Article

Evaluation of environmental, social, and behavioral modulations of the circadian phase of dancers trained in shifts

ECOLOGICAL MODEL FOR THE STUDY OF SOCIAL, ENVIRONMENTAL, AND BEHAVIORAL MODULATIONS OF HUMAN CIRCADIAN PHASE

DETERMINANTS OF THE CIRCADIAN PHASE

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Highlights

Circadian phase was earlier in morning than in night dancers in real-life conditions

Sleep time (and night-shift chronotypes) correlated with the circadian phase

In the phase-advance and phase-lag windows, light was not associated with DLMO

In the phase-lag time window, the longer the intense exercise the later the phase
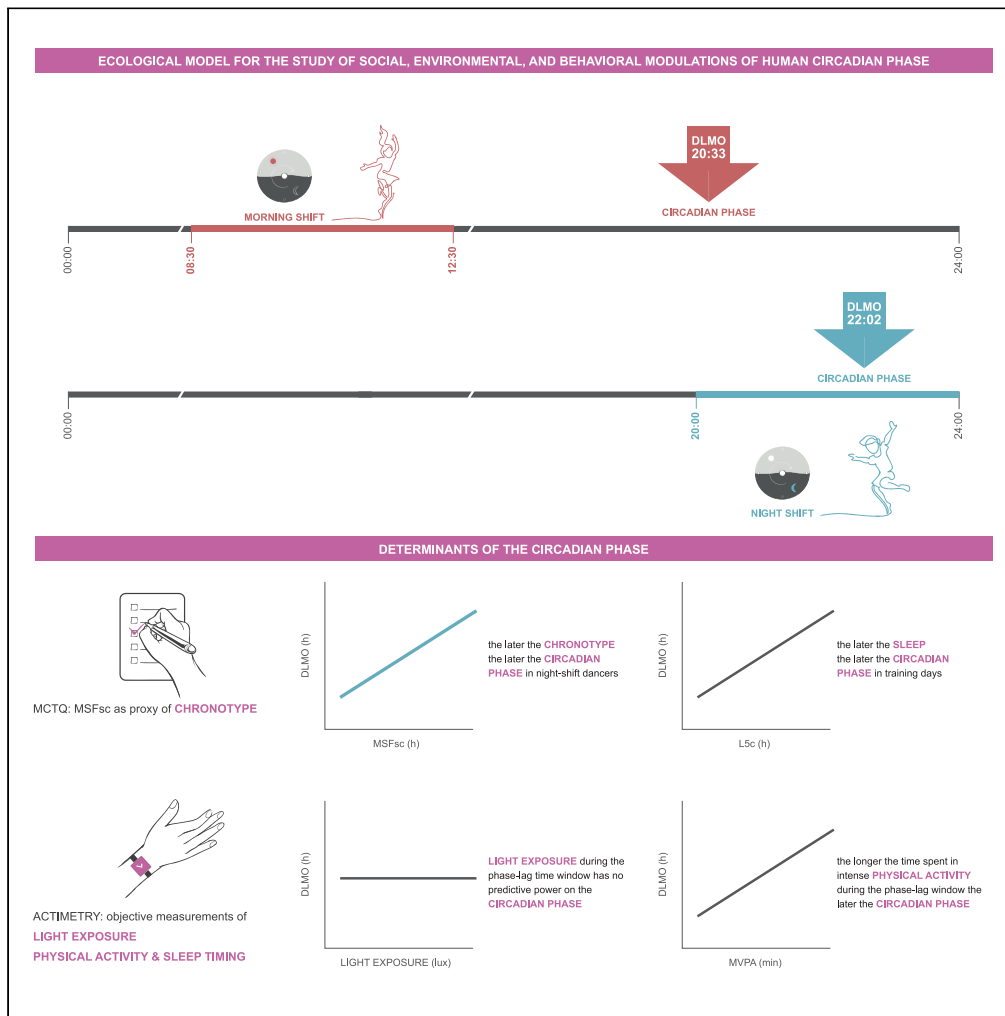
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Article

Evaluation of environmental, social, and behavioral modulations of the circadian phase of dancers trained in shifts

Natalia Coirolo,[1] Cecilia Casaravilla,[2] Bettina Tassino,[1,3] and Ana Silva[1,4,5,*]

SUMMARY

The interplay of environmental, social, and behavioral factors influencing human circadian phase in ecological conditions remains elusive. The Uruguayan national dance school END-SODRE operating in two shifts (morning: 8:30–12:30 and night: 20:00–24:00) allowed us to evaluate how social demands, chronotype, environmental light, physical activity, and sleep patterns affected individual circadian phase measured by the onset of the nocturnal increase of melatonin (DLMO) in a single study. The DLMO was 1.5 h earlier in morning-shift dancers (n = 7) compared to night-shift dancers (n = 11). Sleep time and chronotype (only in night-shift dancers) were associated with the circadian phase. In training days, during each participant's phase-advance and phase-delay time windows, light exposure was similar between morning and night-shift dancers and did not correlate with DLMO. In contrast, the time spent in moderate-vigorous physical activity during each participant's phase-lag time window was higher in night-shift dancers than in morning-shift dancers and positively correlated with DLMO.

INTRODUCTION

The circadian rhythms of most animals (including humans) synchronize to environmental cues to survive in the real-world day after day (Bhadra et al., 2017). Daily cycles of light and darkness, ambient temperature, and food availability, among others, act as entrainers (zeitgebers), and the circadian system adaptively uses this information to reset its circadian clock to match its period with that of the zeitgeber (Daan, 1998). Understanding the interplay of environmental, social, and behavioral factors influencing human circadian phase in ecological conditions is a major challenge of current research. Recent studies have contributed promising strategies to evaluate human circadian timekeeping in real-world situations at the population level (Bowman et al., 2021; Facer-Childs et al., 2020), but there is a long way to go to fully understand this matter.

The rhythm of melatonin secretion provides the best available measure of the timing of the internal clock (Arendt, 2005). The dim light melatonin onset (DLMO) is the start of the evening rise of melatonin in plasma or saliva and has been identified as the best proxy of individual human circadian phase (Lewy et al., 1999; Lewy and Sack, 1989). The DLMO has proven to be useful for determining whether an individual is entrained or not, and for assessing phase delays or advances of rhythms in entrained individuals (Pandi-Perumal et al., 2007). However, DLMO shifts have seldom been explored in real-life situations (Stothard et al., 2017; Wright et al., 2013).

The light-dark cycle is the most important zeitgeber of the circadian system across evolution, whose mechanisms of action are well understood (Prayag et al., 2019; Rea et al., 2021). In mammals, the inputs of retinal circadian photoreceptors directly modulate the firing of the circadian master clock neurons in the hypothalamic suprachiasmatic nuclei (Meijer et al., 1998), which in turn influence outputs of the clock including the pineal secretion of melatonin (Gastel et al., 1998). Bright light during daytime (especially in the morning) increases the amplitude of nocturnal melatonin secretion (Park and Tokura, 1999), whereas light exposure at night suppresses melatonin secretion (Lewy et al., 1980). The DLMO itself is affected by light, so it can either be advanced by bright light exposure in the morning (during the circadian phase-advance time window) (Hashimoto et al., 1997; Khalsa et al., 2003; Kozaki et al., 2016; Kripke et al., 2007; Takasu et al., 2006), or delayed by light exposure in the evening (during the circadian phase-lag time window) (Akacem et al.,

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2018; Gooley et al., 2011). Besides, age and individual differences have been reported to influence the sensitivity of the circadian system to light, and thus have an impact on individual circadian phase (Akacem et al., 2018; Phillips et al., 2019). Although the DLMO is an endogenous and rather stable trait (McHill et al., 2021), it can also be an accurate proxy of the daily resetting of the circadian phase (Stothard et al., 2017; Wright et al., 2013; Zerbini et al., 2021). However, how light exposure affects the human circadian phase in daily life has yet to be fully explored.

Human modern life has challenged the biological clock by making ambient light a less reliable timekeeper (Czeisler, 2013; Roenneberg et al., 2013). As an indirect result, circadian rhythms have become more variable and less synchronized among individuals (Roenneberg, 2013). In this world with no clear light contrasts, early melatonin onset is associated with early chronotypes, whereas late chronotypes show later DLMO values (Burgess and Eastman, 2005; Kantermann et al., 2015; Kitamura et al., 2014); and chronotype-dependent circadian phase shifts have also been identified (Silva et al., 2019; Wright et al., 2013). On the other hand, social demands often force a chronic misalignment between the inner and social clocks that may also impose circadian phase changes. This occurs, for example, in adolescents, whose DLMO is later in vacation than during the school term (Crowley et al., 2006); or in health shift workers, whose melatonin peak is later in night-shift workers than in day-shift ones (Razavi et al., 2019). In addition, as social demands have led to irregular daily meal patterns, the timing of nutrient income has become an important entrainer of the circadian phase (McHill et al., 2019).

Although the photic input is the main signal of entrainment for the circadian master clock, secondary behavioral circadian rhythms alone, such as the sleep-wake cycle, feeding regime, stress, and scheduled exercise, are also capable of entraining the mammalian circadian system (Czeisler, 1980; Lax et al., 1998, 1999; Tahara et al., 2017). To avoid confounding factors, the best way to test the light-independent effect of non-photic zeitgebers is to evaluate them in total darkness (Mistlberger and Skene, 2005). Studies in humans under controlled light conditions have identified that the practice of physical activity in the morning induces a circadian phase advance and that physical activity during the evening provokes a circadian phase delay (Barger et al., 2004; Buxton et al., 2003; Thomas et al., 2020; Youngstedt et al., 2019). To date, it has been very difficult to evaluate the impact of non-photic entrainers in real-life conditions given the compelling evidence of a nonlinear, although partially additive, integration of the influences of the photic and nonphotic cues on the circadian phase (Challet and Pévet, 2003). For example, (Youngstedt et al., 2016) showed an additive effect of light and exercise on the circadian phase delay with respect to that observed by each of these stimuli separately.

From a chronobiological perspective, professional dancers can be conceived as a special case of elite athletes, who undergo intensive and regular physical training, but who are also challenged emotionally to accomplish artistic excellence. Although training requirements for dancers usually threaten their regular chronobiological patterns and normal sleep-wake rhythms, dancers remain extensively underexplored in chronobiological studies (Fietze et al., 2009). The Uruguayan national dance school END-SODRE operates a morning shift from 8:30 to 12:30, and a night shift from 20:00 to 24:00. It thus offers a natural laboratory to evaluate how dancers' circadian systems deal with the social pressure of being trained at different times of the day. We have already identified training shift-dependent changes in dancers' chronotype and self-reported sleep patterns (Coirolo et al., 2020). In this real-life study, we measured dancers' DLMO as a proxy of individual circadian phase and evaluated how it is influenced by photic and non-photic entrainers. We first analyzed the dependence of the circadian phase on training shifts, chronotype, and activity-rest patterns. Besides, we evaluated the associations of light and exercise with the circadian phase in both the circadian phase-advance and phase-lag time windows.

RESULTS

Eighteen students (7 attending the morning shift and 11 attending the night shift) of the dance college training program END-SODRE, Uruguay, participated in this study (Table 1). The END-SODRE is organized as a 4-year training program with classes taught from Monday through Friday. Although first and second grade students attend the night shift (20:00 to 24:00) and third and fourth grade students attend the morning shift (8:30 to 12:30), the age of participants was not different between shifts. Average chronotype, proxied by self-reported mid-sleep point on free days corrected for sleep debt on workdays (MSFsc) corresponded to 05:28 ± 01:38 and was not significantly different in dancers attending the night shift with respect to morning-shift dancers, while social jetlag (SJL) was (Table 1). As expected, MSFsc correlated

Table 1. Demographic and chronobiological and characterization of participants

	Total	Morning-shift	Night-shift	p
Participants (males)	18 (1)	7	11 (1)	
Age	22.61 ± 4.03	22.57 ± 2.82	22.64 ± 4.78	0.974
Chronotype MSFsc	05:28 ± 1:38	05:37 ± 1:17	05:22 ± 1:53	0.759
Social Jet Lag	1.64 ± 1.50	2.71 ± 1.02	0.97 ± 1.38	**0.011**

Number of participants, gender, age, and chronobiological characterization of morning-shift dancers and night-shift dancers. Data are presented as mean ± SD and compared by Unpaired Student's t-tests. Statistically significant test (p < 0.05) is shown in bold.

with SJL (R = 0.56, p = 0.016). Sleep log data showed that dancers of both training shifts delayed their sleep in weekends with respect to training days, whereas night-shift dancers scheduled their sleep significantly later than morning-shift ones in training days (Table S1). Morning-shift dancers slept longer than night-shift dancers on weekends, but the average weekly sleep duration was above 7 h per day and similar across shifts (Table S1).

The timing of the activity-rest cycle and light exposure differs between shifts

Activity actograms during 18 days of consecutive recording (12 training and 6 weekend days) in two representative participants attending either the morning or the night shift are presented in Figure 1 (additional actograms are shown in Figure S1). These actograms illustrate the main characteristics of dancers' daily rhythms of activity statistically tested in Tables 2 and S2. First, all participants showed robust circadian rhythms of activity with significant fit to a cosine function over 24 h period, whose acrophase was significantly earlier in morning-shift dancers compared to night-shift dancers during training days (Table 2). Second, the timing of activity and rest, measured by M10c (the midpoint of the most active 10 h period) and L5c (the midpoint of the least active 5 h period), respectively, was also earlier in morning vs. night shift dancers during training days (Table 2). Third, the timing of activity and rest, measured by the acrophase, M10c, and L5c, was later at weekends compared to training days for morning-shift dancers, whereas only L5c showed this pattern in night-shift dancers (Table 2).

In contrast to timing, the intensity of daily physical activity, measured by M10 (average activity of the most active 10 h period) and L5 (average activity of the least active 5 h period), was not significantly different between shifts during both training days and weekends (Table S2), although M10 was marginally higher at weekends for night-shift dancers. Morning-shift dancers displayed more intense activity during training days with respect to weekends (higher M10, Table S2), whereas night-shift dancers had less rest during weekends with respect to training days (higher L5, Table S2). Despite these dissimilarities across shifts, the robustness of the activity-rest rhythm was high (with relative amplitudes above 0.6 in all the conditions) and significantly higher on training days than weekends for dancers, irrespective of shift (Table S2).

The pattern of light exposure was also different across shifts in timing (Table 3) but not in intensity (Table S3). The timing of the midpoint of the most brilliant 10 h period (MB10c) and the midpoint of the least brilliant 5 h period (LB5c) was earlier in morning vs. night shift dancers during training days, whereas light acrophase was not different across shifts (Table 3). Only LB5c was significantly later in weekends compared to training days for morning-shift dancers, whereas only light acrophase showed this pattern in night-shift dancers (Table 3).

As expected, when the time of analysis was restricted to the time in which shifts were scheduled during the 12 training days of actimetry recordings, we confirmed that morning-shift dancers displayed more intense physical activity (97.03 ± 19.99 mg) than night-shift dancers (64.39 ± 26.13 mg) during the morning shift (08:30 to 12:30; unpaired Student's t test, p = 0.013). Vice versa, night-shift dancers displayed more intense physical activity (107.67 ± 13.74 mg) than morning-shift dancers (55.66 ± 9.94 mg) during the night shift (20:00 to 24:00; unpaired Student's t test, p < 0.0001). In addition, school training meant a similar physical challenge for participants of both shifts as the physical activity of morning-shift dancers during the morning shift was not different than the physical activity displayed by night-shift dancers during the night shift (unpaired Student's t-test, p = 0.198). We carried out a similar analysis of the intensity of light exposure restricted to the time in which shifts were scheduled during the 12 training days of actimetry recordings.
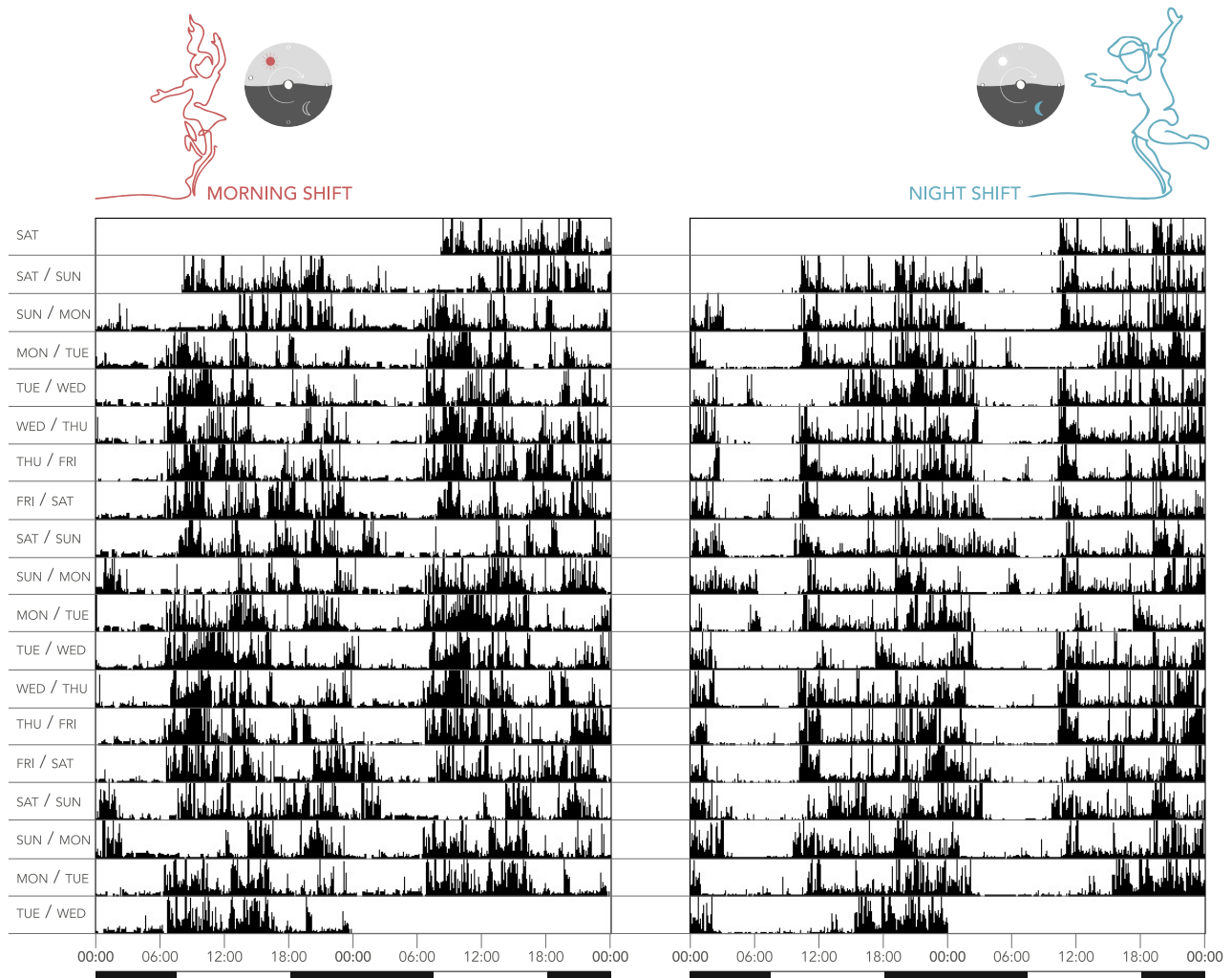
Figure 1. Actograms of the activity-rest cycle during the 18 consecutive days of recording (12 training and 6 weekend days) of 2 representative participants attending either the morning or the night shift

Data are presented in rows of 48 h. The light-dark cycle is represented by the white-black bars. These actograms illustrate the main characteristics of dancers' daily rhythms of activity and their differences between shifts.

See also Figure S1.

Obviously, both morning and night-shift dancers were 15–40 times more exposed to light during the morning (627.93 ± 378.12 lux for morning-shift dancers and 566.53 ± 397.5 lux for night-shift dancers) than during the night (15.67 ± 7.54 lux for morning-shift dancers and 32.99 ± 15.93 lux for night-shift dancers; paired Student's t-test, $p = 0.005$ for morning-shift students and $p = 0.001$ for night-shift students). In addition, we confirmed that morning-shift and night-shift dancers were exposed to a similar light intensity during the morning shift (unpaired Student's t test, $p = 0.75$); on the other hand, night-shift dancers were more exposed to light than morning-shift dancers during the night shift (unpaired Student's t-test, $p = 0.017$).

The effect of training shifts, chronotype, and sleep timing on dancers' circadian phase

The absolute melatonin levels from 18:00 to 24:00 are plotted for two representative participants attending either the morning or the night shift in Figures 2A and 2B, respectively (additional melatonin curves are presented in Figure S2). We observed the expected nocturnal increase in melatonin levels in all participants, although the calculated threshold for the onset of this nocturnal increase was variable among individuals (ranging from 2.5 to 11.36 pg/mL; Figure S2) and not significantly different between shifts (unpaired

Table 2. Timing of the activity-rest cycle measured by actimetry records

Type of day	M10c			L5c			Acrophase		
	training	free	p[1]	training	free	p[1]	training	free	p[1]
Morning-shift	12:15 (0:42)	17:26 (0:45)	**0.018**	03:29 (0:37)	06:33 (1:05)	**0.018**	13:49 (0:49)	17:52 (0:56)	**0.018**
Night-shift	19:15 (0:41)	17:08 (2:55)	0.722	05:03 (0:54)	06:59 (1:01)	**0.010**	18:26 (0:28)	18:27 (1:43)	0.722
p[2]	**0.001**	0.928		**0.030**	0.277		**0.001**	0.856	

Circular variables: the midpoint of the more active 10 h period (M10c), the midpoint of the least active 5 h period (L5c) and the moment of the day in which the cosinor function reaches its maximum physical activity (acrophase). Data are presented as median $\pm$ MAD and compared statistically by Wilcoxon signed-rank test (1) and Mann-Whitney U test (2). Statistically significant tests (p < 0.05) are shown in bold.

Student's t-test, p = 0.92). Noticeably, as shown in Figure 2C, the DLMO, as a marker of the individual circadian phase, was significantly earlier in morning-shift dancers (20:33 $\pm$ 00:35) than in night-shift dancers (22:02 $\pm$ 01:06; unpaired Student's t-test, p = 0.005).

We analyzed the influence of MSFsc (proxy of individual chronotype) and L5c (proxy of individual sleep timing) on the circadian phase. MSFsc, which was not different between morning and night shift participants (Table 1), had no predictive power on DLMO overall (p = 0.151), although DLMO and MSFsc were positively associated in night-shift dancers (R = 0.74, p = 0.01) and not correlated in morning-shift dancers (p = 0.32). L5c of training days (L5cw) was different between participants of the morning and night shifts (Table 2) and positively associated with DLMO (R = 0.66, p = 0.003). In contrast, L5c of weekends (L5cf.), which was not different across shifts (Table 2), had no significant predictive power on DLMO (p = 0.154).

The effect of light and physical activity on dancers' circadian phase

Individual DLMO data gave us the opportunity to analyze the effects of light and physical activity on the circadian phase occurring during each participant's phase-sensitive time windows. During the phase-advance time window at the antipode of the DLMO (−13 h to −10 h with respect to DLMO) of the last day of actimetry recordings, light exposure was not different across shifts (morning: 1158.43 $\pm$ 1621.15 lux, night: 1100.98 $\pm$ 1725.22 lux, unpaired Student's t-test, p = 0.944) and did not correlate with DLMO (p = 0.388; Figure 3A). In this same phase-advance time window, the time spent in displaying moderate-vigorous physical activity was also not different across shifts (morning: 64.14 $\pm$ 44.57 min, night: 51.45 $\pm$ 47.43 min, unpaired Student's t test, p = 0.579) and did not correlate with DLMO (p = 0.479; Figure 3B). During the phase-lag time window around the DLMO (−2 h to +1 h with respect to DLMO) on the day before melatonin sampling, which overlapped at least partially with the timing of the night shift in all participants, all dancers were similarly exposed to a low light intensity (morning: 25.61 $\pm$ 37.43 lux, night: 33.53 $\pm$ 20.03 lux, unpaired Student's t test, p = 0.564) that did not correlate with DLMO (p = 0.745; Figure 3C). In this same phase-lag time window, the time spent in displaying moderate-vigorous physical activity was higher in night-shift dancers than in morning shift dancers (morning: 23.24 $\pm$ 15.95 min, night: 75.55 $\pm$ 18.00 min, unpaired Student's t test, p < 0.001) and positively correlated with DLMO (R = 0.65, p = 0.003; Figure 3D), indicating that later DLMO values were associated with more intense exercise during this time window.

DISCUSSION

The ultimate goal of human chronobiology is to comprehensively understand how the biological clock works in normal daily life. Despite this question has been recently approached in epidemiological studies integrating self-reported data and objective measurements (Bowman et al., 2021; Facer-Childs et al., 2020), the multiple pressures of urban life make this task very difficult. One strategy to overcome these difficulties is to find an advantageous model system or conditions resembling a natural chronobiology laboratory. This is the case of the model system presented here, in which a population of young dancers is trained at the national dance school END-SODRE in Uruguay either during an early morning shift or a late night shift. This compelling social demand, which also involves a substantial challenge in terms of physical training, makes this real-life situation an advantageous chronobiological model to evaluate the interplay of photic and non-photic factors influencing the circadian phase. We have previously identified differences in chronotype and self-reported sleep timing between morning-shift and night-shift dancers (Coirolo et al., 2020). In this study, we first confirmed that shift training also involved differences in dancers' endogenous circadian phase as the

Table 3. Timing of the light exposure cycle measured by actimetry records

Type of day	MB10c			LB5c			Acrophase		
	training	free	p[1]	training	free	p[1]	training	free	p[1]
Morning-shift	12:55 (0:10)	13:26 (0:39)	0.128	03:26 (0:17)	05:32 (0:19)	**0.018**	13:07 (0:26)	13:26 (1:08)	0.866
Night-shift	13:41 (0:47)	14:08 (0:42)	0.424	04:30 (0:37)	05:26 (1:10)	0.075	13:52 (0:08)	14:29 (0:24)	**0.008**
p[2]	**0.030**	0.341		**0.030**	0.651		>0.9	0.147	

Circular variables: the midpoint of the more brilliant 10 h period (MB10c), the midpoint of the least brilliant 5 h period (LB5c) and the moment of the day in which the cosinor function reaches its maximum exposure to light (acrophase). Data are presented as median $\pm$ MAD and compared statistically by Wilcoxon signed-rank test (1) and Mann-Whitney U test (2). Statistically significant tests (p < 0.05) are shown in bold.

onset of melatonin nocturnal increase was earlier in morning-shift than in night-shift dancers. We also found that chronotype, sleep patterns, and physical activity also influenced dancers' circadian phase. Finally, and remarkably for an ecological study, we found that in the environmental conditions they are habitually exposed to, the average light levels during either the phase-advance or phase-delay window were not associated with DLMO but the amount of exercise specifically during the phase-delay window was. Based on these findings, it is tempting to speculate that the intense nighttime exercise experienced by the night-shift dancers compared to the morning-shift dancers, rather than the light levels these dancers are exposed to in their daily life, contributes to the difference in DLMO in the two groups.

The consequences of dance training in shifts

The participants in this study belong to a larger population of dancers being trained at END-SODRE in Montevideo, Uruguay, whose circadian preferences, and self-reported sleep patterns have been previously described (Coirolo et al., 2020). For this work, we selected the 18 dancers whose DLMO values were earlier than 24:00, and who had validated actimetry recordings. We consider this subpopulation of dancers to be representative of the larger one given that, although chronotype was not different between shifts as previously reported, there was a strong association between chronotype (MSFsc) and circadian disruption (SJL) as observed in the original population (Coirolo et al., 2020). In addition, the activity-rest patterns obtained via actimetry recordings in this study showed differences between shifts, and differences between training and weekends within shifts, compatible with those reported by means of sleep logs in this sample of participants (Table S1) and similar to what was observed in the original population (Coirolo et al., 2020).

Objective actimetry recordings confirmed the advantages of this clean model system in which the intensity of neither light exposure nor daily physical activity differed across shifts (Tables S2 and S3), whereas their timing did (Tables 2 and 3). Obviously, morning-shift dancers displayed more physical activity than night-shift dancers during the morning of training days; on the other hand, night-shift dancers displayed more physical activity than morning-shift dancers at night. Although the timing was different between shifts, the overall daily physical activity intensity was similar in dancers of both shifts. Interestingly, actimetry indicators of the timing of the activity-rest cycle contributed objective evidence of what shift training involves as social pressure and of how plastic this influence can be. Acrophase, M10c, and L5c were consistently delayed in night-shift dancers with respect to morning-shift dancers on training days, whereas no timing differences were observed across shifts during weekends (Table 2). This pattern is in line with self-reported sleep timing reported here and in a previous study (Coirolo et al., 2020). With respect to light exposure, objective actimetry recordings confirmed that, as expected, all participants were exposed to much higher light during the morning than during the night of training days. In addition, although light acrophase was not different across shifts, the moment of the day in which they were exposed to the most brilliant light (MB10c) and to the least brilliant light (LB5c) during training days was later in night-shift dancers than in morning-shift ones (Table 3). Interestingly, during the time of the night-shift training, all participants were exposed to very dim light (below 35 lux in average), but significantly higher in night-shift dancers than in morning-shift ones.

As previously reported in shift workers (Cheng et al., 2017; Razavi et al., 2019), dance training in shifts were associated to significant differences in dancers' endogenous circadian phase as measured by DLMO, which has been recognized as its best proxy (Lewy, 1999; Lewy and Sack, 1989). Although morning and night-shift dancers were similar in age and were submitted to the same physical challenge, the DLMO of night-shift dancers was significantly later than the DLMO of morning-shift dancers (Figure 2). This robust
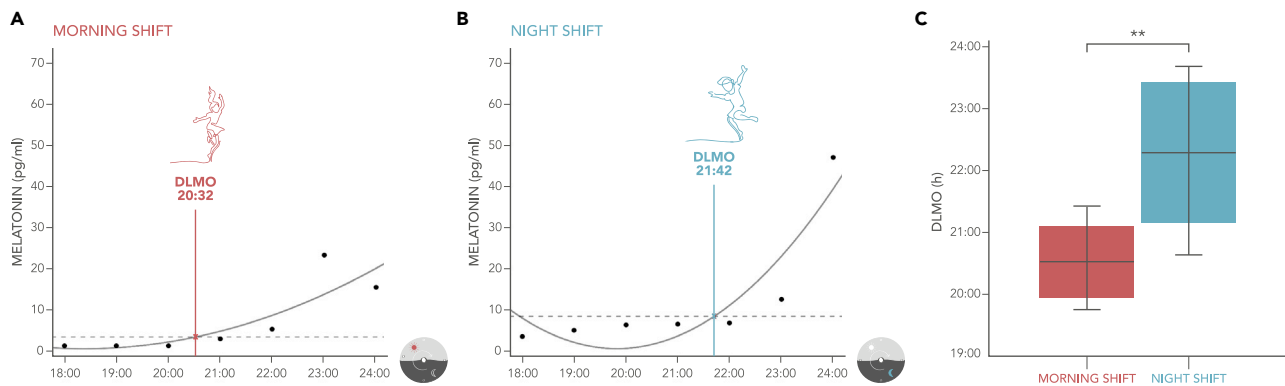
Figure 2. Dancers' circadian phase

(A–C) The absolute melatonin levels from 18:00 to 24:00 are plotted for 2 representative participants attending either the morning (A) or the night shift (B). Data were fitted to a quadratic curve (solid line). Dashed line indicates the individual melatonin onset threshold. Dim light melatonin onset (DLMO) is estimated as the time of the intersection of both lines. The DLMO of the morning-shift dancers was significantly earlier than the night-shift dancers (C) Data are presented as boxplots, (Student's *t*-test p = 0.005).

See also Figure S2.

result reinforces the advantages of this real-life model system, in which social demands (training shifts) can be translated into measurable significant differences of an objective physiological circadian trait such as the DLMO. We probably underestimated the strength of this socially induced difference in dancers' circadian phase between shifts as two night-shift dancers were excluded from the analysis given their melatonin levels never increased within the sampling time window likely because their DLMO occurred after midnight. In line with previously reported data on the enormous plasticity of DLMO in response to sudden changes in light exposure (Silva et al., 2019; Stothard et al., 2017; Wright et al., 2013; Zerbini et al., 2021) and given the lack of changes between shifts in the activity-rest patterns and in light exposure during weekends, we speculate that the circadian phase would not be different between morning-shift dancers and night-shift dancers if we had measured DLMO on Sunday night.

Light exposure is not associated with the circadian phase

Light is the universal circadian zeitgeber, whose direct actions on the mammalian hypothalamic master clock are well documented (Meijer et al., 1998; Prayag et al., 2019; Rea et al., 2021). Regarding the influence of light on DLMO, two sensitive windows are recognized: a) the phase-advance window, located in the morning (2–3 h after awakening or sunrise) (Hashimoto et al., 1997; Khalsa et al., 2003; Kozaki et al., 2016; Takasu et al., 2006), and the phase-lag window, located around sunset (Akacem et al., 2018; Gooley et al., 2011; Silva et al., 2019). Social, environmental, behavioral, and genetic influences shape the DLMO resulting in ample differences among individuals. In particular, there is a marked individual variation in the sensitivity to light, specifically at the phase-lag window (Akacem et al., 2018; Phillips et al., 2019). Therefore, these sensitive time windows, traditionally described in the phase response curve of melatonin to light (Lewy et al., 1998), must be individually adapted to adjust the day to schedules based on DLMO and not on clock or solar time (Phillips et al., 2019; Thomas et al., 2020). In this study, we evaluated the effect of light exposure in both the phase-advance and the phase-lag windows adjusted for all participants with respect to their DLMO. As expected, light in the phase-advance window was approximately 100 times higher than in the phase-lag window but not different between morning-shift dancers and night-shift dancers in either time window. Interestingly, in neither of the two phase-sensitive time windows did the light have predictive power on DLMO (Figure 3). This lack of the effect of light on the circadian phase in these sensitive time windows is a remarkable negative result, which is difficult to interpret. Given the wide range of DLMO values among participants (20:00–23:40), the time of the day in which the phase-advance window occurred for each participant, and thus the intensity of light that participants were exposed to were also very different (0–6000 lux). For late-DLMO participants, who of course corresponded to night-shift dancers, some of them were asleep during this time window (light exposure ≈ 0 lux) and missed the chance to receive the phase-advanced influence of light. However, two of the latest participants were exposed to light above 1000 lux during their phase-advance window. In contrast, during the phase-lag window, all participants were exposed to very dim light (0–80 lux) that may not have reached the detection threshold of circadian
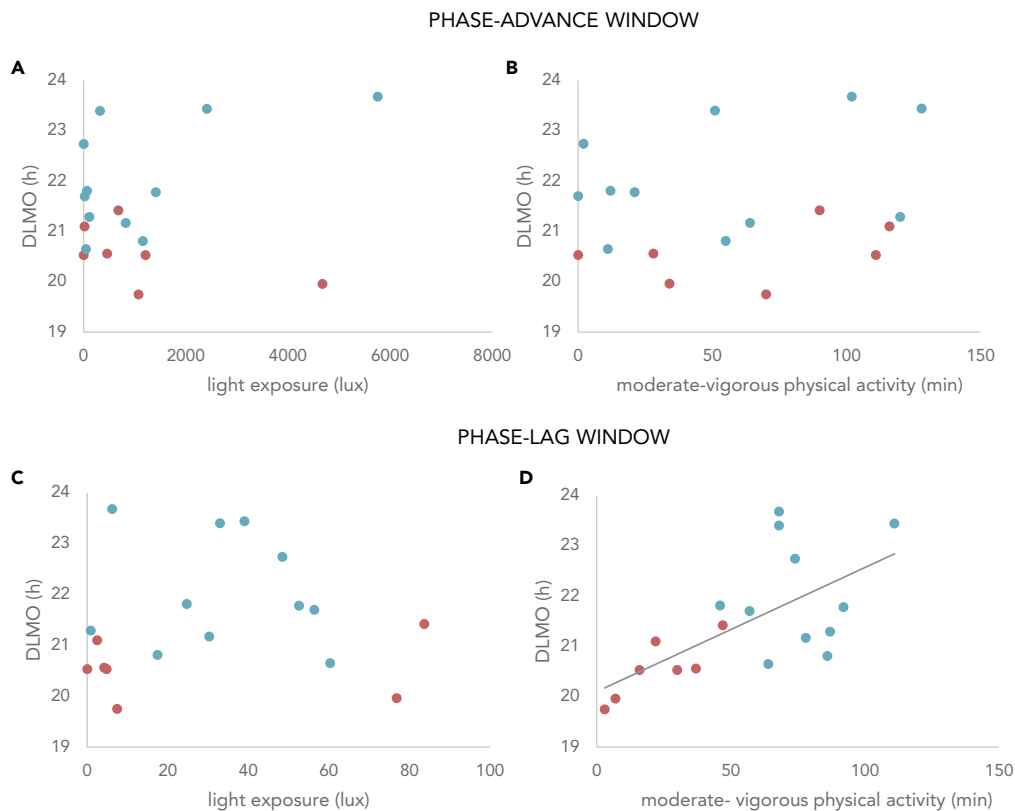
PHASE-ADVANCE WINDOW

PHASE-LAG WINDOW

Figure 3. The effect of light and physical activity on dancers' circadian phase

(A–D) During the individual phase-advance window around the antipode of DLMO, the DLMO showed no correlation (p = 0.388) with light exposure measured in lux (A) nor with the time spent in moderate-vigorous physical activity measured in min (B). During the individual phase-lag window around the DLMO, the DLMO showed no correlation (p = 0.745) with light exposure measured in lux (C) but was significantly associated (R = 0.65, p = 0.003) with the time spent in moderate-vigorous physical activity measured in min (D). Red and blue dots correspond to morning-shift dancers and to night-shift dancers, respectively.

photoreceptors to exert its delaying effect (Duffy and Czeisler, 2009; Duffy and Wright, 2005; Hopkins et al., 2017; Wright et al., 2013).

Chronotype and sleep patterns are associated with the circadian phase

Strong and numerous studies support the association of individual circadian phase and chronotype, showing that morning oriented people have earlier DLMO than evening oriented ones (Adan et al., 2012; Burgess and Eastman, 2005; Kantermann et al., 2015; Kitamura et al., 2014). In this study, only night-shift dancers showed the expected correlation between chronotype and circadian phase. The absence of association between MSFsc and DLMO in morning-shift dancers is likely because of the low number of participants in this group. However, these results also allow us to speculate on the reasons that prevented the circadian phase of morning-shift dancers to behave as expected. The biological clock of these dancers is extremely socially oriented as confirmed by the strong effect of the training shift on SJL and circadian phase. Dancers attending the morning shift exhibit an earlier DLMO and a stronger SJL than night-shift dancers. We can thus speculate that individual chronotype is less important than the huge circadian misalignment these morning-shift dancers suffer to impact on their DLMO. On the other hand, in night shift dancers, who are less disrupted, the expected effect of chronotype on DLMO emerges (Taillard et al., 2021). A bidirectional association between individual circadian phase and sleep timing has been extensively documented (Papatsimpa et al., 2021; Prayag et al., 2019). We used the actimetry parameter L5c (the midpoint of the least active 5 h period) as a reliable proxy of sleep timing in both training days (L5cw) and weekends (L5cf.; (Mitchell et al., 2017). Because we measured melatonin levels at the end of one training day and therefore estimated the individual circadian phase in the middle of the training

week, we confirmed the expected strong predictive power of L5cw on DLMO. In other words, sleep timing on training days was strongly associated with the individual circadian phase, whereas sleep timing on weekends was not. Zerbini et al. (2021) have recently shown changes in DLMO between work and free days associated with changes in light exposure and secondary to changes in sleep patterns. In this study, the sleep of both morning and night-shift dancers was delayed on weekends with respect to training days resulting in no differences across shifts in sleep timing during weekends (Tables 2 and S1). These results suggest that morning-shift dancers are probably delaying their circadian phase between training days and weekends in response to the important changes in their activity-rest patterns.

Physical activity is associated with the circadian phase

Besides photic entrainment, other external stimuli, including food intake, temperature, arousal, stress, and exercise can adjust the period of the internal circadian clock acting on the central master clock or through peripheral oscillators (Bass and Takahashi, 2010; Hamaguchi et al., 2015; Schroeder et al., 2012; Tahara et al., 2017; Wolff and Esser, 2012). There is abundant evidence of the effects of exercise phase-shifting on commonly measured proxies of the circadian rhythm in humans (Lewis et al., 2018) including the onset of melatonin nocturnal increase (Baehr et al., 2003; Barger et al., 2004; Buxton et al., 2003; Miyazaki et al., 2001; Thomas et al., 2020). This modulation of the circadian phase by exercise depends on the time of day or internal time of stimulus application and additively affects the potent photic entrainment (Baehr et al., 2016; Thomas et al., 2020; Youngstedt et al., 2019). Although light-independent actions of exercise as circadian entrainers have been demonstrated in experimental controlled conditions (Barger et al., 2004; Buxton et al., 2003; Youngstedt et al., 2019), it has been very difficult to test by separate physical activity and light in real life situations. In a seminatural experiment (Thomas et al., 2020), showed that evening exercise intervention had either a phase-delay effect in early chronotypes or a phase-advance effect in late chronotypes, but they were unable to confirm whether these effects were independent of light associated phase-shifts. We also did not experimentally control light exposure in the present ecological study, but we were able to identify that during both the phase-advance and the phase-lag windows light had no predictive power on the circadian phase. In contrast, the time spent in moderate-vigorous physical activity around individual DLMO (but not around its antipodes) was positively correlated with DLMO (Figure 3). The significantly higher light exposure recorded during the night shift in night-shift dancers with respect to morning-shift dancers may be a factor that contributes to delaying the circadian phase of night-shift dancers with respect to morning-shift ones. It has been documented that even dim lights with intensities around 50 lux can induce phase shifts in sensitive individuals (Philips et al., 2019). However, we did not find this phase-delay light effect when we restricted the analysis to the moment of the individual phase-delay time window (Figure 3). In this real-life situation, we thus confirmed that intense physical training at night delays the circadian phase in dancers who already have a late chronotype. In other words, the amount of physical activity displayed during the phase-lag window, which was higher in night-shift dancers, was correlated to later dancers' circadian phase, whereas light exposure was not.

Conclusion

We currently understand that multiple dynamic interactions between photic and non-photic stimuli are important for the daily adjustment of the circadian phase. Thus, we argue that natural models are needed to test chronobiological hypotheses in realistic scenarios. This advantageous model system of dancers who are trained in two extreme shifts allowed us to address this question employing a comprehensive approach that includes the interplay of natural environmental, social, and behavioral cues that influence the circadian phase. We showed that dance training at night, along with late chronotypes, late sleep, and late intense physical activity were associated with late circadian phase. Moreover, this natural model system offered us the opportunity to speculate on a distinctive impact of light and physical activity on delaying the circadian phase. During each participant's phase-lag window, the time spent in intense physical activity was longer in night-shift dancers than in morning-shift dancers and positively correlated with the circadian phase, whereas light exposure was similar between shifts and did not correlate with the circadian phase. Even though photic stimuli are the most potent entrainers of the circadian clock, we present a clear-cut example of the phase-delay effect of exercise in real-life conditions in a natural model system in which environmental light is not associated with the modulation of the circadian phase.

Limitations of the study

We recognize several limitations in our work mostly related to the fact that it is an ecological study. First, as the participants belong to a very specific group of dancers, we cannot easily extrapolate our results to the

general population. Second, given that we designed the study to examine dancers under free-living conditions, we did not control for other stimuli that could affect the internal circadian phase (food intake, stimulant substance use, stress, etc.). In particular, it would have been important to assess the effect of the timing of food intake, which is most likely different between shifts. Third, our comprehensive approach included several instruments and methodologies, resulting in a small number of participants having validated data from all of them at the same time, particularly those in the morning shift. Therefore, the small sample size prevented a global statistical analysis and forced us to analyze each variable separately. Fourth, given that this is not a longitudinal study, we cannot discount that the differences in phase might have existed before the dance training, because baseline measurements were not taken. Finally, the analysis of the impact of light and physical activity during the phase-advance and phase-lag windows is a reliable way to assess circadian timekeeping. However, we were only able to reach a consistent interpretation with the data obtained during the phase-lag window around the DLMO, in which all participants were subjected to dim lighting but had a training-shift-dependent physical activity. In contrast to the phase-lag window, during the phase-advance window, light exposure and physical activity were highly variable and non-shift-dependent. This prevented us from reaching any conclusion about the interplay of light and exercise in changing the circadian phase when acting on the phase-advance window.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

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AUTHOR CONTRIBUTIONS

Conceptualization, A.S., B.T., and N.C.; Methodology, N.C., C.C., B.T., and A.S.; Investigation, N.C., C.C., and B.T.; Writing – Original Draft, A.S., N.C., and B.T.; Writing – Review & Editing, A.S., N.C., C.C., and B.T.; Funding Acquisition, B.T. and A.S.; Resources, B.T. and A.S.; Supervision, A.S. and B.T.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Biological samples		
Human saliva samples	This paper	N/A
Critical commercial assays		
Salivary melatonin ELISA kit	Salimetrics™	#1-3402
Software and algorithms		
GENEActiv Windows Software	Activinsights Ltd, Cambridge, UK	version 3.2 https://www.activinsights.com/
Actigraphy integrated software El Temps	© Antoni Díez-Noguera, Barcelona, CA, Spain Diez Noguera, 2007	http://www.el-temps.com/
Other		
Actimeters	Activinsights Ltd, Cambridge, UK	GENEactiv Original accelerometers

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources, data, and codes should be directed to and will be fulfilled by the lead contact, Ana Silva (asilva@fcien.edu.uy).

Materials availability

This study did not generate new unique reagents.

Data and code availability

All data reported in this paper will be shared by the lead contact upon request. Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Eighteen dancers (1 male) from the Uruguayan public school for professional training in contemporary and folkloric dance (Escuelas de Formación Artística, END-SODRE, Ministerio de Educación y Cultura, Uruguay) participated in this study carried out in August 2019 (winter in the southern hemisphere, Table 1). To maximize school infrastructure usage, the END-SODRE is organized as a 4-year training program with classes taught from Monday through Friday in two shifts. First and second grade students attend the night shift (20:00 to 24:00) while students of the third and fourth grade attend the morning shift (8:30 to 12:30) (Coirolo et al., 2020). Therefore, dance students do not select their training shift and there is no institutional bias in the allocation of dance students to their respective training shift. Each training year begins in March and ends in December. We studied 18 dancers attending either the morning (n = 7) or the night shift (n = 11) in mid-August 2019 (in the middle of the training year); i.e., in a period in which each participant was already acclimated to the timing of her/his training shift. The 18 participants of this study are a subset of the sample presented in a previous study (Coirolo et al., 2020), in which the chronobiological characterization of 56 dancers (29 of the morning shift and 27 of the night shift) was presented. We used 18 (out of 56 dancers) in the present study with asymmetric distribution between shifts (7 participants of the morning shift versus 11 participants of the night shift) because only these 18 dancers had validated melatonin measurements and actimetry data in addition to the previously self-reported data (Coirolo et al., 2020).

All participants gave written informed consent. This study was evaluated by the Ethics Committee of the School of Psychology, Universidad de la República, and complied with the principles outlined by the Declaration of Helsinki (World Medical Association, 2013).

METHOD DETAILS

Chronobiological characterization

The chronobiological characterization was assessed using the Spanish version of the Munich Chronotype Questionnaire (MCTQ, (Roenneberg et al., 2019, 2003). Validated MCTQ reports were used to assess the mid-sleep point on free days corrected for sleep debt on workdays (MSFsc) as a proxy of individual chronotype (Roenneberg et al., 2004), and the social jetlag (SJL) as the absolute difference between the mid-points of sleep on training and weekend days (Wittmann et al., 2006).

Sleep patterns

Participants were also instructed to answer daily WhatsApp messages every morning for the 18 days of actimetry recordings (see below) to record their daily sleep timing following Coirolo et al. (2020). Sleep logs allowed us to estimate the average individual midsleep point of training (MSW) and weekend days (MSF), and to calculate the average sleep duration for training days (SDw), weekends (SDf) and the average of weekly sleep duration per day (AVSD).

Melatonin measurements

Hourly saliva samples (1–2 mL, 18:00–24:00) were collected in dim light (<30 lux) on the night after the last day of the actimetry recording (Wednesday, August 28, 2019). The melatonin-sampling day was a normal training day for morning-shift dancers while night-shift dancers skipped training that day to adhere to the saliva sampling protocol. Participants remained in resting position except for brief trips to the also dark bathroom and were not allowed to use light-emitting devices during the sampling period. They rinsed their mouth with water before each sample and received a light meal between the 20:00 and 21:00 and between the 22:00 and 23:00 samples. Saliva samples were frozen, stored at −80°C, and later assayed for melatonin using the salivary melatonin competitive ELISA kit from Salimetrics™ (#1-3402) following the manufacturer's instructions. The tests were carried out by the same technician, at the same time during 3 consecutive days, and the morning shift and night shift samples were combined in each ELISA plate. The concentration-response curves obtained were indistinguishable from those reported by the manufacturer. The same applied to the IC10 values (the melatonin concentrations inhibiting 10% of the absorbance signal observed in the absence of melatonin), taken as an indication of assay sensitivity. This validates the use of the analytical sensitivity value reported by the manufacturer (1.37 pg/mL melatonin).

Circadian timing was determined by calculating the DLMO as marker of the individual circadian phase; i.e., as proxy of the individual circadian phase that indicates the beginning of the internal biological night (Lewy et al., 1999). Following Silva et al. (2019), individual basal melatonin level was calculated as the average of the melatonin levels measured before the nocturnal increase. Individual DLMO onset was calculated in R Statistical Software (RStudio Team, 2017) as the interpolated point in time at which the quadratic fit surpassed 2 standard deviations above the individual basal melatonin level and this value was considered as the individual threshold of the nocturnal melatonin increase (dashed line in Figures 1A and S2). This study includes data of the 18 participants whose melatonin curve adjusted to the quadratic fit with R > 0.45 and whose DLMO were thus earlier than 24:00.

Actimetry

Participants' activity-rest pattern as well as their light exposure pattern were assessed from August 10 08:00 to August 27 23:59, 2019 including training days (12 days for 15 dancers and 10 days for 3 dancers) and weekend days (6 days for all dancers) using wrist-worn GENEactiv Original accelerometers (Activinsights Ltd., Cambridge, UK). All devices were configured on the same computer and set to record at 10 Hz with the same starting clock time. Light and activity data were exported and converted to 1-min epochs using GENEActiv Windows Software from Activinsights (version 3.2). Light exposure was expressed in lux. For physical activity, the raw 10-Hz triaxial data were first summarized into the gravity-subtracted sum of vector magnitudes for each 1-min epoch intervals. The resulting units for this outcome variable are $g \cdot min$ (Esliger et al., 2011; Phillips et al., 2013). We then calculated the average gravity-subtracted signal vector magnitude for each minute, thus converting physical activity values from a time-dependent unit ($g \cdot min$) to the time-independent milligravitational units (mg) in order to compare with previously reported cut-points (moderate-vigorous physical activity above 100 mg; (Goldin et al., 2020; Hildebrand et al., 2014; Okely et al., 2018). We analyzed actigraphy data with the integrated software El Temps (© Antoni Díez-Noguera, Barcelona, CA, Spain) (Diez Noguera, 2007). We obtained individual actograms for the

whole recording period (Figures 1 and S1). We also performed cosinor and non-parametric analyses on physical activity (Tables 2 and S2) and light exposure (Tables 3 and S3). These analyses yielded several variables, including the average activity of the most active 10 h period (M10), the average activity of the least active 5 h period (L5), the average light exposure of the most brilliant 10 h period (MB10), and the average light exposure of the least brilliant 5 h period (LB5) (McGowan and Coogan, 2018; Van Someren et al., 1999). We also measured the relative amplitude as M10-L5/M10+L5 (Lyall et al., 2018; Van Someren et al., 1999). We estimated circular variables from physical activity and light actimetry data, including acrophase (the moment of the day in which the cosinor function of physical activity/light exposure reaches its maximum); M10c (the midpoint of the most active 10 h period); L5c (the midpoint of the least active 5 h period); MB10c (the midpoint of the most brilliant 10 h period); and LB5c (the midpoint of the least brilliant 5 h period) (McGowan and Coogan, 2018; Van Someren et al., 1999). In addition, we analyzed by separate the actimetry recordings of the training days and weekends to calculate L5cw and L5cf.

We calculated the average of both the physical activity and the light exposure for each participant during the occurrence of the morning (08:30–12:30) and night (20:00–24:00) training shifts from actimetry data of the 12 recorded training days. We also calculated the average of light exposure and the time spent in moderate-vigorous physical activity for each participant during the 3h-phase-delay window around individual DLMO from 2 h before to 1 h after the DLMO and during the 3h-phase-advance window around individual DLMO antipode from 13 h to 10 h before the DLMO of the last day of recording before saliva samples collection (Khalsa et al., 2003; Silva et al., 2019).

QUANTIFICATION AND STATISTICAL ANALYSIS

Data were globally analyzed with no distinction among genders. Data are expressed as mean values ± standard deviation throughout the text except otherwise stated. Unpaired Student's t-test was used for comparisons between shifts of variables that complied with normality and homoscedasticity criteria (MCTQ data, DLMO, physical activity, and light recordings). When data did not comply with normality and/or homoscedasticity, statistical comparisons were analyzed by non-parametric tests: the Wilcoxon signed-rank test for comparisons between work and free days in the same individuals, the Mann–Whitney U test for comparisons across participants between shifts (actimetry indexes). Associations between MCTQ parameters and DLMO and its determinants were assessed by Pearson correlations. Statistical procedures were carried out using the software PAST (Hammer et al., 2001). Values of $p < 0.05$ were considered statistically significant throughout.