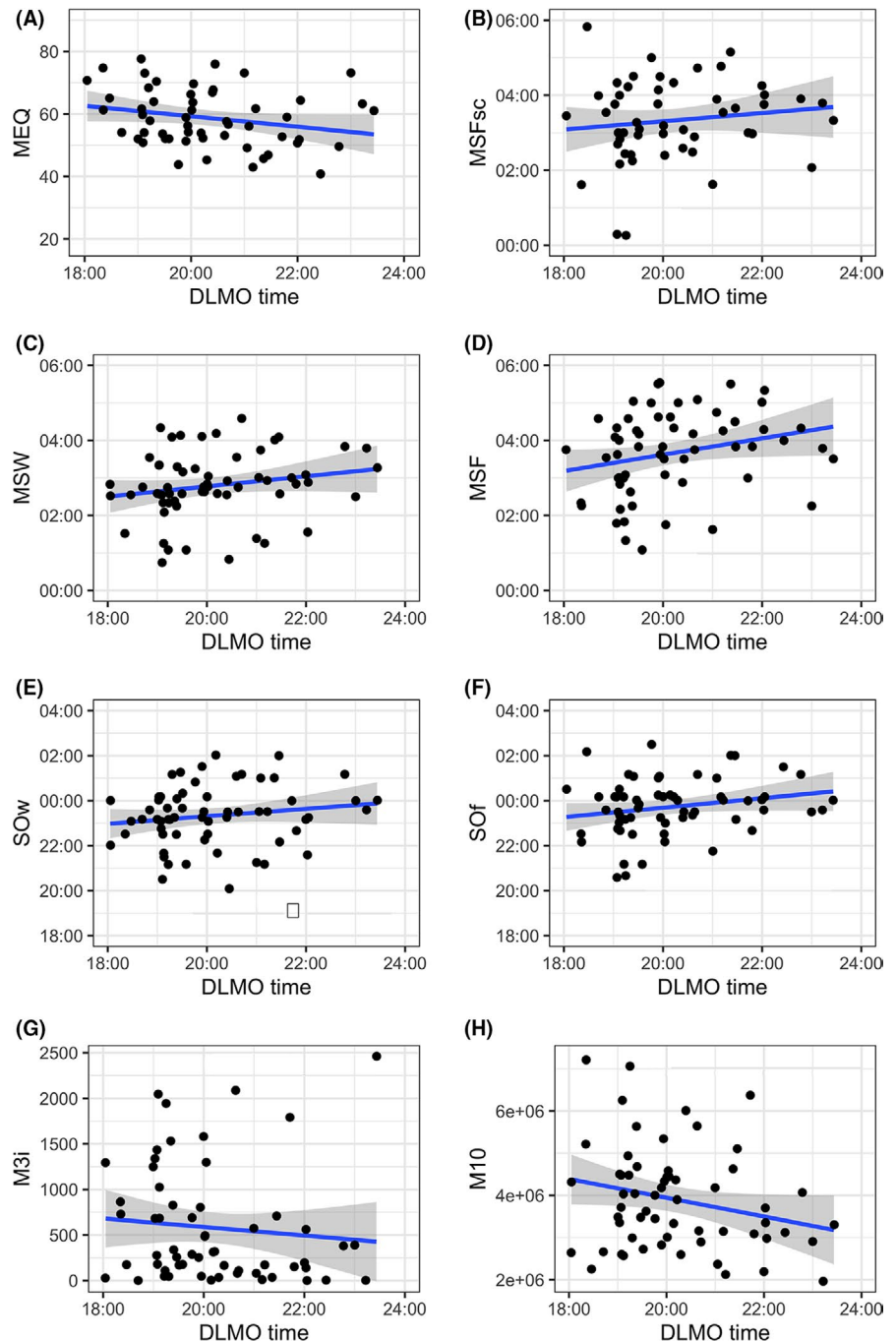
activity during the most active ten-hour period (M10) was correlated with objective sleep onset ($r = -0.31$; $P = .003$), MSF ($r = -0.47$; $P = .001$) and MSFsc ($r = -0.48$; $P = .001$), and RA ($r = -0.41$; $P = .01$), as shown in Figure S1E-H.

As DLMO was correlated with light exposure, chronotype and sleep timing, we applied a regression analysis to assess which of these variables would better predict the DLMO timing. The model using MEQ, MSF, MSW, SOw, SOf, M10 and light exposure during the 3 hours after sunrise (M3i), age and sex to predict DLMO explained 44% of the variance in DLMO ($P = .02$). The strongest significant predictor of DLMO was MEQ (beta $= -0.63$, $P = .004$), followed by age

(beta $= 0.52$, $P = .01$); Durbin-Watson $= 1.69$. Sex, light exposure, sleep onset and mid-sleep were not significant predictors.

4 | DISCUSSION

We show that the tendency towards morningness in Baependi cohort is not only behavioural, but has physiological correlates. Notably, to the best of our knowledge, this is the largest study accessing the circadian phase under habitual sleep/wake schedules using the gold standard

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	Total (n = 67)	Female (n = 35)	Male (n = 32)
Sleep onset time (hh:min)	23:46 ± 01:09	23:42 ± 01:12	23:50 ± 01:05
Sleep offset time (hh:min)	06:56 ± 01:20	7:10 ± 01:22	6:41 ± 01:17
Sleep duration (hh:min)	06:56 ± 00:53	7:11 ± 00:44	6:39 ± 00:57*
Diurnal motor activity	$3.9 \times 10^6 \pm 1.2 \times 10^6$	$3.3 \times 10^6 \pm 0.9 \times 10^6$	$4.7 \times 10^6 \pm 1.3 \times 10^6$ **
Nocturnal motor activity	$3.7 \times 10^4 \pm 2.0 \times 10^4$	$3.5 \times 10^4 \pm 1.9 \times 10^4$	$4.1 \times 10^4 \pm 2.1 \times 10^4$
Intradaily variability	0.93 ± 0.30	0.97 ± 0.17	0.90 ± 0.40
Interdaily stability	0.60 ± 0.49	0.61 ± 0.49	0.60 ± 0.49
Relative amplitude	0.9764 ± 0.0154	0.9770 ± 0.01573	0.9759 ± 0.01528
Beginning of rest phase	24:47 ± 01:13	24:53 ± 01:24	24:40 ± 00:59
Beginning of active phase	08:39 ± 01:55	08:57 ± 02:00	08:19 ± 01:49
Diurnal light exposure	349.2 ± 747.1	3195.1 ± 602.9	3808.1 ± 763.1**
Total light exposure within 3 h after sunrise	601.6 ± 654.2	359.1 ± 520.1	877.5 ± 689.2**
Phase angle between DLMO and sleep onset	3:42 ± 1:46	3:16 ± 1:53	4:11 ± 1:31**
Phase angle between DLMO and beginning of rest phase	4:43 ± 1:49	3:25 ± 2:00	5:02 ± 1:33
Phase angle between DLMO and beginning of active phase	11:24 ± 2:26	11:29 ± 2:32	11:18 ± 2:21

TABLE 2 Activity, light and sleep characteristics derived from actigraphy

Note: Data are presented as mean ± SD; GLM: **$P < .01$.

measure, DLMO, and describing its association with sleep and nonparametric derived variables from actigraphy during a short and defined period (the one larger sample presented in Figure 1 represents pooled data from multiple studies performed over a number of years in the same location).[5] Based on methodological differences and on the large inter-individual differences in melatonin production,[5] the comparison between DLMO time across different studies requires some caution, and the figure has been annotated with information about the different methods used in each study. Our observations confirm the hypothesis that morningness in Baependi is associated with an early phase in objective measurements (DLMO, actigraphy) and with early light exposure. The only previously reported population-based DLMO earlier than the one observed here

was observed in rubber tappers living in the Amazonian rainforest.[9]

Additionally, we present objective data describing the sleep characteristics (timing and duration), light exposure and activity patterns of the same subset of participants from the Baependi Heart Study. Together, the DLMO evaluation and sleep description by actigraphy are in agreement with our previous findings described based on self-reports of both morningness-eveningness[13] and sleep timing.[16] Located 1.5° North of the Tropic of Capricorn, the Baependi population is extensively exposed to the natural light/dark cycle, with high exposure to morning sunlight, as we will discuss below.

The role of light as *zeitgeber* is very well established, and humans naturally synchronise to sun time[27]. We have demonstrated clear associations between DLMO and light
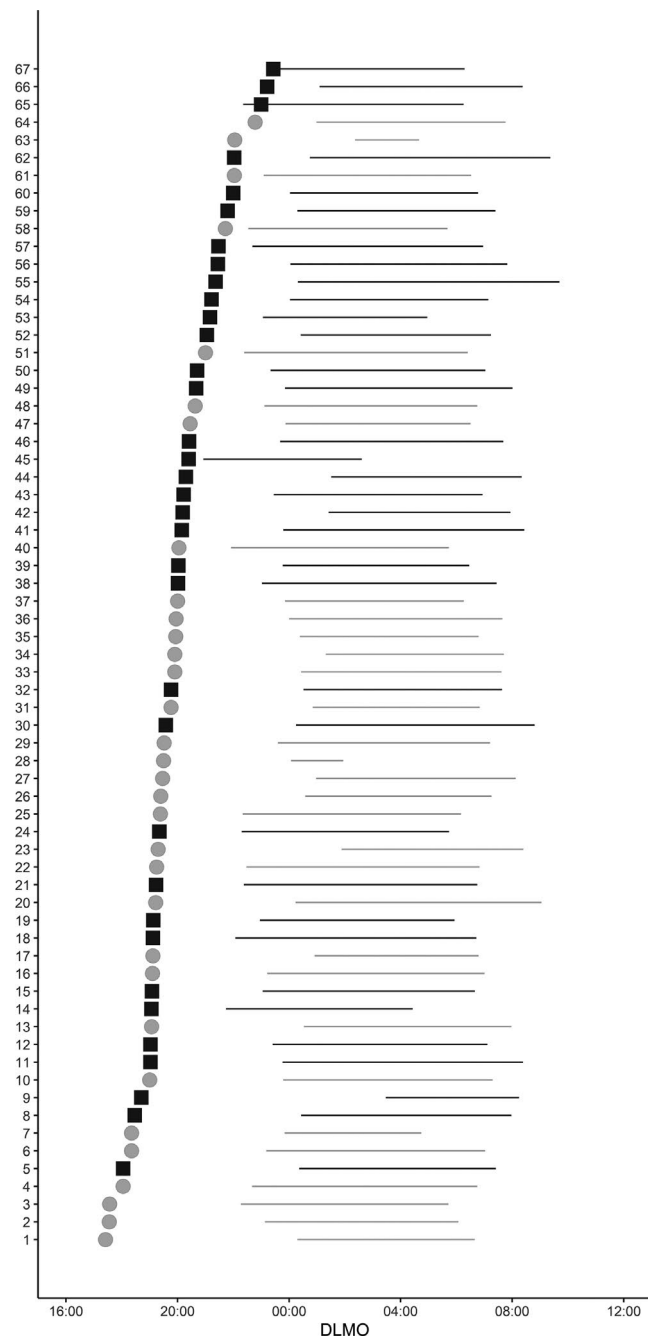
FIGURE 4 Individual differences in DLMO and its relationship to habitual nighttime sleep. DLMO time is represented by filled squares for women (black) and filled circles for men (grey). To represent the habitual sleep schedule, the average of sleep episodes obtained using actigraphy plotted using horizontal bars. Bar heights are proportional to the number of individuals with DLMO, sleep onset and offset time from actigraphy within each hour. Data are ordered from earliest to latest DLMO. Four participants were excluded from the figure due to missing actigraphy data

exposure pattern (the diurnal light exposure—M16lu) and higher exposure to light in the early morning (M3i). This is consistent with circadian phase, and thus DLMO, being entrained to the solar light-dark cycle.[1,2] The nonparametric analysis of actigraphy allows us to infer properties of

circadian timing system. According to Gonçalves and colleagues,[24] the most active 10-hour period and the least active 5-hour period represent key behavioural outputs of the circadian timing, and indices of the synchronisation of the circadian timing system to the external world. We found robust correlation between M10 and DLMO, as well as between M10 and sleep onset. The evidence of association between the nonparametric variables and DLMO substantially supports the Gonçalves model.[24]

We observed earlier average DLMO times in men than in women. This was concomitant with earlier activity and higher morning light exposure. Thus, our study does not provide any evidence suggesting that this difference is biological as opposed to behaviour-dependent. Indeed, our findings contrast to the published literature, which either reports no sex difference in DLMO or earlier timings in women.[5,28-31]

DLMO is an extremely precise measure of circadian phase. Although we were able to collect it from a relatively large sample of the population, collecting DLMO data is resource intensive and not feasible in many studies. We therefore sought to investigate how well the questionnaire data from the same population were able to predict our measured DLMO values. Only two studies published to date have compared MEQ, MCTQ and DLMO in the same individuals. Kitamura and colleagues[32] assessed DLMO in 37 middle-aged adults (both sexes) from hourly saliva samples that the participants individually collected unsupervised in their homes. The results indicated a stronger association between MSFsc and DLMO than MEQ and DLMO, suggesting that MSFsc better reflects endogenous circadian timing compared with the MEQ score. Another study conducted by Kantermann and colleagues[33] assessed DLMO in more controlled sampling conditions, but included not only normal controls (n = 36) but also individuals diagnosed with delayed sleep phase disorder (DSPD) reported similar results. MEQ is a scale that is based on questions around preferred timings of sleep and waking activities, whereas MCTQ asks about actual timings during weeks and weekends. As DLMO was assessed during workdays, the phase of entrainment reported by the MSF$_{sc}$ was not associated with the biological marker of phase (DLMO). Our results highlight that diurnal preference (MEQ) better reflects endogenous circadian timing than MCTQ in the conditions reported here. A possible explanation is that the chronotype measured by MCTQ will be more likely to change depending on social cues (work or entrainment).[34] It has been proposed that industrialisation has caused a chronic phase delay of rest-activity rhythms.[8,10-12] A phase difference in sleep timing has been described when groups of people were compared who lived in very similar conditions apart from the presence or absence of electricity.[6,8,9] The only population study showing an earlier average DLMO than what we report here was performed in participants living
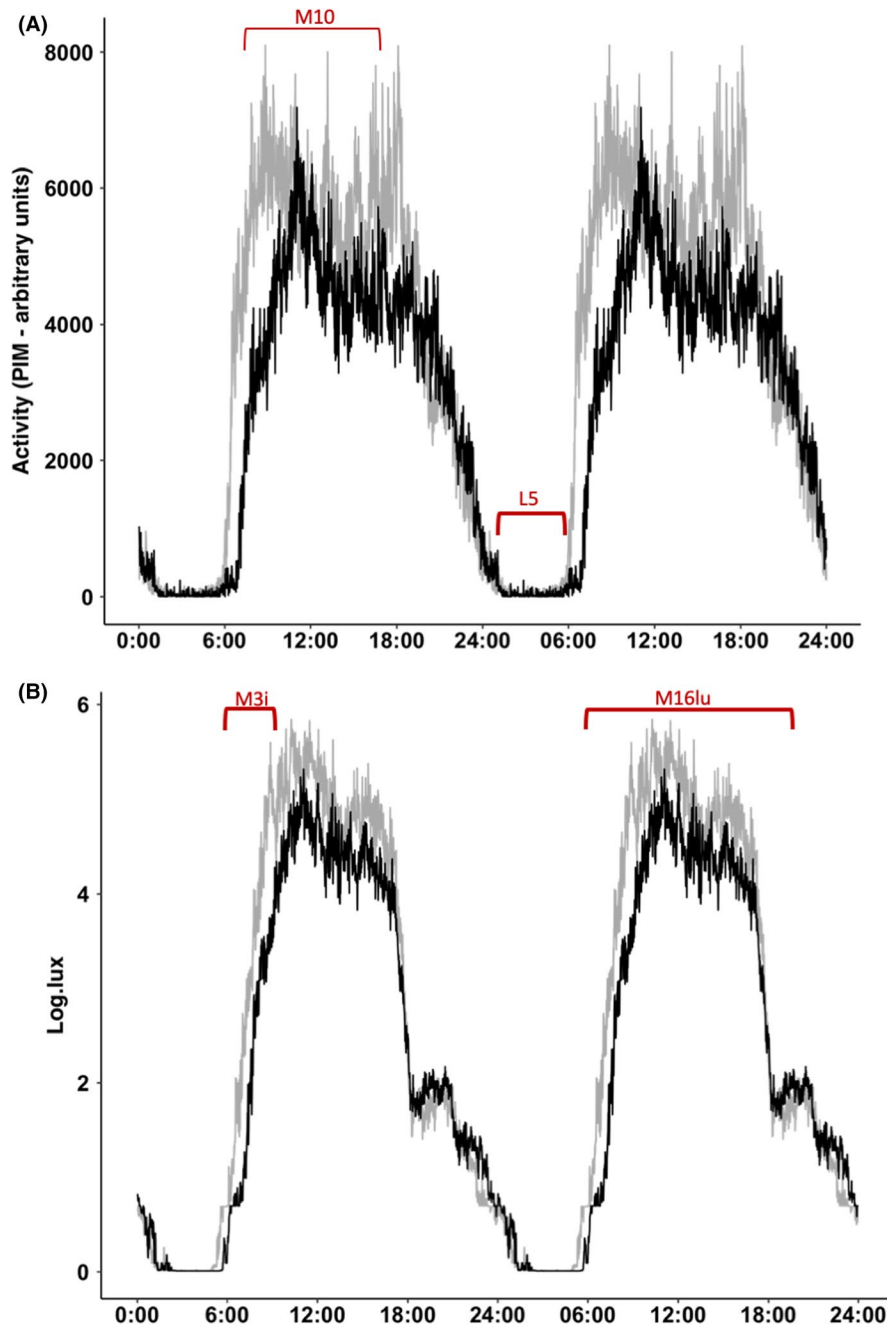
FIGURE 5 Double plotted 24-h average activity (arbitrary units) and light exposure (lux) measured in 60-s epochs using actigraphy devices. Means of each epoch for 5 d of recordings are presented. Men (grey) began their activity earlier than women (black) and displayed higher amount of activity during the most active ten-hour period (M10) (A). Light exposure during the first three hours after sunrise was higher for men in comparison with women, and total light exposure during consecutive 16 h (M16lu) also was higher for men in comparison with women (B)

without access to electricity (see Figure 1).[9] Although fully electrified, Baependi is a small rural town whose inhabitants experience a high degree of exposure to the daylight. In summary, we observed an early circadian phase in Baependi, which is consistent with multiple previous behavioural observations of sleep-wake timing and chronotype.

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CONFLICT OF INTEREST

The authors declare no competing financial interests.

AUTHOR CONTRIBUTIONS

FB, JEK, HV, JA, ACP, KLK, MP and MvS designed the study. FR, FB, TPT, BM, JA, ACP, KLK, MP and MvS developed methods and collected data. FR, FB, ADB, BSBG, DV, TPT, BM, JEK, HV, JA, ACP, KLK, MP and MvS analysed and interpreted the data. FR, FB and MvS prepared the manuscript. All authors approved the final version of the manuscript.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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