# Circadian Regulation of Sleep in *Drosophila*: Insights from a Two-Process Model

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## Abstract

Sleep is largely controlled by two processes – a circadian clock that regulates its timing and a homeostat that regulates the drive to sleep. *Drosophila* has been a useful model for understanding the molecular and neuronal control of circadian rhythms, and more recently, also for the homeostatic control of sleep. Consequently, mechanisms underlying both the circadian and the homeostatic processes regulating sleep have been characterized. Borbély and Daan's two-process model has provided a powerful quantitative framework for understanding how circadian and homeostatic processes converge to regulate sleep for four decades. Despite the clear utility of this model for understanding mammalian sleep regulation, the field of fly sleep has not employed a formal two-process model as a framework for the investigation of sleep control. To this end, we have adapted the two-process model to fly sleep. In this study, we establish the utility of this model by showing that it can provide empirically testable predictions regarding both the circadian and homeostatic control of fly sleep. Using this model, we show that the ultradian rhythms previously reported for loss-of-function clock mutants are a predictable consequence of a functional sleep homeostat in the absence of a functioning circadian system. Though a two-process model in which the circadian and homeostatic components act independently captures most aspects of sleep remarkably well, we found that increasing the rates of sleep pressure changes when circadian clocks run faster produced better fits between predicted and observed sleep rhythms. Finally, our results indicate that longer sleep bouts better reflect the homeostatic process than the current unitary definition of sleep as any bout of inactivity lasting five minutes or more. This simple two-process model represents a powerful theoretical framework for future work on the molecular and physiological regulation of fly sleep.



*Keywords: Drosophila, sleep, circadian, sleep homeostat, two-process model, free-running, period, ultradian rhythms*

## Introduction

Sleep-like states are ubiquitous within the animal kingdom [1]. The complexity of sleep behavior has prompted many attempts at mathematically modeling its regulation. Such models provide a powerful quantitative framework for the experimental investigation of sleep regulation across taxa. They are particularly useful by virtue of their provision of testable hypotheses [2–9].

Some of the most successful models of sleep regulation have been variants of Borbély's two-process model [2,4–7], which posits the presence of a sleep pressure that builds during wakefulness (Fig. 1A). Once this sleep pressure reaches an upper threshold, sleep is induced. During sleep, pressure falls until it reaches a lower threshold, at which point wakefulness is initiated. According to this model, the build-up and discharge of sleep pressure is driven by one process – the sleep homeostatic component or the S-process, whereas the upper and lower thresholds change predictably throughout the day and are set by a second process– the circadian clock or the C-process (Fig. 1A). Though myriad factors impact sleep [10], the regulation of typical sleep/wake cycles can be explained by just these two processes acting in concert but operating independently [6,7]. This model has been a major conceptual framework in the field for nearly four decades owing to its ability to predict the timing and amount of sleep in mammals, both during normal sleep/wake cycles and in the aftermath of sleep deprivation [5–7]. The model has also been used over the years to inform sleep clinicians in the treatment of sleep disorders associated with major depression, seasonal affective disorders, irregular sleep patterns, and the application of bright light therapy [7]. Additionally, the model is useful for assessing potential interactions between the two processes and how they might contribute to the control of sleep [11].

Although *Drosophila* has proved to be a critical model system for understanding the genetic, neuronal and physiological bases of circadian rhythms since the 1970s, it has become an informative model for understanding sleep only over the past two decades [12–14]. Furthermore, because of the availability of a powerful array of genetic tools and the ability to spatially and temporally regulate gene expression in the fly, the *Drosophila* sleep field has made significant advances in understanding the regulation of sleep [15–17]. While much of the focus of fly sleep research has been on the identification of genes and neural networks that regulate the amounts of sleep [14,16,18–27] relatively little is known about the regulation of sleep timing (e.g., [28]). Therefore, much remains to be learned about how circadian clocks and homeostatic control mechanisms interact to regulate sleep.

While there are significant gaps in our understanding of sleep regulation, the fly continues to provide insights into its mechanisms [14]. A major reason for this is the apparent similarity (behavioral, genetic, and physiological) between fly and mammalian sleep [29]. For example, as in mammals, flies display bouts of extended inactivity characterized by increased arousal thresholds, rapid reversibility, and homeostatic rebound in response to sleep deprivation [12,15,30]. In addition, a major feature of mammalian sleep is the occurrence of distinct stages of sleep, i.e., rapid eye movement (REM) and several non-REM (NREM) sleep stages [31]. Although various durations of inactivity have previously been classified as distinct epochs of sleep [12,32], fly sleep is now almost universally treated as a unitary state, with any period of inactivity lasting more than five minutes being considered a sleep like state [14,32]. However, recent work has provided strong evidence for multiple sleep stages in flies, with longer bouts of inactivity representing physiologically, metabolically, and behaviorally distinct sleep states compared to

shorter bouts [33–39]. Owing to the predictive power of the two-process model of human sleep regulation, we sought to establish a quantitative theoretical framework for a two-process model of fly sleep. Here we describe the development of such a model, identify the sleep homeostatic parameters that best explain fly sleep, and use it to reveal interesting relationships between sleep and the circadian clock. Based on this work, we propose that longer durations of inactivity, which have been recognized by others as reflecting relatively deep sleep, are a likely a stronger reflection of sleep homeostatic processes in *Drosophila* than the currently employed unitary definition of fly sleep.

## Results

### Circadian time-series analysis of *Drosophila* sleep

Though sleep is considered a major output of the circadian system, sleep behavior in flies has rarely been subjected to circadian time-series analysis. The vast majority of studies use locomotor activity, typically measured as infrared beam crossings, for such analyses. However, we argue that to fully understand the circadian control of sleep, time-series analyses should be performed directly on sleep data. For this reason, we have developed an analysis package [40], which is now available on the Comprehensive R Archive Network (CRAN; https://cran.r-project.org/web/packages/phase/index.html). Similar to double plotted actograms for locomotor activity, sleep rhythms can be visualized as double plotted somnograms (Fig. 1B). Consistent with previous work [12,14,30], somnograms reveal two distinct phases of increased sleep under light/dark (LD) cycles, one during the daytime and one during the nighttime (Fig. 1B). Although the field has long recognized that fly sleep is bimodal, in that there are two major episodes of sleep in each diurnal or circadian cycle [12,14,30], traditional time-series methods to quantitatively

assess bimodality and periodicity in sleep time-series have not been employed. We used the Chi-squared ($\chi^2$) periodogram [41] on sleep time-series data to analyze the two daily peaks of sleep under both LD cycles and constant darkness (DD), revealing two clear peaks in the periodogram, one at 12-h and the other at 24-h (Fig. 1C). Though largely similar, there are subtle differences between the periodograms of sleep and activity data measured under LD cycles and DD (Figs. 1C and 1D, compare across panels and see Table 1). Generally speaking, the powers of average sleep time-series are higher than the powers of locomotor activity time-series (see Table 1). The 12-h peak is typically lower than the 24-h peak in periodograms, but this difference is much smaller for the locomotor activity periodograms – in other words, the 12- and 24-h periodogram peaks are of more comparable height for locomotor activity (compare Figs. 1C and 1D). Using the last four cycles under entrainment, we estimated the percentage of a 24-h day that a fly spends sleeping. Across the three genotypes, we found that flies spend ~70 to 80% of their day sleeping (Fig. 1E). We used these two general features of sleep under LD conditions, the presence of two daily peaks in the periodogram (at 12- and 24-h under LD) with approximately 75% of time spent sleeping within a 24-h cycle, to develop a two-process model of fly sleep.

**Formulating the *Drosophila* two-process model**

Although the schematic of the two-process model described above (Fig. 1A) produces a single daily peak of sleep, some animals, including humans, often show biphasic sleep with a nap during mid-day and deeper sleep during the night, much like sleep in *Drosophila* [15,42]. S and C parameters that could produce bimodal sleep (e.g., daytime, and nighttime sleep episodes) were identified in an earlier two-process model (Fig. 2A) [5]. We therefore began building our model with previously published parameter values defining the C-process that were shown to produce

bimodal sleep and created various models consisting of different combinations of build-up and decay rates of sleep pressure. For each of these models we first asked if they produced unimodal, bimodal, complex, or arrhythmic sleep patterns. We found that a large region of the parameter space of S-process (all values that S can have, as defined by the input to the model) produced complex sleep rhythms (Fig. 2B), meaning they produced periodograms with three or more peaks above the significance line. A significant region of the parameter space failed to produce coherent sleep rhythms (Fig. 2B), meaning that there were no detectable periodicities revealed by our $\chi^2$ analysis. Small regions of the parameter space produced coherent unimodal (one 24-h periodogram peak) or bimodal (peaks at 12-h and 24-h) sleep rhythms (Fig. 2B; also see Suppl. Fig. 1).

Given that we were interested in parameter values that produce two daily episodes of sleep, we focused on combinations that yielded two periodogram peaks. Among these, we quantified the percentage of time spent sleeping per cycle. For each set of parameters, we then compared the values they produced with the percent of time that three fly genotypes, *Canton-S*, $w^{1118}$ and *yw*, slept per cycle, using sum of squared differences (SSD; see Materials and Methods; Fig. 2C). We found that the lowest SSD (best fit) was attained when the value for the build-up of sleep pressure was 0.09 and the decay of sleep pressure was 0.94 (Fig. 2C). Therefore, we chose these parameter values as the best descriptors for daily fly sleep under standard LD cycles. The model generated using these parameter values is shown in Fig. 2D. Though it appears to underestimate daytime sleep and somewhat overestimate nighttime sleep, the percentage time spent sleeping/24-h cycle predicted by the model was ~80%, which is close to the percentage of time that the three fly strains spend asleep (Fig. 2E).

## Predictions from the two-process model

We built a two-process model of *Drosophila* sleep in order to systematically predict the effects of altering either the S- or the C-process on sleep rhythms. We first asked what would happen to daily sleep if the circadian modulation of sleep thresholds were abolished. To model this, we set the amplitude of the upper and lower thresholds to zero, such that they were held constant at their mean values. Our model predicted that eliminating the contribution of the circadian system would result in ultradian sleep rhythms (Figs. 3A and B). Ultradian rhythms in the locomotor activity of flies with no functional circadian clocks have been reported previously [43]. Our model suggests that such rhythms are a predictable consequence of a functional S-process in the absence of circadian modulation of sleep thresholds and predicts ultradian periodicities of sleep of eight- to nine-hours per cycle in the absence of a circadian clock. We tested this prediction by examining sleep rhythms in wildtype and loss-of-function clock mutants ($per^{01}$) under constant darkness using continuous wavelet transforms (CWT; a method to detect wide range of periodicities at local timescales) as have been used previously for circadian time-series data (see Materials and Methods) [44–47].

We found strong 12- and 24-h periodicity in the sleep rhythms of wildtype (*CS*) flies, as expected (representative CWT spectrograms in Fig. 3C), along with weak ultradian components around seven to eight hours (Fig. 3E). As expected, there were no detectable periodicities in the circadian range for $per^{01}$ mutants (representative CWT spectrograms in Fig. 3D). However, there was a broad distribution of ultradian periodicities ranging from four to ten hours (Figs. 3D and E) in these flies. The range of ultradian periodicities that we detected for sleep was similar to that

detected previously for locomotor activity [43], albeit the variance in periodicities reported were larger than those we observed here for sleep. Dowse et al., 1987 [43] reported ultradian periodicities ranging from four to 22-h. Our results indicate that sleep/wakefulness rhythms are indeed ultradian and driven by a functional S-process in the absence of circadian clock-controlled thresholds.

**How independent are the S- and C-processes in *Drosophila*?**

Based on the persistence of the C-process in the absence of sleep and the maintenance of the S-process in the absence of circadian timekeeping, the two-process model assumes that they act without influencing aspects of each other (reviewed in [11]). For example, rhythms in the propensity to sleep appear to be independent of the duration of prior waking or sleep duration, implying the independent action of the clock and the homeostat [31]. Furthermore, homeostatic increases in NREM sleep and slow wave activity are observed after sleep deprivation in the absence of the brain's central circadian pacemaker, the suprachiasmatic nuclei (SCN) [48–51]. Though these results support the independence of the S- and C-processes, recent studies have suggested that these two processes influence one another (reviewed in [11]). For example, loss-of-function modifications to the circadian clock appear to have significant impact on sleep homeostatic markers in mice and flies [32,52–56]. Furthermore, variation in human chronotypes is associated with variation in sleep homeostatic markers (reviewed in [57]). Thanks to the genetic tools available for the manipulation of the C-process, we were able to generate flies with a wide range of circadian periodicities and assess the potential relationships between the period of the C-process and the amount and waveform of sleep. Thus, we can compare the model's predictions to the experimental effects of C-process manipulation on sleep rhythms. We first made predictions

about the power of sleep rhythms across a wide range of free-running periods (Figs. 4 and 5C) by altering the free-running periods of the upper and lower thresholds (see Materials and Methods), leaving all other parameters unchanged (Suppl. Fig. 2).

Our model predicted that specific ranges of free-running periodicities would be associated with relatively weak sleep rhythms. Specifically, that sleep rhythms would display relatively low periodogram powers for circadian periods between 15- and 19-h (unhighlighted somnograms), relatively higher power for periods between 19.5 and 25-h (green highlighted somnograms), and relatively low power for periods between 25.5 and 30-h (Fig. 4). Interestingly, power was predicted to be relatively high again at periods longer than 30.5-h (Fig. 4). We then determined the proportion of time spent sleeping/circadian minute for this entire range of circadian periods. We scaled sleep to circadian minutes to account for the difference in the total number of circadian sleep cycles that different free-running periods would complete for a fixed simulation time (see Materials and Methods). Our model predicted that the proportion of time spent sleeping would increase linearly with lengthening of circadian period (Fig. 5D; see subsequent sections).

To test these predictions, we recorded locomotor activity in flies with a wide range of circadian periods under DD and constant temperature conditions. We combined five *period* alleles (located on the X-chromosome): $per^{T}$ (free-running period – 15-h; [58]), $per^{S}$ (free-running period – 21-h; [59]), $per^{+}$ (free-running period – 23.5-h), $per^{L}$ (free-running period – 27.75-h; [59]) and $per^{01}$ (arrhythmic; [59]). Individually, these alleles produce significantly different free-running periods (see Table 2). By creating various combinations of these alleles (by including heterozygous females), we generated large numbers of flies with a continuous and wide range of free-running

circadian periods between ~15- to ~30-h, as previously described by others (see Table 2; [58,59]). We performed this experiment twice, pooled flies from both experiments, and quantified power and percentage time spent sleeping for all rhythmic flies observed (Tables 2 and 3). The sleep rhythms displayed by these flies revealed relatively weak power in flies with free-running periods between 15- and ~19-h (Fig. 5A). Power was relatively high for flies with periods between 19- and 25-h. Power dropped as periods increased beyond 25-h and increased again for periods between 28- and 30-h before falling again (Fig. 5A). Thus, the model accurately predicted a complex dependence of sleep rhythm power on free-running period (Fig. 5C), though the specific ranges of periods associated with weak sleep rhythms were not predicted precisely (Fig. 5C). We also quantified the relationship between free-running period and the power of locomotor activity rhythms. Interestingly, we found that there is a reasonable match in normalized power of rhythm between locomotor activity and sleep time-series in the short period range (between ~15- to ~21-h), but there is quite a mismatch for period values beyond ~21-h (Suppl. Fig. 3A). Consequently, the degree of match between power of locomotor activity time-series and predictions from the model is different from that with sleep time-series (compare Figs. 5C and Suppl. Fig. 3B), thereby highlighting the importance of using sleep time-series to study the circadian regulation of sleep rather than extrapolate from periodogram analyses on locomotor activity time-series. Interestingly, the proportion of time spent sleeping/circadian minute seems to increase with lengthening free-running period as predicted, but, in contrast to predictions, did so in a non-linear manner (Fig. 5B), with an unpredicted dip in the proportion of time spent sleeping for a narrow range of periods around ~18-h, after which it increased until a small dip at periods around 26-h (Fig. 5B). These dips were not predicted by the model (Fig. 5D), which predicted a somewhat linear increase in proportion of time spent sleeping with lengthening free-running period (Fig. 5D).

The model also tended to overestimate the time spent sleeping compared to the sleep measured in flies (Fig. 5D).

As discussed above, although the S- and C-processes were initially thought to act without influencing each other [31], recent work indicates that they do in fact influence each other [11,32,52–57]. We therefore adjusted the S-process parameters for each free-running period to maximize the fit with our experimental data once again using the SSD-based approach which, unsurprisingly, produced a much better fit for power and proportion of time spent sleeping (see Materials and Methods; Figs. 5C-D and 6A-B; also see Suppl. Fig. 4). The S-process values that produced this fit are shown in Figs. 6C and 6D. The relationship between these values and free-running period was complex and non-linear (Figs. 6C and 6D). Our adjusted model suggests that the build-up of sleep pressure is increased for periods between 16.5- and 19.5-h periodicities (Fig. 6C). For periods of 20-h and longer, our model indicates that S build-up rates are more or less constant until period of ~30-h, at which point the model predicts a small dip (Fig. 6C). The model also implies that the rate of sleep pressure decay will fall between periods of 15- and 21-h, beyond which it increases and stabilizes at around 24-h (Fig. 6D). Though a simple two-process model, in which the S- and C-processes unfold independently, captures a number of features of fly sleep, our analyses predict that the two processes are indeed interdependent in a way that has implications for our understanding of the regulation of sleep.

**To what extent is *Drosophila* sleep unitary?**

The original two-process model made predictions regarding the amount of total sleep time by modeling the S-process based on the exponential decay of the power of sleep EEG (0.75- to 25-

Hz). This wide range of EEG frequencies corresponds to all sleep types, i.e., slow wave and REM sleep. Interestingly, the decay constant derived from such a wide range of EEG frequencies was very similar to the constant derived from slow wave sleep alone (0.5- to 2-Hz) [5,60], thereby suggesting that although the two-process model accounts for total sleep, the S-process properties are most closely reflected by NREM sleep. Though sleep has largely been treated as a unitary state in the fly, several independent approaches support the notion that long durations of inactivity correspond to a relatively "deep-sleep" stage. For example, metabolic rates of flies that have been inactive for ~35-min are significantly lower than flies that have been inactive for the standard five-minute definition of fly sleep [33]. Furthermore, experiments tracking arousal thresholds and brain physiology identify deeper sleep intensities when flies have been inactive for ~30-min [34]. Since decay constants best reflect NREM sleep, we assessed the degree to which model predictions would describe empirical data when increasing durations of inactivity were used as the defining criterion for a sleep like state, thereby biasing our sleep analysis to longer, presumably deeper, bouts of sleep in experimental flies.

We systematically altered the inactivity duration definition of sleep, i.e., the duration of inactivity used to define the sleep state, and examined the correlation between the proportion of time flies spent sleeping and our model's prediction (Fig. 7). Traditionally, any bout of inactivity that is five minutes or more is defined, in flies, as sleep. It is clear that the correlation between our model's prediction and measurements of proportion of time spent sleeping in our series of allelic combinations of *period* changes very little with increasing inactivity definitions, at least until the criterion for sleep reaches 60-min of inactivity (Fig. 7). This result implies that shorter bouts of

inactivity contribute very little to how the proportion time spent sleeping varies with circadian period.

To further test this idea, we performed the same analysis as above but with specific ranges of inactivity durations used for the definition of sleep (see Materials and Methods for details). Briefly, a five to ten-minute interval definition of sleep would mean that any bout of inactivity that is equal to or more than five minutes but fewer than ten minutes would be considered sleep. We found that the correlation between empirical results and model predictions is relatively low when five to ten, five to 20 and five to 30 minutes of inactivity were used as the definitions of sleep (Fig. 7B). Correlations improved a little when the inactivity definition was set to 30 to 60-minutes and became almost as high as the standard definition of sleep (five minutes or longer) when set to 30 to 120 or 60 to 120-minutes (Fig. 7B). These results are consistent with recent work suggesting that different durations of inactivity are unlikely to represent the same state with respect to sleep in *Drosophila* [33–39].

**Inactivity bouts longer than 60-min reflect the S-process better than the standard definition of fly sleep**

As discussed above, our results suggest that homeostatic S-processes in the absence of a functional circadian clock are expected to produce ultradian sleep rhythms (Fig. 3). If longer bouts of inactivity better reflect the S-process than the standard definition of fly sleep, these ultradian rhythms would be unaltered in loss-of-function clock mutants when longer durations of inactivity are used to define sleep. This prediction stems from the idea that shorter bouts of inactivity are dispensable for the observed temporal patterns of sleep behavior. Indeed, we found that ultradian

periodicities in sleep rhythms are observed even when inactivity duration definitions of up to 60 minutes of inactivity are used for sleep (Figs. 8A and 8B-D). Interestingly, when we examined the CWT spectrograms for wildtype *CS* flies, it is clear that, as expected from decades of previous work, 12- and 24-h components strongly dominate the range of periodicities detected under DD (Figs. 8A and 8B-D). With increasing durations of inactivity as our definition of sleep, the ultradian components become more dominant while the circadian components become less dominant (Figs. 8B-D; see Discussion). Here, we use dominance to refer to the height of the histograms corresponding to each periodicity window (trace the dotted lines in Figs. 8B-D). Our results further strengthen the notion, recently proposed by others [33–39] that sleep is not a unitary state in flies, and that longer bouts of inactivity appear to better reflect the action of the S-process.

In fitting our two-process model to sleep observed over a range of circadian periods, our results suggested that the S- and C-processes, though largely independent, likely interact, with changes in the S-process predicted to accompany changes in the free-running period of the C-process. For example, we increased the fit of our model by changing the rates of S-process increases and decreases as a function of free-running period (Figs. 5 and 6). We therefore sought to examine the rates of build-up and decay of the homeostatic sleep pressure in flies with different free-running circadian periods. The relatively high values of S-process build-up and lower values of S-process decay both of which correspond to faster rates of build-up and decay (see Fig. 6), for flies with free-running periods around 20-h, suggest that flies with these circadian periods will display sharper increases and declines in sleep pressure compared to flies with longer periods (Figs. 6C and 6D). This leads to the prediction that, in the absence of the circadian cycling of sleep thresholds, ultradian rhythms in sleep/wake would be faster in $per^{s}$ mutants compared to $per^{+}$ flies.

*Drosophila* is arrhythmic under continuous white light (LL) owing to the constitutive CRYPTOCHROME mediated degradation of TIMELESS [61–63]. We tested our prediction by comparing ultradian periodicities under LL between $per^S$ and $per^+$ flies. Although the average time-series of sleep does not appear to have any ultradian periodicity under LL, individual traces hint at them which we quantified using CWT (Fig. 8E). We found that indeed, $per^S$ flies had significantly faster ultradian rhythms in sleep compared to $per^+$ flies (Fig. 9F; one-tailed Wilcoxon's $W = 360.5$, $p < 0.05$). Our results therefore support the model's predictions that free-running period and the rates of change of homeostatic sleep pressure components are interdependent.

**How does fly sleep compare to sleep across the animal kingdom?**

Our results suggest that short bouts of inactivity contribute only modestly to the overall daily pattern of sleep observed under varying free-running periods, and that longer bouts of inactivity fit the predictions of our two-process model of fly sleep well. We next examined the extent to which varying the inactivity duration definition of sleep might alter its temporal organization across the day. Environmental light promotes arousal and, when very bright, can suppress locomotor activity in flies (e.g., [64]). We therefore compared the temporal distribution of sleep across three entrained day and night cycles and across four subjective days and nights under DD, with varying definitions of sleep (Fig. 9A). As previously shown, the standard definition of sleep reveals two episodes of sleep per cycle under both LD and DD conditions (Fig. 9A), although this is less pronounced in DD. Interestingly, increasing the inactivity duration criterion for sleep produced no major effect on the timing of sleep under LD, but resulted in a near complete loss of

sleep during the subjective day (Fig. 9A). In other words, under free-running conditions the longest bouts of inactivity/sleep occur predominantly during the subjective night. Thus, the temporal regulation of short duration and long duration bouts of sleep appear to be different in the absence of an environmental cycle, suggesting that the circadian system likely regulates shallow and deep sleep differently. This result is consistent with previous work showing that subjective nighttime rest bouts are predominantly longer than 30 minutes [12]. For relatively long inactivity duration definitions of sleep, the mean and median profiles of sleep begin to diverge significantly, which is not seen for the standard definition of sleep (five minutes of inactivity or more). This is important to consider because averages may not be representative of central population tendencies when longer bouts of inactivity are considered [65]. The clear difference between the temporal organization of standard measures of fly sleep and longer bouts of sleep under constant darkness is consistent with the notion that short and long bouts of inactivity represent different kinds of sleep that are subject to distinct modes of regulation.

To gain perspective on how *Drosophila* compares to other animal species, we compared the percentage of time that flies spend sleeping to a wide range of animal taxa (Fig. 9B). A majority of this data set was curated from a review by Campbell and Tobler (1984) [1]. The remaining data were collected by a separate literature survey [66–74]. In all, we compared fly sleep to sleep in 187 species/populations (which includes *Drosophila* species from different geographical locations, other non-*Drosophila* invertebrates, non-mammalian vertebrates and mammals). There is considerably large variation in amount of sleep within the *melanogaster* species itself, depending on their geographical origin [74]. When using the standard definition of sleep, *CS* flies from our laboratory sleep more than that of 174 of these species/populations (Fig. 9B), 50% of which are

asleep for ~30 to ~60% of the day (Fig. 9B). If we define sleep as 60-min or more of inactivity, a definition that fits the S-process well in our model, flies sleep for approximately ~40% of the day, which would place flies closer to the middle of the distribution of sleep amount for the animal species surveyed here (Fig. 9B). It is important to note that it is challenging to directly compare the percentage of time spent asleep across such a wide range of taxa, given that a wide range of methods were used to measure sleep. However, in case of bats and opossums, among the animals that sleep more than our laboratory's *CS* flies in this survey, basis of the sleep measurement was electrical recordings [1]. Therefore, large proportions of time spent asleep are not necessarily a reflection of the use of behavioral measurements to quantify sleep amounts. Therefore, while it is possible that *Drosophila* as a genus is a long sleeping group, the close match of longer bouts of inactivity to the S-process mediated sleep (Figs. 7 and 8), and the amount of long bout sleep relative to other groups (in Fig. 9B) suggest distinct regulation of short and long bouts of inactivity.

## Discussion

*Analyzing Drosophila sleep*

Although sleep is recognized as a major output of the circadian system, fly sleep itself has rarely been subjected to time-series analysis and we are not aware of easily accessible tools for such analysis. We have therefore developed an open-access R package [40] (different from our recently published MATLAB software, PHASE [75]), that supports the quantitative assessment of sleep rhythms in the fly (https://cran.r-project.org/web/packages/phase/index.html). An important feature of this package is the ability to easily alter the inactivity duration-based definition of sleep (including specific intervals of inactivity durations), which, as we have shown through this manuscript, has significant impact on the temporal distribution, particularly under free-running

conditions (Figs. 7-9). We are currently developing a GUI for this package to increase the accessibility of its functionality for the fly community. Recent work by others has revealed that inactivity bouts of more than ~30-min represent a stage of sleep that is significantly different from shorter bouts of sleep and likely represent a deep sleep state [33–39]. Our results are consistent with this idea and show that longer bouts of inactivity best reflect sleep driven by the homeostatic S-process. The relatively long bout lengths implicated in this study relative to the ~30-min of inactivity described previously for deep sleep states are likely a product of the use of single beam activity monitors, which are known to overestimate sleep compared to multi-beam recordings or video tracking [76]. Given that NREM and REM stages of human sleep appear to be differentially controlled by the clock and the homeostat [77], future work on fly sleep should consider various definitions of sleep when examining the effects of experimental manipulations of the circadian clock or the sleep homeostat. We suspect that analyses of short and long bouts of sleep separately will provide important new insights into the regulation of sleep by the circadian and homeostatic systems that would be missed if only the standard unitary definition of sleep is employed in the analysis of sleep behavior. We also suggest that constant conditions are likely more appropriate for the study of the circadian regulation of sleep. The apparent absence of long-duration sleep during the subjective daytime DD conditions (Fig. 9A) suggests that long periods of immobility during the daytime under LD cycles may reflect the action of bright light on locomotor activity and may not reflect homeostatic and/or circadian control of sleep. Furthermore, given the considerably large inter- and intra-specific variation reported in the amount of *Drosophila* sleep (see Table 4 and Fig. 9; [74]), the most appropriate inactivity durations used to define distinct kinds of sleep will likely differ between fly strains.

*A simple two-process model assuming the independence of the S- and C-processes captures the general patterns of Drosophila sleep*

The theoretical framework around Borbély and Daan's two-process models has played a large part in the advances made in our understanding of mammalian sleep [2,4–7]. The conservation of sleep-like states across taxa and the power of *Drosophila* as a genetically tractable organism has made the fly a fruitful model system for the study of sleep regulation. However, the field of fly sleep has not benefitted from the strong theoretical framework provided by a formal two-process model. Our goal, therefore, was to adopt this model to *Drosophila* sleep and evaluate the extent to which it can guide our understanding of its circadian and homeostatic regulation.

An implicit assumption of the two-process model was that while the S- and C-processes cooperate to determine the timing and amount of sleep, they do not influence each other's properties and previous work is consistent with the conclusion that that and S- and C-processes can operate independently. For instance, loss of the circadian clock does not prevent homeostatic sleep rebound following deprivation and the circadian clock continues to function normally during prolonged sleep deprivation (reviewed in [11]). Based on this apparent independence, our initial model assumed that the two processes governing sleep act independently in the fly. This simple model predicted the presence of ultradian sleep rhythms in the absence of a functional circadian clock, as consequence of the rise and fall of the S-process between the flat upper and lower sleep thresholds (Fig. 3B) and correctly predicted that $per^{01}$ mutants, which lack a functional circadian oscillator, would display prevalent ultradian periodicities in sleep (Figs. 3D and 3E). Previous work on loss-of-function *period* mutants detected the presence of ultradian rhythms in locomotor activity [43], which were hypothesized to be due to an uncoupling of ultradian oscillators

[43,78,79]. However, our results suggest that ultradian rhythms are a predicable outcome of a functional S-process in the absence of a cycling C-process. It is important to note here that although the model predicts an ultradian sleep rhythm of ~eight to ~nine hours, our results in *per*$^{01}$ flies reveal a much shorter period, albeit with a wide distribution of periods. Among the possible explanations for this discrepancy are a reduced distance between the thresholds in the mutants, a steeper rise or decay of sleep pressure or a combination of both. Enhanced sensitivity of *per*$^{01}$ mutants to sleep deprivation has been reported previously, suggesting a pleiotropic effect of the loss of *period* on the sleep homeostat [32].

To further test the extent to which an independent S- and C-process model could account for sleep rhythms in flies, we generated predictions for the power of sleep rhythms for a wide range of free-running periods and found, surprisingly, that some free-running periods were predicted to be associated with relatively weak sleep rhythms (Fig. 4). Remarkably, when we examined sleep rhythms in an allelic series of *period* mutants, we found, as predicted by the model (Fig. 4), that some ranges of free-running circadian period were indeed characterized by relatively weak and unstable sleep rhythms (Fig. 5A and 5C). Thus, the simple two-process model not only predicted the daily amount and waveform of sleep and the presence of ultradian sleep rhythms in loss-of-function clock mutants, but it also predicted an unexpected relationship between circadian period and strength of sleep rhythms. However, the predicted ranges of periods that should be associated with weakened sleep rhythms did not precisely match our experimental results. Furthermore, the model failed to predict the reduction in the percentage of time spent sleeping within certain ranges of free-running period that we observed in our *period* allelic series and generally overestimated the amount of time spent sleeping (Fig. 5D).

*Adjusting the model to better fit experimental data suggest complex and non-linear relationships between the S- and C-processes*

Based on these discrepancies we sought to make simple changes to our model that would better capture the sleep behavior of flies over a wide range of circadian periods. We therefore adjusted our model by varying homeostatic parameters for each free-running period to increase the model's fit to our experimental data (Figs. 6A and 6B). These adjustments lead to the prediction that the rates of S increase and decrease have a complex relationship with the period of the C-process. Specifically, our adjusted model predicts that the parameter regulating S-process increases are relatively high (i.e., faster build-up) for flies with periods between approximately 18- and 20-h. Further, the parameter regulating S-process decreases were low (i.e., a faster decay) for flies with short periods of approximately 19- and 22-h (Figs. 6C and 6D). We tested this prediction by comparing sleep behavior in wildtype ($per^{+}$) and short period ($per^{S}$) flies under constant light, conditions which the C-process is rendered arrhythmic, and a functional S-process produces ultradian sleep rhythms (Figs. 3 and 8). If, as predicted by our model, $per^{S}$ has a faster rate of S-process increase and decrease (Fig. 6C and D), we would predict that the ultradian sleep rhythms would have a decreased period in the $per^{S}$ mutant. This prediction was supported by our experimental results (Figs. 8E and 8F), highlighting the utility of our model as a framework for understanding sleep regulation in the fly.

The shorter ultradian periods of $per^{S}$ flies under LL is remarkable because PER protein is constitutively low under continuous light [80]. This implies that there may be a fundamental relationship between clock speed and the rates of change of the sleep homeostatic system. It is,

however, possible that the free-running circadian period present during development shapes homeostatic systems so as to coordinate the build-up and decay of sleep pressure with clock speed. It is also possible that *period* alleles have different effects on the homeostatic control of sleep via epigenetic mechanisms. Recent studies have reported differential epigenetic regulation associated with cell populations with divergent periods and distinct levels of clock gene expression [81,82]. Our results in flies may be broadly relevant to animal sleep regulation, as similar results have been reported in humans [57]. Specifically, the human $PER3^{5/5}$ polymorphism is highly enriched in morning chronotypes, and such early types have faster running circadian clocks. Remarkably, $PER3^{5/5}$ individuals experience higher sleep pressures and a faster build-up of non-REM activity during sleep deprivation compared to another human *PER* polymorphism, $PER3^{4/4}$ [57,83].

*The utility of the two-process model of fly sleep for understanding previous findings and the generation of new testable hypotheses*

Our results highlight the utility of a formal two-process model with regard to the circadian control of fly sleep. An examination of this model, particularly in the context of the growing body of knowledge regarding the relationships between circadian clock neurons and sleep timing, leads to new specific and testable hypotheses. Under normal circumstances (i.e., the absence of physiological or social challenges), the model suggests that the onset of daytime and nighttime bouts of sleep are timed by the rising phase of the upper threshold (Fig. 10A). Furthermore, the model suggests that the timing of morning and afternoon waking are both regulated by the rising phase of the lower threshold (Fig. 10A). Finally, the falling phase of the lower threshold is predicted to play an important role in the maintenance of nighttime sleep (Fig. 10A). For the sake of simplicity, we assume that the upper and lower thresholds are regulated in unison by the

circadian clock neuron network, with a constant distance between them. Work over the last decade has assigned sleep- or wake-promoting functions to specific classes of circadian clock neurons (reviewed in [14]). For example, the small ventral lateral neurons (s-LNvs) and the dorsal lateral neurons (LNds) are thought to be wake-promoting, whereas the dorsal neuron 3 class (DN-3) is thought to be sleep-promoting (reviewed in [14,16]). In these cases, the daily timing of neural activity displayed by these cell types, as measured by relative $Ca^{2+}$ levels, fits these proposed roles in sleep control well (Fig. 10B). The s-LNvs display $Ca^{2+}$ peaks that coincide with the morning bout of locomotor activity, the LNds display peaks that coincide with the evening bout of locomotor activity, and the DN-3s display their highest $Ca^{2+}$ levels during the nighttime bout of sleep [84]. Integrating these patterns with our two-process model suggests that the s-LNvs control the timing of morning wakefulness by driving the initial rise of the lower sleep threshold, that the LNds promote the timing of evening wakefulness by the continued rise in the in the lower sleep threshold, and that the DN-3s promote the maintenance of nighttime sleep by driving the fall of the lower threshold throughout the night (Fig. 10B). Though we opted for a simple model for the rise and fall of the upper and lower threshold and have proposed that the action of specific subsets of clock neurons contribute either to the rise or fall of both, the model also suggests that the timing and duration of daytime and nighttime sleep windows could be controlled in other ways. For example, the distance between the two thresholds, the phase relationships between the two thresholds, or the relative amplitudes of the upper and lower threshold cycles might all be modulated by the circadian system to shape the timing and amount of sleep.

In addition to generating the above testable hypotheses regarding the specific roles of circadian clock neurons in the regulation of daytime and nighttime sleep, our model may reconcile

previously conflicting results. Several studies come to opposing conclusions regarding the role of the dorsal neurons 1 (DN-1) in sleep regulation: they have been proposed to be both wake- [85,86] and sleep-promoting [87,88]. Though these apparently incompatible conclusions may ultimately be explained by the different technical approaches used in these studies, our model may provide an explanation for how similar experimental manipulations of the same clock neuron classes might lead to contradictory conclusions. According to Liang et al., 2016 [84] the DN1ps display peak levels of neuronal activity in the middle of the night, a time associated with nighttime sleep, a pattern that would appear to fit a sleep promoting role for these clock neurons. However, our model predicts that the effect of acutely exciting the DN-1ps would depend on the timing of the manipulation on neural signaling. For instance, if the DN-1ps promote the fall in upper and lower sleep thresholds, as suggested by the relationship between the sleep thresholds and DN-1p $Ca^{2+}$ profiles (Figs. 10B), exciting these neurons during the late afternoon would lead to an early and prolonged fall in thresholds (Fig. 10C). According to the model, this would increase daytime sleep and cause a slight delay and decrease in nighttime sleep (Fig. 10C). An increase in daytime sleep is used by Guo et al., 2016 [88] to support the conclusion that exciting DN-1ps promotes sleep. In contrast, Kunst et al., 2014 [86], present evidence for increased daytime sleep (Fig. 10C), but highlight a reduction in nighttime sleep, which, while not observed by Guo et al., 2016 [88], is predicted by the two-process model. Blocking synaptic transmission in the DN-1ps has been shown to decrease sleep [88]. If we assume that this manipulation results in sustaining sleep thresholds at high levels, our model predicts this effect well (Suppl. Fig. 5).

*Speculations and Concluding Remarks*

Our time-series analysis of *Drosophila* sleep detected the presence of ultradian periodicities under constant conditions in both wildtype flies and mutants lacking circadian timekeeping. In wild-type flies these ultradian rhythms were dwarfed by the presence of strong circadian gating of sleep/wake cycles that produce the characteristic 12- and 24-h periodicities seen in normal sleep cycles in the fly (Figs. 8A-D). Might these relatively weak ultradian sleep rhythms reflect a cycling through different stages during the two daily bouts of sleep? Might such cycling occur at specific times of the circadian cycle? Answers to these questions await further detailed analyses of sleep time-series using the methods of analysis described here.

Here we have established a formal, quantitative model of sleep regulation in *Drosophila* and have used it to guide the development of testable hypotheses regarding the circadian control of sleep. We've also made this accessible to the wider sleep community by generating a Shiny-based GUI to facilitate simulations. We propose that this model can serve as a powerful framework for investigating the contributions of the various clock neuron to sleep regulation. Our model also makes quantitative predictions regarding the inter-dependence of the clock and homeostatic processes, which we have begun to test here. At first glance, these predictions appear to be accurate and are reminiscent of human studies on the relationships between circadian timekeeping and the homeostatic control of sleep. This suggests that the relationships between these two processes discovered in the fly will be broadly relevant. Our analysis also suggests that simple changes to the way we define sleep-like states will support significant insights into the homeostatic regulation of fly sleep. Because the two-process model has been a major driving force for our understanding of sleep regulation in mammals, we propose that the model described in this

manuscript along with the adoption of varied definitions of sleep-like states will support significant new insights into the mechanisms controlling sleep.

## Materials and Methods

### Fly husbandry

*Canton-S (CS*; BDSC stock number: 64349*)*, *white*$^{1118}$ (*w*$^{1118}$; [89]), *yellow, white* (*yw*; BDSC stock number: 1495), *period*$^{TAU}$ (*per*$^{T}$; [58]), *period*$^{SHORT}$ (*per*$^{S}$; [59]), *period*$^{LONG}$ (*per*$^{L}$; [59]), and *period*$^{01}$ (*per*$^{01}$; BDSC stock number: 80928) flies were used for the behavioral experiments reported in this manuscript. *CS* flies are also referred to as *period*$^{+}$ (*per*$^{+}$) throughout the text, as all the *period* alleles used here were in a *CS* background. Flies were reared on Corn Syrup/Soy media made by Archon Scientific (Durham, North Carolina). Flies were approximately five days old on day one of all behavioral experiments.

### Generation of flies with a wide range of free-running periods

For the generation of flies with a wide range of period values, virgin females, and males from all five *period* allele cultures (*per*$^{T}$, *per*$^{S}$, *per*$^{+}$, *per*$^{L}$ and *per*$^{01}$) were collected over the span of approximately five days. Reciprocal crosses were performed, resulting in a total of 25 distinct crosses. Male and female offspring from each of these 25 crosses, representing 50 distinct sex and genotype combinations (see Tables 1 and 2), were collected and assayed for locomotor activity as described below.

### Assay of locomotor activity

Locomotor activity was recorded using the *Drosophila* Activity Monitor (DAM) system (Trikinetics, Waltham, MA; https://trikinetics.com). Beam crossing counts were collected every minute for all experiments. Flies were recorded under LD12:12 cycles, constant darkness (DD) or constant bright white light (LL). In all experiments reported in this manuscript, flies were assayed under 25 °C and the intensity of white light was between ~400 and ~500 lux.

**Data analyses and visualization**

*Circadian time-series analysis of Drosophila sleep*

Locomotor activity of approximately 32 each of *CS*, $w^{1118}$ and *yw* male flies was recorded under LD12:12 for nine days, after which activity was recorded under constant darkness (DD) for seven days. Beam crossing data from these recordings were used to determine the pattern and amount sleep for each fly. Individual fly locomotor activity and sleep time-series data, binned in 1-minute intervals, were subjected to (Chi-squared) $\chi^2$ periodogram analyses. To visualize sleep profiles as averaged time-series, flies from three independent experiments under LD12:12 were pooled, which included more than 90 flies for each genotype.

*Calculating the percentage/proportion of time spent sleeping*

For fly sleep under LD cycles in this study, the percentage of time spent sleeping was calculated by first computing the total sleep over the entire duration of the experiment for each fly (last four cycles of entrainment). This total was divided by the number of 24-h cycles (i.e., 4), and then multiplied by $\frac{100}{24}$.

Percentage of time spent sleeping for other species were computed based on the number of hours of sleep reported in Campbell and Tobler (1984) [1]. In addition, sleep amounts were estimated from published figures for a few other species whose sleep has been more recently quantified [66–71]. In all these cases the averaged/reported amount of sleep is multiplied by $\frac{100}{24}$.

For flies under DD in this study and for simulations of free-running sleep, the total amount of sleep (in minutes) over the entire duration of the experiment or simulations (for each fly in case of the experiment) was calculated. Because flies with different free-running periods would differ in the number of sleep/wake cycles experienced during free run – for example periods shorter than 24-h would complete more than 10 cycles in 10, 24-h days and clocks with periods longer than 24-h would complete fewer – we accounted for clock speed in our calculations. We did this by estimating the number of circadian cycles each fly, or model output completed over the entire duration of the experiment/simulation. In the case of a 10-day experiment, a fly with a 23-h period would have free-run for $10 \times \frac{24}{23} = \sim 10.44$ cycles or $10.44 \times 240 \times 60 = 150336$ circadian minutes, as compared to $10 \times 240 \times 60 = 144000$ circadian minutes for a fly with a period of exactly 24-h. The proportion of a circadian minute that a fly (real or simulated) spent sleeping was then calculated as the ratio of total minutes of sleep and the total number of circadian minutes over the entire duration of the experiment/simulation.

*Continuous wavelet transforms as a tool to identify ultradian rhythms in sleep*

Biological oscillations are often non-stationary time-series, i.e., their amplitude and period vary significantly over time. Such variations are often not of interest when estimating period and power of circadian rhythms. However, when attempting to identifying multiple periodic components

from non-stationary time-series traditional methods like the $\chi^2$ periodogram often fail to accurately reflect the presence of multiple periodicities, especially when they are restricted to certain windows of time throughout the length of the time-series. Recently, analytical tools to resolve circadian rhythms in both the time and frequency domain (i.e., provides period and amplitude information locally over a long time-series) have been developed and utilized [44–47]. We made use of one such tool, the continuous wavelet transforms (CWT), to examine periodic components in sleep time-series of our flies. We used functions from the WaveletComp package in R, which use a Morlet mother wavelet and resolves biological time-series in the time-frequency domain [44,90]. Complex-valued wavelet transforms such as the Morlet wavelet is useful because it provides amplitude and phase over time and generally preserves information about these locally [44,90]. Estimates of ultradian period values for comparisons across $per^S$ and $per^+$ flies were drawn by computing the median values across all occurrences of periods under nine hours because that was the maximum value of ultradian period predicted by the model under conditions of non-oscillating thresholds (see Fig. 3).

*Time-series analysis of various definitions of sleep*

Traditionally, *Drosophila* sleep has been defined as any bout of inactivity (usually the absence of beam crossings in DAM monitors) that lasts five-min or longer [12,13,15]. Though this method has been shown to overestimate the amount of sleep [76], its easy implementation and consistency over decades of research have made it an integral approach to measuring sleep in the fly. In addition to the traditional definition of sleep, we systematically changed the inactivity duration criterion for sleep to examine how the temporal regulation of sleep was affected by biasing our analysis to longer bouts of inactivity or specific ranges of inactivity. For example, a ten-minute

inactivity duration definition of sleep would mean that any bout of inactivity lasting ten minutes or more would be considered sleep and subjected to time-series analysis, whereas a five to 20-minute definition of sleep would mean only bouts of inactivity that are more than five minutes but fewer than 20 minutes would be considered as sleep and subject to the analysis. Chi-squared periodogram analyses in these cases were performed on data binned in 120-minute intervals.

*Statistical tests and comparisons*

Correlations between the proportions of time spent sleeping predicted from the model and the proportions measured in experimental flies using various inactivity duration definitions of sleep were tested using the Pearson's product-moment correlation implemented in R [91]. Since the overall levels of sleep change as a function of the definition of sleep, all data were normalized to maxima to restrict the dynamic range of values between 0 and 1. This reduces the extent of variation in levels across both axes and therefore is better amenable to correlation analyses. Differences in ultradian periodicities under LL between genotypes were statistically compared using a one-tailed Wilcoxon's rank sum test implemented in R [91].

*Figure generation*

All figures were generated using custom scripts in R. We used the "wesanderson" package [92] to generate color palettes used in the figures, except in case of the acto-/somnograms, time-series plots in Fig. 1, the CWT spectrograms, and the Suppl. Figs. In these three cases, default color palettes were used to generate the plots.

**Creating the two-process model and generating predictions**

A major strength of the original two-process model was that it used EEG data to derive the parameter values of the sleep homeostat [4,5]. Because no equivalent physiological correlate of sleep is, as yet widely employed in flies, we relied on best-fitting approaches to find model parameters that best explain the waveform and amount of sleep in *Drosophila*. The basic structure of the two-process model used here is taken from a two-process model proposed by Daan et al., in 1984 [5]. The upper and lower thresholds are modeled as simple sinusoidal oscillations:

$$U = M_u + A_u \cos\,(\omega_u t + \varphi)$$

$$L = M_l + A_l \cos\,(\omega_l t + \varphi)$$

$U$ and $L$ are the upper and lower thresholds, respectively. $M_u$ and $M_l$ are the mean levels around which $U$ and $L$ oscillate. $A_u$ and $A_l$ are the amplitudes of the upper and lower threshold oscillations. $\omega_u$ and $\omega_l$ are the angular velocities of the oscillations. The angular velocities are determined by the period of the oscillation, which is expressed as $\omega = \frac{2\pi}{\tau}$. $\tau$ is the period of the oscillation, which in this study, was fixed at the same value for both thresholds. $\varphi$ is the phase angle by which the starting phase of the oscillations are adjusted and was likewise fixed at the same value for the upper and lower thresholds. The build-up of sleep pressure is modeled as:

$$s_t = (1 - i) \times (1 - s_{t-1})$$

$s_t$ is the value of sleep pressure at any given time $t$. $i$ is the parameter determining the increase of build-up of sleep pressure. $s_{t-1}$ is the value of sleep pressure at any given time $t - 1$. The decay of sleep pressure is modeled as:

$$s_t = \delta s_{t-1}$$

$s_t$ is the value of sleep pressure at any given time $t$. $\delta$ is the decay rate of sleep pressure. $s_{t-1}$ is the value of sleep pressure at any given time $t - 1$. The upper and lower thresholds were computed for the entire duration of the simulation, which was typically 21-days. The simulations for sleep

pressure build-up and decay were performed in discrete 30-min time-steps under the following conditions: At every time-step $t$, the program will increase sleep pressure as long as its value at the previous time-step $(t-1)$ was lower than the higher threshold at $t$. If at $t-1$, the sleep pressure value is higher than the upper threshold at time $t$, the program will release the sleep pressure at $t$. Sleep pressure will continue to be released (i.e., fall) until it reaches the lower threshold value at $t$. The window of time in the simulation where sleep pressure is being released is designated as the window of sleep. Using this window, the number of hours of sleep predicted by the model is computed for all predictions. This allows for the estimation of percentage of time sleeping across the circadian cycle. The sleep pressure time-series thus generated was subjected to $\chi^2$ periodogram analyses to produced estimates of number of peaks in the periodogram and to assess the power of sleep rhythms. Power was defined as the height of the periodogram peak above the 0.05 significance cut-off. To facilitate easy access to predictions from these simulations, we have created a GUI which is available here:

https://abhilashlakshman.shinyapps.io/twoprocessmodel/

*Measuring sum of squared differences (SSD)*

The S-process parameters that best recapitulated the waveform and amount of fly sleep were estimated using a sum of squared differences approach. For each combination of sleep pressure build-up $(i)$ and decay $(\delta)$ parameters, we asked if the model produced two coherent peaks of sleep, and, if so, we assessed the difference between the proportion of time spent sleeping predicted by the model and the proportion of time our experimental flies spent asleep and this difference was squared to remove negative values. For the results reported in Fig. 2, SSD values were calculated across the three genotypes and, in the case of fitting reported in Fig. 6, SSD was computed for both

the percentage time spent sleeping and for the normalized power of the sleep time-series periodogram. Any prediction that is close to empirical value would have small differences with the experimental data and consequently would have low SSD values. The build-up and decay rates that together yielded the minimum SSD were considered the best-fitting parameters for our two-process models. See Suppl. Figs. 1, 2 and 4 for a guide to the algorithms for our model outputs.

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## Figure Legends

**Figure 1: Circadian analysis of *Drosophila* sleep.** (A) A schematic of the two-process model that leads to one episode of sleep/cycle. (B) Somnograms (left) and actograms (right) of *Canton-S* (*CS*; top), $w^{1118}$ (middle) and *yw* (bottom) flies under light/dark 12:12 (LD12:12) cycles and constant darkness (DD). Gray shaded regions indicate the dark phase of the LD cycle. Representative Chi-square periodograms of sleep time-series (C) under LD12:12 (top) and DD (bottom), and of locomotor activity time-series (D) under LD12:12 (top) and DD (bottom) of the three genotypes of flies. The red lines indicate the Chi-square significance line at $\alpha = 0.05$. Height of peaks of the black lines indicate the strength of that periodicity in the time-series. Gray shaded regions indicate the dark phase of the LD cycle. (E) Frequency distributions of percentage time spent sleeping/24-h cycle in the three genotypes under LD12:12.

**Figure 2: Building a *Drosophila* two-process model of sleep regulation.** (A) Schematic of the two-process model that leads to two episodes of sleep/cycle taken from Daan et al. 1984 [5]. (B) Heatmap summarizing the nature of sleep rhythms within the build-up and decay of sleep pressure parameter space. (C) Heatmap showing the sum of squared differences (SSD) between model prediction and empirical values in the same parameter space for the value combinations producing bimodal rhythms. The red arrowhead marks the point in the parameter space that yields minimum SSD, indicating the parameter values that described fly sleep the best. Both panels (B) and (C) share the same *y*-axis. (D) The two-process model with best-fitting parameters. (E) the percentage of time spent sleeping/24-cycle in the three genotypes compared with the predicted value from the model plotted in (D).

**Figure 3: Ultradian periodicities are a predictable consequence of a functioning S-process under a dysfunctional C-process.** (A) A two-process model with the best-fitting homeostatic parameters and a circadian clock speed of 24-h (top). Also shown is the somnogram of simulated sleep data produced by this model (bottom). (B) The same model as in panel (A) but with flat, non-oscillating thresholds to model sleep regulation in a loss-of-function clock mutant (top), and the somnogram it produced (bottom). Four representative continuous wavelet transform (CWT) spectrograms for *Canton-S* (*CS*; C) and $per^{01}$ (D) flies that were recorded under constant darkness (DD). The color bars against each spectrogram are representative of local rhythmic power. Note: the *y*-axes on the spectrograms are on a logarithmic scale. (E) Probability density distributions of detected periodicities across all flies for both genotypes show strong ultradian components in $per^{01}$ flies as opposed to strong 12- and 24-hour periodicities in *CS*.

**Figure 4: The model predicts regions of unstable sleep rhythms across a range of free-running periods.** Somnograms of simulated sleep outputs from the two-process model for different free-running periods of the circadian clock regulated upper and lower thresholds. Green highlighted somnograms with high stability and relatively high sleep rhythm power. Unhighlighted somnograms indicate sleep rhythms with poor cycle to cycle stability and low periodogram power.

**Figure 5: Empirical estimates of power and amount of sleep modestly match predictions from the two-process model.** (A) Power of sleep rhythms and (B) the proportion of time spent sleeping/circadian minute for flies with different free-running periods, generated through a

combination of five *period* alleles (see Materials and Methods). Panels (A) and (B) share the same *x*-axis. (C) Median power of sleep rhythms and (E) proportion of time spent sleeping/circadian minute across the entire range of free-running periods overlayed with the predictions of the two-process model when only the free-running period was altered in the model.

**Figure 6: Allowing homeostatic parameters to change with free-running periods reveal a complex inter-dependence between the S- and C-processes.** (A) Median power of sleep rhythms and (B) proportion of time spent sleeping/circadian minute across the entire range of free-running periods overlayed with predictions of an altered two-process model with homeostatic sleep pressure parameters covaried with free-running period. (C) The relationship between build-up of sleep pressure and free-running periods, and (D) decay of sleep pressure and free-running periods used in the altered model. The red lines overlaying the boxplots are median values and the dark green line is a loess regression (locally weighted running line smoother or a locally weighted polynomial regression) smoothed fit. Higher values on the *y*-axis of the build-up of sleep pressure panel and lower values on the *y*-axis of the decay of sleep pressure panel indicate steeper rise and fall of sleep pressure in the model, respectively. Panels (C) and (D) share the same *x*-axis. Also shown are the regions along the *x*-axis corresponding to the free-running periods of males bearing the four rhythmic *period* alleles used in our study (see also Table 2).

**Figure 7: Short bout sleep is dispensable for capturing the relationship between free-running period and the amount of sleep predicted by the model.** (A) Scatter plots of the normalized proportion of time spent sleeping/circadian minute across different free-running periods for different inactivity duration definitions of sleep overlayed with the proportions measured using

standard definition of sleep and the proportions predicted by the model. (B) The correlation coefficients from Pearson's product-moment correlations between model predictions of normalized proportion of time spent sleeping/circadian minute and for different inactivity duration definitions of sleep.

**Figure 8: Long bout sleep reflects the S-process mediated sleep better.** (A) Representative continuous wavelet transforms (CWT) spectrograms for $per^{+}$ (left) and $per^{01}$ (right) flies that were recorded under constant darkness (DD) using different inactivity duration definitions of sleep (see Materials and Methods). The color bars against each spectrogram are representative of local rhythmic power. Note: the *y*-axes on the spectrograms are on a logarithmic scale. Probability density distributions of detected periodicities across all flies for both genotypes for (B) five-minute, (C) 30-minute and (D) 60-minute inactivity duration definitions of sleep. Note how the relative probability densities of ultradian and circadian ranges of period values change depending on the definition of sleep, in case of $per^{+}$ flies (dotted lines). All three panels, (B), (C) and (D) share the same *x*-axis. (E) Time-series of 60-min inactivity duration definition of sleep for two *period* alleles for five days under constant white light (LL) and six days of constant darkness (DD). (F) Ultradian periodicities of $per^{S}$ and $per^{+}$ flies under LL. Statistical comparisons were made using the one-tailed Wilcoxon's test. * < 0.05, ** < 0.01, *** < 0.001, and N.S. (Not Significant).

**Figure 9: Temporal organization of sleep under different definitions of sleep and Drosophila sleep as compared to other animals.** (A) Sleep time-series of *Canton-S* (*CS*) flies under three days of LD12:12 and four days of constant darkness (DD) for different definitions of sleep (see Materials and Methods). Dark gray shaded regions in the plots indicate the dark phase of LD

cycles and subjective dark phases under DD (i.e., when darkness would have occurred had the previous LD cycle continued). The light gray shaded regions indicate the subjective day phases under DD. (B) Estimations of the percentage of time spent sleeping/24-h day in a wide range of animal species (also see Table 4).

**Figure 10: Utility of the two-process model in generating testable predictions.** (A) Predicted roles played by the rising and falling phases of the thresholds in regulating the timing and maintenance of sleep, in the context of the two-process model. (B) Hypothetical relationships between $Ca^{2+}$ levels in circadian clock neurons, the two-process model, and sleep behavior. Data for the $Ca^{2+}$ levels were extracted from Liang et al., 2016 and smoothed using custom R scripts. Light gray shaded regions represent the subjective day phase and the dark gray shaded regions represent the subjective night phase of one circadian cycle. Note how peaks of $Ca^{2+}$ of the s-LNvs and LNds map onto the rising phase of the threshold oscillations and the $Ca^{2+}$ peaks of DNs map onto the falling phase of the thresholds of the two-process model. (C) A schematic representation of the effect of activation of DN-1s on sleep previously reported by others. Activating DN-1ps presumably increases $Ca^{2+}$ levels earlier in the day (dotted green line) which concomitantly leads to earlier fall and prolonged suppression of the threshold oscillations of the two-process model (dotted black lines). As a consequence of the earlier falling of the thresholds, the sleep pressure takes longer to hit the lower threshold (dotted red line) and therefore there is an increase in day-time sleep (dotted blue bounded boxes) as was observed in two previously reported studies (bottom panel). The plots in the bottom panel are redrawn from Guo et al. (2016) [88] and Kunst et al. (2014) [86]. Green lines represent sleep in the DN-1p activated flies and the black lines represent

unmanipulated controls. This manipulation also leads to a slightly delayed and earlier end of nighttime sleep as was reported by Kunst et al., (2014) [86].

# Tables

**Table 1:** Adjusted power values (mean±SEM) of *Canton-S* (*CS*), $w^{1118}$ and *yw* flies from Chi-squared periodogram analyses on sleep and locomotor activity time-series under LD and DD conditions. Also reported are the differences in power values between sleep and locomotor activity powers for each genotype. Sample sizes are provided in parentheses.

| | ***CS*** | | ***w*$^{1118}$** | | ***yw*** | |
|---|---|---|---|---|---|---|
| | **LD12:12** | **DD** | **LD12:12** | **DD** | **LD12:12** | **DD** |
| **Sleep** | 4006.7 ± 163.0 (32) | 2119.6 ± 242.1 (31) | 3964.3 ± 138.1 (31) | 3107.3 ± 200.7 (31) | 3022.8 ± 211.1 (31) | 1613.0 ± 140.5 (28) |
| **Locomotor activity** | 1345.3 ± 180.5 (32) | 503.8 ± 89.3 (28) | 2657.2 ± 125.6 (31) | 1200.2 ± 110.1 (31) | 638.8 ± 120.7 (30) | 296.2 ± 60.7 (27) |
| **Δ Power** | 2661.4 | 1615.8 | 1307.1 | 1907.2 | 2384.0 | 1316.8 |

**Table 2:** Median free-running periods of sleep rhythms under constant darkness averaged across two-replicate runs. The values for male (top, blue) and female (bottom, red) progeny from each cross are reported. Outcomes of any cross that had fewer than 12 flies pooled from both replicate runs have not been reported in this table. Sample sizes are provided in parentheses.

| Paternal genotype | Maternal genotype: $per^T$ | $per^S$ | $per^+$ | $per^L$ | $per^{01}$ |
|---|---|---|---|---|---|
| $per^T$ | ♂: 15.25 (44)<br>♀: 17.75 (38) | ♂: 19.75 (30)<br>♀: 18.50 (24) | ♂: 24.25 (63)<br>♀: 20.50 (55) | ♂: 28.75 (62)<br>♀: 21.75 (55) | ♂: NA<br>♀: 19.75 (60) |
| $per^S$ | ♂: 17.00 (63)<br>♀: 19.25 (59) | ♂: 21.00 (34)<br>♀: 20.38 (12) | ♂: 24.25 (59)<br>♀: 22.00 (53) | ♂: 28.25 (60)<br>♀: 23.50 (62) | ♂: NA<br>♀: 21.50 (56) |
| $per^+$ | ♂: 16.25 (51)<br>♀: 20.75 (59) | ♂: 19.63 (60)<br>♀: 22.00 (62) | ♂: 23.50 (47)<br>♀: 24.00 (57) | ♂: 27.50 (59)<br>♀: 24.75 (63) | ♂: NA<br>♀: 25.00 (63) |
| $per^L$ | ♂: 16.50 (30)<br>♀: 22.00 (31) | ♂: 20.00 (61)<br>♀: 23.00 (55) | ♂: 24.00 (59)<br>♀: 24.50 (63) | ♂: 27.75 (42)<br>♀: 27.50 (53) | ♂: NA<br>♀: 29.75 (55) |
| $per^{01}$ | ♂: NA<br>♀: NA | ♂: 20.00 (29)<br>♀: 21.50 (28) | ♂: 24.25 (47)<br>♀: 25.00 (57) | ♂: 28.50 (60)<br>♀: 29.50 (55) | ♂: NA<br>♀: NA |

**Table 3:** Median Chi-squared periodogram power of sleep rhythms under constant darkness averaged across two-replicate runs. The values for male (top, blue) and female (bottom, red) progeny from each cross are reported. Outcomes of any cross that had fewer than 12 flies pooled from the replicate runs have not been reported in this table. Sample sizes are provided in parentheses.

| Paternal genotype \ Maternal genotype | $per^T$ | $per^S$ | $per^+$ | $per^L$ | $per^{01}$ |
|---|---|---|---|---|---|
| $per^T$ | ♂: 33.0 (44)<br>♀: 33.6 (38) | ♂: 187.5 (30)<br>♀: 143.9 (24) | ♂: 136.1 (63)<br>♀: 79.5 (55) | ♂: 135.5 (62)<br>♀: 98.4 (55) | ♂: NA<br>♀: 119.4 (60) |
| $per^S$ | ♂: 98.0 (63)<br>♀: 110.7 (59) | ♂: 109.7 (34)<br>♀: 40.7 (12) | ♂: 95.9 (59)<br>♀: 105.1 (53) | ♂: 137.5 (60)<br>♀: 70.8 (62) | ♂: NA<br>♀: 103.3 (56) |
| $per^+$ | ♂: 51.5 (51)<br>♀: 76.4 (59) | ♂: 161.0 (60)<br>♀: 117.0 (62) | ♂: 79.4 (47)<br>♀: 92.6 (57) | ♂: 64.9 (59)<br>♀: 118.0 (63) | ♂: NA<br>♀: 130.3 (63) |
| $per^L$ | ♂: 93.7 (30)<br>♀: 108.4 (31) | ♂: 81.1 (61)<br>♀: 55.5 (55) | ♂: 53.9 (59)<br>♀: 134.0 (63) | ♂: 72.0 (42)<br>♀: 85.4 (53) | ♂: NA<br>♀: 92.8 (55) |
| $per^{01}$ | ♂: NA<br>♀: NA | ♂: 114.6 (29)<br>♀: 114.4 (28) | ♂: 32.7 (47)<br>♀: 102.4 (57) | ♂: 56.1 (60)<br>♀: 72.7 (55) | ♂: NA<br>♀: NA |

**Table 4:** Percentage of time spent sleeping/24-h day in a range of animal species. Animals are arranged in descending order of % time spent sleeping. Data were compiled from the following sources: [1,66–74].

| *Species* | *Category in plot* | *% time spent sleeping* | *Source* |
|---|---|---|---|
| *Terrapene carolina (North American Box Turtle)* | Non-Mammalian Vertebrates | 85.00 | Campbell and Tobler, 1984 |
| *Myotis lucifugus (Little Brown Bat)* | Mammals | 82.92 | Campbell and Tobler, 1984 |
| *Aedes aegypti (Mosquito)* | Invertebrates | 81.72 | Ajayi et al., 2022 |
| *Eptesicus fuscus (Big Brown Bat)* | Mammals | 81.25 | Campbell and Tobler, 1984 |
| *Didelphis marsupialis (North American Opossum)* | Mammals | 80.83 | Campbell and Tobler, 1984 |
| *Lutreolina crassicaudata (Little Water Opossum)* | Mammals | 80.83 | Campbell and Tobler, 1984 |
| *Drosophila melanogaster (Fruitfly; from Bermuda)* | Invertebrates | 77.58 | Brown et al., 2018 |
| *Anopheles stephensi (Mosquito)* | Invertebrates | 76.87 | Ajayi et al., 2022 |
| *Drosophila melanogaster (Fruitfly; from Bogota, Colombia)* | Invertebrates | 76.41 | Brown et al., 2018 |
| *Drosophila melanogaster (Fruitfly; from Kariba Dam, Zimbabwe)* | Invertebrates | 76.14 | Brown et al., 2018 |
| *Priodontes giganteus (Giant Armadillo)* | Mammals | 75.42 | Campbell and Tobler, 1984 |
| *Drosophila melanogaster (Fruitfly; from American Samoa)* | Invertebrates | 75.21 | Brown et al., 2018 |
| *Python sebae (African Python)* | Non-Mammalian Vertebrates | 75.00 | Campbell and Tobler, 1984 |
| *Culex pipiens (Mosquito)* | Invertebrates | 72.60 | Ajayi et al., 2022 |
| *Dasypus novemcinctus (Nine-banded Armadillo)* | Mammals | 72.50 | Campbell and Tobler, 1984 |

| | | | |
|---|---|---|---|
| *Drosophila melanogaster (Fruitfly; from Ogasawara Islands, Japan)* | Invertebrates | 72.45 | Brown et al., 2018 |
| *Dipsosaurus dorsalis (Desert Iguana)* | Non-Mammalian Vertebrates | 71.04 | Campbell and Tobler, 1984 |
| *Aotus trivirgatus (Owl Monkey)* | Mammals | 70.83 | Campbell and Tobler, 1984 |
| *Drosophila melanogaster (Fruitfly; from Cape Town, South Africa)* | Invertebrates | 69.81 | Brown et al., 2018 |
| *Citellus undulates parryi (Arctic Ground Squirrel)* | Mammals | 69.17 | Campbell and Tobler, 1984 |
| *Choloepus hoffmanni (Two-toed Sloth)* | Mammals | 68.33 | Campbell and Tobler, 1984 |
| *Tamias striatus (Eastern Chipmunk)* | Mammals | 67.92 | Campbell and Tobler, 1984 |
| *Drosophila melanogaster (Fruitfly; from Crete, Greece)* | Invertebrates | 66.98 | Brown et al., 2018 |
| *Srix aluco (Tawny Owl)* | Non-Mammalian Vertebrates | 66.67 | Campbell and Tobler, 1984 |
| *Microtus ochrogaster (Prairie Vole)* | Mammals | 66.25 | Campbell and Tobler, 1984 |
| *Bradypus infuscatus (Three-toed Sloth)* | Mammals | 65.83 | Campbell and Tobler, 1984 |
| *Centetes ecaudatus (Tenrec)* | Mammals | 65.00 | Campbell and Tobler, 1984 |
| *Drosophila melanogaster (Fruitfly; from Cebu, Phillipines)* | Invertebrates | 64.40 | Brown et al., 2018 |
| *Drosophila melanogaster (Fruitfly; from Queensferry, Scotland)* | Invertebrates | 64.26 | Brown et al., 2018 |
| *Merionis unguiculatus (Mongolian Gerbil)* | Mammals | 63.75 | Campbell and Tobler, 1984 |
| *Drosophila melanogaster (Fruitfly; from Monkey Hill, St. Kitts)* | Invertebrates | 62.44 | Brown et al., 2018 |

| | | | |
|---|---|---|---|
| *Blarina brevicauda (Greater Short-tailed Shrew)* | Mammals | 62.08 | Campbell and Tobler, 1984 |
| *Eutamias dorsalis (Cliff Chipmunk)* | Mammals | 62.08 | Campbell and Tobler, 1984 |
| *Drosophila ananassae (Fruitfly)* | Invertebrates | 61.87 | Prabhakaran and Sheeba, 2012 |
| *Drosophila melanogaster (Fruitfly; from Bahia, Brazil)* | Invertebrates | 61.13 | Brown et al., 2018 |
| *Bufo boreas (Western Toad)* | Non-Mammalian Vertebrates | 60.83 | Campbell and Tobler, 1984 |
| *Neofiber affeni (Round-tailed Muskrat)* | Mammals | 60.83 | Campbell and Tobler, 1984 |
| *Citellus lateralis (Golden-mantled Ground Squirrel)* | Mammals | 60.42 | Campbell and Tobler, 1984 |
| *Onychomys leucogaster (Northern Grass-hopper Mouse)* | Mammals | 60.42 | Campbell and Tobler, 1984 |
| *Tinca tinca (Tench)* | Non-Mammalian Vertebrates | 60.00 | Campbell and Tobler, 1984 |
| *Aplodontia rufa (Mountain Beaver)* | Mammals | 60.00 | Campbell and Tobler, 1984 |
| *Mesocricetus auratus (Golden Hamster)* | Mammals | 60.00 | Campbell and Tobler, 1984 |
| *Speotyto cunicularis hypugaea (Burrowing Owl)* | Non-Mammalian Vertebrates | 59.58 | Campbell and Tobler, 1984 |
| *Drosophila melanogaster (Fruitfly; from Plainville, Connecticut)* | Invertebrates | 59.11 | Brown et al., 2018 |
| *Drosophila melanogaster (Fruitfly; from Athens, Greece)* | Invertebrates | 58.53 | Brown et al., 2018 |
| *Leucophea maerae (Cockroach)* | Invertebrates | 58.33 | Campbell and Tobler, 1984 |
| *Citellus tridecemlineatus (Thirteen-lined Ground Squirrel)* | Mammals | 57.92 | Campbell and Tobler, 1984 |
| *Drosophila melanogaster (Fruitfly; from Blacksburg, Virginia)* | Invertebrates | 57.70 | Brown et al., 2018 |

| | | | |
|---|---|---|---|
| *Trichosurus vulpecula (Phalanger)* | Mammals | 57.08 | Campbell and Tobler, 1984 |
| *M. pennsylvanicus (Meadow Vole)* | Mammals | 57.08 | Campbell and Tobler, 1984 |
| *Rattus norvegicus (Lab Rat)* | Mammals | 57.08 | Campbell and Tobler, 1984 |
| *Drosophila melanogaster (Fruitfly; from Chiapas, Mexico)* | Invertebrates | 56.97 | Brown et al., 2018 |
| *Neomys fodiens (Water Shrew)* | Mammals | 56.67 | Campbell and Tobler, 1984 |
| *Drosophila melanogaster (Fruitfly; from San Luis Potosi, Mexico)* | Invertebrates | 56.19 | Brown et al., 2018 |
| *Rattus norvegicus (Lab Rat)* | Mammals | 55.00 | Campbell and Tobler, 1984 |
| *Rattus norvegicus (Lab Rat)* | Mammals | 55.00 | Campbell and Tobler, 1984 |
| *Felis domestica (Domestic Cat)* | Mammals | 55.00 | Campbell and Tobler, 1984 |
| *P. polionotus (Oldfield Mouse)* | Mammals | 54.58 | Campbell and Tobler, 1984 |
| *Drosophila melanogaster (Fruitfly; from Pyrenees, Spain)* | Invertebrates | 54.28 | Brown et al., 2018 |
| *Canis lupus (Arctic Wolf)* | Mammals | 54.17 | Campbell and Tobler, 1984 |
| *P. maniculatus bairdi (Deer Mouse)* | Mammals | 53.75 | Campbell and Tobler, 1984 |
| *Canis domesticus (Domestic Dog; Beagle)* | Mammals | 53.75 | Campbell and Tobler, 1984 |
| *Drosophila melanogaster (Fruitfly; from La Jolla, California)* | Invertebrates | 53.54 | Brown et al., 2018 |
| *Aythia ferina (European Pochard)* | Non-Mammalian Vertebrates | 53.33 | Campbell and Tobler, 1984 |
| *Spheniscus mendiculus (Galapagos Penguin)* | Non-Mammalian Vertebrates | 53.33 | Campbell and Tobler, 1984 |

| | | | |
|---|---|---|---|
| *Suncus murinus (Musk Shrew)* | Mammals | 53.33 | Campbell and Tobler, 1984 |
| *Mus musculus (Lab Mouse)* | Mammals | 53.33 | Campbell and Tobler, 1984 |
| *Drosophila melanogaster (Fruitfly; from Ica, Peru)* | Invertebrates | 53.24 | Brown et al., 2018 |
| *Drosophila melanogaster (Fruitfly; from Queenslan, Australia)* | Invertebrates | 52.93 | Brown et al., 2018 |
| *Cavia porcellus (Guinea Pig)* | Mammals | 52.50 | Campbell and Tobler, 1984 |
| *Caiman latirostris (Caiman)* | Non-Mammalian Vertebrates | 52.08 | Campbell and Tobler, 1984 |
| *Chinchilla laniger (Chinchilla)* | Mammals | 52.08 | Campbell and Tobler, 1984 |
| *Alopex lagopus (Arctic Fox)* | Mammals | 52.08 | Campbell and Tobler, 1984 |
| *Phrinosoma regali (Horned Lizard)* | Non-Mammalian Vertebrates | 51.25 | Campbell and Tobler, 1984 |
| *Cygnus c. cygnus (Whooper Swan)* | Non-Mammalian Vertebrates | 50.00 | Campbell and Tobler, 1984 |
| *Heterometrus longimanus (Scorpion)* | Invertebrates | 49.51 | Tobler and Stalder, 1988 |
| *Macaca mulatta (Rhesus Monkey)* | Mammals | 49.17 | Campbell and Tobler, 1984 |
| *Gallus domesticus (White Leghorn Chicken)* | Non-Mammalian Vertebrates | 48.75 | Campbell and Tobler, 1984 |
| *Parus rufescens (Chestnut-backed Chickadee)* | Non-Mammalian Vertebrates | 48.75 | Campbell and Tobler, 1984 |
| *Potorous apicalis (Kangaroo Rat)* | Mammals | 48.33 | Campbell and Tobler, 1984 |
| *Testudo marginata (Margined Tortoise)* | Non-Mammalian Vertebrates | 47.92 | Campbell and Tobler, 1984 |
| *Phaner furcifer (Lemur)* | Mammals | 47.92 | Campbell and Tobler, 1984 |
| *M. montanus (Mountain Vole)* | Mammals | 47.50 | Campbell and Tobler, 1984 |

| | | | |
|---|---|---|---|
| *Geochelone carbonaria (Red-footed Tortoise)* | Non-Mammalian Vertebrates | 47.29 | Campbell and Tobler, 1984 |
| *Thryomanes bewickii (Bewick Wren)* | Non-Mammalian Vertebrates | 47.08 | Campbell and Tobler, 1984 |
| *Sigmodon hispidus (Cotton Rat)* | Mammals | 47.08 | Campbell and Tobler, 1984 |
| *Calypte anna (Anna's Humming-bird)* | Non-Mammalian Vertebrates | 46.25 | Campbell and Tobler, 1984 |
| *Drosophila melanogaster (Fruitfly; from Southwest Harbor, Maine)* | Invertebrates | 45.85 | Brown et al., 2018 |
| *Triturus vulgaris (Smooth Newt)* | Non-Mammalian Vertebrates | 45.83 | Campbell and Tobler, 1984 |
| *Nycticebus coucang (Slow Loris)* | Mammals | 45.83 | Campbell and Tobler, 1984 |
| *Procambarus clarkii (Crayfish)* | Invertebrates | 45.83 | Ramón et al., 2004 |
| *Peromyscus erimicus (Cactus Mouse)* | Mammals | 45.42 | Campbell and Tobler, 1984 |
| *Anas platyrhynchos domesticus (Domestic Duck)* | Non-Mammalian Vertebrates | 45.00 | Campbell and Tobler, 1984 |
| *Microtus canicaudus (Gray-tailed Vole)* | Mammals | 45.00 | Campbell and Tobler, 1984 |
| *Aptenodytes forsteri (Emperor Penguin)* | Non-Mammalian Vertebrates | 44.58 | Campbell and Tobler, 1984 |
| *Drosophila melanogaster (Fruitfly; from Bangalore, India)* | Invertebrates | 44.35 | Prabhakaran and Sheeba, 2012 |
| *Columba livia (Domestic Pigeon)* | Non-Mammalian Vertebrates | 44.17 | Campbell and Tobler, 1984 |
| *Apus apus (Common Swift)* | Non-Mammalian Vertebrates | 44.17 | Campbell and Tobler, 1984 |
| *Talpa europaea (European Mole)* | Mammals | 43.75 | Campbell and Tobler, 1984 |
| *Tursiops truncatus (Bottlenosed Dolphin)* | Mammals | 43.33 | Campbell and Tobler, 1984 |
| *Paraechinus hypomelas (Desert Hedgehog)* | Mammals | 42.92 | Campbell and Tobler, 1984 |

| | | | |
|---|---|---|---|
| *Condylura cristata (Star-nosed Mole)* | Mammals | 42.92 | Campbell and Tobler, 1984 |
| *Streptopelia capicola (Ring-necked Dove)* | Non-Mammalian Vertebrates | 42.50 | Campbell and Tobler, 1984 |
| *Erinaceus europaeus (European Hedgehog)* | Mammals | 42.08 | Campbell and Tobler, 1984 |
| *Drosophila melanogaster (Fruitfly; from Madeira, Portugal)* | Invertebrates | 42.05 | Brown et al., 2018 |
| *Panthera onca (Jaguar)* | Mammals | 41.67 | Campbell and Tobler, 1984 |
| *Vulpes vulpes (Red Fox)* | Mammals | 40.83 | Campbell and Tobler, 1984 |
| *Drosophila melanogaster (Fruitfly; from Israel)* | Invertebrates | 39.57 | Brown et al., 2018 |
| *Aratinga canicularis (Parakeet)* | Non-Mammalian Vertebrates | 39.17 | Campbell and Tobler, 1984 |
| *Lemur macaco fulvus (Lemur)* | Mammals | 39.17 | Campbell and Tobler, 1984 |
| *Macaca radiata (Bonnet Monkey)* | Mammals | 38.54 | Campbell and Tobler, 1984 |
| *Heterometrus spinnifer (Scorpion)* | Invertebrates | 38.44 | Tobler and Stalder, 1988 |
| *Cryptotis parva (Lesser Short-tailed Shrew)* | Mammals | 37.92 | Campbell and Tobler, 1984 |
| *Tupaia glis (Tree Shrew)* | Mammals | 37.08 | Campbell and Tobler, 1984 |
| *Papio papio (Baboon)* | Mammals | 36.67 | Campbell and Tobler, 1984 |
| *Oryctolagus cuniculus (Rabbit)* | Mammals | 36.67 | Campbell and Tobler, 1984 |
| *Tachyglossus aculeatus (Echidna)* | Mammals | 35.83 | Campbell and Tobler, 1984 |
| *Sorex minutus (Pygmy Shrew)* | Mammals | 35.42 | Campbell and Tobler, 1984 |
| *Rhabdomys prinulus (African Striped Mouse)* | Mammals | 35.42 | Campbell and Tobler, 1984 |

| | | | |
|---|---|---|---|
| *Scalopus aquaticus (Eastern Mole)* | Mammals | 35.00 | Campbell and Tobler, 1984 |
| *Canis domesticus (Domestic Dog; Pointer)* | Mammals | 35.00 | Campbell and Tobler, 1984 |
| *Eudyptula minor (Little Penguin)* | Non-Mammalian Vertebrates | 34.58 | Campbell and Tobler, 1984 |
| *Anas crecca carolinensis (Green-winged Teal)* | Non-Mammalian Vertebrates | 34.17 | Campbell and Tobler, 1984 |
| *Anas acuta (Pintail)* | Non-Mammalian Vertebrates | 33.75 | Campbell and Tobler, 1984 |
| *Parus m. major (Great Tit)* | Non-Mammalian Vertebrates | 33.33 | Campbell and Tobler, 1984 |
| *Aplysia californica (Marine Mollusc)* | Invertebrates | 32.98 | Vorster et al., 2014 |
| *Nyctea scandiaca (Polar White Owl)* | Non-Mammalian Vertebrates | 32.92 | Campbell and Tobler, 1984 |
| *Sorex araneus (Common Shrew)* | Mammals | 32.50 | Campbell and Tobler, 1984 |
| *P. gossypinus (Cotton Mouse)* | Mammals | 32.50 | Campbell and Tobler, 1984 |
| *P. leucopus (White-footed Mouse)* | Mammals | 32.50 | Campbell and Tobler, 1984 |
| *Sus domesticus (Pig)* | Mammals | 32.50 | Campbell and Tobler, 1984 |
| *Hydra vulgaris* | Invertebrates | 32.29 | Kanaya et al., 2020 |
| *Honeybees* | Invertebrates | 31.67 | Kaiser, 1988 |
| *Perca fluviatilis (Perch)* | Non-Mammalian Vertebrates | 31.25 | Campbell and Tobler, 1984 |
| *Tilapia mossambica (Guppy)* | Non-Mammalian Vertebrates | 29.17 | Campbell and Tobler, 1984 |
| *Calomys callosus (Launcha De Campo)* | Mammals | 29.17 | Campbell and Tobler, 1984 |
| *Platanista indi (Indus Dolphin)* | Mammals | 29.17 | Campbell and Tobler, 1984 |
| *Emys orbicularis (European Pond Turtle)* | Non-Mammalian Vertebrates | 28.75 | Campbell and Tobler, 1984 |
| *Anas rubripes (Black Duck)* | Non-Mammalian Vertebrates | 28.75 | Campbell and Tobler, 1984 |

| | | | |
|---|---|---|---|
| *Drosophila melanogaster (Fruitfly; from Fukushima, Japan)* | Invertebrates | 28.69 | Brown et al., 2018 |
| *Astyanax mexicanus (Mexican Cavefish; surface dwelling)* | Non-Mammalian Vertebrates | 27.84 | Jaggard et al., 2017 |
| *Perognathus longimembris (Pocket Mouse)* | Mammals | 27.29 | Campbell and Tobler, 1984 |
| *Aythia fuligula (Tufted Duck)* | Non-Mammalian Vertebrates | 26.67 | Campbell and Tobler, 1984 |
| *Bucephala clangula (European Golden-eye)* | Non-Mammalian Vertebrates | 26.67 | Campbell and Tobler, 1984 |
| *Rissa tridactyla (Kittiwake)* | Non-Mammalian Vertebrates | 26.25 | Campbell and Tobler, 1984 |
| *Anser anser (Landaise Goose)* | Non-Mammalian Vertebrates | 25.83 | Campbell and Tobler, 1984 |
| *Cygnus c. buccinator (Trumpeter Swan)* | Non-Mammalian Vertebrates | 23.75 | Campbell and Tobler, 1984 |
| *Heterophyrax brucei (Rock Hyrax)* | Mammals | 23.75 | Campbell and Tobler, 1984 |
| *Globicephala scammoni (North Pacific Pilot Whale)* | Mammals | 22.08 | Campbell and Tobler, 1984 |
| *Larus argentatus (Herring Gull)* | Non-Mammalian Vertebrates | 20.83 | Campbell and Tobler, 1984 |
| *Procavia johnstoni (Rock Hyrax)* | Mammals | 20.42 | Campbell and Tobler, 1984 |
| *Dendrohyrax validus (Tree Hyrax)* | Mammals | 20.42 | Campbell and Tobler, 1984 |
| *Oenanthe oenanthe (Wheatear)* | Non-Mammalian Vertebrates | 20.00 | Campbell and Tobler, 1984 |
| *Plectrophenax nivalis (Snow Bunting)* | Non-Mammalian Vertebrates | 19.17 | Campbell and Tobler, 1984 |
| *Astyanax mexicanus (Mexican Cavefish; from Tinaja)* | Non-Mammalian Vertebrates | 19.13 | Jaggard et al., 2017 |

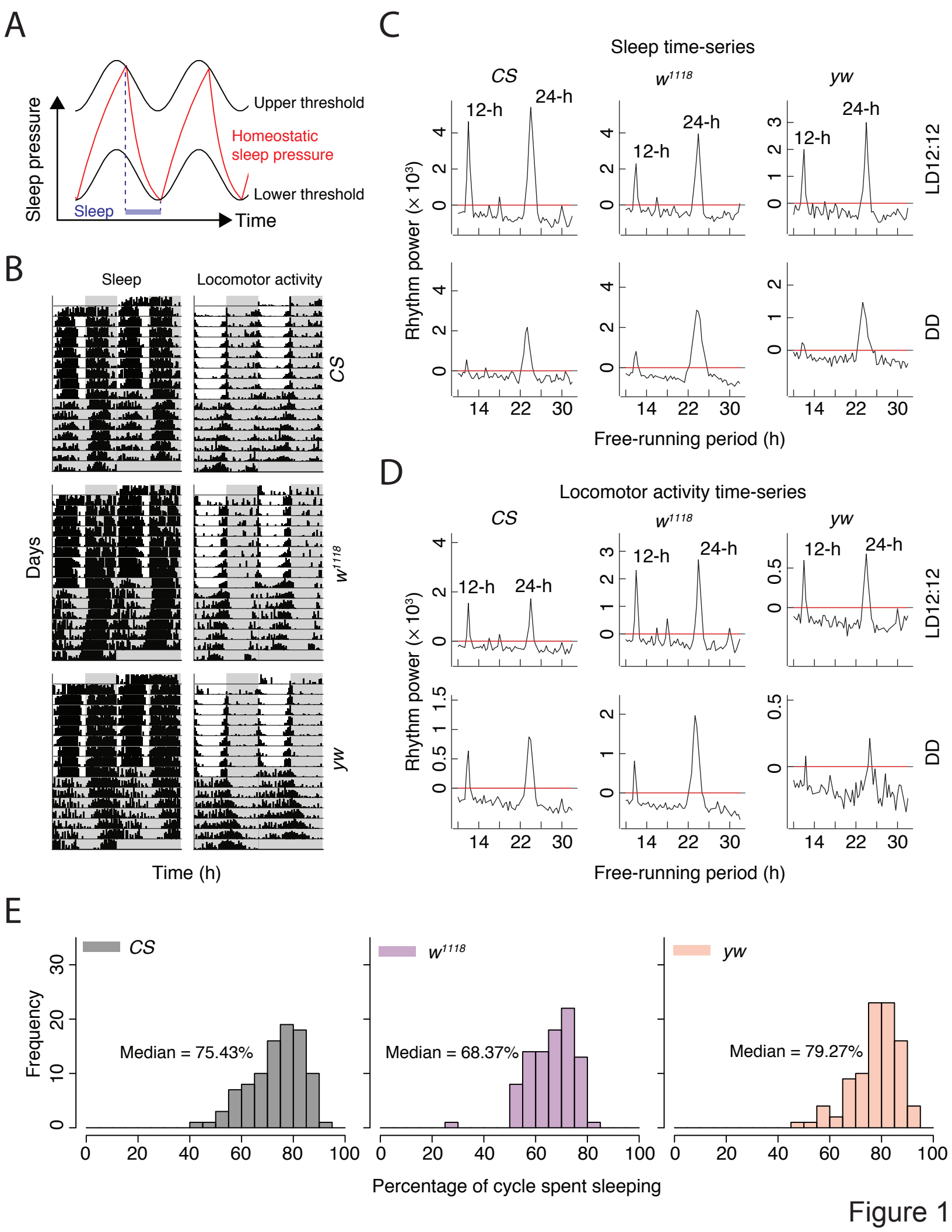
A
Sleep pressure
Upper threshold
Homeostatic sleep pressure
Lower threshold
Sleep
Time
B
Sleep
Locomotor activity
CS
w1118
yw
Days
Time (h)
C
Sleep time-series
CS
w1118
yw
12-h
24-h
LD12:12
DD
Rhythm power (× 10³)
Free-running period (h)
D
Locomotor activity time-series
CS
w1118
yw
12-h
24-h
LD12:12
DD
Rhythm power (× 10³)
Free-running period (h)
E
CS
w1118
yw
Frequency
Median = 75.43%
Median = 68.37%
Median = 79.27%
Percentage of cycle spent sleeping

Figure 1

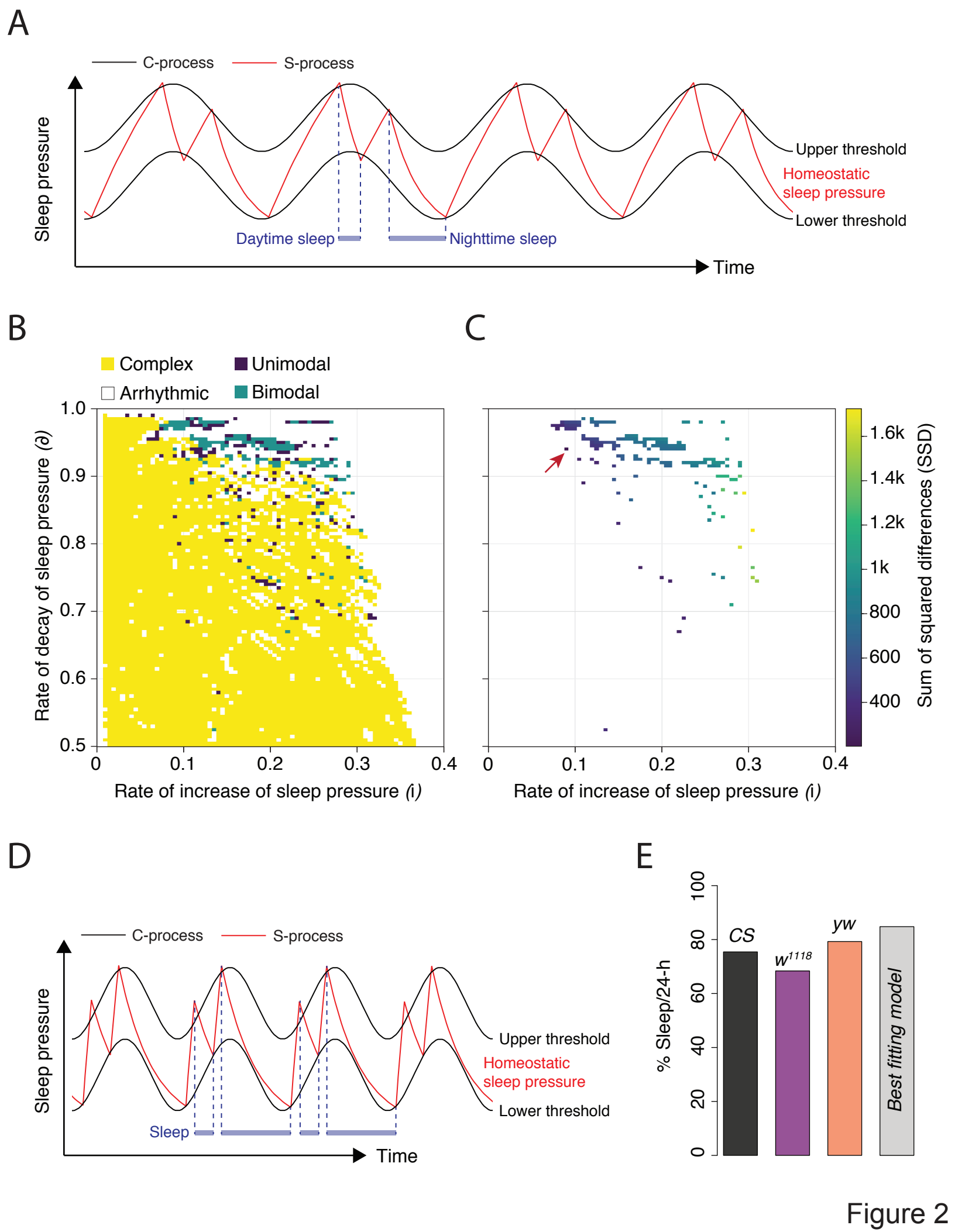
A
C-process
S-process
Sleep pressure
Upper threshold
Homeostatic sleep pressure
Lower threshold
Daytime sleep
Nighttime sleep
Time
B
Complex
Unimodal
Arrhythmic
Bimodal
Rate of decay of sleep pressure (∂)
Rate of increase of sleep pressure (i)
C
Sum of squared differences (SSD)
1.6k
1.4k
1.2k
1k
800
600
400
D
C-process
S-process
Sleep pressure
Upper threshold
Homeostatic sleep pressure
Lower threshold
Sleep
Time
E
% Sleep/24-h
CS
w1118
yw
Best fitting model

Figure 2

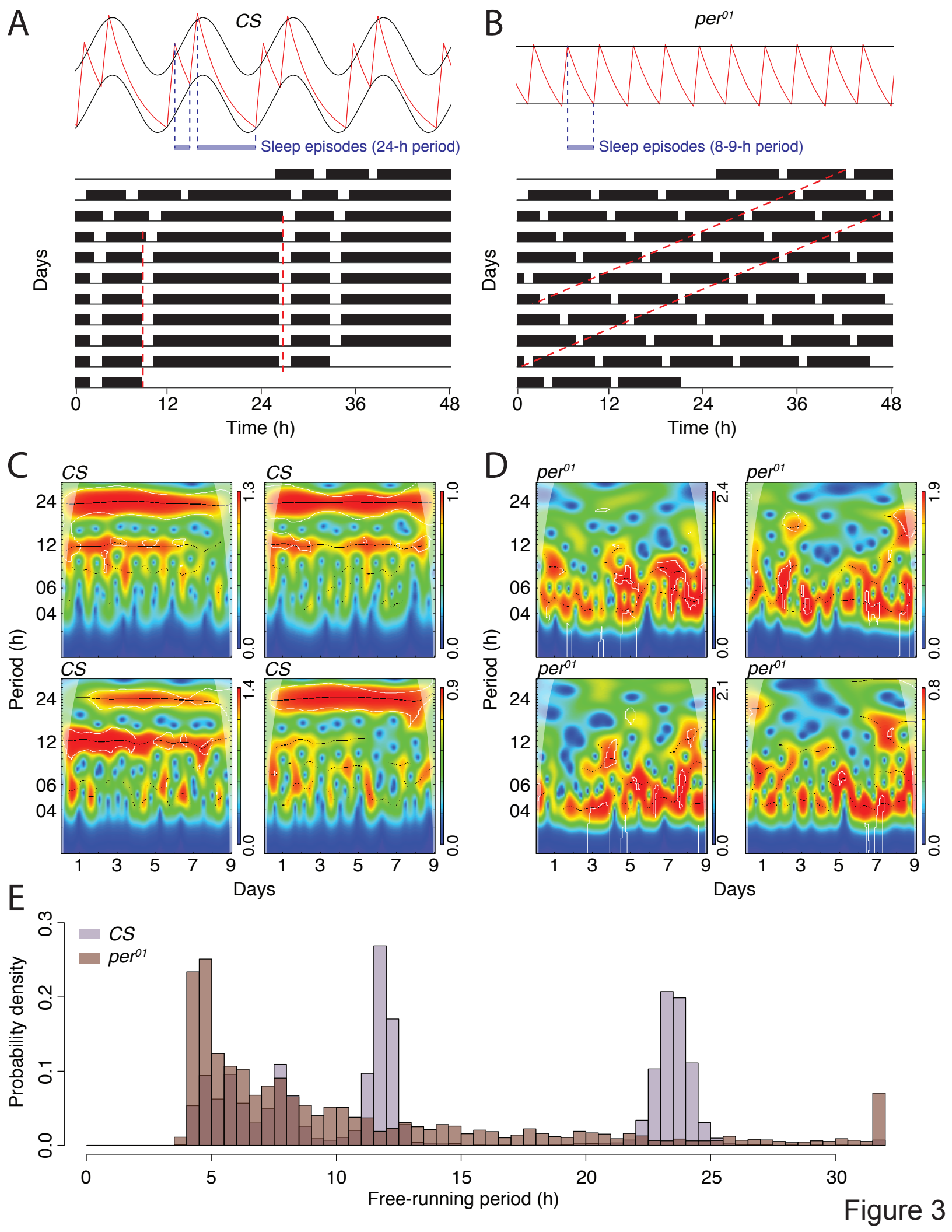
A
CS
Sleep episodes (24-h period)
Days
0
12
24
36
48
Time (h)
B
per01
Sleep episodes (8-9-h period)
Days
0
12
24
36
48
Time (h)
C
CS
CS
CS
CS
Period (h)
24
12
06
04
1.3
0.0
1.0
0.0
1.4
0.0
0.9
0.0
1
3
5
7
9
Days
D
per01
per01
per01
per01
Period (h)
24
12
06
04
2.4
0.0
1.9
0.0
2.1
0.0
0.8
0.0
1
3
5
7
9
Days
E
CS
per01
Probability density
0.0
0.1
0.2
0.3
0
5
10
15
20
25
30
Free-running period (h)

Figure 3

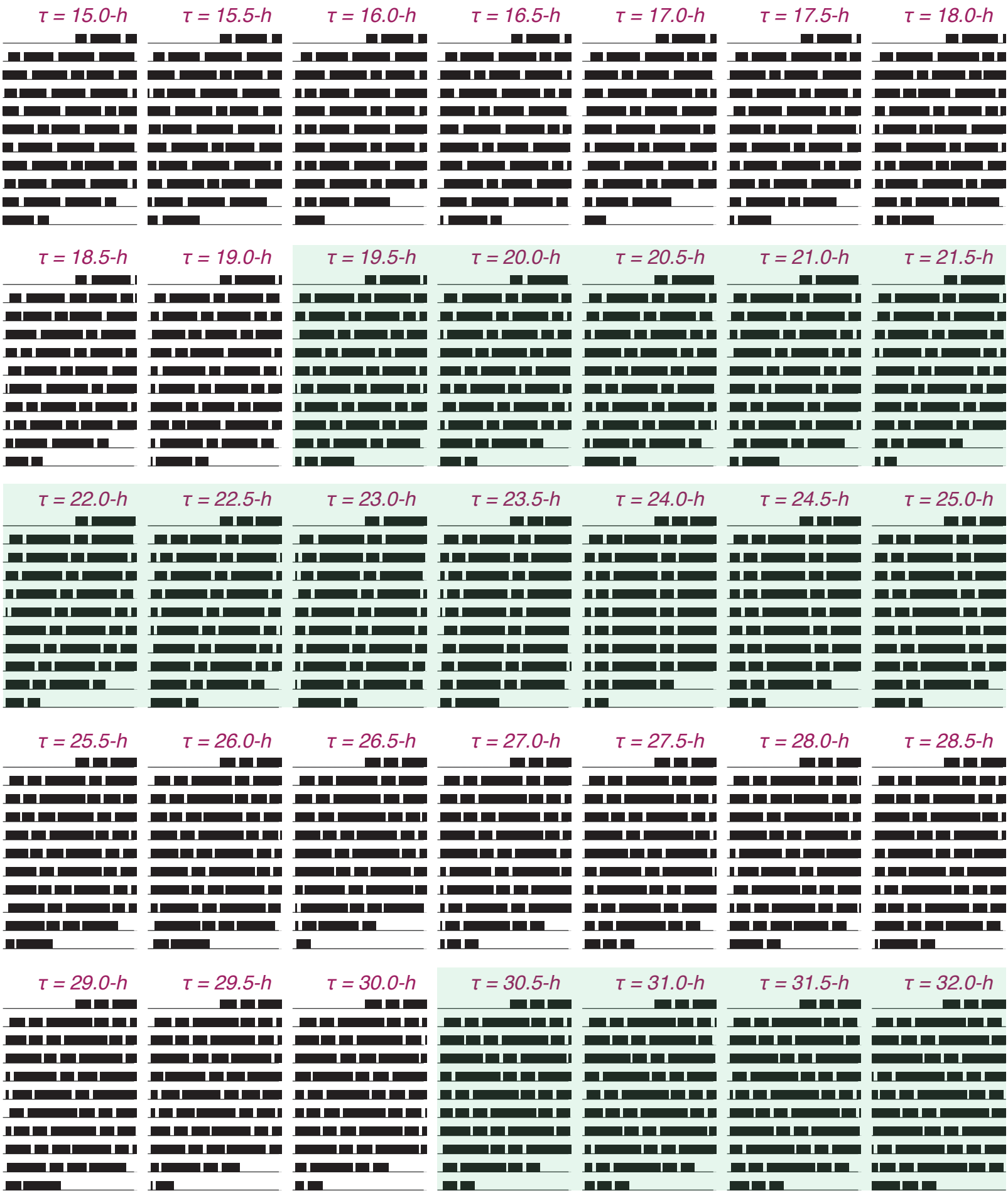
τ = 15.0-h
τ = 15.5-h
τ = 16.0-h
τ = 16.5-h
τ = 17.0-h
τ = 17.5-h
τ = 18.0-h
τ = 18.5-h
τ = 19.0-h
τ = 19.5-h
τ = 20.0-h
τ = 20.5-h
τ = 21.0-h
τ = 21.5-h
τ = 22.0-h
τ = 22.5-h
τ = 23.0-h
τ = 23.5-h
τ = 24.0-h
τ = 24.5-h
τ = 25.0-h
τ = 25.5-h
τ = 26.0-h
τ = 26.5-h
τ = 27.0-h
τ = 27.5-h
τ = 28.0-h
τ = 28.5-h
τ = 29.0-h
τ = 29.5-h
τ = 30.0-h
τ = 30.5-h
τ = 31.0-h
τ = 31.5-h
τ = 32.0-h

Figure 4

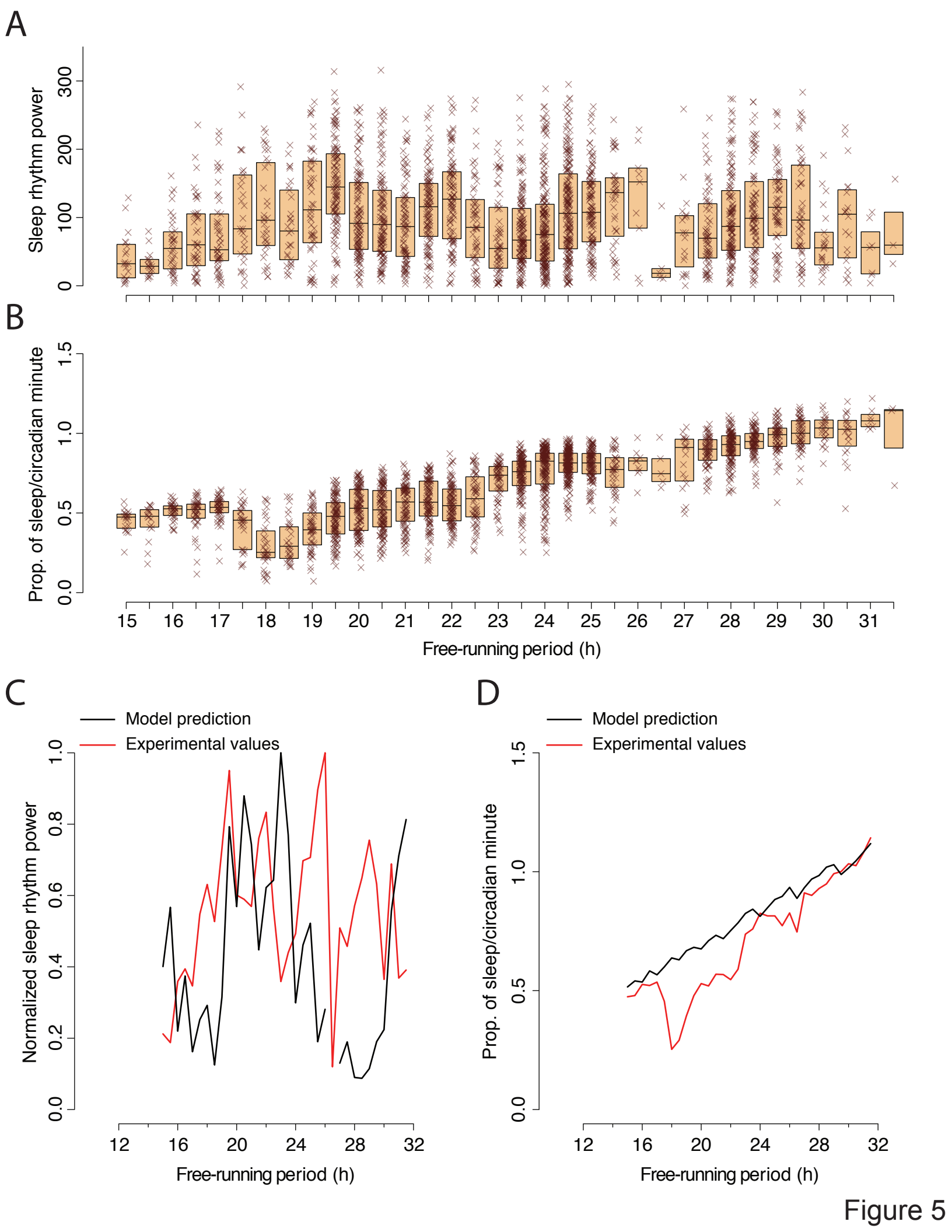
A
Sleep rhythm power
0
100
200
300
B
Prop. of sleep/circadian minute
0.0
0.5
1.0
1.5
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
Free-running period (h)
C
Model prediction
Experimental values
Normalized sleep rhythm power
0.0
0.2
0.4
0.6
0.8
1.0
12
16
20
24
28
32
Free-running period (h)
D
Model prediction
Experimental values
Prop. of sleep/circadian minute
0.0
0.5
1.0
1.5
12
16
20
24
28
32
Free-running period (h)

Figure 5

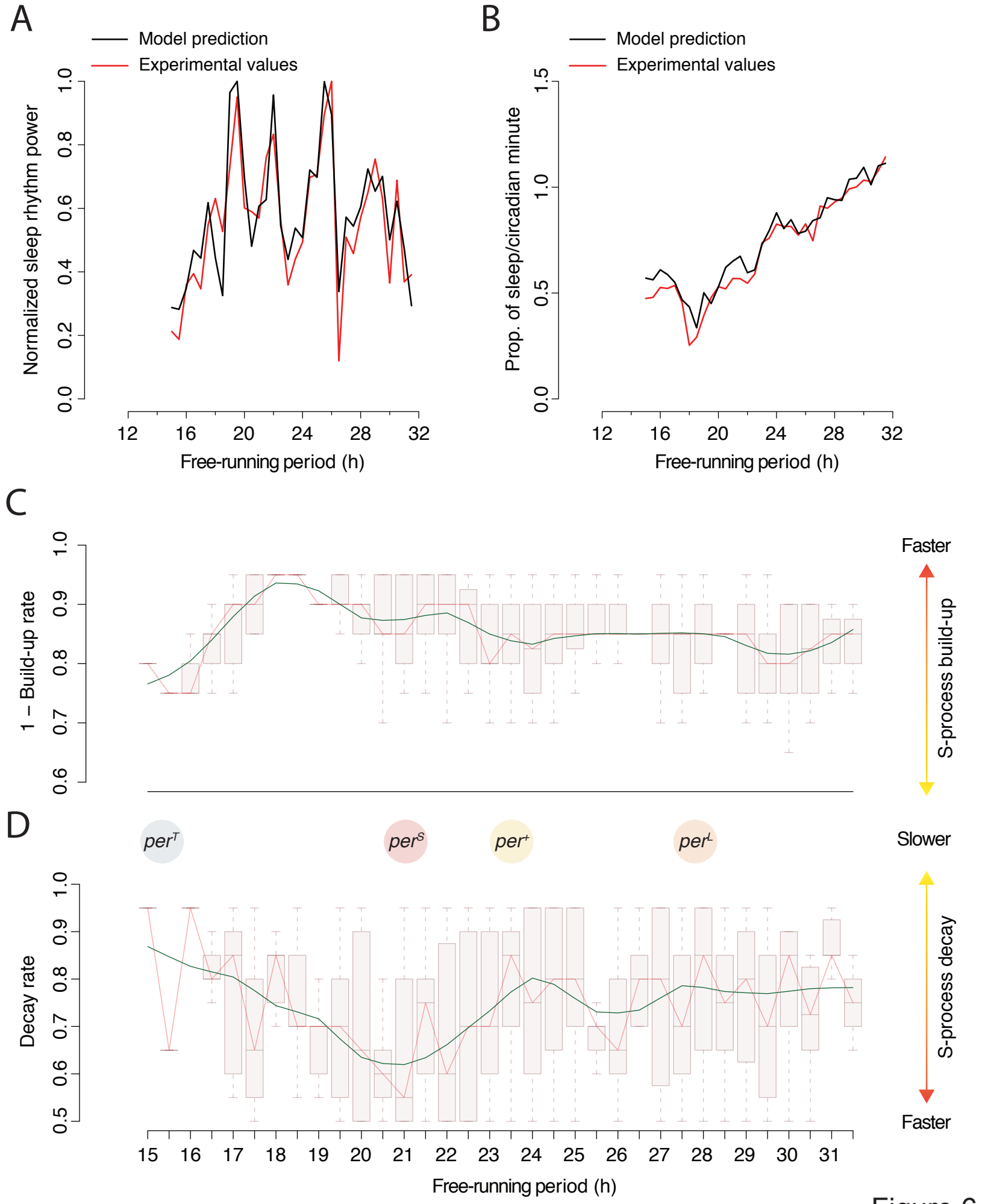
A
Model prediction
Experimental values
Normalized sleep rhythm power
0.0
0.2
0.4
0.6
0.8
1.0
12
16
20
24
28
32
Free-running period (h)
B
Model prediction
Experimental values
Prop. of sleep/circadian minute
0.0
0.5
1.0
1.5
12
16
20
24
28
32
Free-running period (h)
C
1 – Build-up rate
0.6
0.7
0.8
0.9
1.0
Faster
S-process build-up
D
perT
perS
per+
perL
Slower
Decay rate
0.5
0.6
0.7
0.8
0.9
1.0
S-process decay
Faster
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
Free-running period (h)

Figure 6

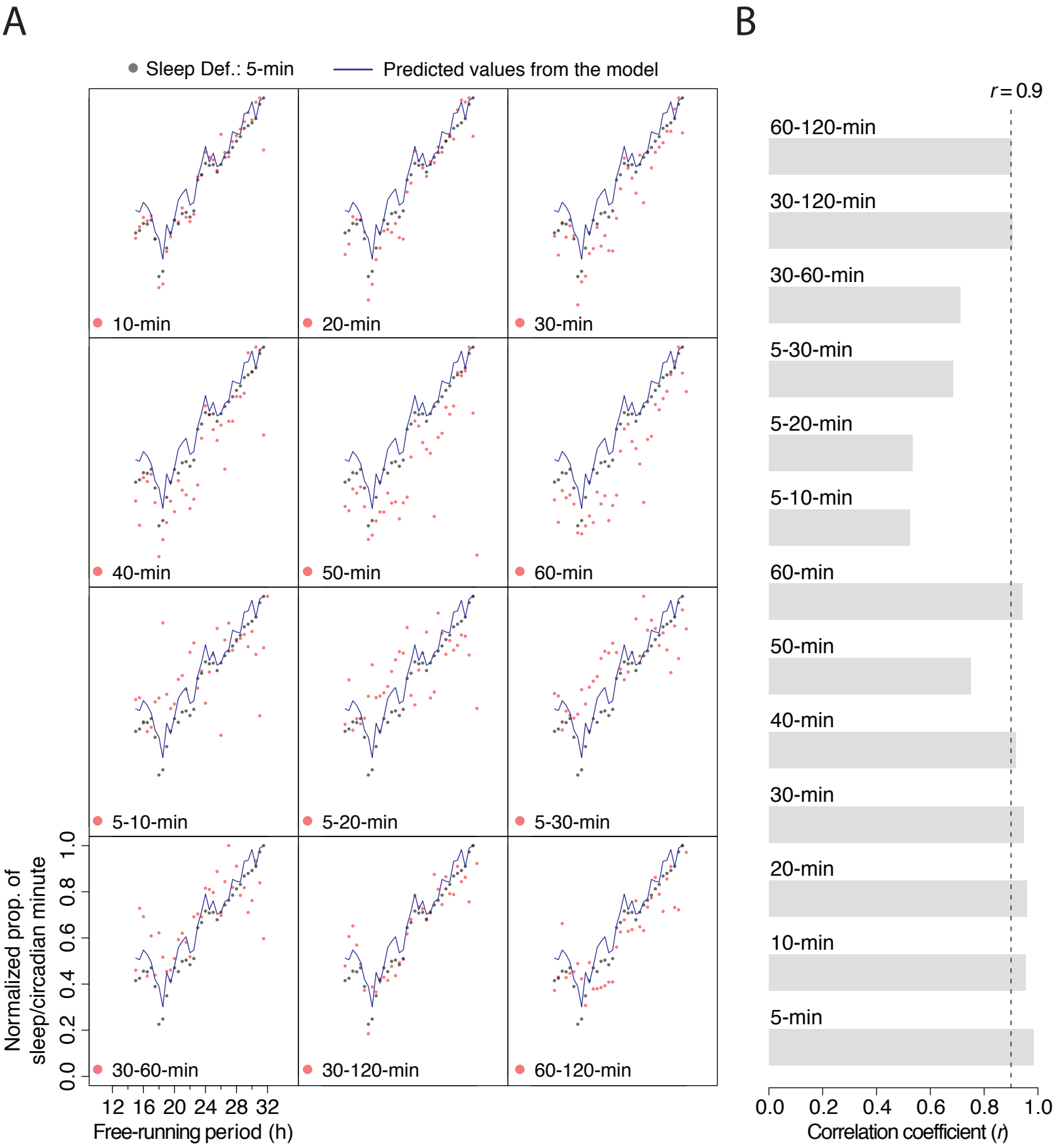
A
Sleep Def.: 5-min
Predicted values from the model
10-min
20-min
30-min
40-min
50-min
60-min
5-10-min
5-20-min
5-30-min
30-60-min
30-120-min
60-120-min
Normalized prop. of sleep/circadian minute
0.0 0.2 0.4 0.6 0.8 1.0
12 16 20 24 28 32
Free-running period (h)
B
r = 0.9
60-120-min
30-120-min
30-60-min
5-30-min
5-20-min
5-10-min
60-min
50-min
40-min
30-min
20-min
10-min
5-min
0.0 0.2 0.4 0.6 0.8 1.0
Correlation coefficient (r)

Figure 7

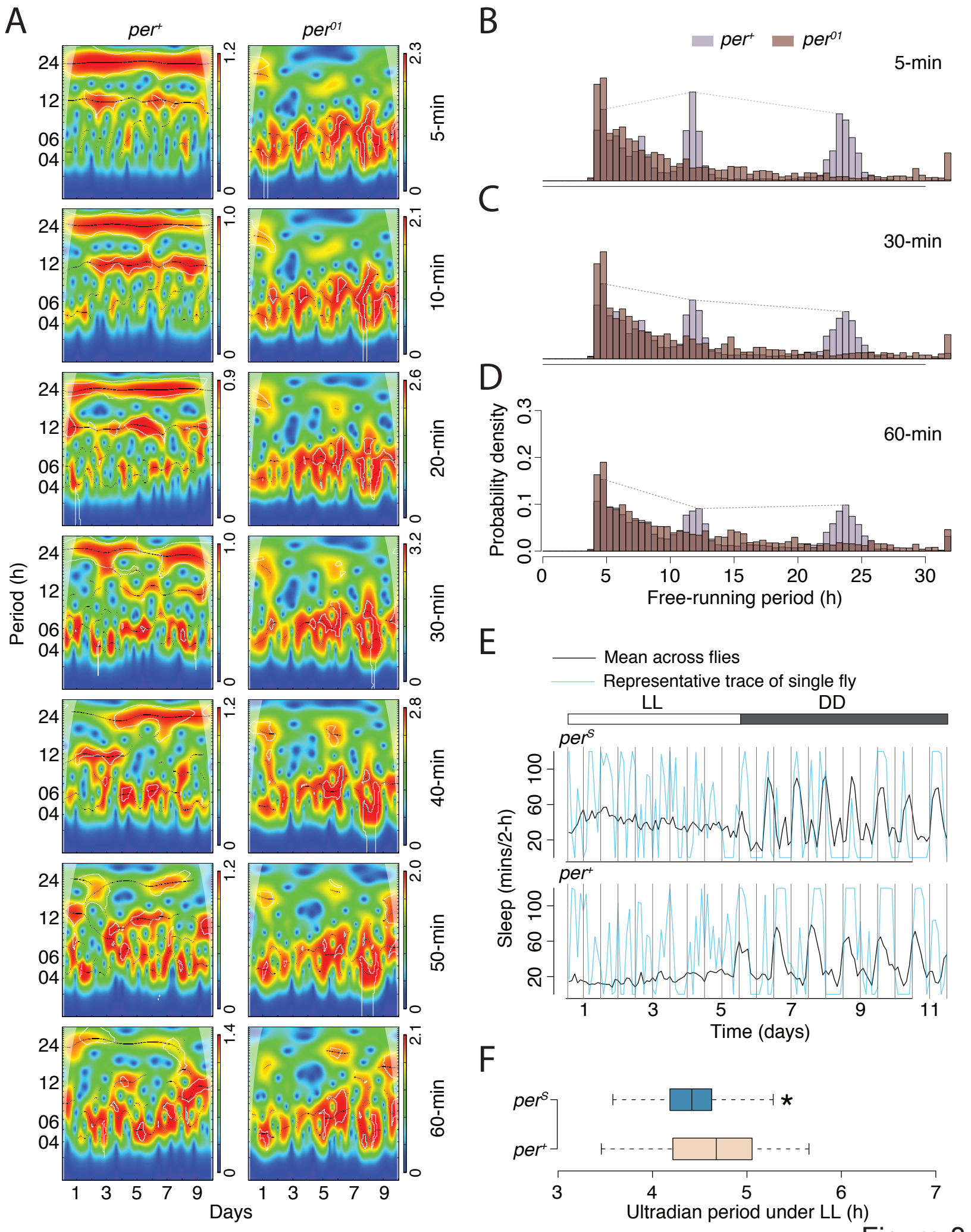
A
per+
per01
Period (h)
24
12
06
04
5-min
10-min
20-min
30-min
40-min
50-min
60-min
Days
B
per+
per01
5-min
C
30-min
D
60-min
Probability density
Free-running period (h)
E
Mean across flies
Representative trace of single fly
LL
DD
perS
per+
Sleep (mins/2-h)
Time (days)
F
perS
per+
Ultradian period under LL (h)

Figure 8

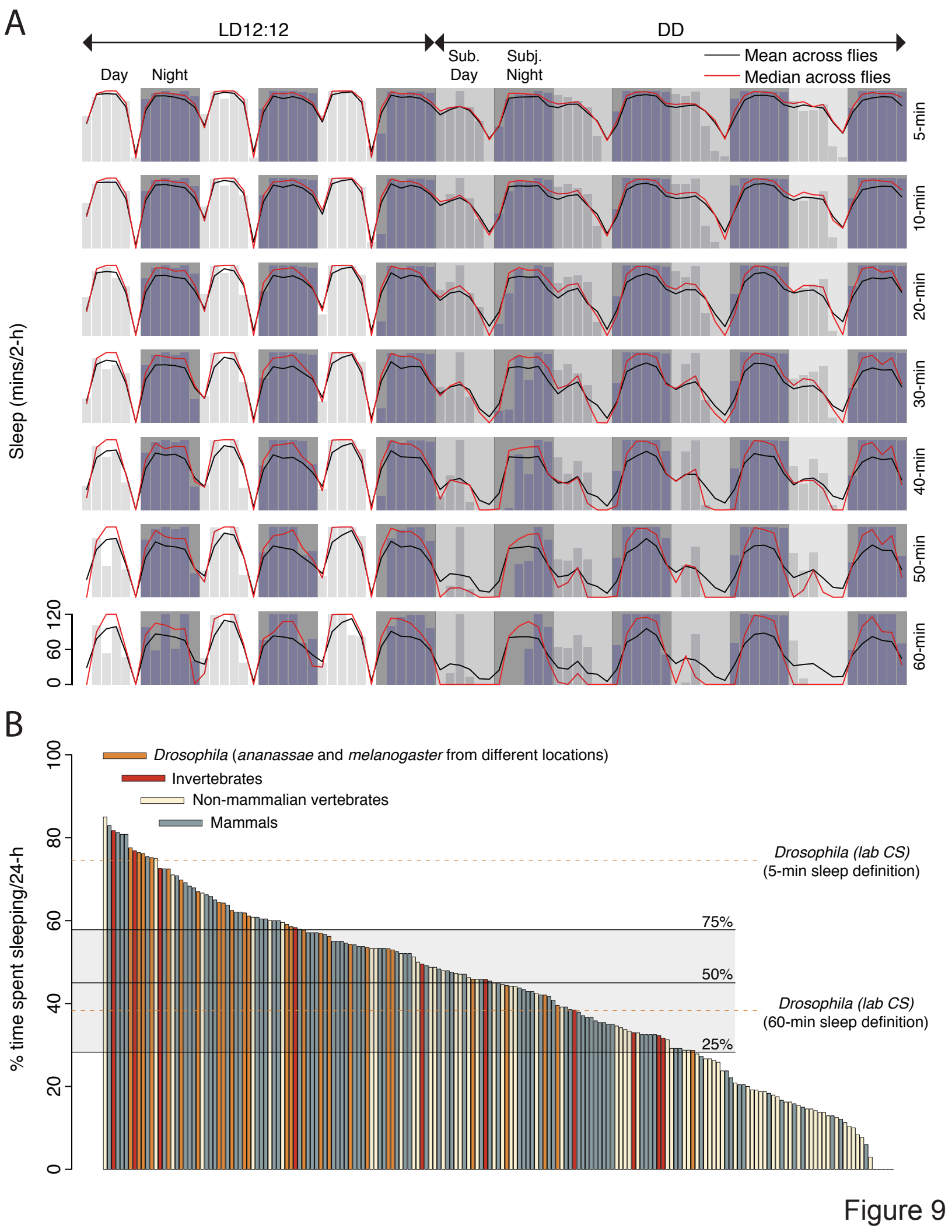
A
LD12:12
DD
Day
Night
Sub. Day
Subj. Night
Mean across flies
Median across flies
Sleep (mins/2-h)
5-min
10-min
20-min
30-min
40-min
50-min
60-min
0
60
120
B
% time spent sleeping/24-h
0
20
40
60
80
100
Drosophila (ananassae and melanogaster from different locations)
Invertebrates
Non-mammalian vertebrates
Mammals
Drosophila (lab CS)
(5-min sleep definition)
75%
50%
25%
Drosophila (lab CS)
(60-min sleep definition)

Figure 9

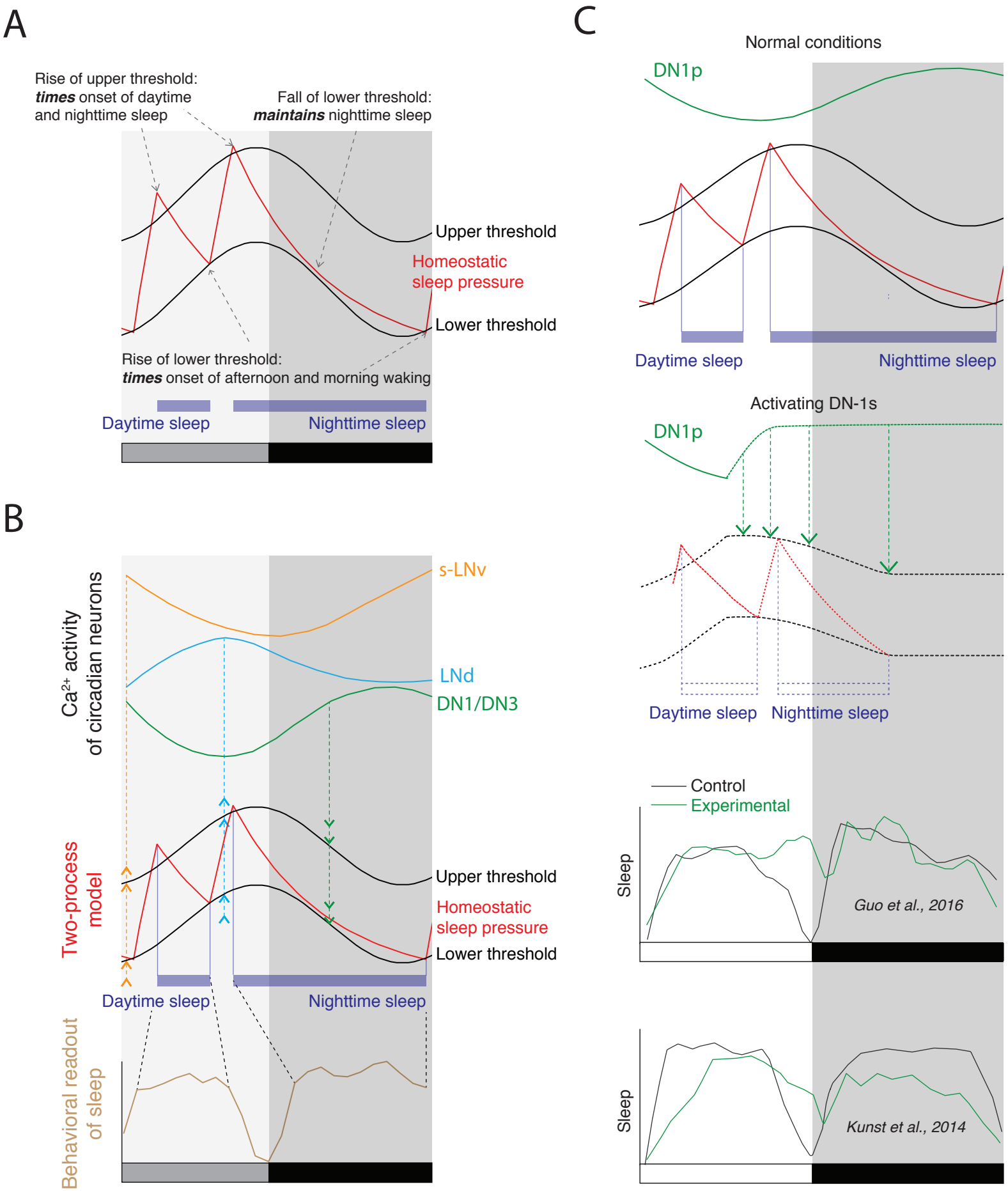
A
Rise of upper threshold:
times onset of daytime
and nighttime sleep
Fall of lower threshold:
maintains nighttime sleep
Upper threshold
Homeostatic
sleep pressure
Lower threshold
Rise of lower threshold:
times onset of afternoon and morning waking
Daytime sleep
Nighttime sleep
B
Ca2+ activity
of circadian neurons
s-LNv
LNd
DN1/DN3
Two-process
model
Upper threshold
Homeostatic
sleep pressure
Lower threshold
Daytime sleep
Nighttime sleep
Behavioral readout
of sleep
C
Normal conditions
DN1p
Daytime sleep
Nighttime sleep
Activating DN-1s
DN1p
Daytime sleep
Nighttime sleep
Control
Experimental
Sleep
Guo et al., 2016
Sleep
Kunst et al., 2014

Figure 10