Supporting Information

De et al. 10.1073/pnas.1220960110

Materials and Methods

Fly Strains. All experiments were performed on virgin *CS* male flies (unless specified) starting at the age of 3–4 d. To examine the role of circadian clocks in the regulation of activity peaks under seminatural conditions (SN), we used the null mutant of circadian *period* gene (per^0) in w^{1118} genetic background and short- (per^S) and long-period mutants (per^L).

Assay Conditions. ***SN.*** All behavioral assays were done within the Jawaharlal Nehru Centre for Advanced Scientific Research campus, Bangalore (12°59'N, 77°35'E), inside an enclosure constructed under a leafy canopy (1). The enclosure was an iron cage ($122 \times 122 \times 122$ cm^3) with grids (6×6 cm^2) allowing free flow of air and covered only on top with a sloping translucent plastic sheet. Locomotor activity was recorded using *Drosophila* activity monitor (DAM) system (Trikinetics). The daily profiles of light, temperature, and humidity were also monitored simultaneously using DEnM (Trikinetics). Additionally, light intensity was measured using LiCor luxmeter. Two sets of experiments were performed: June to July 2012 and January to February 2013.

SN light-filtering experiments. Monitors were covered with neutral density filters (Lee Filters) to create three protocols where the naturally varying light was reduced by 90% (SN_{90}), 75% (SN_{75}), and 50% (SN_{50}). Other than causing reduction in the amplitude of light waveform, these filters do not cause any alteration in the qualitative profile of light.

Light-blocking experiments. The activity monitors were kept under SN and covers were placed for different time intervals of the day, every day, for 7 d—morning cover (MC) (0400–1000 hours), afternoon cover (AC) (1000–1600 hours), evening cover (EC) (1600–2200 hours), and morning-plus-evening cover (MEC) (0400–1000 and 1600–2200 hours). These intervals were chosen based on the average light intensity profiles under SN recorded for several days, just before the assays. MC blocked the rising part of light profile, whereas AC and EC blocked the plateau and decreasing parts, respectively, and MEC blocked both the rising and falling phases of the natural light profile while allowing midday light to reach the flies.

Constant light or constant darkness under otherwise SN condition. To create constant light (LL) of varying intensities, under SN, activity monitors were placed inside light-tight metal boxes ($44 \times 27 \times 20$ cm^3) fitted with LEDs, light baffles, and a small fan allowing temperature and humidity inside the box to match the outside. Temperature and humidity inside and outside the boxes were recorded continuously with DEnM and found to be concordant. The LL intensities used were 10 (LL_{10}+SN), 100 (LL_{100}+SN), and 1000 lux (LL_{1000}+SN). Light intensity was measured using LiCor luxmeter. Similar boxes were used to create constant darkness (DD) under SN (DD+SN).

Observing behaviors under SN. Visual observation of DAM2 monitors. Single male flies placed in glass activity tubes ($n = 32$) in DAM2 monitors kept under SN were manually observed every 1 h from 0700 to 1900 hours for 11 consecutive days during January 2013 while automated recording occurred in parallel (Fig. 2*B*). Three consecutive visual scans were made at every time point and the location of flies (near food, middle, and near cotton plug) and whether they were moving was noted. The proportion of flies in each zone at each time point, was used as the basic unit of data.

Visual observation of flies in activity tubes. Single male flies in glass activity tubes were placed flat on a tray in the same SN enclosure. Tubes were either left completely unshaded, or shaded near food, in the middle, or near cotton plug ($n = 4$ tubes each) using a black tape (~18-mm wide). Tubes were manually observed every 2 h throughout the day for 5 consecutive days during July 2012. Three consecutive visual scans were made at every time point and the location of flies (near food, middle, and near cotton plug) was noted. The proportion of flies in each zone at each time point was used as the basic unit of data.

Visual observation of flies in petri dishes. Flies were housed in petri dishes with a thin layer of standard cornmeal fly food at the bottom. Flies were either assayed in groups of three males and three females per dish, or as solitary males, with six replicates of each type. At every 2-h interval, the number of instances of behaviors (locomotion, rest, wing expansion, chasing, and copulation) was recorded by visually scanning each dish. Three consecutive visual scans were made at every time point and the behavior was noted. The proportion of flies performing a particular behavior at each time point was used as the basic unit of data. Before these assays, all experimenters involved in conducting behavioral observations made a series of observations in parallel and ensured that experimenter bias was kept to the minimum by using similar criteria for all behaviors to be scored.

Statistical Analyses. We designated specific intervals of time as morning (M) (0400–1000 hours), afternoon (A) (1000–1600 hours), and evening (E) (1600–2200 hours). To determine objectively the presence of M, A, and E peaks, average activity profiles (15-min bin) for each genotype or protocol were plotted. An interval was considered to have a peak based on qualitative assessment of the activity profiles averaged across flies and days of recording. Strains or light protocols where there was no distinguishable peak in the average profile were not analyzed further. Dawn anticipation index (AI) was calculated as the ratio of activity counts for 3-h duration before dawn (defined as the time point when light intensity first rose above 0 lux) over activity counts for 6-h duration before dawn (2). The same definition for dawn was also applied to those protocols where natural light was blocked, because this point also coincided with temperature minimum and humidity maximum of the day. Unlike dawn, a similar index for dusk anticipation could not be calculated as there was no such point when a clear phase marker for all three environmental variables coincided.

The total daily activity was estimated for each fly by first obtaining average activity profiles across 7 d, and then for each protocol or genotype, data from up to 32 flies were averaged. One-way ANOVA was followed by post hoc multiple comparisons using Tukey's test. Activity levels during M, A, and E intervals were calculated as the sum of the activity counts in the three intervals for each fly averaged across ~32 flies. Two-way ANOVA was followed by post hoc multiple comparisons using Tukey's test on activity counts in different intervals under different protocols, with protocol and interval treated as fixed factors.

For visual observation of flies in tubes kept in DAM2 monitor, one-way ANOVA was done on proportion of flies found in the middle zone of activity tubes to test for time-dependent preference for middle zone of the tube. Similar analysis was done on proportion of flies exhibiting locomotion. For visual-observation data from the experiment where tubes were not placed in monitors and were shaded, one-way ANOVA was done on arcsine-transformed proportion of flies found in the shaded region of activity tubes to test for time-dependent preference for

shaded part of the tube. For chronoethogram data from grouped flies, separate one-way ANOVA was carried out on courtship-associated movements and on general locomotion or rest to test for time-of-day dependence. For solitary flies, similar test was done for general locomotion.

In all figures depicting waveforms, the error bars are standard error of mean (SEM) whereas all other plots for quantification of phase, amplitude, total activity, and activity in different intervals, error bars are 95% confidence interval (95% CI), allowing visual hypothesis testing of differences between means.

1. De J, Varma V, Sharma VK (2012) Adult emergence rhythm of fruit flies *Drosophila melanogaster* under seminatural conditions. *J Biol Rhythms* 27(4):280–286.

2. Harrisingh MC, Wu Y, Lnenicka GA, Nitabach MN (2007) Intracellular Ca^{2+} regulates free-running circadian clock oscillation in vivo. *J Neurosci* 27(46):12489–12499.

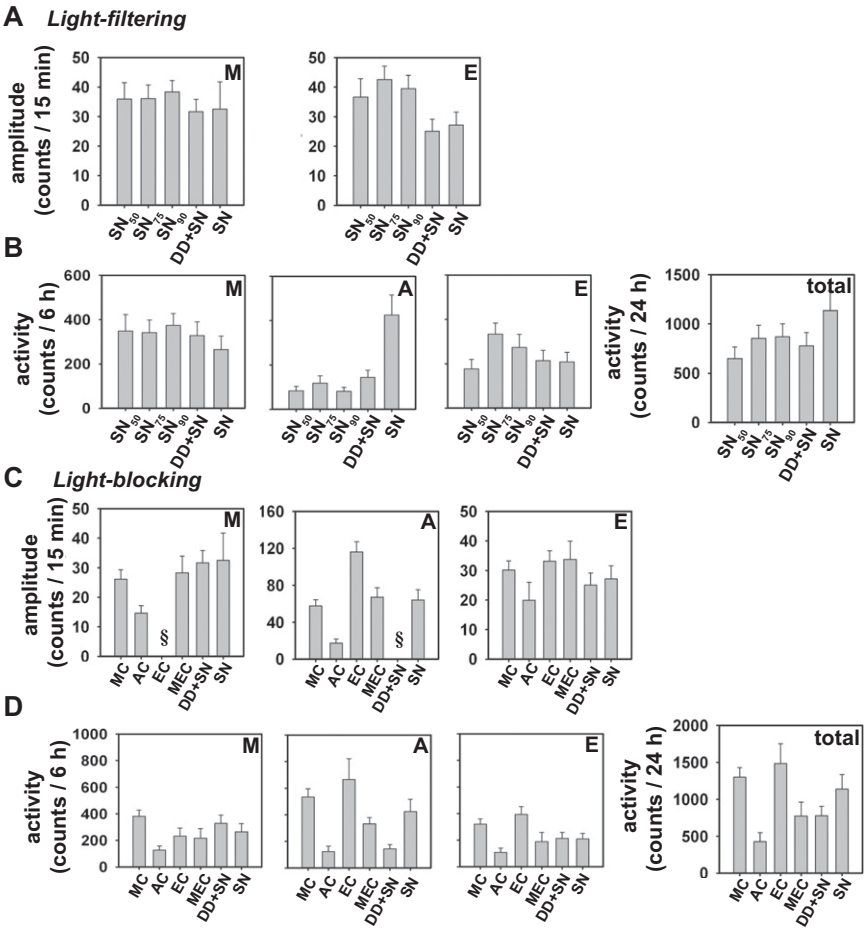

Fig. S1. Amplitudes of morning (M), afternoon (A), and evening (E) peaks and total daily activity of flies under light-filtering and light-blocking protocols. (*A*) Average activity recorded at the M and E peaks under SN with light intensity filters reducing the intensity of naturally varying light by 50% (SN_{50}), 75% (SN_{75}), and 90% (SN_{90}) compared against flies with no exposure to natural light (DD+SN) and full exposure to natural light (SN). Although amplitude of the evening peak (*Right*) was significantly affected by light-filtering protocols [$F_{(4,137)} = 10.89$, $P < 0.001$], M peak (*Left*) was not [$F_{(4,142)} = 0.94$, $P > 0.05$]. (*B*) Activity levels in the M, A, and E intervals in light-filtering protocols were significantly modulated by the protocol [$F_{(4,465)} = 6.17$, $P < 0.000$], time interval [$F_{(2,465)} = 48.47$, $P < 0.0001$], and protocol by time interval interaction [$F_{(4,465)} = 17.88$, $P < 0.0001$]. (*Right*) Total daily activity under light-filtered protocols showed a significant effect of protocol [$F_{(4,149)} = 6.21$, $P < 0.001$]. (*C*) Average activity recorded at the M, A, and E peaks under SN with morning cover (MC) ($n = 24$), afternoon cover (AC) ($n = 26$), evening cover (EC) ($n = 32$), and morning-plus-evening cover (MEC) ($n = 30$), where sections of natural light profile were blocked, showed a significant effect of protocol on the M [$F_{(4,130)} = 6.10$, $P < 0.001$], A [$F_{(4,129)} = 53.58$, $P < 0.001$], and E peaks [$F_{(5,147)} = 5.89$, $P < 0.001$] compared with DD+SN and SN. (*D*) In light-blocking protocols, activity levels in the M, A, and E intervals were significantly modulated by protocol [$F_{(5,536)} = 38.79$, $P < 0.0001$], time interval [$F_{(2,536)} = 29.18$, $P < 0.0001$], and protocol by time interval interaction [$F_{(10,536)} = 11.07$, $P < 0.0001$]. (*Right*) Total daily activity was also significantly affected by protocol [$F_{(5,171)} = 18.55$, $P < 0.001$]. The section symbol (§) denotes the protocols in which no distinct peak could be detected during that interval. All error bars represent 95% CIs.

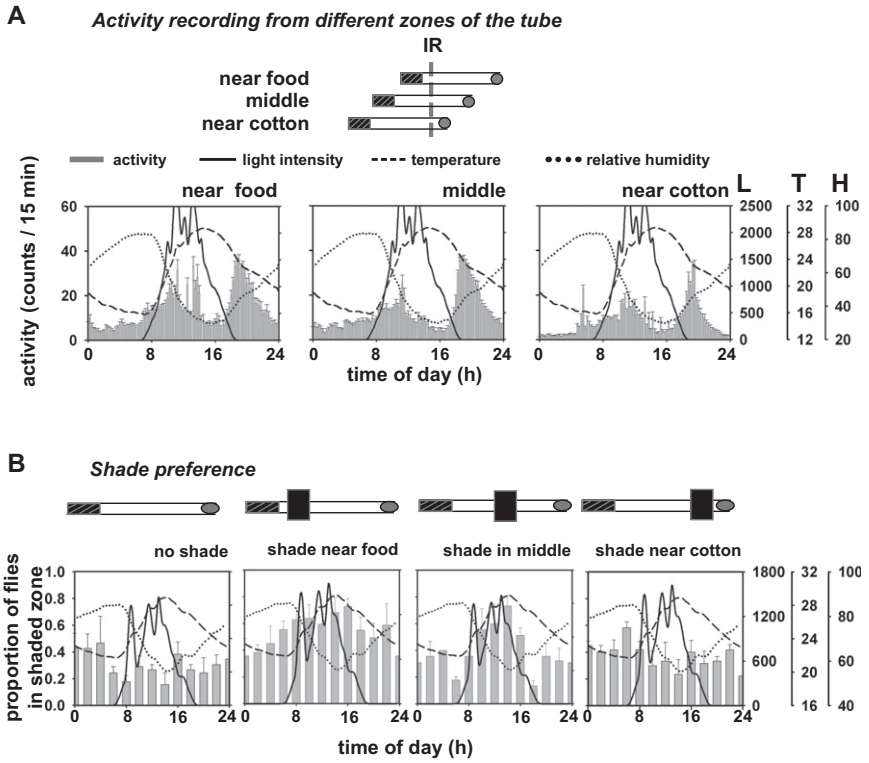

Fig. S2. (*A*) Schematic representation (*Upper*) of protocols for recording activity from different zones of the tubes by placing the tubes in DAM2 monitors in such a manner that the IR beam detects activity near the food, in the middle as usual, or near the cotton plug. Average activity profile (*Lower*) of the activity recording near the food, in the middle, and near cotton plug of the activity tube are similar. (*B*) Results of visual observation of flies in tubes with shade provided in different zones. Proportion of flies in the middle zone is plotted for the unshaded tubes (extreme left panel), whereas when the shade was provided near the food, in the middle, or near the cotton plug (all other panels), proportion of flies in the shaded region is plotted. Individual flies were placed in the locomotor activity tubes with food at one end and cotton plug at the other and black tape was used to shade different zones of the tube ($n = 4$ tubes for each type of shaded protocol). Three replicate observations were made at 2-h intervals throughout the day for 5 d and the position of the fly in the tube was noted (whether near the food, in the middle, or near the cotton plug). These data were averaged across days for each time point. The instances of occurrence in each zone were averaged for each zone across days. All error bars indicate SEM.

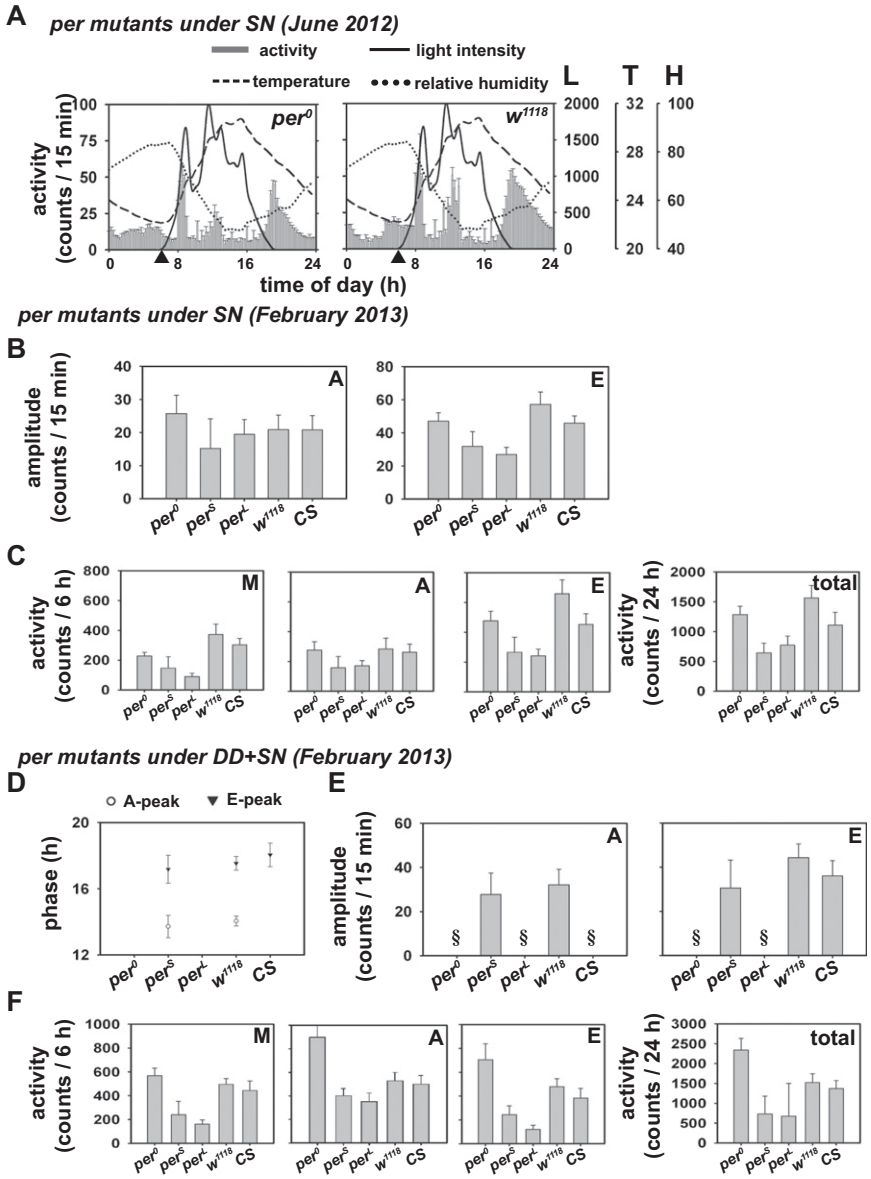

Fig. S3. Waveforms, phase, and amplitude of the M, A, and E peaks, activity levels in the M, A, and E intervals, and total daily activity of *per* mutants and controls under SN, and DD in otherwise SN conditions. (*A*) Average activity profile (counts per 15 min) of per^0 flies and its control, w^{1118} under SN recorded in the month of June 2012 in parallel with the light-modified protocols. The per^0 flies differed from their controls w^{1118} only in terms of the amplitude of the A peak in that they show lower amplitude than w^{1118} [$F_{(1,30)} = 16.27$, $P < 0.003$], whereas amplitude of other peaks and their phases did not differ significantly. The total activity of per^0 flies was also not different from w^{1118}. Error bars are SEM. (*B*) Average activity recorded at the A peak of *per* mutant flies with null (per^0), short-period (per^S), and long-period (per^L) alleles and their wild-type controls, w^{1118}, and *CS* under SN conditions (February 2013) showed no significant effect of genotype [$F_{(4,81)} = 1.71$, $P > 0.05$], whereas E peak was affected [$F_{(4,87)} = 17.19$, $P < 0.001$]. (*C*) Average activity in the M, A, and E intervals showed a statistically significant effect of strain [$F_{(4,258)} = 39.35$, $P < 0.001$], time interval [$F_{(2,258)} = 52.23$, $P < 0.001$], and genotype by time interval interaction [$F_{(8,258)} = 4.37$, $P < 0.001$]. (*Right*) Total daily activity showed a statistically significant effect of genotype [$F_{(4,85)} = 15.56$, $P < 0.001$]. (*D*) Average phase of the A [$F_{(1,23)} = 1.14$, $P > 0.05$] and E peaks [$F_{(2,38)} = 1.63$, $P > 0.05$] of *per* mutant flies and their controls did not show a statistically significant effect of genotype under DD+SN. (*E*) Average activity recorded at the A [$F_{(1,23)} = 1.18$, $P > 0.05$] and E peaks [$F_{(2,38)} = 3.17$, $P = 0.05$] of locomotor activity in the *per* mutant flies were not different from their controls under DD+SN. (*F*) Average activity in the M, A, and E intervals showed a statistically significant effect of strain [$F_{(4,243)} = 75.19$, $P < 0.001$], time interval [$F_{(2,243)} = 15.61$, $P < 0.001$], and genotype by time interval interaction [$F_{(8,243)} = 3.01$, $P < 0.005$]. (*Right*) Total daily activity also showed a statistically significant effect of strain [$F_{(4,94)} = 26.57$, $P < 0.001$]. The section symbol (§) denotes the protocols in which no distinct peak could be detected during that interval. All error bars represent 95% CIs.

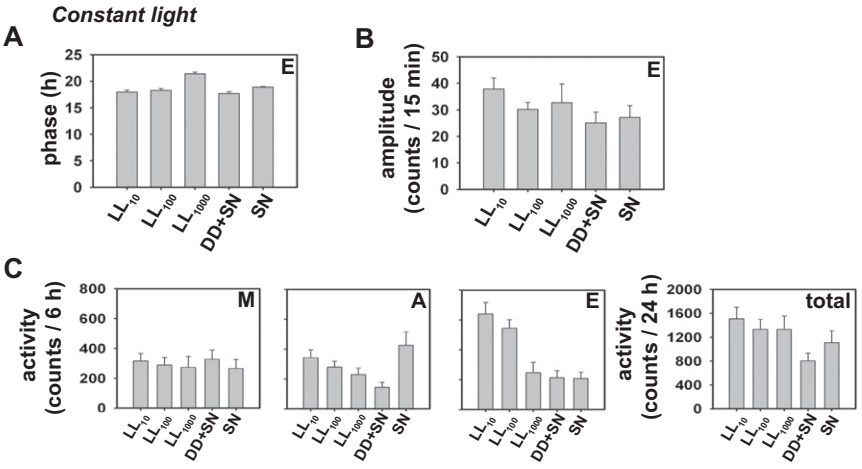

Fig. S4. Phase and amplitude of the E peak, activity levels in the M, A, and E intervals, and total daily activity of flies in constant light protocols under otherwise SN conditions and waveforms of per^0 and w^{1118} under SN. (*A*) Average phase of the E peak under different constant light intensities 10 (LL_{10}+SN), 100 (LL_{100}+SN), and 1000-lux (LL_{1000}+SN) in otherwise SN conditions compared against flies exposed to no light (DD+SN) or SN showed significant effect of protocol [$F_{(4,131)} = 84.75$, $P < 0.001$]. (*B*) Average activity recorded at the E peak showed significant effect of protocol [$F_{(4,130)} = 4.91$, $P < 0.005$]. (*C*) Average activity in the M, A, and E intervals showed main effects of protocol [$F_{(5,537)} = 23.10$, $P < 0.001$], time interval [$F_{(2,537)} = 28.73$, $P < 0.001$], and protocol by time interval interaction [$F_{(10,537)} = 18.36$, $P < 0.001$], and (*Right*) total daily activity was also significantly affected by protocols [$F_{(4,134)} = 9.40$, $P < 0.001$]. All error bars represent 95% CIs.

Table S1. Dawn anticipation indices of fly strains under different experimental protocols

	AI		
	June 2012	February 2013	
Strain/protocol	SN	SN	DD+SN
CS	0.57 ± 0.06	0.55 ± 0.08	0.60 ± 0.05
w^{1118}	0.59 ± 0.12	0.54 ± 0.06	0.60 ± 0.06
per^0	0.56 ± 0.05	0.47 ± 0.03	0.48 ± 0.03
per^S	—	0.66 ± 0.14	0.52 ± 0.21
per^L	—	0.21 ± 0.04	0.48 ± 0.06
SN_{50}	0.81 ± 0.07	—	—
SN_{75}	0.77 ± 0.07	—	—
SN_{90}	0.78 ± 0.07	—	—
MC	0.79 ± 0.08	—	—
AC	0.74 ± 0.12	—	—
EC	0.66 ± 0.09	—	—
MEC	0.66 ± 0.09	—	—
DD+SN	0.77 ± 0.07	—	—
LL_{10}+SN	0.59 ± 0.05	—	—
LL_{100}+SN	0.53 ± 0.04	—	—
LL_{1000}+SN	0.51 ± 0.07	—	—

Shown are anticipation indices (AIs) of wild-type flies in different protocols and mutants under seminatural (SN) and constant darkness in otherwise SN (DD+SN). Dawn AI under light-filtering, light-blocking, and constant-light (LL+SN) protocols under otherwise SN and mutant flies under SN and DD+SN was calculated as the ratio of activity counts for 3-h duration before dawn (light intensity >0 lux) over activity counts for 6-h duration before dawn. One set of experiments with the light-filtered, light-blocked, and constant-light protocols in otherwise SN was conducted in June 2012. The *per* mutants and their controls were assayed in February 2013 under SN and DD+SN to determine the role of molecular circadian clock in timing of peaks and anticipation to dawn. All error values are 95% CIs.