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Melatonin alleviates circadian system disruption induced by chronic shifts of the light-dark cycle in *Octodon degus*

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Abstract

Modern 24-h society lifestyle is associated with experiencing frequent shifts in the lighting conditions which can negatively impact human health. Here, we use the degus, a species exhibiting diurnal and nocturnal chronotypes, to: (a) assess the impact of chronic shifts of the light:dark (LD) cycle in the animal's physiology and behaviour and (b) test the therapeutic potential of melatonin in enhancing rhythmicity under these conditions. Degus were subjected to a "5d + 2d" LD-shifting schedule for 19 weeks. This protocol aims to mimic lighting conditions experienced by humans during shift work: LD cycle was weekly delayed by 8h during 5 "working" days (Morning, Afternoon and Night schedule); during weekends (2 days), animals were kept under Morning schedule. After 9 weeks, melatonin was provided daily for 6h in the drinking water. The "5d + 2d" shifting LD schedule led to a disruption in wheel-running activity (WRA) and body temperature (Tb) rhythms which manifested up to three separate periods in the circadian range. This chronodisruption was more evident in nocturnal than in diurnal degus, particularly during the Afternoon schedule when a phase misalignment between WRA and Tb rhythms appeared. Melatonin treatment and, to a lesser extent, water restriction enhanced the 24-h component, suggesting a potential role in ameliorating the disruptive effects of shift work.

KEYWORDS

body temperature rhythm, chronotype, circadian disruption, degu, diurnal, melatonin, shift work

1 | INTRODUCTION

The endogenous circadian system generates near 24-h rhythms in physiology and behaviour, allowing organisms to anticipate daily changes in their environment. In mammals, the master circadian pacemaker is located in the hypothalamic suprachiasmatic nucleus (SCN).[1,2] The SCN activity is reset daily by environmental synchronizers, with the light:dark (LD) cycle being the most potent.[3,4] Optimal alignment between the animals' physiology and behaviour and environmental time cues is a necessary condition for good health and well-being.[5]

Following an abrupt phase shift in the LD cycle, there is a transitory period of internal desynchrony within the SCN[6,7] and between the SCN and extra-SCN oscillators.[8] This is due to the different rates of resynchronization of the different brain and peripheral regions constituting the extended circadian system. This results in a transient disruption in the normal phase relationship between different output rhythms, as well as in a misalignment between internal timing and the

external environment. In our modern 24-h societies, these abrupt shifts in the LD cycle have become frequent for many individuals, such as flight attendants, pilots and intercontinental travellers, and rotating shift workers.[9] Importantly, the repeated disruption of the circadian system, or chronodisruption (CD), is associated with a wide range of health problems and pathologies. For example, epidemiological studies have shown that CD resulting from long-term shift work in nurses and from frequent time-zone travelling (flight attendants and pilots) is associated with a higher incidence of breast,[10,11] colorectal[12] and prostate[13,14] cancer, cognitive impairments,[15] coronary heart disease,[16] alteration in the inflammatory responses, higher overall mortality rates[17] and dysregulation of reproduction.[18] Therefore, understanding the impact and mechanisms involved in the internal temporal order desynchronization is important in order to develop useful therapies and treatments for CD.

One of the many challenges in studying how circadian system disruption impacts human health is that the human population shows a continuum of chronotypes, ranging from larks to owls.[19] As such, there have been renewed interests to use animal models to better understand the human circadian system beyond the commonly used nocturnal laboratory rodents (mice and rats). Unlike most animal species used in chronobiological studies, the *Octodon degus,* as humans, is primarily a diurnal species.[20,21] However, a subset of individuals can switch their diurnal activity pattern and become nocturnal when access to running wheels is provided.[22,23] Thus, the degus show heterogeneity in terms of chronotypes (a continuum from strictly diurnal to nocturnal).[23-26] Therefore, this animal species can be a well-suited animal model in which to assess the detrimental effect of CD on human health, and possible strategies to cope with shift work.[27] In addition, degus exhibit some of human-like age-related diseases, such as atherosclerosis, diabetes, cataracts and Alzheimer-like disease (see [28] for review).

Here, we investigated the desynchronizing effects of chronic LD cycle phase shifts on the circadian system of diurnal and nocturnal degus; this LD-shifting schedule aims to experimentally mimic the lighting regime experienced by shift workers. We also assessed the therapeutic potential of exogenous melatonin treatment in enhancing circadian rhythmicity in animals experiencing these LD cycle shifts.

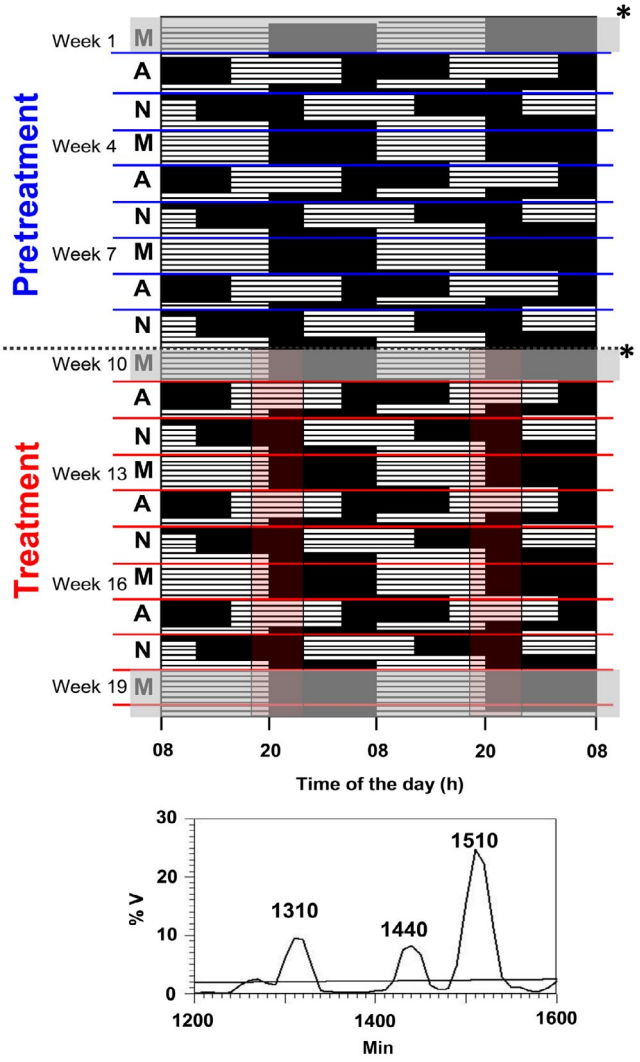

FIGURE 1 Shifting light protocol "5d + 2d". Double-plotted actogram detailing the experimental design. Degus were subjected to a "5d + 2d" lighting schedule for a total of 19 weeks. The light:dark (LD) cycle was delayed weekly by 8h during the 5 "working days," placing the degus into a Morning (M), Afternoon (A) and Night (N) schedule (lights-on from 08:00-20:00, 16:00-04:00 and 00:00-12:00 h). In the weekends (2 days), the degus were always returned to the Morning schedule. White and dark areas represent the light and dark phases, respectively. Red boxes indicate the period of time (6h, from 18:00 to 00:00 h) when the treatments (vehicle or melatonin) were provided daily from week 10 onwards. Asterisks indicate timing when surgeries for implanting body temperature sensors were performed. Grey boxes specify those weeks excluded from data analysis (see Material and Methods for more details). Sokolove-Bushell periodogram for the lighting schedule during pretreatment stage is included under the actogram. %V = percentage of variance. Values on the periodogram indicate significant periods detected

2 | MATERIAL AND METHODS

2.1 | Animals and housing

A total of 35 male degus between 25 and 35 months of age were obtained from a colony maintained at the Animal Facilities of the University of Alicante (Spain). Each degu was individually housed in a Plexiglas cage (52 × 15 × 27 cm, length x height x width), each equipped with a running wheel (25 cm diameter, 10.5 cm width). Animals were kept in isolated chambers with controlled temperature (26.3 ± 0.8ºC), relative humidity (60% ± 10%) and fluorescent lights (350 to 400 lux at the level of the cage). Animals were fed with

commercial rat chow (A04-rat-mouse maintenance Panlab, Barcelona, Spain) and provided with drinking water ad libitum unless stated otherwise. All experimental procedures were performed in accordance with the guidelines issued by the Spanish Ministry of Agriculture, Fishing, and Feeding (Royal Decree 1201/2005 of 21 October 2005) and were approved by the Bioethical Committee at the University of Murcia.

2.2 | Experimental design

Degus were kept with running wheels under a 12:12 light:dark (LD) cycle for 2 months before any experiments began. The animals were then released into continuous darkness (DD) for 18 days before returning to 12:12 LD conditions for 7 more days. After this acclimation period, 18 degus were subjected to a "5d + 2d" light:dark shifting schedule for a total of 19 weeks (Figure 1).

Each shift lasted one week, with 5 "working" (Monday to Friday) and 2 "weekend" (Saturday and Sunday) days. A complete cycle of shifting schedules consisted of 3 weeks: Week 1 (Morning schedule): 5 "working" days with lights-on from 08:00-20:00 h and 2 weekend days with lights-on also at 8:00 h; Week 2 (Afternoon schedule): 5 "working" days with lights-on from 16:00-04:00 h and 2 weekend days with lights-on at 8:00 h; and Week 3 (Night schedule): 5 "working" days with lights-on from 00:00-12:00 h and 2 weekend days with lights-on at 8:00 h. Thus, the 5d + 2d paradigm consisted of a weekly 8 h delay in their LD cycle during the 5 "working" days, placing degus into a Morning, Afternoon and Night rotating schedule. In the weekends (2 days), the LD cycle always returned to a Morning schedule. Each "5 working days" shift (Morning/ Afternoon/ Night) was done by delaying the time of light onset with the appropriate number of hours with respect to the weekend-morning schedule. Thus, the transition from the "2 morning-weekend days" to the "first afternoon-shift day" involved extending the dark phase by 8 h, while the transition from the "2 morning-weekend days" to the "first night-shift day" involved extending the dark phase by 16 h.

After three cycles (9 weeks in total, pretreatment stage), drinking water availability was restricted to 6 h per day (from 18:00-00:00 h). During this time (treatment stage), half of the animals were treated with melatonin dissolved in ethanol (2.5 mg/kg BW/day) (Shift-MEL group), while the other half received vehicle alone (Ethanol 0.04% in water) (Shift-CON group). As control group, 17 degus were maintained under a nonshifting LD condition (Standard conditions) throughout 19 weeks, with lights-on from 08:00-20:00 h. After 9 weeks, half of these nonshifting degus were treated with melatonin (Standard-MEL) and the other half with vehicle (Standard-CON), as described above.

2.3 | Melatonin treatment

Stock solution for melatonin (Fagron Iberica, 33457-24; Barcelona, Spain) was prepared in 100% ethanol every 2 days. Final melatonin concentration (2.5 mg/kg BW/day) was achieved by adding stock solution to the drinking water. Considering an average weight of the degus (~250 g) and a daily water intake of ~ 20 mL, the concentration of melatonin in the drinking water was initially set to 0.031 mg/mL. Final ethanol concentration in drinking water was maintained at 0.04%. Water with melatonin was supplied in light-protected bottles for a period of 6 h per day (from 18:00 to 00:00 h) during the treatment stage (see Experimental design section above). Water intake was measured throughout the experiment, and the concentration of melatonin in the stock solution adjusted accordingly on group basis. Control groups were supplied with drinking water containing 0.04% ethanol for the same six-hour period. The average weight of the degus during the treatment stage showed almost no change (<5% and not statistically significant) and was ~ 250-260 g for the different groups. Note that the animals used in these experiments were adults/mature whose body weight in a period of 2 months showed little or no variation.

2.4 | Data recording

Wheel-running activity (WRA) was recorded as wheel revolutions/10-min intervals using a data acquisition system (Electronic Service at the University of Murcia, Spain, design information is available upon request).

Body temperature (Tb) was recorded every 60 min using a datalogger (ThermoChron®, Data loggers iButton, Maxim Integrated Products, Sunnyvale, California), which has an accuracy of ± 0.125ºC. Sterile data loggers were implanted intraperitoneally under aseptic surgical conditions. Fluothane was used as anaesthetic (Forane®, Abbot Laboratories SA, Madrid, Spain) and iodine solution (Betadine®, Viatris, Madrid, Spain) as a surgical scrub. Absorbable sutures (2/0, Safil®Quick B/Braun, Barcelona, Spain) were used to suture the abdominal muscle layer, and nonabsorbable silk (2/0, Silkam®, B/Braun, Barcelona, Spain) was used to suture the skin. No mortality or morbidity was observed after the surgery in any of the animals. Animals were allowed to recover for a week. Data collected during the recovery period were excluded from data analysis. Surgeries were performed before starting the pretreatment experimental stage, and new surgeries were performed at the beginning of the treatment stage to replace the data loggers (see Figure 1). Once the temperature sensors were removed, temperature data were transferred to a computer. Some of the temperature data from the pretreatment and/or treatment stages were missing due to a failure of the iButton sensors.

2.5 | Chronotype characterization and data analysis

Degus were classified as diurnal or nocturnal based on the percentage of their daily activity during the light phase while being maintained under a 12:12 LD cycle, before starting the experimental stage. Degus with < 40% of activity during the day were included in the nocturnal group, as previously described.[29,30] Mean WRA waveforms from animals categorized as diurnal or nocturnal within the "shifting" groups are shown in Figure 5A. On average, in nocturnal animals, 83.8% of their total daily WRA occurred during the scotophase, while a 70.6% of the activity appeared during the photophase for the diurnal chronotype. When released under DD conditions, the majority of nocturnal animals (77.7%) started to free-run from their former LD phase, confirming their entrainment to the dark period. In line with a previous publication,[23] although diurnal degus tended to show a longer circadian period under DD conditions compared with nocturnal degus, this difference was not statistically significant (diurnal: 1429 ± 5.122 min vs nocturnal: 1419 ± 3.093 min; $T(16) = 1.671$, $P = .114$, unpaired t test).

Sokolove-Bushell periodograms with Bonferroni correction[31] were performed for each animal during the two experimental stages (pretreatment and treatment) for WRA and Tb rhythms. Data from weeks 1 and 10 were excluded from the periodogram analysis to avoid residual effects of the surgery/anaesthesia (see Figure 1). Therefore, a total of 8 weeks was used for analysis within each stage (data from Weeks 2-9 (pretreatment) and Weeks 11 −18 (treatment)).

This analysis allows us to identify different rhythmic components under the "5d + 2d shifting schedule." The percentage of variance (%V) explained by each period was used as an indicator of the importance of each component. To evaluate the power of the 1440 min component with respect to other potential periods (1510 and 1310 min) of WRA and Tb rhythms, a ratio between the %V at 1440 min respect to $\%V_{total}$ (computed as $\%V_{1440}+\%V_{1510}+\%V_{1310}$) was calculated. In this configuration, animals with a robust 24-h component show a high ratio, whereas a less prominence of the 24-h component in favour of the other two components results in low ratio values.

Acrophases of WRA and Tb overt rhythms were defined for each schedule (Morning, Afternoon and Night) by cosinor analysis. Double-plot actograms, mean waveforms, cosinor and Sokolove-Bushell periodograms of WRA and Tb rhythms were performed using El Temps (version 1.228: © Diez-Noguera, University of Barcelona, www.el-temps.com).

Two- tailed paired Student's t test was performed to compare the $\%V_{1440}/\%V_{total}$ ratio for WRA and Tb between the two experimental stages (pretreatment vs treatment) in each group. Unpaired Student's t test was used to compare the %V explained by the different periods between diurnal and nocturnal chronotypes. One-way ANOVA with repeated measures was performed to address differences in the WRA and Tb rhythm acrophases across the different schedules (Morning, Afternoon or Night), for each chronotype (diurnal and nocturnal), or experimental group (Shift-CON and Shift-MEL). Where appropriate, ANOVA tests were followed by Tukey's post hoc multiple comparisons. Acrophases from those temporal patterns in which cosinor analysis did not detect a significant rhythmicity were excluded from the analysis. Values of $P < .05$ were considered statistically significant. Data are expressed as mean ± SEM. Specific sample sizes can be found in the figure legends and results section.

All statistical analysis was performed using GraphPad Prism 7.04 (Prism, GraphPad Software Inc, San Diego, CA).

3 | RESULTS

3.1 | Circadian components under the "5d + 2d" shifting LD protocol

Periodogram analysis of the "5d + 2d" shifting LD schedule data revealed three significant circadian peaks: a long-period component (1510 min) related to the weekly 8-h LD delay; a short-period component (1310 min) associated with the advances of the LD cycle during the weekends and a 1440-min component. This latter emerged as a result of the Morning schedule being the dominant lighting paradigm across our experimental protocol. That is, in a three-week period, a total of 11 days had the Morning schedule—5 working days plus 3 weekends (2 days each), which always had Morning schedule, whereas Afternoon and Night schedules amounted to only 5 days each (Figure 1).

3.2 | Pretreatment stage

Control animals maintained under standard nonshifting conditions displayed a stable pattern of entrainment to the external LD cycle, with a sole period of 24 h (Figure 2E-F). However, in degus subjected to the shifting "5d + 2d" LD schedule, a disruption in their wheel-running activity (WRA) and body temperature (Tb) patterns emerged (Figures 2A-D and 3A-D). Visual inspection of actograms and periodograms shows that the WRA and Tb patterns of the shifting degus closely followed their light:dark schedule (Figure 1), displaying up to three circadian components: 1440, 1510 and 1310 min peaks (Figures 2A-D, 3A-D and Figure 4: pretreatment stage). However, and by contrast to the lighting paradigm which had a strong 1510 min component, overall, the degus showed a prominent component at 1440 min, both for WRA and Tb rhythms, which explained a higher percentage of variance (%V) compared with 1510

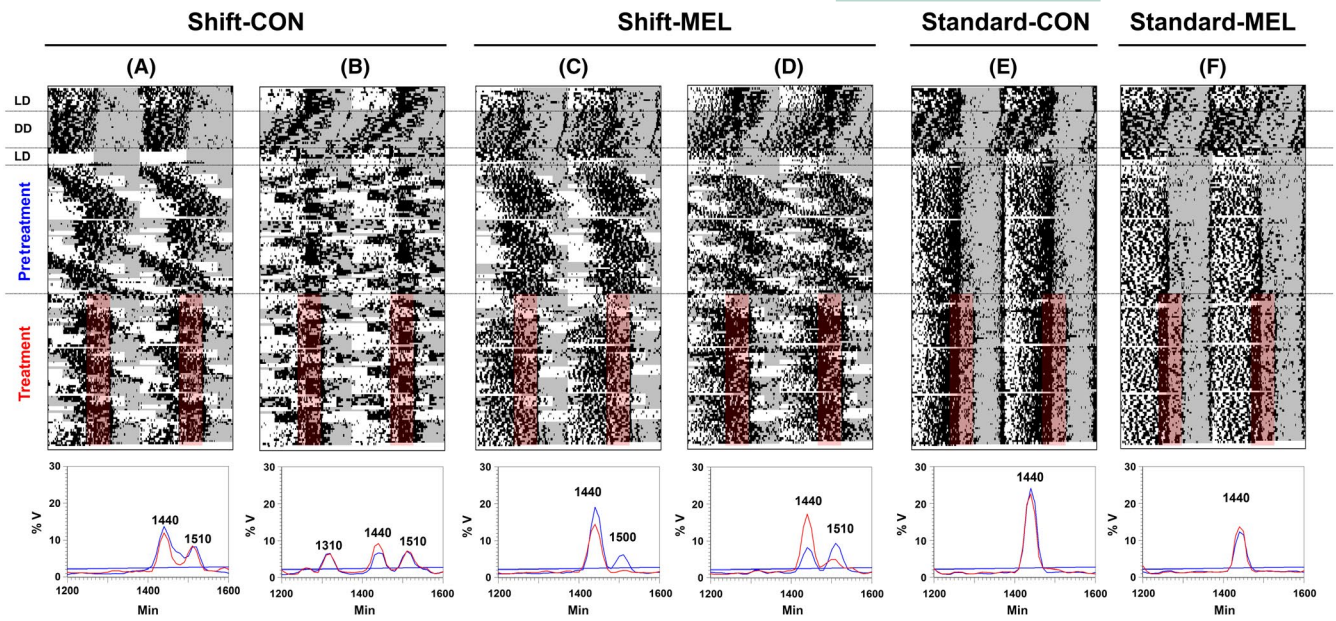

FIGURE 2 Representative double-plotted wheel-running activity (WRA) actograms of degus in the shifting groups, Shift-CON (treated with vehicle) and Shift-MEL (treated with melatonin), and from standard groups treated with melatonin (Standard-MEL) or vehicle (Standard-CON). Before starting the experiment, the degus were kept under a 12:12 LD cycle, followed by constant darkness (DD) to determine their chronotype (Diurnal: A and C; Nocturnal: B and D). The two experimental stages, pretreatment and treatment, are indicated on the left side of the actograms. Grey background area in the WRA actograms indicates the lights-off phase. Red boxes indicate the period of time (6 h) in which the treatments were provided. The periodograms for each experimental stage are shown under each actogram, (blue line for pretreatment and red line during treatment). Significance threshold in periodograms is set to $P = .05$. %V = percentage of variance. Values in the periodogram indicate significant periods

and 1310 min periods (Figures 2A-D, 3A-D and Figure 4: pretreatment stage).

When the %V at these three periods (1440, 1510 and 1310 min) were compared, significant differences were found between nocturnal and diurnal chronotypes (Figure 5). For WRA, diurnal degus exhibited a higher 1440 min peak than nocturnal animals ($T(15) = 2.286$, $P = .037$). However, the opposite situation was observed for the %V at the 1310 component, with nocturnal animals showing higher values than diurnal degus ($T(15) = -2.54$, $P = .023$). No differences were found between the two chronotypes for the $\%V_{1510}$ ($P > .05$) (Figure 5B). As a result, the ratio $\%V_{1440}/\%V_{total}$ for WRA was higher in diurnal than in nocturnal degus ($T(15) = 2.374$, $P = .03$; Figure 5D). A similar trend was also observed for the Tb rhythmicity (Figure 5C,E).

During the first days in the Morning schedule (Baseline-week 1, Figure 6A,B), diurnal animals showed a typical diurnal profile of activity and Tb, exhibiting the majority of their WRA during the light phase, whereas nocturnal degus displayed higher WRA levels during the dark period. A peak of WRA also occurred at dusk in both chronotypes. However, in the 7th week, after two complete chronic "5d + 2d" LD schedule shifts (six weeks in total; see Figure 1), diurnal animals exhibited high WRA and Tb during the dark period (Figures 6C,D and 7A). This therefore resulted in a misalignment between the WRA and Tb rhythms with the external light:dark cycle (compare Figure 6A,B with C,D, and Figure 7A: Baseline 1st week vs Morning 7th week). As a result, acrophase differences between diurnal and nocturnal animals during baseline conditions (WRA: $T(13) = -2.804$, $P = .015$) disappeared in the 7th week (Figure 6A-D). A similar observation was seen in animals during the Night schedule, where both nocturnal and diurnal degus exhibited high WRA and Tb levels during the dark phase with no differences in their acrophases (Figure 6G,H and Figure 7, Night 9th week).

When the WRA and Tb acrophases were compared among the 3 schedules (Morning, Afternoon and Night), internal desynchronization appeared when degus were kept under the Afternoon schedule (lights-on from 16:00 to 04:00 h) (Figure 6E,F), which was more apparent in nocturnal degus, since their WRA acrophase occurred during the dark phase (14:56 ± 02:07 h) and their Tb acrophase during the light period (23:40 ± 02:11 h) (Figure 7).

3.3 | Treatment stage

Melatonin treatment in the drinking water, restricted to 6 h every day (18:00-00:00 h), to animals under "5d + 2d" shifting LD schedule, strengthened the 1440 min component in WRA and Tb, while attenuating the 1510-min component (Figures 2, 3 and Figure 4A,B). This resulted in a significant increase

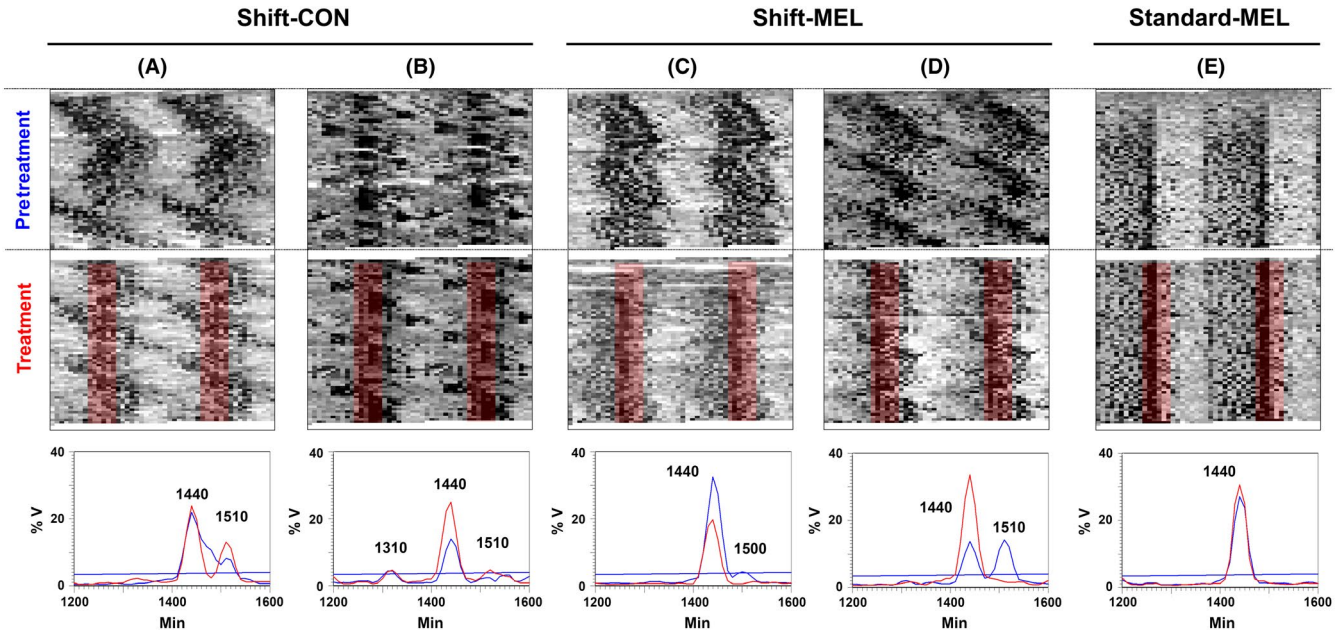

FIGURE 3 Representative double-plotted body temperature actograms from the same degus shown in Figure 2: Shifting groups, Shift-CON (treated with vehicle) and Shift-MEL (treated with melatonin), and standard group treated with melatonin (Standard-MEL). The two experimental stages, pretreatment and treatment, are indicated on the left side of the actograms. Red boxes indicate the period of time (6 h) in which treatments were provided. The periodograms for each experimental stage are shown under each actogram, (blue line for pretreatment and red line during treatment). Significance threshold in periodograms is set to $P = .05$. %V = percentage of variance. Values in the periodogram indicate significant periods

in the $\%V_{1440}/\%V_{total}$ ratio, both in WRA ($T(7) = -2.428$, $P = .046$) and Tb ($T(6) = -2.573$, $P = .042$) of treated animals (Figure 4C,D). In some animals, melatonin treatment even completely removed the components that lie outside the 24-h cycle (eg Figures 2C and 3C). However, despite causing a significant increase in the $\%V_{1440}/\%V_{total}$ ratio, melatonin treatment could not restore these values to levels seen in nonshifting control animals kept under standard conditions (Figure 4C,D). Interestingly, there was a trend for restricted drinking alone (water containing vehicle) to strengthen the 24-h component (increase in the $\%V_{1440}/\%V_{total}$ ratio) in WRA (Figure 4C, $T(8) = -2.264$, $P = .053$) although not in Tb ($P > .05$, Figure 4D).

Analysis of the mean waveform both for WRA and Tb rhythms between different lighting schedules suggests that restricted drinking (with melatonin or vehicle) increased the 24-h component by synchronizing the peak values in Tb, causing them to occur within the animal's drinking period for Morning, Afternoon and Night lighting schedules (Figure 8B,D,F). This was also true for WRA in all except for the Afternoon lighting schedule (Figure 8A,C,E), where WRA peaked outside the time window of drinking availability. However, the overall robustness of the WRA rhythm under this condition (Figure 8C) was higher than during ad libitum drinking (Figure 6E).

Comparisons of WRA and Tb rhythm acrophases across the different lighting schedules (Morning, Afternoon and Night) demonstrate that melatonin treatment maintained the phase alignment between these two rhythms, especially during the Afternoon lighting schedule (Figure 9B). This restoration in the phase relationship of WRA and Tb rhythms was not seen in vehicle-treated animals (Figure 9A).

4 | DISCUSSION

Our results show that the "5d + 2d" shifting light:dark (LD) schedule is an effective paradigm in disrupting WRA and Tb rhythms in the degus. During these shifting conditions, up to three separate circadian components emerged: one longer than 24 h (~25h) that followed the weekly 8-h delays; a short-period (~22h) resulting from the weekend's phase advances; and, finally, a 24-h component. The chronodisruption was more evident in nocturnal than in diurnal degus and during the Afternoon schedule. Exogenous melatonin treatment and, to a lesser extent, water restriction strengthened the 24-h component while attenuating the other two periods. Our results, therefore, suggest that melatonin treatment could be used as a therapeutic strategy to ameliorate the disruptive effects of shift work.

Modern 24-h societies are associated with frequent shifts in our lighting conditions, and this is particularly magnified for shift workers. Different experimental procedures to assess the impact of repeated LD shifts on the circadian system function and its relationship with health have been used under laboratory conditions. Most of these studies are based

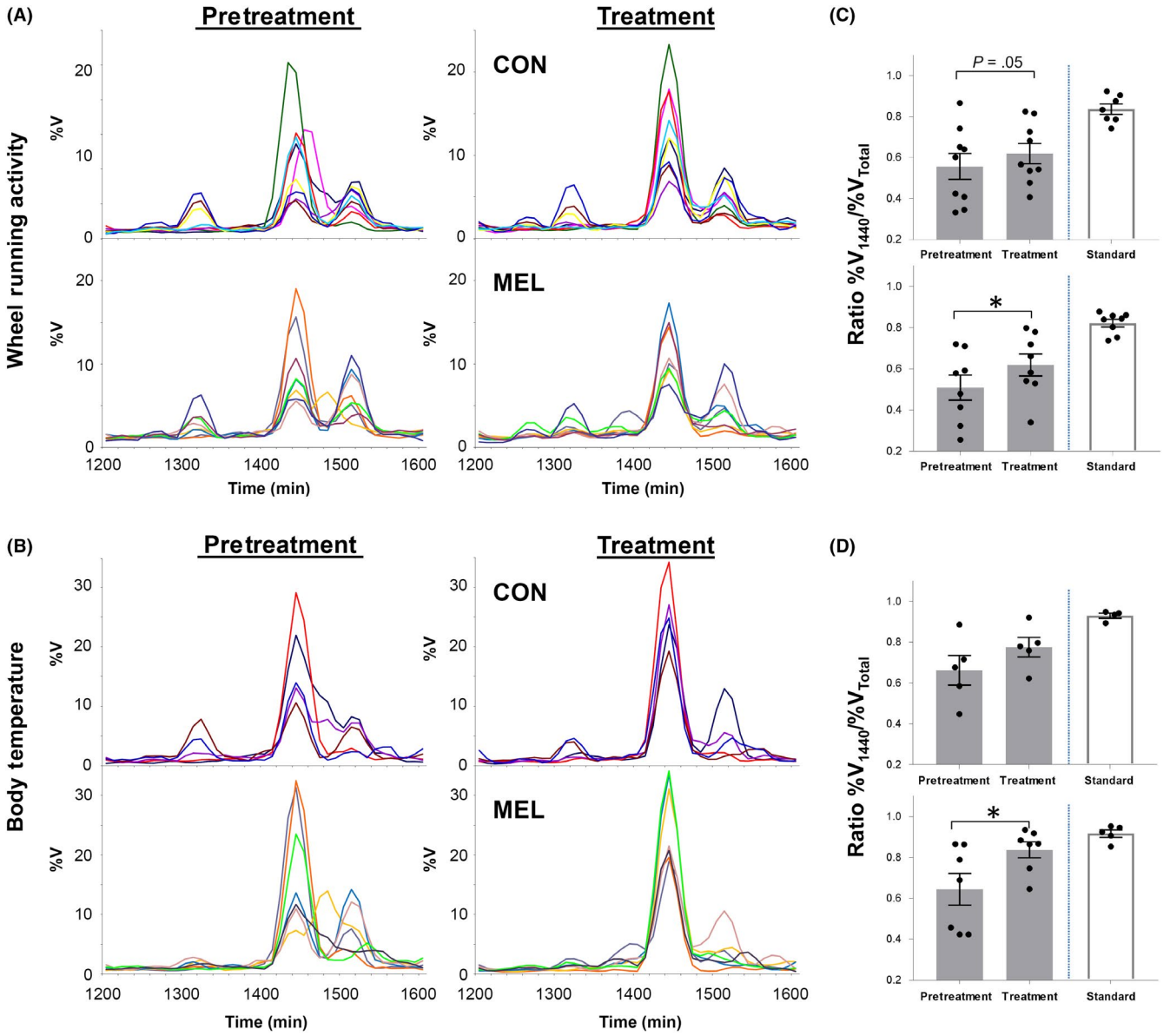

FIGURE 4 Sokolove-Bushell periodograms for (A) wheel-running activity (WRA) and (B) body temperature (Tb) recordings from animals subjected to the "5d + 2d schedule" during the pretreatment and treatment stages. Different colour lines represent one individual animal. %V = percentage of variance. (C, D) Ratio %V at 1440 min respect to the total %V (including 1440, 1510 and 1440 min) calculated for shifting groups at pretreatment and treatment stages for WRA (C) and Tb (D) rhythms. Asterisks indicate significant differences between the two stages for the shifting group treated with melatonin (*$P < .05$, paired t test). Shift-CON: n = 9 and 5; Shift-MEL: n = 8 and 7 for WRA and Tb rhythms, respectively. White bars on the left show the %V ratio for the standard groups during the pretreatment stage as reference

on exposing experimental animals, mainly nocturnal rodents (mice and rats), to jetlag-like shifting paradigms (eg chronic phase advances or delays in their LD cycles with a determined inter-shift intervals.[32-44] However, considerably less of such studies have been performed in diurnal animals,[45,46] and to the best of our knowledge, no studies have been performed in dual-phasing species, like the degus. Although the *Octodon degus* is primarily a diurnal species,[20,21] it also has the ability to switch its WRA and Tb patterns from a diurnal to nocturnal phase when running wheels are provided.[22,23] Interactions between the mechanisms involved in entrainment of the pacemaker to diurnal or nocturnal phases and those causing masking by light have been associated with chronotypes heterogeneity seen in this species (a continuum gradient from strictly diurnal to nocturnal).[23,47] Although humans are diurnal, a continuum of chronotypes ranging from matutine (larks) to vespertine (owl) is also observed.[19] Therefore, the degu provides a useful animal model in which to deepen our understanding of the circadian system functionality in humans and how environmental perturbations, such as rotating shift work,[9] disrupt its normal function.

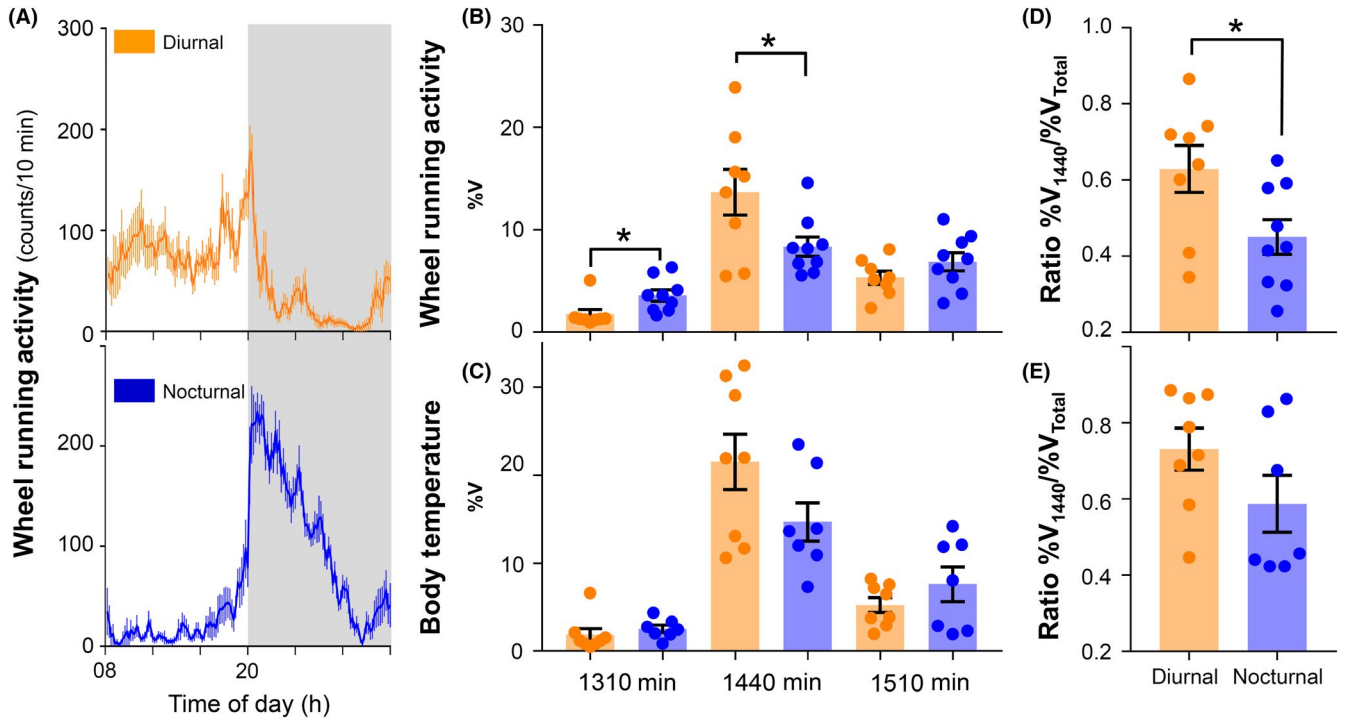

FIGURE 5 Physiology and behaviour of diurnal and nocturnal degus under the "5d + 2d" shifting light protocol. (A) Wheel-running activity mean waveforms from degus classified as diurnal (orange line, n = 9) or nocturnal (blue line, n = 9) based on their activity patterns while maintained under 12:12 LD cycle, before starting the experiments. Grey background area indicates the dark phase. Percentage of variance explained by each of the three periods (1310, 1440 and 1510 min) resulting from Sokolove-Bushell periodograms analysis for (B) wheel-running activity (WRA) and (C) body temperature (Tb) rhythms in diurnal and nocturnal degus kept under the "5d + 2d schedule" during the pretreatment stage. %V ratio at 1440 min respect to the total %V (including 1440, 1510 and 1440 min) calculated for diurnal and nocturnal degus for WRA (D) and Tb (E) rhythms. Asterisks indicate significant differences between the two chronotypes (*$P < .05$, unpaired t test). Diurnal, n = 8 and 8; Nocturnal, n = 9 and 7 for WRA and Tb rhythms, respectively

In this study, we subjected degus to a "5d + 2d" shifting LD schedule in an attempt to mimic the lighting conditions experienced by rotating shift workers during the working weekdays and weekends. This shifting paradigm combines different shift work lighting schedules (Morning, Afternoon and Night) during the 5 working days, and a fixed Morning schedule during weekends (which is generally more frequently adopted by humans in order to increase compatibility of social and familial life). It must be stressed that here we did not engage the animal into forced work, as described elsewhere,[48-50] but merely manipulating their LD cycles.

When the periodogram analysis for our lighting schedule was performed, three significant peaks emerged at 1510, 1440 and 1310 min periodicities, with 1510 min being the strongest component of the three. Our results show that the degus also manifested these three periodic components in the circadian range in their WRA and Tb rhythms, with the 24-h periodicity being the most important for both variables. This is not surprising since the 24-h component is closer to the degu's endogenous free-running period (around 1410-1430 min,[23]), when compared with the 1510- and 1310-min periods.

Under standard nonshifting lighting conditions, animals displayed a robust 24-h rhythmicity and a stable alignment between the internal timing in physiology and behaviour and the external LD environment. In order to accurately assess the severity of the shifting light:dark schedule disruption on the WRA and Tb rhythms in the degus, we proposed an index based on the periodogram analysis. Here, the ratio between the %V at 1440 min with respect to $\%V_{total}$ ($\%V_{1440}+\%V_{1510}+\%V_{1310}$) was calculated. Based on this ratio, our results indicate that the highest disruption occurred in nocturnal animals, probably because they exhibit a stronger negative masking by light than the diurnals,[23,26,47] driving them to follow the shifted lighting paradigm. This disruption was more evident in the WRA than in the Tb pattern, an observation that corresponds with previous studies showing that in this species WRA is more strongly masked by light than Tb.[47] In addition, this masking by light would explain the misalignment between the WRA and Tb rhythms seen in nocturnal degus in the Afternoon schedule. This raises the possibility that other body rhythms affected or modulated by activity or/and temperature patterns could indirectly become out of synchrony too and have a negative impact on well-being in the long term.

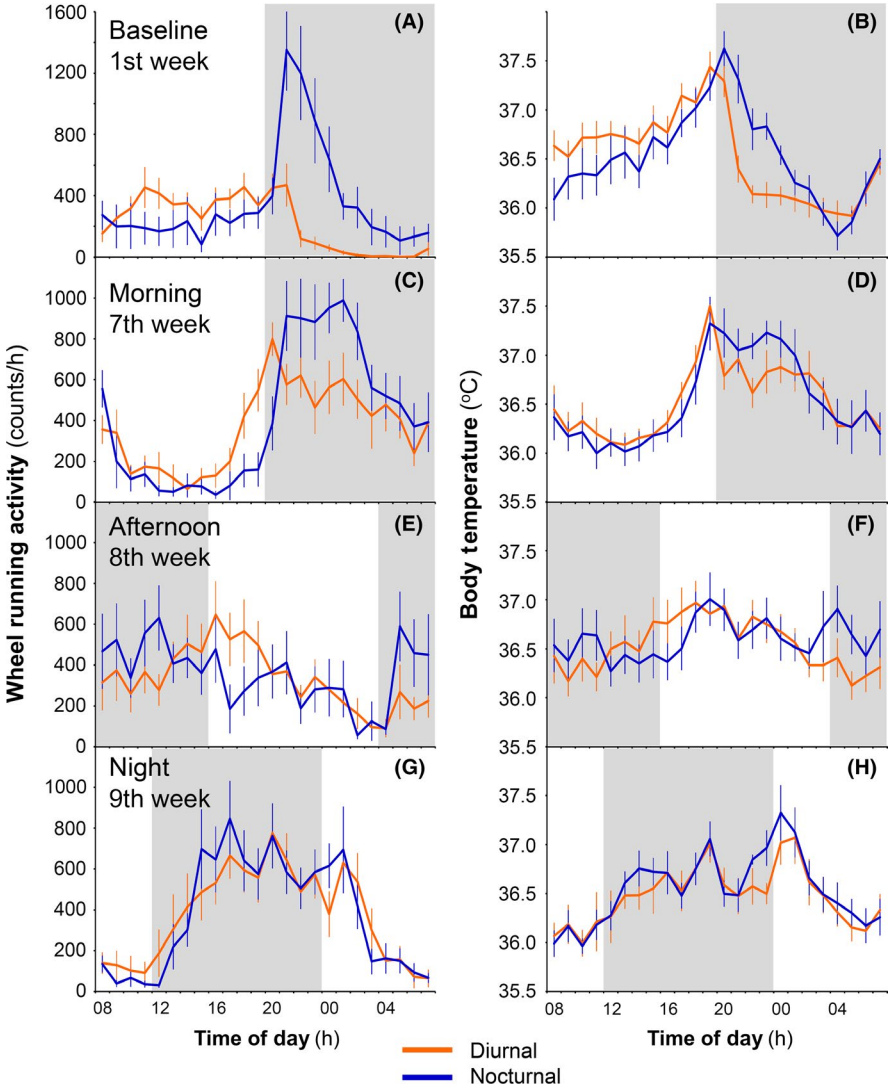

FIGURE 6 Mean waveforms for wheel-running activity (WRA, left panel) and body temperature (Tb, right panel) rhythms for diurnal (orange line) and nocturnal (blue line) degus during the 1st week under Morning schedule (at the beginning of the experiment, Baseline), and from the period of "5 working days" in the 7th, 8th and 9th week of the experiment (Morning, Afternoon and Night schedule, respectively). Grey background area indicates the dark phase. Values are expressed as mean ± SEM (Diurnal: n = 8; Nocturnal: n = 7). Note that Baseline mean waveforms (A, B) are calculated using only 4 days due to the proximity of the surgery for implantation of the temperature sensors

In the modern Western societies, the repeated disruption of our circadian system is associated with a wide range of health impairments, so much so that chronodisruption is becoming a public health issue. This situation calls for the development of appropriate interventions and therapies to strengthen the circadian rhythmicity. Melatonin has chronobiotic properties since it can phase-shift the circadian clock and adjust the timing of internal biological rhythms.[51] Based on these properties, melatonin is widely used as a pharmacological agent to treat circadian rhythms disturbances, both in animals and humans.[52] For example, melatonin is used to entrain free-running rhythms in animals kept under constant environmental conditions[53,54] or in blind people.[55,56] On the other hand, in a study with degus maintained under normal LD cycles, Vivanco et al. (2007)[30] have shown that melatonin can effectively be used to improve impaired rhythmicity associated with ageing in this species, with very similar effects independently of the animal's chronotype.

In our study, we administered exogenous melatonin at a regular time point every day regardless of the lighting schedule. With this, we aimed to strategically boost the 24-h periodicity in WRA and Tb rhythms in animals subjected to the "5d + 2d" shifting schedule to near control levels (non-shifting animals) and re-align the internal phase synchrony between WRA and Tb rhythms across the different schedules. The treatment was restricted to a 6-h period, (from 18:00 to 00:00 h) coinciding with the WRA peak around light-to-dark transition during the Morning schedule. With this strategy, our results show that melatonin treatment not only strengthened the existing 24-h period in WRA and Tb, but attenuated any periods that occurred outside the 24-h periodicity, resulting in an increased $\%V_{1440}/\%V_{total}$ ratio. Our data also showed that scheduling water availability can act as a weak *zeitgeber* on WRA and Tb rhythms in the shifting degus, as described in other species.[57] Its synchronizing effects on these rhythms were significantly weaker than melatonin. This was more evident during the Afternoon schedule where, for example, melatonin, but not water restriction alone, facilitated the re-alignment of WRA and Tb rhythm acrophases. However, the positive effect of scheduling water

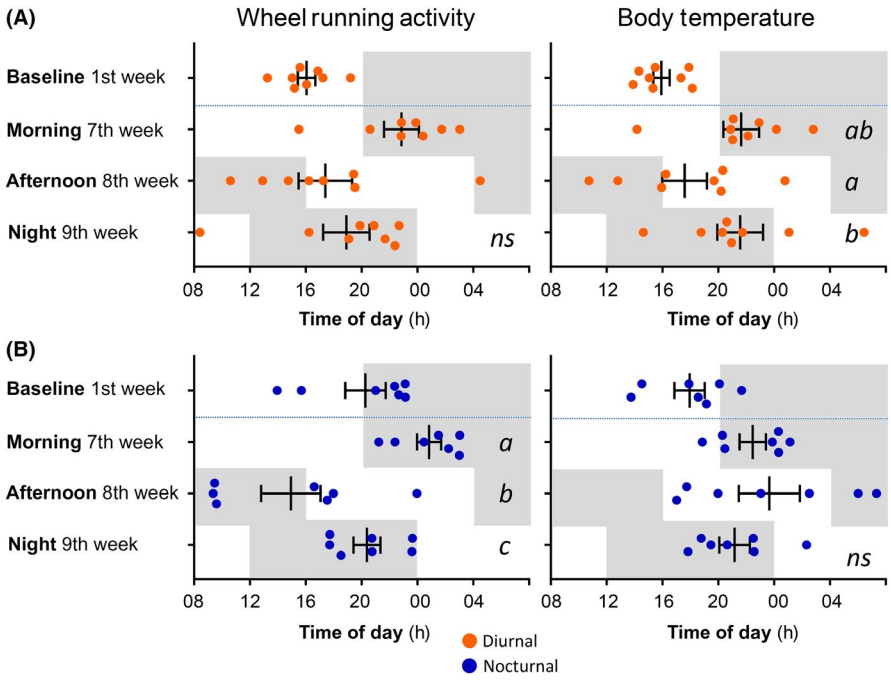

FIGURE 7 Acrophases of wheel-running activity (WRA) and body temperature (Tb) rhythms for diurnal (A, n = 8) and nocturnal (B, n = 7) degus from a period of "5 working days" during the 1st week under Morning schedule (at the beginning of the experiment, Baseline), and at the 7th, 8th and 9th week of the experiment (Morning, Afternoon and Night schedule, respectively). Grey background area indicates the dark phase. Values are expressed as mean ± SEM with each dot representing one animal. Different letters indicate significant differences ($P < .05$) throughout the different shifts for diurnal or nocturnal degus. (Repeated measures ANOVA, Tukey's post hoc analysis; *ns*, nonsignificant). Baseline data were excluded from the statistical analysis

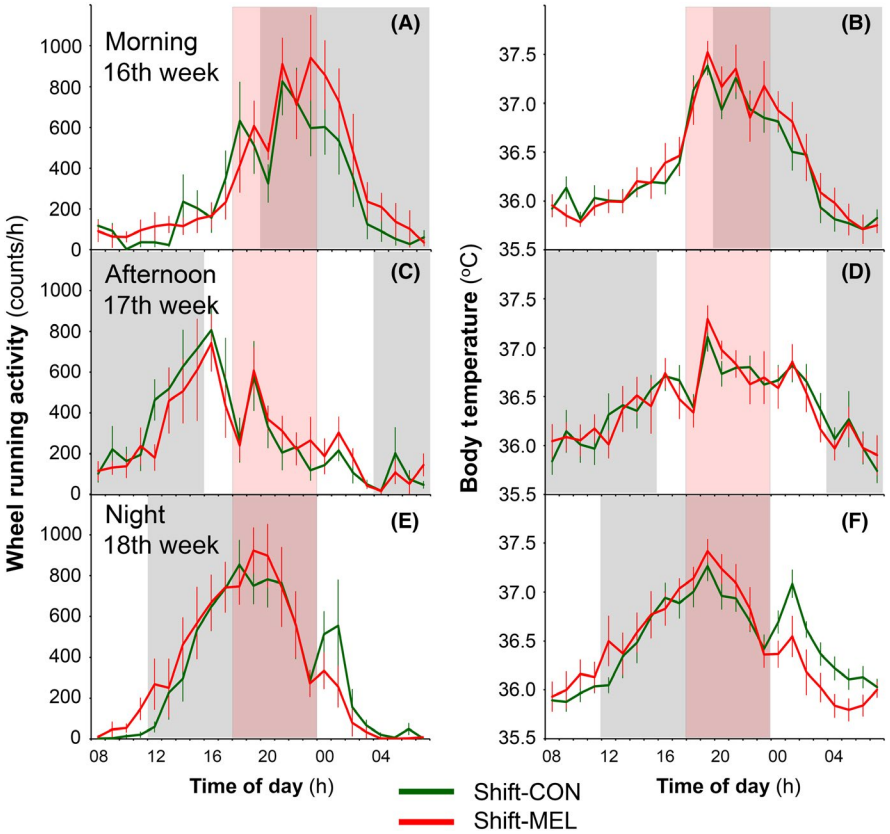

FIGURE 8 Mean waveforms for wheel-running activity (left panel) and body temperature (right panel) rhythms for animals treated with vehicle (Shift-CON, green line) or with melatonin (Shift-MEL, red line) from a period of "5 working days" during the 16th, 17th and 18th week of the experiment (Morning, Afternoon and Night schedule, respectively). Grey background area indicates the dark phase. Red boxes indicate the period of time (6 h) when treatments were provided. Values are expressed as mean ± SEM (Vehicle: n = 8; Melatonin-treated: n = 7)

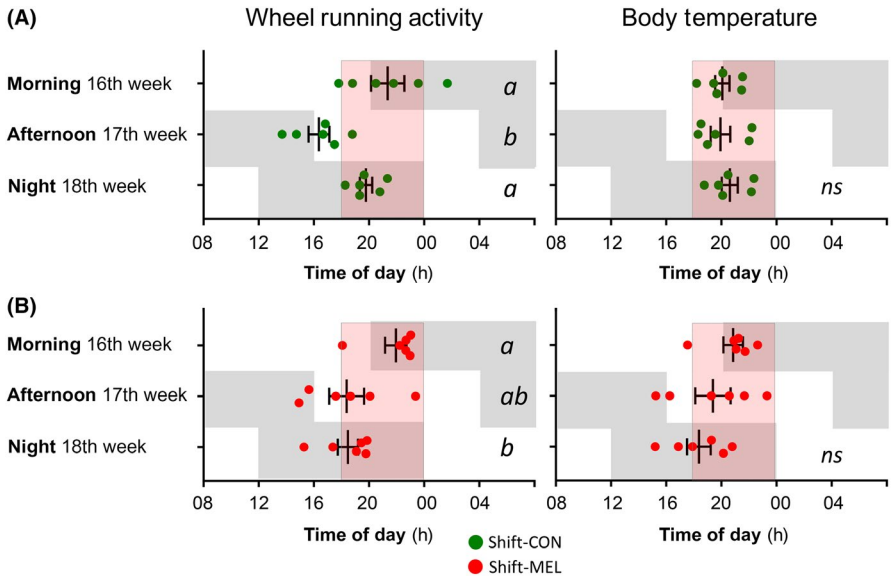

FIGURE 9 Acrophases of wheel-running activity (WRA) and body temperature (Tb) rhythms for animals treated with vehicle (A, Shift-CON, n = 6) or with melatonin (B, Shift-MEL, n = 6) from a period of "5 working days" during the 16th, 17th and 18th week of the experiment (Morning, Afternoon and Night schedule, respectively). Grey background area indicates the dark phase. Red boxes indicate the period of time (6 h) when treatments were provided. Values are expressed as mean ± SEM with each dot representing one animal. Different letters indicate significant differences ($P < .05$) between schedules for Shift-CON or Shift-MEL groups (repeated measures ANOVA, Tukey's post hoc analysis). Acrophases from three animals (2 from the Shift-CON and 1 from the Shift-MEL groups) were not included due to the lack of rhythmicity revealed by cosinor analysis

access cannot be neglected. In a previous study by Vivanco et al., 2007,[30] restricting water to a period of 2 h in degus maintained under nonshifting LD cycle proved to be effective at inducing some improvement in synchronization, at least in those animals with low activity rhythm stability. It is possible that part of the positive effects of water restriction on rhythm robustness is the result of the induced arousal state related with the moment when water becomes available. Indeed, nonphotic stimuli associated with locomotor activity and/or arousal represents a well-established mechanism for an endogenous behaviour to feedback and influence subsequent circadian function.[58] It is reasonable therefore to consider that, in addition to melatonin treatment, these nonphotic stimuli could be a useful strategy to ameliorate the disruptive effects of shift work.

Based on the positive feedback loop between physical exercise[58] (eg in a running wheel) and the circadian pacemaker in promoting rhythm robustness, it can be argued that the effects of melatonin would be more relevant in animals with no wheel access and presumably with less robust rhythmicity to begin with. Future studies subjecting degus to the 5d + 2d shifting protocol while treated with melatonin or restricted water access and no wheel will be needed to address this hypothesis.

In conclusion, we propose the "5d + 2d" shifting LD schedule as a useful protocol to experimentally cause circadian disruption in the degus' physiology and behaviour in order to study the effects of chronodisruption. Future studies are needed to investigate the long-term effects of this shifting protocol on the animal's health status, and the molecular and neurophysiological mechanisms underlying them. Our results also indicate that melatonin treatment at specific time points in the circadian cycle could be a useful therapeutic strategy in cushioning the disruptive effects of shift work, acting by boosting the robustness of the 24-h periodicity.

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CONFLICT OF INTEREST

The authors declare that they have no competing interests.

AUTHORS' CONTRIBUTIONS

BBO, MAR and JAM designed the study. BBO performed the experiments. BBO, MAR and JAM wrote the manuscript. JAM, MAR and BBO acquired funding.

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DATA AVAILABILITY STATEMENT

The data sets generated and analysed during the current study are available from the corresponding author on reasonable request.

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SUPPORTING INFORMATION

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

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