The recognition of behaviorally distinct sleep stages in *Drosophila melanogaster* uncovers previously obscured homeostatic and circadian control of sleep.

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Abstract

Understanding the mechanisms underlying homeostatic sleep regulation is a central unmet goal of sleep science. Our understanding of such regulation in mammals has required recognizing distinct sleep stages. *Drosophila melanogaster* is an important genetic model system for understanding sleep. Since the discovery of sleep-like states in the fly 25 years ago, the field has treated sleep as a unitary state consisting of any inactivity lasting 5 minutes or longer, despite convergent work suggesting the existence of multiple sleep stages. Here, we establish that three distinct sleep stages in flies can be classified based on simple inactivity duration criteria. We show that the daily initiation of these sleep stages is temporally distinct, with long sleep occurring immediately following the largest daily period of wakefulness. We show that rebound in response to mechanical sleep deprivation is present only in long sleep and comes at the expense of shorter sleep stages. Deprivation-induced decreases in shorter sleep stages obscure homeostatic sleep rebound when sleep is measured using traditional methods. We observe distinctly timed ultradian oscillations of fly sleep stages, reminiscent of mammalian sleep cycles. Our results indicate that the field's unitary definition of fly sleep has outlived its usefulness and call for the re-examination of sleep regulation in the fly using methods that reflect the unique relationships among sleep stages and their homeostatic and circadian control. The recognition of these sleep stages will be necessary to fully realize the promise of the *Drosophila* model system for identifying conserved genetic mechanisms underlying such regulation.

Keywords: Drosophila, activity, sleep, DAM, shiny app, phase, circular statistics, somnograms, actograms, periodograms, hypnograms, sleep stages.

Significance

The fact that sleep episodes comprise distinct stages in humans and mammalian model systems has been a fundamental discovery in sleep science. This recognition enabled researchers to study the physiological underpinnings of sleep stages and examine the comorbidities and consequences associated with disrupted sleep. The mechanisms underlying sleep homeostasis remain an open problem, and the fruit-fly *Drosophila melanogaster* continues to be a powerful genetic and neurobiological model to study sleep regulation. Despite recent convergent evidence for distinct sleep stages in the fly, the field continues to treat sleep as a unitary state consisting of any inactivity lasting five minutes or longer. This practice likely obscures important aspects of sleep regulation in the fly, limiting its impact on our understanding of sleep regulation. Inspired by recently developed wearable technologies, which score sleep stages based on motion detection and accelerometry, we asked if distinct fly sleep stages can be identified based on durations of inactivity bouts, and, if so, whether stages of fly sleep are differentially regulated by the two major processes governing sleep: sleep homeostasis and circadian timekeeping. We show that discrete sleep stages can be identified and have distinct relationships amongst each other and to homeostatic sleep regulatory and circadian control centers. Importantly, we find that sleep deprivation produces increases in longer, deeper sleep stages while producing decreases in shorter sleep stages. These opposing responses to deprivation obscure homeostatic sleep responses when the traditional, unitary definition of sleep is used, a reality that has likely hampered the identification of homeostatic regulatory mechanisms in the fly. We concluded that using the simple behavioral criterion we describe here to differentiate sleep stages will be critical for understanding homeostatic and circadian sleep regulation in *Drosophila*, which remains an important model organism for the discovery of mechanisms of sleep regulation.

Introduction

With every rotation of the earth, we cycle from the "*unrecorded fantasies of solitary dreaming, to the collective fantasy of daily social and commercial life*," as Arthur Winfree describes the mysterious and everlasting process of rhythms in sleep and wakefulness (1). We spend about a third of our lives sleeping, about 8 hours a day. Is all this sleep functionally the same? Indeed not. Early research in humans and other mammalian models recognized the existence of distinct stages of sleep during a full night's sleep (2). Studies of mammalian sleep have made significant progress in understanding the physiological basis, functions, and consequences of sleep, progress that has depended critically on the identification and recognition of such sleep stages (3). This is predominantly because sleep stages have distinct relationships to homeostatic and circadian regulation and correspond to different physiological states (4–12). However, despite the identification of sleep stages in humans and other mammals, the basic biology of the sleep homeostasis remains elusive (13). In this context, the fruit fly is a promising genetic model to probe the anatomical, molecular, cellular, neural, and physiological basis of sleep homeostasis and its circadian regulation. Despite recent convergent work indicating the presence of distinct sleep stages in the fly (14–22), sleep in this species has been treated as a unitary state, and recent work from our lab suggests that a failure to recognize sleep states obscures homeostatic sleep regulation (21, 22).

The fruit fly, *Drosophila melanogaster,* sleeps 70-80% of its life (22, 23). This estimate is based on the well-established 25-year-old behavioral correlate of fly sleep being any bout of inactivity that lasts longer than five consecutive minutes (23, 24). For the rest of this manuscript, we will refer to this definition of sleep as "standard" sleep. Previous work by independent groups has suggested that not all sleep is the same in the fly (14–22). About a decade ago, physiological evidence suggested that flies may have distinct stages of sleep (17). Specifically, the power of local field potentials recorded from fly brains fell significantly after the fly had been inactive for 5 minutes, with periods of inactivity beyond 5 minutes associated with even lower powers (17). A subsequent study showed that metabolic rate is significantly reduced under extended periods of sleep, providing evidence for a deep sleep stage characterized by low metabolism in the fly (15). More recently, studies have shown stereotypical proboscis extension and retraction as a marker of

a discrete sleep stage (18) and studies have demonstrated that the number of active neurons in the brain declines with the duration of sleep (19, 20).

Studies using statistical and computational approaches have also provided strong evidence in support of flies sleeping in stages (14, 16). For example, one study examined the dependence of sleep bout length on its likelihood of transitioning from sleep to wake and found that sleep may occur in at least two stages (16). The other used Hidden Markov Models to characterize distinct sleep states in flies using behavioral activity data (14). This study identified four stages of fly sleep and wake. In addition to wakefulness, the authors identified "early wake", "light sleep", and "deep sleep" states (14).

Based on the work of Daan and Borbély (11, 12, 25), we recently modeled *Drosophila* sleep using a two-process model of sleep regulation (22). The two-process model posits that a homeostatic process (process S; reflecting the pressure to sleep) builds while animals are awake and falls while they sleep. The switch between wake and sleep happens when process S hits an upper threshold, and the switch between sleep and wake happens when process S hits a lower threshold. The circadian clock regulates these thresholds, and the oscillating thresholds are called process C (11, 12, 25, 26). We showed that this model captures the relationship between the circadian clock's internal speed (free-running period) and total amount of standard sleep (22). This relationship was also well captured by the model when we biased our analyses to include only longer episodes of sleep, which suggested that longer bouts of sleep were likely a better reflection of homeostatic sleep regulation than shorter bouts of sleep (22).

In another recent study, we showed that only when long bouts of sleep were analyzed were we able to capture statistically significant and biologically meaningful homeostatic rebound in response to mechanical sleep deprivation in male flies (21). When we deprived flies only of long bouts of sleep, there was a significant rebound in long sleep alone. Remarkably, when flies were deprived only of long sleep, the time course of standard sleep looked no different from that of their stimulus-matched controls. When using the standard definition of sleep, the complete loss of long-duration sleep was not detectable during the deprivation day. This result is consistent with the idea that long sleep is a more sensitive behavioral marker of homeostatic sleep regulation. It also

suggests that changes in briefer sleep states can obscure homeostatic processes if not accounted for. A critical takeaway from this study was that treating sleep by the standard unitary criterion of five minutes or more of inactivity obscured homeostatic sleep regulation, and that this challenge can be easily overcome by recognizing a distinct, longer (and presumably deeper) sleep stage (21).

These convergent studies prompted us to re-examine the behavioral criterion for sleep and ask if simple behavioral criteria can identify distinct sleep stages in the fly. While powerful and accurate, technical challenges are associated with the physiological and analytical observations previously used to identify deep sleep states. For instance, the physiological measurements are logistically challenging. Very few laboratories can use these systems to routinely gauge sleep stages in flies, and those that do cannot use such methods for high-throughput analyses. On the other hand, complex statistical tools such as distribution fitting and analytical tools such as Hidden Markov Models are computationally intensive, time-consuming, and require programming skills that are likely to deter widespread use. We therefore reasoned that the field would benefit if simple behavioral criteria could be used to identify fly sleep stages.

In this study, we provisionally identified sleep stages based on simple inactivity criteria and showed that they strongly resemble those identified using Hidden Markov Models. We then sought to determine if these stages displayed the distinct homeostatic and circadian regulation expected of unique behavioral states. Our results established that failing to recognize sleep stages in the fly obscures essential features of homeostatic and circadian sleep regulation. Thus, a full understanding of the molecular, genetic, and neuronal basis of sleep regulation will require the recognition of distinct sleep stages in the fly, as it has in mammals. We conclude that the treatment of fly sleep as a unitary state has outlived its utility. The recognition of sleep states will be necessary to fully realize the fly's promise to reveal conserved mechanisms of homeostatic sleep regulation.

Results

Bout duration-based classification reveals distinct sleep stages in Drosophila.

Given previous work indicating the existence of distinct sleep stages in *Drosophila* (14–20) and the association of longer bouts of inactivity with a deeper sleep state with reduced metabolism (15), we asked if we could define distinct sleep stages based on simple inactivity duration criteria. We first considered the work of Stahl et al. (15)(Fig. 1A), which examined the relationship between sleep bout duration and metabolic rate. The authors reported a statistically significant reduction in metabolic rate, compared to wakefulness, at ~35 minutes into a bout of inactivity, suggesting the initiation of a deeper sleep state. We compared the time course of this metabolic change to studies of local field potential and neuronal activity in the brain in relation to the duration of inactivity (17, 20). These revealed a shift in physiological parameters ~20 to 30 minutes after initiating a sleep bout. Taking these findings together, we reasoned that ~30-min of inactivity might represent a cut-off between a lighter and deeper sleep stage (Fig. 1A). Stahl et al. (15)(Fig. 1A) also reported that the metabolic rate continues to decline beyond 35-min of inactivity, with significant decreases in metabolism continuing until ~50-65 minutes after sleep initiation (15)(Fig. 1A). Based on this, we set a second cut-off at ~60 minutes of inactivity that might mark the initiation of an even deeper sleep state (Fig. 1A).

Using these inactivity duration criteria, we provisionally defined three states of sleep, i.e., "short" sleep: 5-30 minutes inactivity, "intermediate" sleep: 30-60 minutes inactivity, and "long" sleep: > 60 minutes inactivity. But do these represent distinct states of fly sleep? To address this question, we compared the daily waveforms of these three stages with a recent study by Wiggin and colleagues that used Hidden Markov Models to identify four distinct sleep/wake states (14). First, we compared the waveforms of our provisional sleep states under Light/Dark (LD) cycles (Fig. 1B-left) with those of Wiggin et. al., whose observations were made under LD conditions (Fig. 1B-middle). There was a remarkable similarity between the waveforms of our provisional sleep states and those inferred by the Hidden Markov Model. Short sleep displayed two small peaks during the daytime much like the two peaks in early wake inferred by the Hidden Markov Model (Fig. 1B). Intermediate sleep was also high during the daytime, much like the light sleep stage of the Hidden Markov Model (Fig. 1B). Both short and intermediate sleep stages were low during the early part of the night and slowly increased in anticipation of dawn, as was the case in early

wake and light sleep states of the Hidden Markov Model (Fig. 1B). Long sleep, as defined here, and deep sleep of the Hidden Markov Model also showed remarkable similarities. They were both high immediately after the onset of darkness and gradually ramped down in anticipation of dawn (Fig. 1B). However, there were small differences in the levels of daytime long sleep and deep sleep (compare Fig. 1B-left and middle). While the Hidden Markov Model revealed low daytime and high nighttime levels of deep sleep, we found long sleep to be high during both day and night times. Such differences may be caused by differences in light intensities between our studies, which may cause different levels of long/deep sleep, as suggested previously (27). Because sleep rhythms are endogenous, we asked if the provisionally defined sleep stages had similar waveforms under constant darkness (DD; Fig. 1B-right). We found that the waveforms of the sleep stages were largely similar to those observed under LD cycles. Interestingly, the long sleep waveform resembled the deep sleep stage inferred by the HMMs more closely under DD than under LD, suggesting that levels of sleep stages may be differentially sensitive to the light environment.

We take these similarities as strong convergent evidence for multiple sleep stages in the fly. Notably, the temporal waveforms of sleep stages being largely similar under constant darkness and LD cycles indicated their endogenous nature. The distinct temporal waveforms of sleep stages suggest that these stages have different relationships to the major bout of wakefulness and one another.

The probability of initiation of long sleep is highest immediately following the largest bout of wakefulness.

Our recent work indicated that long sleep is a more sensitive reflection of homeostatic sleep than briefer stages of sleep (21, 22). If true, this would predict that long sleep will be more likely than short and intermediate sleep stages to occur immediately following the day's largest bouts of wakefulness. This expectation of long sleep having an intimate temporal relationship with locomotor activity is also based on mammalian sleep studies establishing that different stages of sleep have distinct relationships to the sleep homeostat (11, 12).

To test this prediction, we analyzed temporal patterns of the three sleep stages under various environmental conditions and compared these to the standard sleep measurements traditionally

employed by the field. We examined patterns of locomotor activity and sleep and the probability of initiation of sleep bouts of each of our provisional stages under (i) LD12:12, (ii) DD, and (iii) UV- and blue-blocked ramped light cycles consisting of 12-h of continuously increasing light intensity followed by 12-h of continuously decreasing light intensity. Most previous work on fly sleep has employed LD cycles. We examined sleep under DD to observe endogenous rhythms in the three sleep stages, free from the acute effects of LD cycles on sleep. In addition, we examined rhythms in these sleep stages under a light entrainment condition that does not produce daily startle responses, to study the entrained patterns of sleep that are not disrupted by light-induced masking effects or arousal. We chose to use UV- and Blue-blocked light cycles based on our recent work establishing that wildtype flies entrain robustly and have no startle responses of locomotor activity to any transition in light cues under these conditions (28) and because flies actively avoid blue light during most of the day (29).

If the homeostat regulates a particular stage of sleep, we expect that stage of sleep to immediately follow the major periods of wakefulness within a diurnal or circadian cycle. We estimated the proportion of short, intermediate, and long sleep stages initiated within every 1-h window across the day (see Methods). We refer to this metric as the probability of initiation of sleep bouts. Under LD cycles, we found that the probability of initiation of standard sleep was not significantly elevated following the most prominent daily peak of wakefulness (i.e., the evening peak of activity; Fig. 2A; Tables 1 and 5). Thus, when measured using the standard unitary definition of sleep, it lacked a defining hallmark of homeostatically controlled rest. The probability of initiating short sleep was highest during evening anticipatory activity, which was higher than the probability of initiating short sleep following the evening activity peak (Fig. 2A; Tables 2 and 5). Intermediate sleep initiation was most likely to occur in the hours preceding dawn and was not strongly rhythmic (Fig. 2A; Table 3). Moreover, the probability of initiating intermediate sleep was not different before and after the evening peak of activity (Fig. 2A; Table 5). In stark contrast, the probability of initiating long sleep was highest in the hour immediately following the evening peak of activity and was significantly higher than the probability of entering long sleep in the hours before the evening peak (Fig. 2A; Tables 4 and 5). The probability of entering all three stages of sleep displayed small increases following the relatively brief daily morning peak of activity (Fig. 2A; Tables 1, 2, 3, and 5). Might a homeostat engage all three stages of sleep during morning

wakefulness, or are the small peaks in initiation probabilities following the morning peak the result of light-mediated startle effects? Is the daily peak in the probability of long sleep initiation simply a result of the rapid transition to darkness? To test this, we examined the patterns of initiation likelihood of these sleep stages under DD and UV- and Blue-blocked light cycles, which do not produce the large, light-induced startle responses observed under LD.

Under DD, we observe a low amplitude rhythm in the probability of initiation of standard sleep bouts (Fig. 2B; Table 6), which peaked around mid-subjective day, when activity was increasing. The initiation of standard sleep was lowest during the early subjective night in the hours following the peak of activity (Fig. 2B; Table 10). Short sleep was most likely to be initiated a few hours before the activity peak and was least likely to be initiated in the hours following activity (Fig. 2B; Tables 7 and 10). The likelihood of intermediate sleep initiation was lowest during peak activity but was highest closer to the beginning of the subjective day, in the hours preceding the largest peak of activity (Fig. 2B; Tables 8 and 10). In contrast, the initiation of long sleep was most probable in the hours immediately following the peak of activity and was lowest during times when evening activity was ramping up (Fig. 2B; Tables 9 and 10). These results strongly support the hypothesis that long sleep represented a sleep stage potently controlled by the fly's sleep homeostat and that the short and intermediate sleep stages were not strongly regulated in this way. Furthermore, an examination of standard sleep revealed that it was highest during times within the circadian cycle when locomotor activity was increasing (Fig. 2B). This is not how homeostatically regulated sleep is predicted to unfold. These results suggested that employing the standard, unitary definition of sleep obscured its homeostatic control during regular sleep-wake cycles.

Similar to our results in DD, the probability of initiating standard sleep had a low amplitude rhythm under UV- and Blue-blocked light cycles (Fig. 2C; Table 11). The likelihood of initiating standard sleep was similar before and after the major locomotor activity peak (Table 15). This was also true for short sleep (Tables 12 and 15). Moreover, the probability of initiating short sleep was similarly high throughout the day preceding the ramping up of locomotor activity (Fig. 2C; Tables 12 and 15). Intermediate sleep also had very weak rhythms in its likelihood of initiation (Table 13). The highest probability of initiation of intermediate sleep was around midday, more than 12 hours after the peak of locomotor activity (Fig. 2C; Table 15).

On the other hand, long sleep showed a much higher amplitude rhythm in the likelihood of its initiation (Tables 14 and 15). Locomotor activity under UV- and Blue-blocked light cycles was not unimodal (Fig. 2C). There was a slight morning peak followed by a much larger evening peak. Interestingly, there was a proportionate trend in the likelihood of initiating long sleep immediately following the small morning peak (Fig. 2C; Table 15). The probability of initiating long sleep was much higher immediately after the major peak of locomotor activity (Fig. 2C; Table 15). These results strongly indicated that the likelihood of initiating long sleep was directly and proportionally related to amounts and time of wakefulness and therefore was a closer marker of homeostatically regulated sleep. Moreover, it is imperative to note that light environments drastically affect the temporal patterns of the likelihood of initiating sleep and therefore must be accounted for when sleep homeostasis is being examined. Indeed, studies in flies have shown effects of light intensity and wavelength on sleep levels (27, 30). More recently, studies in rats have shown that the wavelength of light can differentially affect various stages of sleep (31).

Sleep stages occur in a specific temporal order relative to one another.

Our results reveal that the likelihood of initiating our provisionally defined sleep stages had a specific temporal order within a day. Furthermore, they indicate that long sleep displays the relationship to wakefulness expected of a sleep stage under strong homeostatic regulation. We therefore asked if these sleep stages occur at characteristic times of the diurnal/circadian cycle. In mammals and other organisms in which sleep stages have been identified, the stages of sleep have a specific temporal relationship to each other (32). A critical goal of sleep science is to understand the temporal architecture of these sleep stages and how they are coordinated by the brain (3). Because of the large startle/arousal-mediated effects on sleep stages under LD cycles (Fig. 2A), we examined the temporal organization of sleep stages under DD and UV- and Blue-blocked ramping light cycles.

Unlike locomotor activity or adult emergence rhythms, which have sharp, identifiable peaks, sleep rhythms display broader, plateau-like waveforms with no discernible peak (Figs. 1-2). Thus, estimating peaks using the same measurements used for locomotor activity is unlikely to describe phases of sleep rhythms accurately. In such cases, circular statistics are informative (33). We

therefore used the phase of center of mass as a metric to identify the phase (or timing) and the resultant vector length as a measure of consolidation of the sleep stages we identified here. The centers of mass of sleep rhythms closely track the mid-point of sleep, a behavioral estimate often used to chronotype humans (34). In other words, phases of center of mass reflect the time of day around which a particular sleep stage is consolidated.

As expected, locomotor activity is predominantly consolidated around subjective dusk under DD conditions (Fig. 3A; subjective dusk corresponds to the time of lights-off under the LD cycle preceding transition to DD, and subjective dawn corresponds to lights-on). As expected from previous work, standard sleep was spread across nearly 20 hours of the circadian cycle, corresponding to all times outside of the subjective evening peak of activity (Fig. 3A). Short sleep was consolidated primarily within the subjective day and peaked around three hours before subjective dusk, just before locomotor activity started ramping up (Fig. 3A). Intermediate sleep was also consolidated primarily within the subjective day but occurred mainly in the first half of the subjective day (Fig. 3A). Long sleep was consolidated primarily within the subjective night and started building up soon after the peak of locomotor activity (Fig. 3A). Thus, the timing and amount of the three sleep stages matched the probability of initiation shown above (Fig. 2).

We quantified the center of mass phases for these sleep stages, which revealed the distinct timing of the various sleep stages relative to wakefulness. Locomotor activity was consolidated around CT11 (CT or Circadian Time 00 corresponds to subjective dawn; Suppl. Fig. 1A; Table 16). Long sleep had its center of mass at ~CT18.5, about seven hours after the mid-point of activity (Suppl. Fig. 1A; Table 16). The center of mass of intermediate sleep was at ~CT1.5, about seven hours after the mid-point of long sleep (Suppl. Fig. 1A; Table 16). The short sleep center of mass was at ~CT5, about 3.5 hours after the center of mass of intermediate sleep and about 6 hours before the center of mass of locomotor activity (Suppl. Fig. 1A; Table 16). It is important to note that distinct sleep stages, by definition, cannot co-occur in time, i.e., they are mutually exclusive, within a fly.

When entrained, under UV- and Blue-blocked light cycles, fly sleep stages show similar temporal sequencing as they did under constant darkness. The rose plots reveal that long sleep was

consolidated within the hours following locomotor activity, which was centered on the falling phase of the ramping light cycle (Fig. 3B). There was a slight, proportionate increase in long sleep closely following the meager peak of locomotor activity during the falling phase of the light ramp (Fig. 3B). Most of the short and intermediate sleep states were consolidated to broad windows preceding the major bout of locomotor activity (Fig. 3B), like their timing under constant darkness. Center of mass values reflected this pattern of temporal sequencing and showed remarkable similarities to patterns observed under DD (Suppl. Fig. 1B; Table 16). The temporal segregation between short and intermediate sleep states was less drastic under ramped light cycles than constant darkness. This may have been due to the small secondary peak of locomotor activity during the rising phase of the light ramp (Figs. 2C and 3B), which may have resulted in shorter sleep states being distributed more widely around the clock. Nonetheless, there was a temporal sequencing of sleep stages under both constant darkness and entrainment under ramped light cycles. Long sleep was followed by locomotor activity, which was followed by intermediate sleep, and subsequently by short sleep. The consistent daily timing of the long sleep stage to the hours following peak wakefulness further supports the hypothesis that the sleep homeostat more potently controls this sleep stage than the short and intermediate stages of sleep.

The homeostatic rebound of long sleep occurs at the expense of shorter sleep states, obscuring rebound measures when the standard, unitary definition of sleep is used.

Long sleep was unique among the sleep stages in its tendency to follow the day's major bout of wakefulness, an expected hallmark of a sleep stage potently regulated by homeostatic mechanisms. One of the most direct ways of examining homeostasis is examine sleep after sleep deprivation (23, 24). We further explored the relationship between sleep stages and homeostatic regulation by asking if rebound following sleep deprivation produces differential effects on short, intermediate, and long sleep stages. Existing approaches of sleep deprivation in the fly produce relatively modest rebounds (21, 35, 36). Using the Ethoscope system (37), we recently showed that homeostatic sleep rebound was severely obscured when sleep is traditionally defined as any bout of inactivity lasting five minutes or longer (21). However, when more prolonged sleep bouts were examined, in this case, defined as periods of inactivity lasting 25 minutes or longer, a significant proportion of lost sleep was recovered following deprivation. Remarkably, when flies were completely deprived of only long sleep, the time courses of standard sleep (five minutes of

inactivity or longer) looked no different from those of their stimulus-matched controls (21). Nevertheless, analysis of long sleep revealed a significant cumulative rebound over multiple cycles following sleep deprivation (21).

Though Ethoscopes are a powerful and flexible method of depriving flies of sleep, they have not yet been adopted widely by the field. A more commonly used method of sleep deprivation is the vortexer-induced mechanical disturbance combined with Trikinetics *Drosophila* Activity Monitors to measure sleep through beam crossing measurements (38). This method has consistently produced a measurable, albeit modest, sleep rebound following deprivation when flies are stimulated three times per minute to prevent sleep. We recently showed that such mechanical disturbances delivered less frequently prevent sleep completely yet produce no statistically significant rebound in standard sleep (21). These results prompted us to ask if standard sleep measurements obscured homeostatic sleep rebound when using the vortexer method of sleep deprivation. In mammalian systems, sleep stages are characterized by differential responses to deprivation (2, 39). We therefore asked if the recognition of sleep stages in the fly might reveal hidden homeostatic responses. Distinct responses to sleep deprivation would be strong evidence that our provisional sleep stages represent distinct behavioral states.

Despite being an important and frequently used experimental approach, methods for depriving flies of sleep and measuring sleep rebound vary significantly between studies and have not been standardized (13, 38). This has made it difficult to compare reported effects on homeostatic sleep regulation between studies (13). Owing to the strong circadian regulation of sleep, we recently suggested the use of 24-h sleep deprivation and a 24-h rebound analysis window (21), as opposed to most previously published studies that deprive for shorter periods and examine sleep rebound within a smaller window of time following deprivation (38). Here we propose a simple method for analyzing homeostatic sleep rebound after sleep deprivation (Fig. 4A). We define sleep rebound as the difference in sleep amount between the post deprivation period and baseline day, adjusted for the changes in sleep displayed by undisturbed controls across the three days during which baseline, deprivation, and recovery sleep are measured (Fig. 4A; see Methods for details).

We calculated sleep rebound for each of our provisional sleep stages to determine if the sleep stages proposed here respond differentially to sleep deprivation. As we previously reported (21), we found no statistically significant rebound in standard sleep over 24-h post deprivation when male flies were kept awake for 24 hours by mechanically vortexing them once every 220-sec (Fig. 4B; Suppl. Fig. 2). In contrast, there was a significant rebound in long sleep (Fig. 4B; Suppl. Fig. 2). Flies showed more than two hours of rebound in long sleep (Fig. 4B; Suppl. Fig. 2). Remarkably, this increased rebound in long sleep occurred at the expense of short and intermediate sleep stages, both of which showed statistically significant reductions following deprivation (Fig. 4B; Suppl. Fig. 2). These results establish that our provisional sleep stages display distinct responses to sleep deprivation, suggesting that they do represent different behavioral states. Though sleep stages are, by definition, mutually exclusive, this does not mean that changes in one stage must occur at the expense of others if the total amount of sleep changes. Our results reveal a distinct homeostatic regulation of the sleep stages defined here and demonstrate the necessity of distinguishing sleep stages when analyzing the homeostatic regulation of fly sleep. They also illustrate a critical point for the field: treating sleep as a unitary state obscures homeostatic regulation.

Long sleep displays robust circadian rhythms, whereas short and intermediate sleep stages display stronger ultradian rhythms.

Distinct sleep states likely have different functions and may, for that reason, be differentially controlled by the circadian system. Previous work on mammalian sleep has shown that REM (rapid eye movement) and NREM (non-REM) sleep have distinct relationships to the circadian clock. While the central circadian system sets the timing of both these sleep stages, and NREM and REM are both homeostatically regulated, the circadian clock appears to have a stronger influence on REM sleep (4–12). Though the circadian clock gates mammalian sleep, once sleep commences, alternations between sleep stages result in ultradian rhythms of sleep, cycling through multiple stages. Thus, circadian rhythms in sleep are accompanied by ultradian rhythms in specific sleep states during particular phases of the circadian cycle. Therefore, we examined both circadian and ultradian periodicities to determine if the three provisional sleep stages display distinct circadian regulation. We employed wavelet analyses to resolve periodic components of sleep time series across days under constant darkness, as we have recently described (22). Wavelet-based

methods of timeseries analyses allow for the identification of periodic components in the timeseries and how they unfold over time (40–42).

Scalograms visualize the power of the rhythm for each periodic component of a timeseries across time (see Methods). The power of a particular periodicity reflects the amplitude of the oscillation. The rhythm's power, strength, and amplitude are used interchangeably here. Scalograms of standard sleep revealed strong 12- and 24-h periodicities, as expected from the fly's biphasic sleep pattern (Fig. 5A). In addition, there were strong ultradian periodicities present under DD, whose predominant periods were in the one to four-hour range (Fig. 5A). These ultradian periodicities largely displayed relatively low amplitudes compared to the 12- and 24-h rhythms in standard sleep (Fig. 5B). We repeated this analysis for the three provisional stages of sleep we identified above to determine if they display distinct circadian control and temporal organization under free running conditions, as would be expected of distinct sleep states.

An examination of short sleep revealed high amplitude cycling in both the 24-h range and ultradian periodicities with relatively low amplitude cycling at the 12-h periodicity (Fig. 5A). When averaged across time, we found that the ultradian rhythm component was comparable in amplitude to the circadian peak (Fig. 5B). In contrast, intermediate sleep displayed relatively low amplitude periodicities in the circadian range and strong periodicities in the ultradian range (Figs. 5A and B). Finally, and in contrast to short and intermediate sleep stages, long sleep displayed strong periodicities in circadian range, moderately strong periodicity in the 12-h range, and relatively low amplitude ultradian periodicities (Fig. 5A and B). These results revealed a distinct regulation of sleep stages by the circadian clock and further support the need to recognize sleep stages in the fly.

The circadian clock sets the phase of ultradian sleep rhythms.

In mammals, sleep cycles through various stages during the night with an ultradian periodicity. For instance, such ultradian rhythms have a periodicity of ~90 minutes in humans (3). An analysis of a long timeseries of a single stage of human sleep would produce a scalogram containing circadian and ultradian components, as we have observed for the fly (Fig. 5). The circadian component would be a consequence of the sleep stage being restricted to the night. The ultradian

component would reflect the cycling in and out of that sleep stage, which would likewise be limited to the night. Therefore, mammalian sleep stages display circadian and ultradian periodic components, with the ultradian components restricted to specific times of the day (3). An examination of the scalograms presented in Fig. 5A suggests that ultradian oscillations in short, intermediate, and long sleep stages occur within distinct windows, which each stage displaying a characteristic timing (magenta arrowheads in Fig. 5A). High amplitude ultradian rhythms of short and intermediate sleep states appeared to be timed to the subjective day, whereas high amplitude ultradian rhythms in long sleep were restricted to the subjective night (Fig. 5A). This prompted us to ask whether the circadian clock gated these ultradian rhythms to specific times of the day by comparing wildtype flies to clock loss-of-function *period*^{01} (*per*^{01}) mutants under constant darkness. If the circadian clock is responsible for the proper daily timing of ultradian cycling of sleep stages in the fly, we would expect *per*^{01}mutants to lack the consolidation of ultradian rhythms we observed in wildtype flies (Fig. 5A).

We found the strength (i.e., amplitudes) of ultradian periodicities in short sleep was clearly rhythmic for wildtype flies but not for *per*^{01}mutants (Fig. 6A). We determined this by comparing the mean power across subjective days and nights and found that the power of ultradian rhythms was significantly higher during the subjective day for wildtype flies (Fig. 6B-left; Table 17). No such day-night difference was found in the *per*^{01} mutants (Fig. 6B-right; Table 17). Intermediate sleep displayed apparent rhythms in the strength of ultradian periodicity in wildtype flies (Fig. 6C), but there was no statistically significant difference between the amplitudes of ultradian periodicities between the subjective day and subjective night (Fig. 6D-left; Table 18). Upon careful inspection of the time-course we found that the apparent rhythmicity in the amplitude of ultradian rhythms in intermediate sleep was not neatly segregated to the subjective day or night phase. It spanned across the later part of the subjective night to early subjective day (subjective dawn), which may be one reason we found no significant difference between the day and night. Moreover, intermediate sleep had weak circadian regulation (Fig. 5B), which could be one reason for the lack of strong gating of ultradian periodicities in wildtype flies. As expected, there was no apparent rhythmicity (Fig. 6C) or significant day-night difference (Fig. 6D-right; Table 18) in the strength of ultradian sleep rhythms in *per*^{01} mutants. The strength of long sleep ultradian rhythms in wildtype flies was robustly rhythmic with peak power values timed to the middle of the

subjective night, and there was no indication of such rhythmicity in *per*^{01} mutants (Fig. 6E; Table 19). We found a statistically significant increase in subjective night power for wildtype flies (Fig. 6F-left; Table 19), and, as expected, there was no such difference in the *per*^{01} mutant flies (Fig. 6F-right; Table 19). Thus, these three sleep stages display distinct modes of circadian regulation, further supporting the conclusion that they represent distinct behavioral states. Our results further highlight the fact that standard measures of sleep obscure important aspects of its regulation.

Discussion

Do flies have distinct sleep stages?

The recognition of human sleep stages was a critical discovery for sleep science, and our current understanding of sleep and its impact on physical and mental health would be significantly less advanced if human sleep had been treated as a unitary state (2, 3). Based on recent convergent work suggesting the presence of distinct sleep stages *in Drosophila melanogaster*, we have asked if distinct stages can be defined behaviorally in this species. The answer to this question seems clear: yes. We have identified four stages of fly sleep/wake: wakefulness (i.e., activity), short sleep, intermediate sleep, and long sleep, which are based on previous work examining the relationships between inactivity and physiology (14–22) and correspond remarkably well to the sleep/wake states previously identified using Hidden Markov Modeling (Fig. 1B). The stages we have identified here have distinct relationships to both circadian and homeostatic regulators, as would be expected of discrete sleep states. We have also established simple inactivity-based criteria can be used to provisionally identify these sleep stages (Fig. 1).

Results described here, which converge with our recent work (21, 22), reveal that, among the three sleep stages, long sleep is uniquely subject to homeostatic control (Figs. 2-4). Importantly, long sleep's relationship to homeostatic processes is clear for both daily sleep rhythms and for rebound sleep following deprivation. Previous work by multiple groups using the standard unitary definition of sleep has observed an apparent disconnect between the regulation of daily sleep and homeostatic rebound based on manipulations that affect one but not the other (13). Based on these results, investigators have suggested that baseline sleep and homeostatic rebound are governed by different molecular/cellular mechanisms. However, the two-process model of sleep regulation, which we recently showed captures fly sleep quite well (22), posits that the same process that produces sleepiness during normal wakefulness is the same process engaged by sleep deprivation. Here we show that the long sleep stage, uniquely among the stages identified in the study, maintains both the predicted temporal relationship to wakefulness and responds to sleep deprivation with substantial rebound (Figs. 2-4). Identifying a sleep stage potently regulated by the sleep homeostat that has been obscured by the field's unitary definition of sleep calls for the reassessment of previous work identifying genetic and cellular components of the fly's sleep homeostat.

Our work also establishes that fly sleep consists of identifiable states that display unique temporal sequencing across the circadian cycle (Figs. 2-3), as would be expected of distinct sleep stages. Moreover, we found that all three sleep stages display both circadian and ultradian rhythms (Fig. 5), and that ultradian rhythms of each stage are gated by the clock to specific windows within the circadian cycle (Fig. 6). Future work will be required to determine if these gated ultradian rhythms represent something akin to programmed sleep cycles in the fly.

One potential objection to our definition of sleep stages in the fly is that it is simply a variation on the field's longstanding practice of examining sleep bout duration as a measure of sleep consolidation. Sleep consisting of fewer, but longer bouts of sleep, is said to be more consolidated than the same amount of sleep divided into shorter and more frequent bouts. One might argue that the use of inactivity criteria to define distinct sleep states is simply another way of measuring consolidation. But simple arithmetic can show that the amount of long sleep can be identical in two flies with significantly different average sleep bout durations (Suppl. Fig. 3). For example, changes in the relative amounts of short and intermediate sleep can produce changes in average sleep bout duration when no changes have occurred in long sleep (e.g., Suppl. Fig. 3).

One important caveat of our provisional definition of sleep stages is the data acquisition method used to examine sleep. Our definition of short (5 to 30 minutes), intermediate (30 to 60 minutes), and long (> 60 minutes) sleep is applicable only to data collected using the IR beam-based acquisition method through the Trikinetics *Drosophila* Activity Monitoring (DAM) systems (Waltham, MA, USA). Compared to video-based measurements, the DAM systems overestimate sleep (21, 37, 43, 44), and therefore, the corresponding inactivity criteria will be lower for video-based data acquisition methods. Careful calibration will likely be required to standardize criteria across different systems. However, based on our previous work (21) we suggest that ~30 minutes of inactivity in a video-based data acquisition method likely corresponds to the long sleep we define here. Remarkably, a founding study in the field of *Drosophila* sleep science, which used a video-based behavioral assay, suggested that a large percentage of all sleep was > 30-min and the longest bouts of sleep occurred soon after subjective dusk (23). In hindsight, a 30-min inactivity criterion for sleep, based on one of the earliest reports of fly sleep (23), our recent work (21, 22),

and the results presented here, would likely have been a more sensitive metric for examining sleep homeostasis.

It is difficult and unwise to compare sleep stages across phyla directly.

The circadian gating of ultradian rhythms in sleep stages is reminiscent of the temporal patterning of sleep cycles in mammalian sleep (45). It is important to state that we are not suggesting that the sleep stages identified herein are akin to REM (Rapid Eye Movement)/NREM (non-REM) sleep in mammals, though there are clear similarities in two regards. First, like mammalian sleep stages, fly sleep stages are distinctly sensitive to homeostatic regulation (21, 22). Second, different sleep stages in the fly are distinctly timed to occur at different phases of the circadian cycle, much like sleep stages in mammals. Although REM/NREM sleep stages have been identified in rodent systems, they have been shown to differ somewhat from human REM/NREM stages, in that human NREM has substages (46). There are also physiological differences between rodent sleep stages and human sleep stages (46). Interestingly, while it is true that birds show REM and NREM sleep stages, they appear to differ from mammalian REM/NREM in important ways (47, 48). For instance, bird NREM sleep lacks substates and specific brain oscillations (47, 48). Reptilian sleep also has electrophysiologically distinct sleep states, but REM and NREM-like sleep stages in the bearded dragon do not completely match REM and NREM sleep states seen in mammals (49, 50). Therefore, while there may be similarities in sleep states between species, there are also likely to be significant differences across phyla, and directly comparing them, without understanding their function and regulation, is unwise (51). In comparing sleep stages across phyla, we must be mindful of the "*misuse of nomenclature, and lack of openness towards potentially intriguing differences*" as warned by Rayan and colleagues (46).

Our results call for an end to the treatment of fly sleep as a unitary state.

Inactivity-based measures of sleep correlate strongly with electroencephalograms (EEGs) and electromyograms (EMGs) and have been suggested as a first-pass behavioral criterion to examine sleep in the pursuit of high-throughput screening programs in mice (52). The use of similar strategies in the fly, especially in the absence of easily accessible physiological correlates, can be readily used by the field in its effort to identify the neural and molecular genetic correlates of sleep regulation. For over 25 years, fly sleep research has used a unitary definition of sleep, i.e., any

bout of inactivity equal to or longer than five continuous minutes. Despite half a century of sleep scoring using standards set by Rechtschaffen and Kales (53), the classification of sleep remains an active area of research in mammalian sleep (46). Why should the same not be true of sleep research in the fly?

Despite a quarter century of work and the identification of hundreds of "sleep genes," the field has failed to produce a widely accepted model for sleep homeostasis (13). Our results here, along with other recent work from our lab, converge to provide a possible explanation for this failure: (i) the employment of a unitary definition of fly sleep and (ii) the use of bright light/dark cycles with very little information on intensity or spectral composition of the light have obscured potent homeostatic sleep processes. Specifically, we have shown that using the standard definition of sleep obscures the detection of sleep rebound because manipulations used to deprive flies of sleep have opposing effects on different sleep stages (Fig. 4). Although every bout of inactivity longer than 5-min meets the behavioral criteria for sleep, they are not all homeostatically regulated, and most certainly not in the same manner. Lines written by Sneha Khanwalkar and Varun Grover for a cult classic Bollywood film, *Gangs of Wasseypur*, capture this issue quite accurately:

"हर यार वफ़ादार नहीं होता (every lover/friend is not faithful/loyal)

हर पत्थर चमकदार नहीं होता (every stone is not shiny/precious)

ना जाने बन में कितने फूल खिले हैं (there may be innumerable flowers blooming in the forest)

हर फूल ख़ुशबूदार नहीं होता (but every flower is not fragrant)"

The recognition and wide-spread acceptance of sleep stages in the fly will be required if the *Drosophila* model's promise for identifying conserved mechanisms of sleep homeostasis is to be realized. Realizing this promise will require the field to closely examine the sleep states most strongly subjected to homeostatic regulation. Treating all epochs of inactivity lasting five minutes or more as a unitary sleep state has likely outlived its utility for discovering the mechanisms underlying sleep regulation. This is particularly true for understanding the homeostatic regulation of sleep, which remains a primary unmet goal of sleep science.

Conclusion

Sleep stages in humans are clearly identifiable and have predictable temporal sequencing (3, 45). They correlate with different physiological and behavioral changes (3). Their identification has been critical to understanding the complex phenomenon of sleep. However, much remains to be discovered regarding the molecular and neural regulation of the timing and amount of sleep (13). *Drosophila melanogaster* remains a promising model system for discovering conserved mechanisms of sleep regulation. Our identification of sleep stages in the fly will allow for a more sensitive and accurate accounting of sleep regulation. The stages we have defined here remain provisional and, in contrast to the longstanding unitary definition of fly sleep employed by the field, they should be doubted and debated before a consensus is reached regarding their definitions, their physiological correlates, and the best methods for their measurement. However, our results clearly establish the existence of distinct sleep states in the fly and reveal the necessity of focusing on relatively long durations of inactivity as the most sensitive means of discovering the cellular and molecular mechanisms of sleep homeostasis in *Drosophila*.

Methods

Flies and behavioral recording

Wildtype (*Canton-S*; BDSC stock number: 64349) and a loss-of-function clock mutant (*per*^{01}; BDSC stock number: 80928) flies were used in the experiments reported in this manuscript. Flies were maintained in bottles with Corn Syrup/Soy media (W1; Archon Scientific, Durham, NC) under ~25 °C. Three to five-day-old males were collected from the bottles under CO_2 anesthesia and loaded into glass locomotor activity tubes with sucrose-agar media. About 32 flies each were setup in *Drosophila* Activity Monitors (DAMs; Trikinetics Inc., Waltham, MA, USA) and recorded under light/dark (LD) cycles with 12-h of white light and 12-h of darkness in Tritech incubators at a constant 25 °C (Tritech Research, Los Angeles, CA, USA). During the day, the intensity of light was ~500 lux. Flies were recorded under LD cycles for about eight days, then transferred to constant darkness (DD) and temperature (25 °C) for nine days. All behavioral data were collected in 1-minute intervals/bins. Data for the three runs in Fig. 1 under LD and DD are from three different independent runs where flies were transferred to DD after exposure to LD12:12.

Also, data collected from *Canton-S* flies whose activity rhythms were recorded under UV- and Blue-blocked ramped light cycles and reported previously (28) were used for analyzing the probability of initiation of sleep bouts. ~32 flies were recorded under ~25 °C and the light cycled from 10$\mu W/cm^2$ to 40-50$\mu W/cm^2$ (measured at 600nm). See Abhilash and Shafer, 2023 (28) for more details on the experimental conditions. Results from one representative experiment have been shown in this study.

Data collected from *Canton-S* flies subjected to 24-h sleep deprivation using the vortexer method and a deprivation trigger frequency of 220-sec on homeostatic rebound, as previously reported (21), were re-analyzed here to examine the extent of sleep stage-specific rebound. These flies were also recorded under LD12:12 conditions at 25 °C. See Chowdhury, Abhilash et al., 2023 (21) for more details on the experimental design. ~32 flies were recorded per treatment. Results from one independent experiment have been shown here.

Bout-length-based categorization of sleep stages and computation of sleep bout duration

Based on the meta-analysis of studies suggesting sleep stages in flies, we chose three provisional cutoff points to segregate fly sleep (see Results for details). Any bout of inactivity (i.e., epoch with zero beam crossings) that was equal to or longer than 5 minutes, but less than 30 minutes was designated "short sleep." Any bout of inactivity that was equal to or greater than 30 minutes, but less than 60 minutes was designated "intermediate sleep." Finally, any bout of inactivity that was equal to or longer than 60 minutes was categorized as "long sleep." Standard sleep was defined as any bout of inactivity that was equal to or longer than 5 minutes. For the visualization of waveform shape in Fig. 1, these sleep states were normalized to maxima, such that all the waveforms range from 0 to 1.

Analysis of homeostatic sleep rebound

A schematic of the logic of analyzing sleep rebound has been shown in Fig. 4 (please refer to the figure for this section). Once "undisturbed sleep increase" is measured for each fly, we average these values. Then, we measure the "deprived sleep increase" for each fly. The difference between each fly's "deprived sleep increase" and the averaged "undisturbed sleep increase" yields the individual experimental fly's measure of rebound. These were calculated for standard sleep and for each of the three stages of sleep identified in this study. Statistical comparisons were made using two-tailed, one-sample *t*-tests against a known mean of 0, under the null hypothesis of no rebound in response to sleep deprivation. All analyses were carried out using custom R scripts.

Circular statistics of sleep stages

Rose plots are circular or polar analogs of activity/sleep profiles plotted on a Cartesian (x-y) plane. We calculated sleep time series over the course of eight days under LD and binned the output in 30-minute intervals. A 30-minute interval was chosen as a reasonable compromise between visualizing temporal patterns of activity and sleep without losing nuance to smoothing of the data due to larger interval sizes. These were then averaged over days and across flies. The resulting average activity or sleep profiles were plotted as rose plots. Individual fly's average activity/sleep profiles were used and transformed to polar coordinates to compute properties of the center of mass (33). Center of mass is the mean phase of an oscillation, expressed in polar coordinates. Points on a polar coordinate plane are described by r and θ; r is the distance of the point from the

center of the circle and is reflective of the consolidation of the rhythm and θ is the mean angle or phase of the center of mass. These metrics have been used previously to characterize *Drosophila* rhythms (54, 55).

Probability of initiation of sleep bouts

For each fly and sleep stage, a 1-minute timeseries was generated. From this timeseries, a list of all the standard, short, intermediate, or long sleep bouts that were initiated at each minute was curated, and the CT/ZT at which sleep bouts were initiated was noted. CT (Circadian Time) is a timescale to measure internal time. According to convention, in the fly, CT00 is defined as the time at which lights would have turned ON if the fly had continued to remain under LD cycles from which it was transferred to DD. ZT (Zeitgeber Time) is a timescale that refers to time relative to the zeitgeber (German for *time-giver*). By convention, ZT00 is defined as the time of lights-on or minimum light intensity, in the case of ramped light cycles. Once this data set was generated, we counted the number of sleep bouts that were initiated in every 1-h interval starting at CT/ZT01 and proceeding until CT/ZT24. We chose 1-h intervals to ensure sufficient sampling of bouts of individual sleep stages in each time window. Then the total number of bouts initiated in each 1-h window was divided by the total number of sleep bouts of the respective stage that the fly showed. This metric serves as a measure of the frequency or probability of initiation of standard/long sleep in a 1-h window across the entire length of a 24-h cycle. For each sleep definition, i.e., standard, short, intermediate, and long sleep, a one-way repeated measures ANOVA was carried with time-point as the repeated measures variable and probability of initiation of sleep bout as the dependent variable. Post-hoc analyses were carried out using Tukey's HSD test. All the analyses were carried out using custom R scripts. Each sleep definition under each environmental condition was analyzed using a separate ANOVA.

Continuous Wavelet Transforms

Sleep timeseries for all three stages of sleep were binned in 5-min intervals and subjected to Continuous Wavelet Transforms (CWT). A narrower temporal window was chosen in these analyses to ensure sufficiently high temporal resolution to subject our data to time series analyses. This was important, especially in the context of examining temporal variations in the strength of ultradian rhythms. Owing to biological timeseries being highly non-stationary, it was necessary

to use methods such as the CWT to detect rhythmicity, as previously described (22, 40–42, 56), to examine how multiple periodicities occur and change across and within circadian cycles. CWT allows for sensitively detecting rhythms across various periodicities and captures how these changes occur across timeseries (40–42, 56). The overall method of the wavelet method is like the one recently described (22). Briefly, we used the Morlet mother wavelet to resolve our timeseries in the time-frequency domain. Time-averaged period versus amplitude plots were generated to capture the overall contribution of specific period bands to the overall raw timeseries of the various sleep stages. A 95% Confidence Interval for the time-averaged amplitude of different period components was generated by using the bootstrap method, using 1000 replications.

To estimate the variation in amplitude/power/strength of ultradian rhythms in sleep, we considered period bands of 1 to 4 hours for short and intermediate sleep and 2 to 6 hours for long sleep. These period windows were chosen based on the signal in the scalograms for each of these sleep stages (Fig. 5A). Further, owing to our definition of long sleep being 60 minutes or longer, 1-h ultradian rhythms are impossible to occur. These amplitude time-courses were then parsed into subjective day and night phases, and the average daytime and nighttime cumulative amplitudes were estimated for each fly, similarly to a recent paper that parsed day and night data to understand nuances of ultradian rhythms in rodents (56). The day and nighttime parsed amplitude values were then statistically compared using one-tailed paired *t*-tests, separately for each sleep stage.

Introducing phaseR

phaseR is an RStudio and Shiny-based application that we have developed to facilitate all the analyses we report in this manuscript. It enables the analysis of user-defined sleep stages, and the application of wavelet transforms to activity and sleep time series. The application can be downloaded from its GitHub repository (*Abhilash and Shafer, manuscript in preparation*; https://github.com/abhilashlakshman/phaseR). Most functions used in *phaseR* are part of an R package, called *phase* (57). Functions that are not part of *phase*, have been implemented in the server environment of the Shiny application. Please see the user manual for a detailed description of all the functionalities and instructions on how to use them.

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Figure Legends

Figure 1: Durations of inactivity can be used to identify sleep stages. (A) Plot of sleep bout durations *vs* % change in metabolic rate. Data extracted from Stahl et al., 2017 (15) and replotted here. Physiological changes previously reported by others to accompany sleep bout duration that informed bout duration cutoffs are also indicated (17, 20)(see text for details). (B) Bout length-based classification of sleep reveals waveforms of provisional sleep stages under Light/Dark cycles (left) for wildtype flies. These are remarkably similar in shape to the *Drosophila* sleep stages identified previously using Hidden Markov Models (HMMs; middle)(14). Also shown are the waveforms of the three provisional sleep stages, during the first day of constant darkness (right) following entrainment to LD cycles. The plots under LD (left) and DD (right) show the mean waveform from three independent replicate runs. The error regions represent the Standard Error of Mean (SEM) across the three replicate runs.

Figure 2: Probability of initiating long sleep is highest immediately following major peaks of wakefulness. (A) Activity and profiles of standard sleep and the three provisionally identified sleep stages for wildtype flies under Light/Dark cycles (A-left), constant darkness (B-left), and UV- and Blue-blocked ramped light cycles (C-left). Also shown are plots of initiation probabilities of standard, short, intermediate, and long sleep under Light/Dark cycles (A-right), constant darkness (B-right), and UV- and Blue-blocked ramped light cycles (C-right). For initiation probability plots, the *x*- and *y*-axes represent the same features and therefore have been labeled only in panel A. Gray shaded region in panel A represents the nighttime of the Light/Dark cycle. Light and dark gray shaded regions in panel B represent the subjective day and night times, respectively. The golden dotted line in panel C represents the ramping up and down of the light cycle. All the plots show mean±SEM. Also see Tables 1-15.

Figure 3: Rose plots and hypnograms clearly demonstrate the temporal sequencing of sleep stages in wildtype flies. Rose plots of locomotor activity and sleep stages under constant darkness (A) and UV- and Blue-blocked light cycles (B). Rose plots are averaged over flies. In each sleep stage, the locomotor activity rose plots are overlaid to facilitate visualization of their temporal inter-relationships. Also see Suppl. Fig. 1 and Table 16.

Figure 4: Long sleep displays substantial homeostatic sleep rebound following deprivation, rebound that is obscured by standard sleep, in wildtype flies. (A) Schematic of a proposed method of measuring of sleep rebound following deprivation. (B) Estimated sleep rebound for standard sleep and each of the three provisional stages of sleep. Statistical comparisons were made against 0 for each of the metrices and sleep stages separately using single sample *t*-tests. Also see Suppl. Fig. 2.

Figure 5: Sleep stages display distinct circadian and ultradian regulation. (A) Normalized, averaged scalograms of standard sleep, and the three stages of sleep under constant darkness. Note that the *y*-axis is plotted on a logarithmic scale. Magenta arrowheads are meant to indicate potential gating of high ultradian rhythms as reflected by relatively high amplitude ultradian cycling. Light and dark gray shaded regions represent the subjective day and night times, respectively. (B) Plots of period *vs* amplitude for all the definitions of sleep under constant darkness, averaged over the time-axis. Note that the error regions for each trace represent 95% confidence intervals estimated through bootstrapping. Therefore, any amplitude values that have non-overlapping error regions may be considered statistically significantly different from each other.

Figure 6: Ultradian rhythm amplitudes for each sleep stage are distinctly gated by the circadian clock. Normalized scalograms for short (A), intermediate (C), and long (E) sleep in wildtype and loss-of-function per^{01} mutant flies depicting amplitude values in the ultradian period range used for downstream analyses (see text for details). Also, shown are amplitude values over time for each sleep stage (A, C, E). All scalograms have a *z*-axis scale that ranges from 0 to 1.5. The error regions for each time *vs* amplitude trace represent 95% confidence intervals estimated through bootstrapping. Cumulative amplitude values, parsed into subjective day and night times for both wildtype and loss-of-function per^{01} mutants, are plotted for short (B), intermediate (D), and long (F) sleep. Each datapoint represents a single fly. Daytime values are plotted in yellow and nighttime values in black. The average values are reported in respective colors. Whether the average subjective daytime and nighttime power/amplitude were significantly different from each

other, within each genotype, was tested using paired one-tailed *t*-tests. * < 0.05, ** < 0.01, *** < 0.001, and n.s. (not significant). Also see Tables 17-19.

Supplementary Figure 1: Phases of center of mass and the consolidation of locomotor activity and sleep rhythms in polar coordinates under (A) constant darkness and (B) UV- and Blue-blocked light cycles. Each dot represents a single fly's phase of center of mass and consolidation. The mean phase and consolidation are represented by the straight line and a larger dot. Light and dark gray shaded regions represent the subjective day and night times, respectively. Also see Fig. 3 and Table 16.

Supplementary Figure 2: Averaged timeseries of sleep stages before, during and after mechanical sleep deprivation. Also see Fig. 4.

Supplementary Figure 3: Schematic illustrating the distinction between measurements of sleep fragmentation/consolidation and sleep staging. In this example, the amount of long sleep is not changed despite substantial changes in average bout length, the commonly used metric to assess fragmentation/consolidation.

Table 1: Results of the repeated measures ANOVA to test the effect of time-point on the probability of initiating *standard* sleep under LD cycles, in wildtype flies, under 25 °C. Significant effects are italicized and marked in red. A statistically significant effect of time-point is taken to indicate the presence of a rhythm.

	df Effect	***MS Effect***	***df Error***	***MS Error***	***F***	***p***
Time-point	*23*	*0.00663*	*713*	*0.0006*	*11.14*	*<2e-16*
Channel	31	1.05e-19				

Table 2: Results of the repeated measures ANOVA to test the effect of time-point on the probability of initiating *short* sleep under LD cycles, in wildtype flies, under 25 °C. Significant effects are italicized and marked in red. A statistically significant effect of time-point is taken to indicate the presence of a rhythm.

	df Effect	***MS Effect***	***df Error***	***MS Error***	***F***	***p***
Time-point	*23*	*0.011*	*713*	*0.00102*	*10.75*	*<2e-16*
Channel	31	1.40e-19				

Table 3: Results of the repeated measures ANOVA to test the effect of time-point on the probability of initiating *intermediate* sleep under LD cycles, in wildtype flies, under 25 °C. Significant effects are italicized and marked in red. A statistically significant effect of time-point is taken to indicate the presence of a rhythm.

	df Effect	***MS Effect***	***df Error***	***MS Error***	***F***	***p***
Time-point	*23*	*0.01277*	*713*	*0.00189*	*6.74*	*<2e-16*
Channel	31	2.14e-19				

Table 4: Results of the repeated measures ANOVA to test the effect of time-point on the probability of initiating *long* sleep under LD cycles, in wildtype flies, under 25 °C. Significant effects are italicized and marked in red. A statistically significant effect of time-point is taken to indicate the presence of a rhythm.

	df effect	***MS effect***	***df Error***	***MS Error***	***F***	***p***
Time-point	*23*	*0.0248*	*713*	*0.00189*	*13.16*	*<2e-16*
Channel	31	1.05e-19				

Table 5: Values of probabilities (Avg. Probs.) of initiating standard sleep and each of the sleep stages at different times of the day under LD cycles, compiled from the raw data on which the ANOVAs above were carried out. Post hoc multiple comparisons were carried out using the Tukey's Honestly Significant Difference (HSD) test. Time-points that share letters in the column "Groups" are not significantly different from each other.

	Std. sleep		**Short sleep**		**Inter. sleep**		**Long sleep**	
ZT	*Avg. Probs.*	*Groups*	*Avg. Probs.*	*Groups*	*Avg. Probs.*	*Groups*	*Avg. Probs.*	*Groups*
1	0.0469	bcdef	0.0543	bc	0.0170	ef	0.0412	bc
2	0.0796	a	0.0898	a	0.0365	bcdef	0.0636	b
3	0.0522	bcd	0.0488	bcd	0.0619	abc	0.0599	b
4	0.0359	defg	0.0311	bcde	0.0367	bcdef	0.0553	b
5	0.0307	defg	0.0238	de	0.0380	bcdef	0.0596	b
6	0.0245	gh	0.0211	de	0.0198	def	0.0446	bc
7	0.0272	fgh	0.0254	cde	0.0294	bcdef	0.0327	bcd
8	0.0283	efgh	0.0286	bcde	0.0261	cdef	0.0366	bcd
9	0.0340	defg	0.0375	bcde	0.0581	abcd	0.0074	cd
10	0.0629	ab	0.0906	a	0.0198	def	0.0009	d
11	0.0329	defg	0.0494	bcd	0.0022	f	0.0000	d
12	0.0075	h	0.0106	e	0.0026	f	0.0000	d
13	0.0626	abc	0.0475	bcd	0.0372	bcdef	0.1282	a
14	0.0406	cdefg	0.0365	bcde	0.0542	abcde	0.0410	bc
15	0.0381	defg	0.0343	bcde	0.0428	abcde	0.0427	bc
16	0.0415	bcdefg	0.0371	bcde	0.0478	abcde	0.0564	b
17	0.0455	bcdefg	0.0410	bcd	0.0579	abcd	0.0452	bc
18	0.0425	bcdefg	0.0378	bcde	0.0510	abcde	0.0533	b
19	0.0393	defg	0.0301	bcde	0.0562	abcde	0.0589	b
20	0.0407	bcdefg	0.0343	bcde	0.0486	abcde	0.0557	b
21	0.0434	bcdefg	0.0394	bcde	0.0614	abc	0.0442	bc
22	0.0479	bcdef	0.0442	bcd	0.0802	a	0.0371	bcd
23	0.0499	bcde	0.0488	bcd	0.0675	ab	0.0345	bcd
24	0.0455	bcdefg	0.0578	b	0.0472	abcde	0.0010	d

Table 6: Results of the repeated measures ANOVA to test the effect of time-point on the probability of initiating *standard* sleep under constant darkness, in wildtype flies, under 25 °C. Significant effects are italicized and marked in red. A statistically significant effect of time-point is taken to indicate the presence of a rhythm.

	df Effect	*MS Effect*	*df Error*	*MS Error*	*F*	*p*
Time-point	*23*	*0.0035*	*690*	*0.00023*	*15.06*	*<2e-16*
Channel	30	1.39e-19				

Table 7: Results of the repeated measures ANOVA to test the effect of time-point on the probability of initiating *short* sleep under constant darkness, in wildtype flies, under 25 °C. Significant effects are italicized and marked in red. A statistically significant effect of time-point is taken to indicate the presence of a rhythm.

	df Effect	*MS Effect*	*df Error*	*MS Error*	*F*	*p*
Time-point	*23*	*0.00844*	*690*	*0.00047*	*18.05*	*<2e-16*
Channel	30	2.38e-19				

Table 8: Results of the repeated measures ANOVA to test the effect of time-point on the probability of initiating *intermediate* sleep under constant darkness, in wildtype flies, under 25 °C. Significant effects are italicized and marked in red. A statistically significant effect of time-point is taken to indicate the presence of a rhythm.

	df Effect	*MS Effect*	*df Error*	*MS Error*	*F*	*p*
Time-point	*23*	*0.0056*	*690*	*0.00127*	*4.416*	*6.38e-11*
Channel	30	2.86e-19				

Table 9: Results of the repeated measures ANOVA to test the effect of time-point on the probability of initiating *long* sleep under constant darkness, in wildtype flies, under 25 °C. Significant effects are italicized and marked in red. A statistically significant effect of time-point is taken to indicate the presence of a rhythm.

	df Effect	*MS Effect*	*df Error*	*MS Error*	*F*	*p*
Time-point	*23*	*0.01372*	*667*	*0.00204*	*6.732*	*<2e-16*
Channel	29	1.91e-19				

Table 10: Values of probabilities (Avg. Probs.) of initiating standard sleep and each of the sleep stages at different times of the day under constant darkness, compiled from the raw data on which the ANOVAs above were carried out. Post hoc multiple comparisons were carried out using the Tukey's Honestly Significant Difference (HSD) test. Time-points that share letters in the column "Groups" are not significantly different from each other.

	Std. sleep		**Short sleep**		**Inter. sleep**		**Long sleep**	
CT	*Avg. Probs.*	*Groups*	*Avg. Probs.*	*Groups*	*Avg. Probs.*	*Groups*	*Avg. Probs.*	*Groups*
1	0.0486	abcd	0.0444	cdef	0.0603	a	0.0368	bcdef
2	0.0477	abcd	0.0453	bcde	0.0573	ab	0.0313	cdef
3	0.0507	abc	0.0492	abcd	0.0596	a	0.0451	abcdef
4	0.0494	abcd	0.0459	bcd	0.0588	a	0.0411	bcdef
5	0.0473	abcde	0.0454	bcde	0.0571	ab	0.0284	def
6	0.0532	abc	0.0568	abc	0.0604	a	0.0217	ef
7	0.0576	a	0.0647	ab	0.0461	abcd	0.0231	ef
8	0.0561	ab	0.0663	a	0.0427	abcd	0.0118	f
9	0.0547	ab	0.0680	a	0.0249	bcd	0.0126	ef
10	0.0513	abc	0.0669	a	0.0229	cd	0.0097	f
11	0.0473	abcde	0.0586	abc	0.0176	d	0.0214	ef
12	0.0422	bcdefg	0.0488	abcd	0.0191	cd	0.0250	ef
13	0.0435	abcdefg	0.0439	cdef	0.0243	bcd	0.0494	abcdef
14	0.0336	efghi	0.0296	defgh	0.0400	abcd	0.0489	abcdef
15	0.0308	fghi	0.0258	efgh	0.0350	abcd	0.0707	abcd
16	0.0263	hi	0.0172	h	0.0371	abcd	0.0710	abcd
17	0.0247	i	0.0180	h	0.0384	abcd	0.0545	abcde
18	0.0258	i	0.0194	h	0.0319	abcd	0.0844	a
19	0.0277	hi	0.0209	gh	0.0431	abcd	0.0714	abc
20	0.0298	ghi	0.0250	fgh	0.0396	abcd	0.0763	ab
21	0.0316	fghi	0.0255	efgh	0.0453	abcd	0.0472	abcdef
22	0.0357	defghi	0.0321	defgh	0.0471	abcd	0.0468	abcdef
23	0.0400	cdefgh	0.0405	cdefg	0.0407	abcd	0.0341	bcdef
24	0.0440	abcdef	0.0417	cdef	0.0508	abc	0.0372	bcdef

Table 11: Results of the repeated measures ANOVA to test the effect of time-point on the probability of initiating *standard* sleep under UV- and Blue-blocked ramped light cycles, in wildtype flies, under 25 °C. Significant effects are italicized and marked in red. A statistically significant effect of time-point is taken to indicate the presence of a rhythm.

	df Effect	*MS Effect*	*df Error*	*MS Error*	*F*	*p*
Time-point	*23*	*0.00456*	*621*	*0.00059*	*7.757*	*<2e-16*
Channel	27	2.22e-19				

Table 12: Results of the repeated measures ANOVA to test the effect of time-point on the probability of initiating *short* sleep under UV- and Blue-blocked ramped light cycles, in wildtype flies, under 25 °C. Significant effects are italicized and marked in red. A statistically significant effect of time-point is taken to indicate the presence of a rhythm.

	df Effect	*MS Effect*	*df Error*	*MS Error*	*F*	*p*
Time-point	*23*	*0.00525*	*621*	*0.00099*	*5.287*	*7.73e-14*
Channel	27	1.03e-19				

Table 13: Results of the repeated measures ANOVA to test the effect of time-point on the probability of initiating *intermediate* sleep under UV- and Blue-blocked ramped light cycles, in wildtype flies, under 25 °C. Significant effects are italicized and marked in red. A statistically significant effect of time-point is taken to indicate the presence of a rhythm.

	df Effect	*MS Effect*	*df Error*	*MS Error*	*F*	*p*
Time-point	*23*	*0.01065*	*621*	*0.00402*	*2.65*	*5.14e-05*
Channel	27	1.23e-19				

Table 14: Results of the repeated measures ANOVA to test the effect of time-point on the probability of initiating *long* sleep under UV- and Blue-blocked ramped light cycles, in wildtype flies, under 25 °C. Significant effects are italicized and marked in red. A statistically significant effect of time-point is taken to indicate the presence of a rhythm.

	df Effect	*MS Effect*	*df Error*	*MS Error*	*F*	*p*
Time-point	*23*	*0.03509*	*621*	*0.00562*	*6.246*	*<2e-16*
Channel	27	6.59e-20				

Table 15: Values of probabilities (Avg. Probs.) of initiating standard sleep and each of the sleep stages at different times of the day under UV- and Blue-blocked ramped light cycles, compiled from the raw data on which the ANOVAs above were carried out. Post hoc multiple comparisons were carried out using the Tukey's Honestly Significant Difference (HSD) test. Time-points that share letters in the column "Groups" are not significantly different from each other.

	Std. sleep		**Short sleep**		**Inter. sleep**		**Long sleep**	
ZT	*Avg. Probs.*	*Groups*	*Avg. Probs.*	*Groups*	*Avg. Probs.*	*Groups*	*Avg. Probs.*	*Groups*
1	0.0332	bcdef	0.0223	defg	0.0518	ab	0.1073	ab
2	0.0270	cdef	0.0255	cdefg	0.0217	ab	0.0414	bcde
3	0.0349	bcdef	0.0305	bcdefg	0.0560	ab	0.0317	cde
4	0.0398	bcde	0.0397	abcdefg	0.0431	ab	0.0156	de
5	0.0473	abc	0.0487	abcdefg	0.0486	ab	0.0455	bcde
6	0.0498	abc	0.0508	abcde	0.0624	ab	0.0197	de
7	0.0657	a	0.0656	a	0.0651	ab	0.0487	bcde
8	0.0549	ab	0.0523	abcd	0.0575	ab	0.0680	abcde
9	0.0492	abc	0.0467	abcdefg	0.0624	ab	0.0373	bcde
10	0.0524	ab	0.0490	abcdef	0.0574	ab	0.0453	bcde
11	0.0472	abc	0.0443	abcdefg	0.0541	ab	0.0515	bcde
12	0.0506	abc	0.0464	abcdefg	0.0691	a	0.0456	bcde
13	0.0470	abc	0.0460	abcdefg	0.0561	ab	0.0364	bcde
14	0.0483	abc	0.0506	abcde	0.0523	ab	0.0271	de
15	0.0552	ab	0.0613	ab	0.0504	ab	0.0268	de
16	0.0487	abc	0.0559	abc	0.0321	ab	0.0113	de
17	0.0433	abcd	0.0528	abcd	0.0256	ab	0.0030	de
18	0.0369	bcdef	0.0485	abcdefg	0.0212	ab	0.0000	e
19	0.0208	def	0.0251	cdefg	0.0060	b	0.0090	de
20	0.0156	f	0.0181	g	0.0126	ab	0.0030	de
21	0.0166	ef	0.0198	fg	0.0062	b	0.0095	de
22	0.0463	abc	0.0439	abcdefg	0.0186	ab	0.0758	abcd
23	0.0410	bcd	0.0347	bcdefg	0.0341	ab	0.1379	a
24	0.0282	cdef	0.0215	efg	0.0356	ab	0.1027	abc

Table 16: Phases of Centre of Mass of sleep rhythms under constant darkness and UV- and Blue-blocked ramped light cycles for locomotor activity, standard sleep and all the sleep stages are reported the three stages of sleep, for wildtype flies. Values reported are Means±SEM.

	DD	**Ramps**
Activity	11.07±0.13	18.63±0.11
Standard sleep	23.10±0.09	6.86±0.12
Short sleep	5.03±0.35	8.16±0.37
Intermediate sleep	1.57±0.49	7.82±0.46
Long sleep	18.52±0.20	1.47±0.16

Table 17: Results of one-tailed paired *t*-tests to compare the subjective day *vs* subjective night difference in cumulative power of ultradian rhythms in *short* sleep for wildtype (*Canton-S*) and loss-of-function clock mutants (per^{01}). Statistically significant differences are italicized and marked in red.

	wildtype	***loss-of-function clock mutant***
day	*165.18*	156.54
night	*120.5*	132.18
t	*3.95*	1.36
df	*30*	27
p	*0.0002207*	0.09193

Table 18: Results of one-tailed paired *t*-tests to compare the subjective day *vs* subjective night difference in cumulative power of ultradian rhythms in *intermediate* sleep for wildtype (*Canton-S*) and loss-of-function clock mutants (per^{01}). Statistically significant differences are italicized and marked in red.

	wildtype	***loss-of-function clock mutant***
day	153.6	154.16
night	133.74	131.65
t	1.16	1.67
df	30	27
p	0.1287	0.05368

Table 19: Results of one-tailed paired *t*-tests to compare the subjective day *vs* subjective night difference in cumulative power of ultradian rhythms in *long* sleep for wildtype (*Canton-S*) and loss-of-function clock mutants (per^{01}). Statistically significant differences are italicized and marked in red.

	wildtype	***loss-of-function clock mutant***
day	*121.97*	150.24
night	*158.29*	131.99
t	*-2.06*	1.21
df	*29*	26
p	*0.0244*	0.8818

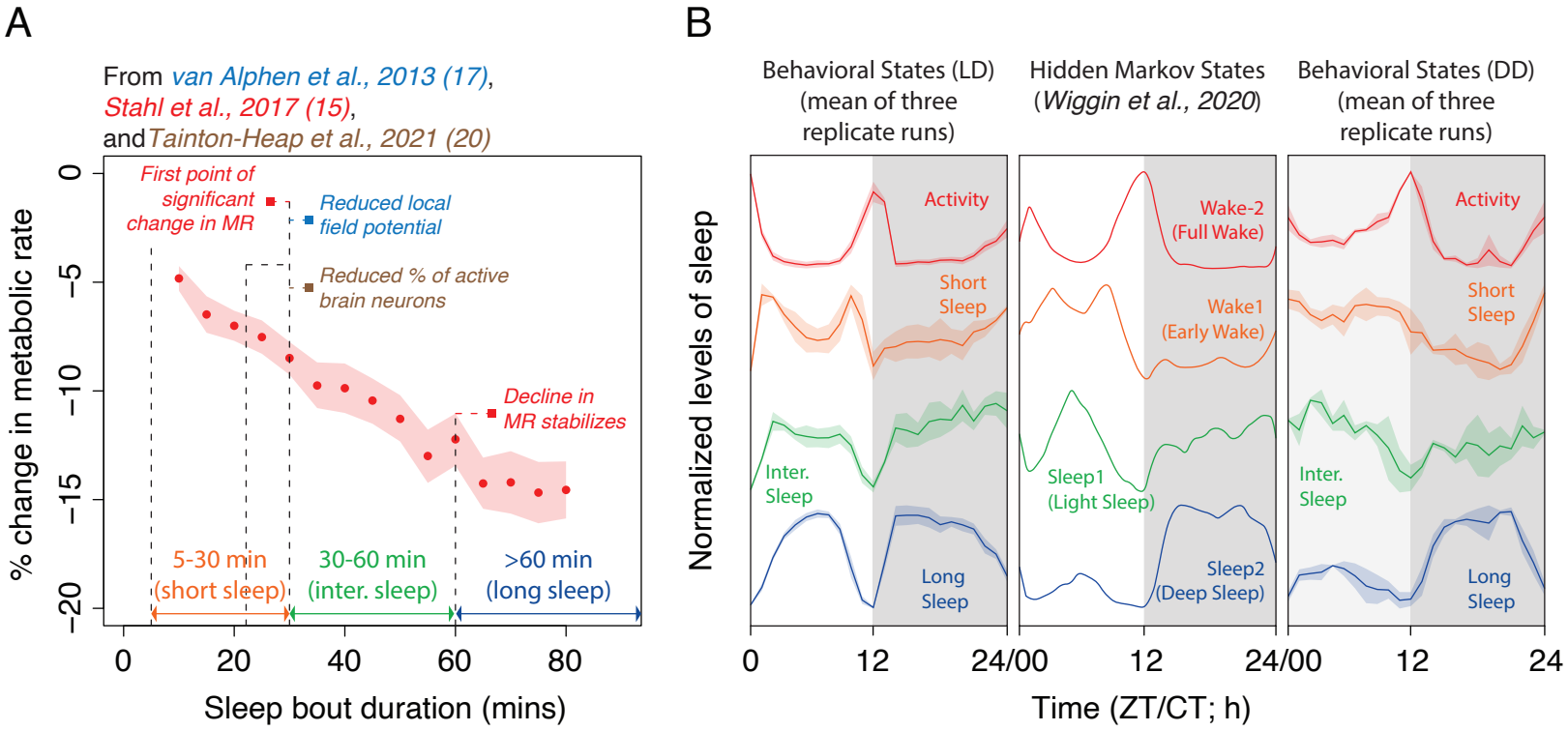
A
From van Alphen et al., 2013 (17),
Stahl et al., 2017 (15),
and Tainton-Heap et al., 2021 (20)
First point of significant change in MR
Reduced local field potential
Reduced % of active brain neurons
Decline in MR stabilizes
5-30 min (short sleep)
30-60 min (inter. sleep)
>60 min (long sleep)
% change in metabolic rate
Sleep bout duration (mins)
B
Behavioral States (LD) (mean of three replicate runs)
Hidden Markov States (Wiggin et al., 2020)
Behavioral States (DD) (mean of three replicate runs)
Activity
Short Sleep
Inter. Sleep
Long Sleep
Wake-2 (Full Wake)
Wake1 (Early Wake)
Sleep1 (Light Sleep)
Sleep2 (Deep Sleep)
Normalized levels of sleep
Time (ZT/CT; h)

Figure 1

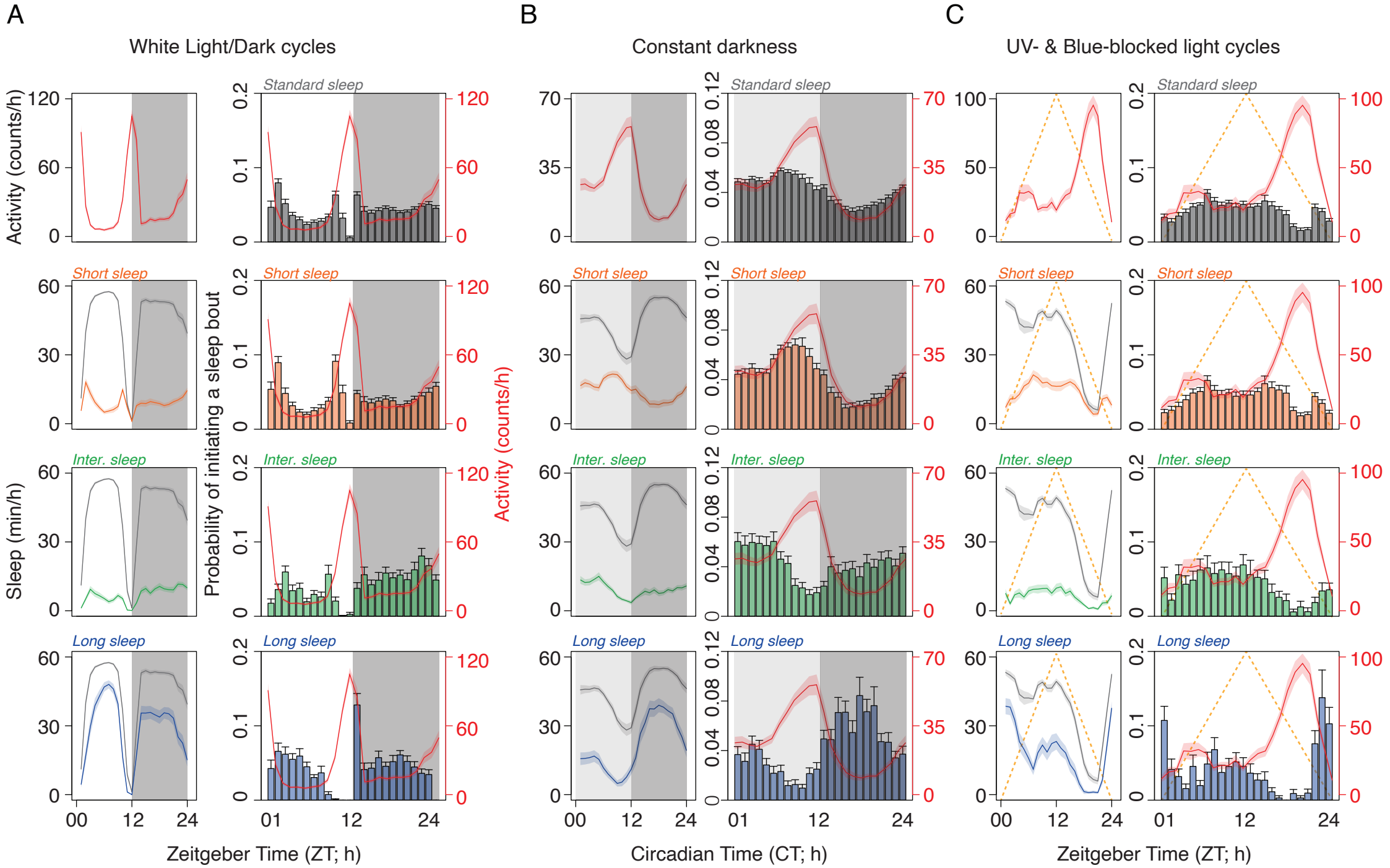
A
White Light/Dark cycles
B
Constant darkness
C
UV- & Blue-blocked light cycles
Activity (counts/h)
Sleep (min/h)
Probability of initiating a sleep bout
Activity (counts/h)
Standard sleep
Short sleep
Inter. sleep
Long sleep
Zeitgeber Time (ZT; h)
Circadian Time (CT; h)
Zeitgeber Time (ZT; h)

Figure 2

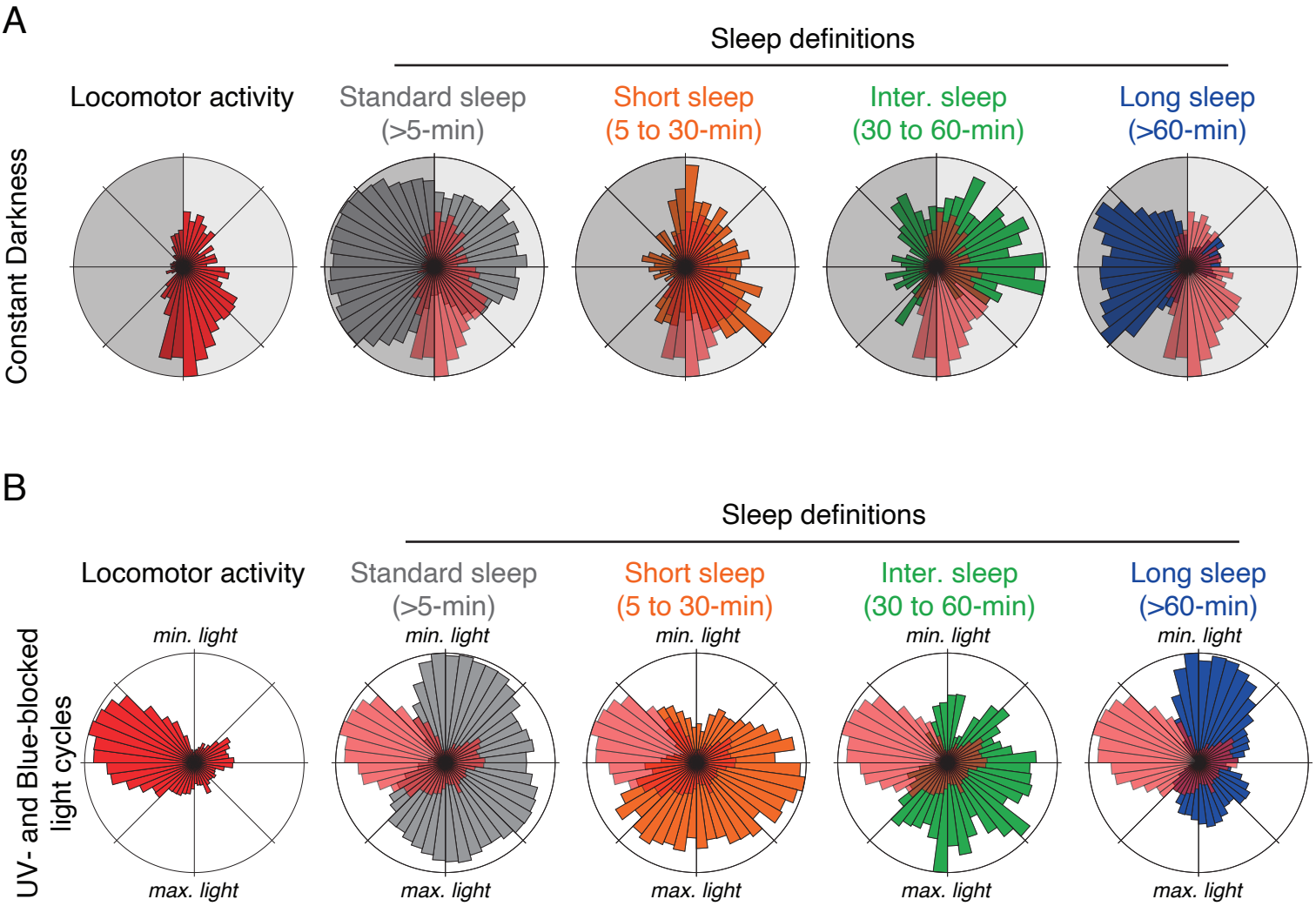
A
Sleep definitions
Locomotor activity
Standard sleep
(>5-min)
Short sleep
(5 to 30-min)
Inter. sleep
(30 to 60-min)
Long sleep
(>60-min)
Constant Darkness
B
Sleep definitions
Locomotor activity
Standard sleep
(>5-min)
Short sleep
(5 to 30-min)
Inter. sleep
(30 to 60-min)
Long sleep
(>60-min)
UV- and Blue-blocked
light cycles
min. light
max. light

Figure 3

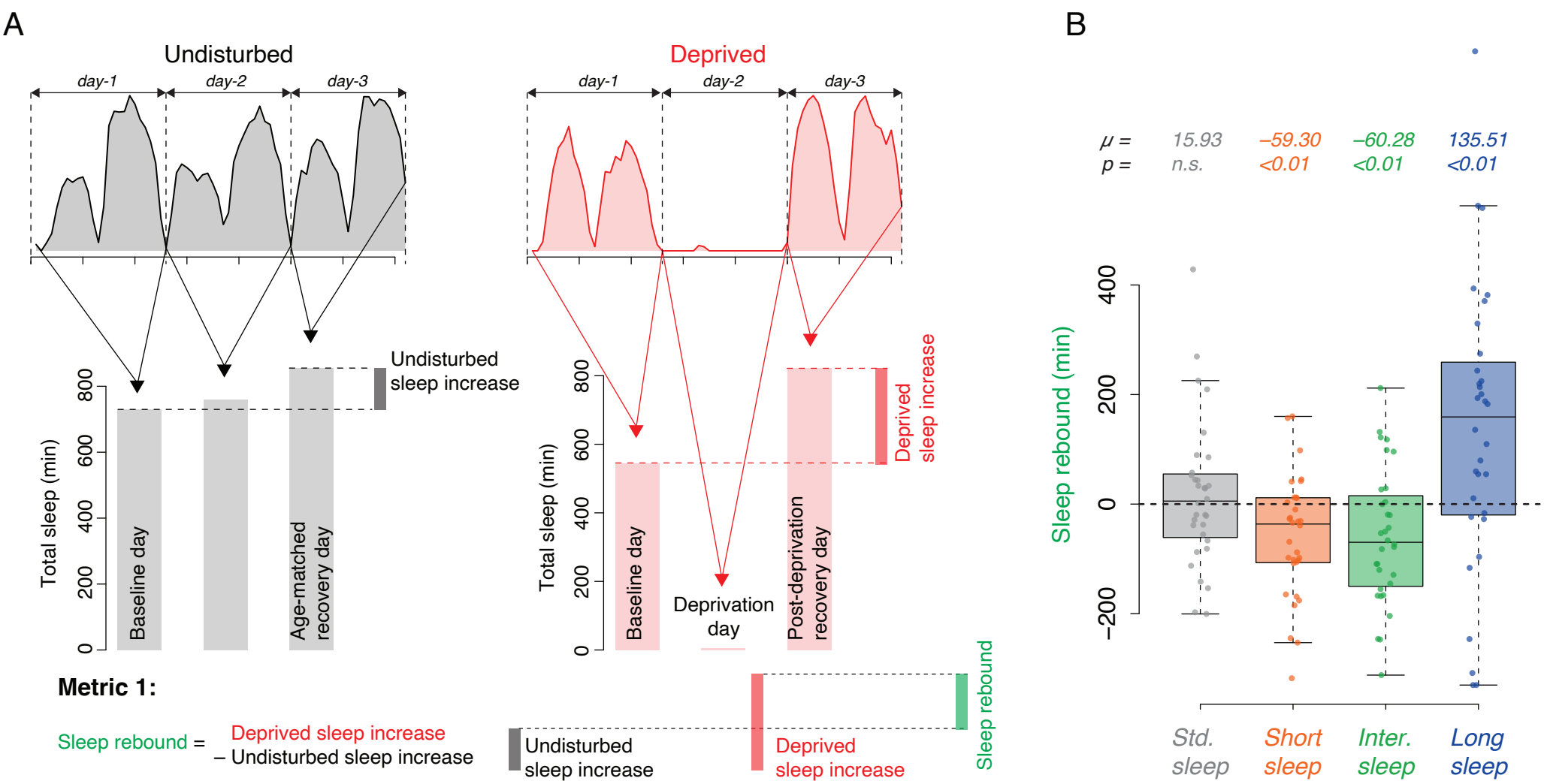
A
Undisturbed
day-1
day-2
day-3
Total sleep (min)
0
200
400
600
800
Baseline day
Age-matched recovery day
Undisturbed sleep increase
Deprived
Deprivation day
Post-deprivation recovery day
Deprived sleep increase
Metric 1:
Sleep rebound = Deprived sleep increase – Undisturbed sleep increase
Undisturbed sleep increase
Deprived sleep increase
Sleep rebound
B
μ = 15.93 −59.30 −60.28 135.51
p = n.s. <0.01 <0.01 <0.01
Sleep rebound (min)
−200
0
200
400
Std. sleep
Short sleep
Inter. sleep
Long sleep

Figure 4

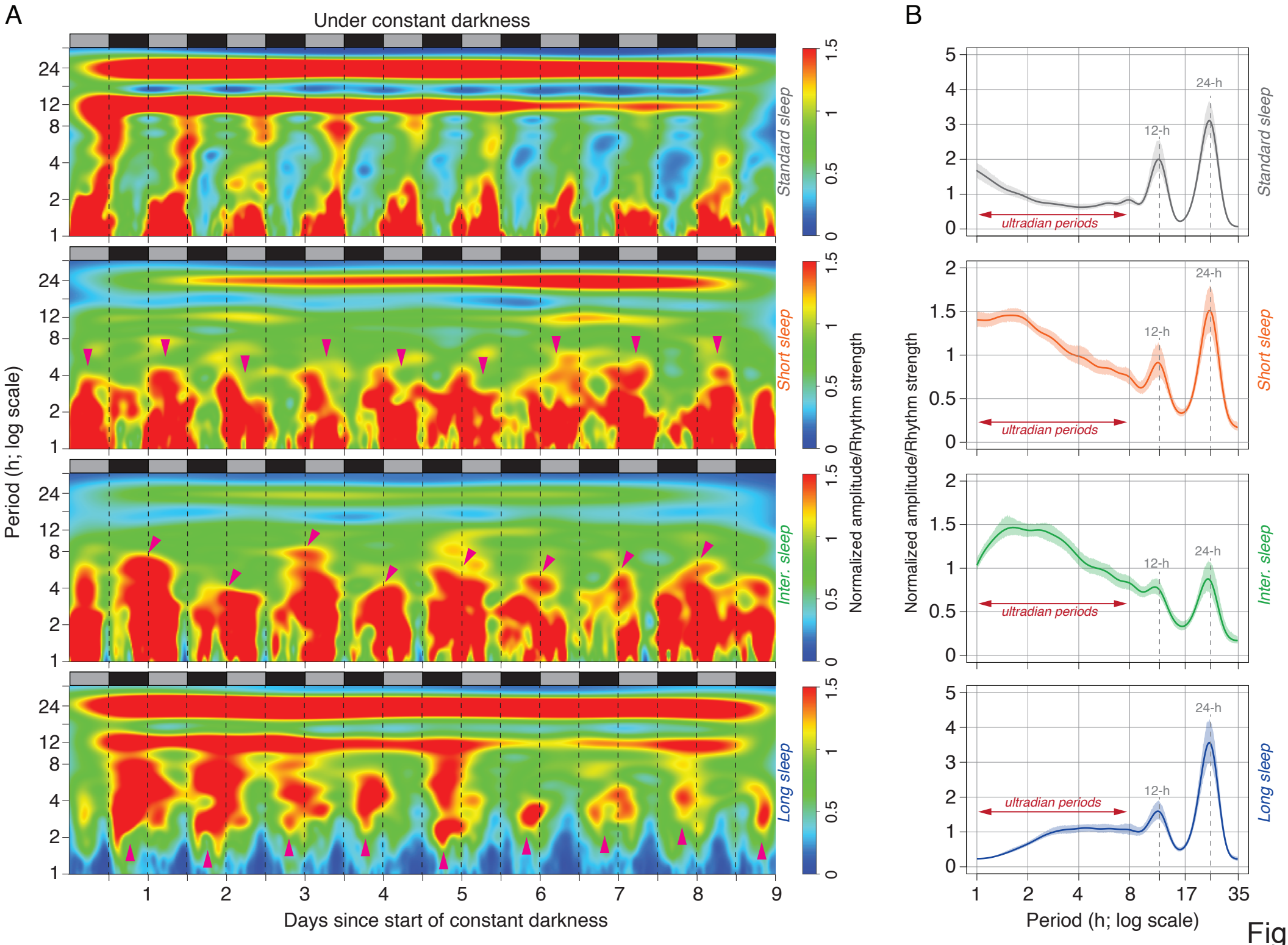
A
Under constant darkness
Period (h; log scale)
Days since start of constant darkness
Standard sleep
Short sleep
Inter. sleep
Long sleep
Normalized amplitude/Rhythm strength
B
ultradian periods
12-h
24-h
Period (h; log scale)

Figure 5

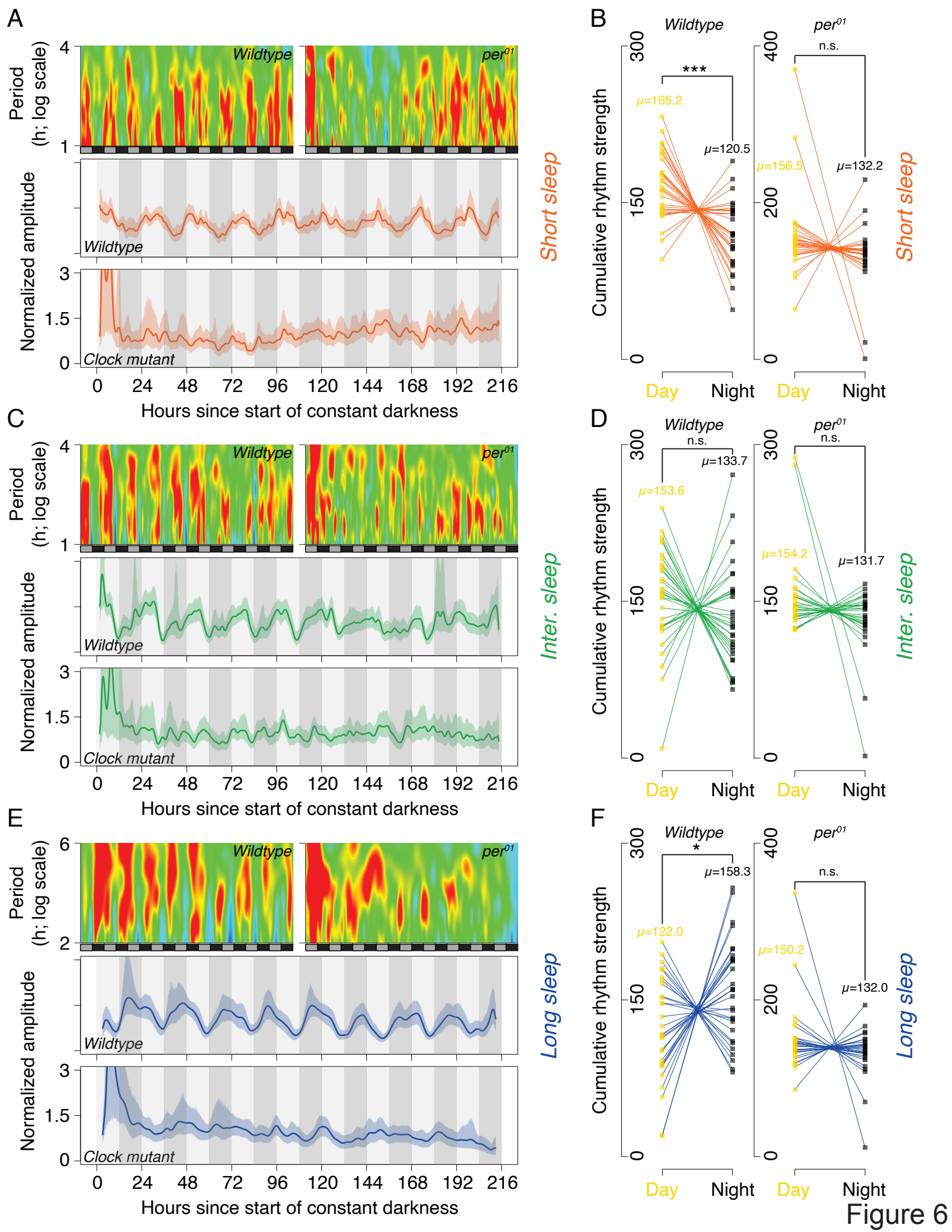
A
Period (h; log scale)
Wildtype
per01
Normalized amplitude
Clock mutant
Hours since start of constant darkness
Short sleep
B
Cumulative rhythm strength
***
n.s.
μ=165.2
μ=120.5
μ=156.5
μ=132.2
Day
Night
C
Inter. sleep
D
μ=153.6
μ=133.7
μ=154.2
μ=131.7
E
Long sleep
F
*
μ=122.0
μ=158.3
μ=150.2
μ=132.0

Figure 6