Scheduled exposures to a novel environment with a running-wheel differentially accelerate re-entrainment of mice peripheral clocks to new light-dark cycles

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Abstract

Effects of scheduled exposures to novel environment with a running-wheel were examined on re-entrainment to 8 hour shifted light-dark (LD) cycles of mouse circadian rhythms in locomotor activity and clock gene, *Per1*, expression in the suprachiasmatic nucleus (SCN) and peripheral tissues. *Per1* expression was monitored by a bioluminescence reporter introduced into mice. The animals were exposed to the novel environment for 3 hours from the shifted dark onset for 4 cycles and released into constant darkness. In the phase-advance shift, the circadian rhythm in locomotor activity fully re-entrained in the exposed group, whereas it was in transients in the control. On the other hand, the circadian rhythm of *Per1* expression in the SCN almost completely re-entrained in both the control and exposed groups. In the skeletal muscle and lung, the circadian rhythm fully re-entrained in the exposed group, whereas the rhythms in the control did not. In the phase-delay shift, the circadian rhythms in locomotor activity and *Per1* expression almost completely re-entrained in both groups. Theses findings indicate that the scheduled exposures to novel environment with a running-wheel differentially accelerate the re-entrainment of the mouse peripheral clocks to 8 hour phase-advanced LD cycles.

Introduction

Circadian rhythms in mammals are regulated by the pacemaker in the suprachiasmatic nucleus (SCN) which entrains to a light-dark (LD) cycle (Rusak & Zucker 1979) and phase-controls the so-called peripheral clocks in a variety of tissues (Yoo *et al.* 2005). On the other hand, non-photic time cues such as timed wheel-running and exposure to novel environment are known to accelerate re-entrainment of circadian behavioral rhythms to phase-shifted LD cycles (Mrosovsky *et al.* 1989). Three hour wheel-running at the first dark onset of phase-advanced LD cycle dramatically increased the rate of re-entrainment of circadian wheel-running rhythm in hamsters (Mrosovsky & Salmon 1987). Furthermore, a single pulse of wheel-running or forced treadmill running produced a phase-dependent phase-shift of behavioral rhythm, indicating that wheel-running is a potential zeitgeber for the circadian clocks in several rodent species (Reebs & Mrosovsky 1989; Marchant & Mistlberger 1996). Wheel-running is also known to change the free-running period of circadian locomotor rhythm under constant darkness (DD) in rats (Yamada *et al.* 1988). Similar effects of non-photic time cues have been demonstrated on the circadian rhythms in humans (Van Reeth *et al.* 1994; Buxton *et al.* 1997; Miyazaki *et al.* 2001; Barger *et al.* 2004). Theses findings have been interpreted as the indication of feedback from the behavioral outputs to the circadian pacemaker in the SCN (Mistlberger & Holmes 2000). There are some indications that the clock genes such as *Per1* and *Per2* in the SCN are suppressed by non-photic stimuli (Maywood *et al.* 1999; Maywood & Mrosovsky 2001; Yannielli *et al.* 2002). However, it remains unknown whether or not non-photic time cues affect directly the circadian pacemaker in the SCN.

Yamazaki *et al.* (2000) demonstrated different rates of re-entrainment of rat circadian rhythms in various tissues following abrupt shifts of LD cycle. The circadian rhythm of *Per1* expression in the SCN shifted immediately, whereas those in the liver, lung and skeletal muscle shifted slowly, taking several days for full re-entrainment. Immediate re-entrainment of the SCN circadian pacemaker was

confirmed by several studies (Hara *et al.* 2001; Abe *et al.* 2002; Nagano *et al.* 2003). Accordingly, the acceleration of re-entrainment by wheel-running is unlikely due to the accelerated phase-shift of the circadian pacemaker in the SCN, since the pacemaker re-entrained to shifted LD cycles immediately, regardless of the presence or absence of non-photic time cues. Alternative hypotheses for the effect of non-photic time cues could be enhancement of phase-control by the SCN over the peripheral clocks, and direct resetting of the peripheral clocks.

The aim of present study was to determine whether or not scheduled exposures to novel environment with a running-wheel could accelerate the re-entrainment of circadian rhythms in mice to 8 h phase-shifted LD cycles. Here we demonstrate differential effects of the scheduled exposures to the novel environment on re-entrainment of the circadian rhythms in locomotor activity and in the expression of clock gene, *Per1*, in peripheral tissues to phase-advanced LD cycle.

Results

Accelerated phase-advance shifts of circadian locomotor rhythm by scheduled exposures to novel environment with a running-wheel

Figure 1 illustrates the experimental protocols in which mice were exposed to novel environment with a running-wheel from the dark onset of the LD cycle which was phase-advanced or phase-delayed by 8 hours. Representative activity rhythms in mice subjected to 2 exposures in the phase-advance protocol were illustrated in Figure 2, and those in mice subjected to 4 exposures in the phase-advance (left) and phase-delay (right) protocols in Figure 3. Table 1 summarizes the phase of activity onset, amount of phase-shift and free-running period of locomotor activity rhythm.

In the phase-advance experiment with 2 exposures, neither the phase of activity onset nor the amount of phase-shift was significantly different between the baseline and shifted LD cycle or between the exposed and control groups (Table 1). In 3 of 10 mice in the exposed group, a large phase-advance shift up to 10 hours was observed (Fig. 2). By contrast, in the phase-advance experiment with 4 exposures, a significant difference was found in the phase of activity onset between the baseline and shifted LD cycle in both the control and exposed groups (Table 1). And, the amount of phase-shift was significantly larger in the exposed group than in the control. In the phase-delay experiment with 4 exposures, the activity onset was significantly phase-shifted in both groups, and the amount of phase-shift was not different between the control and exposed groups.

The free-running period in DD was not significantly different between the control and exposed groups in these experiments (Table 1). However, a significant difference was detected in the free-running period between the phase-advanced and delayed groups. The mice in the phase-delay shift showed a longer period (23.79 ± 0.07 h; mean ± SD, n = 20) than in the phase-advance (23.46 ± 0.14 h, n = 16) (two-way ANOVA with post hoc t-test; $P<0.01$).

There was no statistically significant correlation between the number of wheel-revolution and the amount of phase-shift. Some mice ran a wheel substantially

but did not show an accelerated phase-shift.

Circadian rhythms in *Per1* gene expression in the SCN and peripheral tissues in culture

Figure 4 and 5 illustrate representative *Per1* expression rhythms in the cultured SCN and peripheral tissues (liver, lung and skeletal muscle) in mice subjected to 4 exposures in the 8 h phase-advance and phase-delay protocols, respectively. The *Per1* expression rhythms were monitored continuously by means of a bioluminescence reporter. Table 2 demonstrates the circadian peaks of bioluminescence rhythms in the first cycle of culture.

In the baseline, the circadian peak in the SCN was detected at 12.5 ± 0.9 h (local time), 6.5 hours after the time of light-on in the previous LD cycles. The circadian peaks in the peripheral tissues were 21.1 ± 1.4 h in the liver, 17.8 ± 1.3 h in the lung and 21.8 ± 1.2 h in the skeletal muscle, respectively.

In the phase-advance experiment, the circadian rhythms in the SCN and peripheral tissues of the control group differentially responded to the advanced LD cycles. The circadian peaks in the SCN, lung and skeletal muscle were significantly phase-shifted (Bonferroni test; $P<0.0167$). On the other hand, the circadian peak in the liver was not phase-shifted (Fig. 6).

In the exposed group, one circadian peak in the skeletal muscle was extremely deviated (Smirnov-Grubbs test; $P<0.05$), and excluded from further analyses. The circadian peaks in the SCN, lung and skeletal muscle were significantly phase-advanced (Bonferroni test; $P<0.0167$), whereas the peak in the liver was not shifted (Fig. 6). When compared with the control group, the circadian peaks in the lung and skeletal muscle showed significantly larger phase-shifts, the phases of which were not different from the expected peak phases in full re-entrainment (Fig. 6). On the other hand, the phase-shifts in the SCN were not significantly different between the control and exposed groups.

In the phase-delay experiment, the circadian peaks in the SCN and the peripheral tissues in both groups were significantly phase-delayed (Bonferroni test; $P<0.0167$)

and were not different between the control and exposed groups (Table 2 and Fig. 6).

Discussion

Scheduled exposures to the novel environment with a running-wheel accelerated the re-entrainment of mouse circadian rhythms to 8 hour phase-shifted LD cycles. The effect was differential, depending on the direction of phase-shift and the overt circadian rhythms.

In the case of circadian locomotor rhythm, the re-entrainment to the phase-advanced LD cycle was almost completed by 4 exposures to the novel environment, whereas it was still on the half way without exposure. In some mice, a large phase-shift up to 10 hours was observed even by 2 exposures. On the other hand, the re-entrainment to the phase-delay shift was completed by 4 exposures, regardless of whether the mice were exposed to the novel environment or not. The number of cycles needed for re-entrainment to a phase-shifted LD cycle has been reported to depend on the direction of phase-shift and the phase-marker of circadian rhythm (Aschoff 1981). In rats, the phase-shift of locomotor rhythm was slow in the phase-advance direction but rapid in the phase-delay, when the activity onset was used as a phase-marker (Honma *et al.* 1985; Takamure *et al.* 1991), which was similar to the present results in mice. The free-running period in DD was different between the phase-advance and delay experiment, which was reported as after-effects of phase-shifts (Pittendrigh & Daan 1976).

In the case of the circadian rhythm in *Per1* expression, the rate of phase-shift to the phase-advanced LD cycle was different among tissues (Table 2, Fig. 6). The circadian rhythm in the SCN re-entrained almost completely in 4 cycles, regardless of whether the mice were exposed to the novel environment or not. Immediate phase-shifts of the SCN circadian pacemaker have been observed in response to a phase-shifted LD cycle (Yamazaki *et al.* 2000; Abe *et al.* 2002). In the exposed group, the re-entrainment was also facilitated in the lung and skeletal muscle by the novel environment. In both tissues, the circadian rhythms in the control group were still in transients of full re-entrainment, whereas those in the exposed group were fully re-entrained (Fig. 6). In contrast, the circadian rhythm in the liver did not phase-shift

significantly, regardless of the presence or absence of the novel environment. The slow response of liver circadian clock to a phase-shifted LD cycle was also reported previously (Yamazaki *et al.* 2000; Hara *et al.* 2001). As a result, the so-called internal desynchronization occurred in the control group between the SCN circadian pacemaker and behavioral rhythms or peripheral clocks. Internal desynchronization is regarded as the indication of uncoupling of different circadian oscillators which have been coupled together (Aschoff & Wever 1981). The immediate phase-shift of the SCN pacemaker may be due to high responsiveness to lights, which is described as a type 0 phase-response curve (PRC), whereas the slow phase-shift of the peripheral clocks could be due to a low responsiveness of the oscillator(s) to lights, which is described as a type 1 PRC (Johnson 1999). Or more likely, the slow phase-shift reflect the slow process of re-establishment of an oscillatory coupling with the SCN pacemaker which is disturbed by a rapid phase-shift of the SCN circadian pacemaker.

The circadian oscillator(s) directly responsible for the behavioral rhythms is unknown. Recently, Nagano *et al.* (2003) demonstrated two regions in the SCN, where the circadian rhythms in *Per1* gene expression responded differentially to 6 hour phase-advanced LD cycles. The ventrolateral SCN shifted almost immediately, whereas the dorsomedial SCN shifted slowly. A similar dissociation of phase-shifts was also reported in the *Per1*, *Per2*, and *Cry1* expression rhythms in the SCN (Reddy *et al.* 2002). Although the possibility is not excluded that the oscillator(s) directly responsible for the behavioral rhythms is located in the SCN, the behavioral oscillator(s) is more likely located outside the SCN, as suggested by the methamphetamine-induced or feeding-associated behavioral rhythms in the SCN lesioned animals (Stephan 1983; Honma *et al.* 1987). Recently, several structures in the brain were reported to show the SCN-independent circadian rhythms (Abraham *et al.* 2005) which could be a candidate of peripheral clock for behavioral rhythms.

The mechanism of the differential acceleration of re-entrainment is not known. Exposures to the novel environment may exert their effects not on the SCN circadian pacemaker but on somewhere in the output pathway from the pacemaker to the site of behavior expression, since the *Per1* expression rhythm in the SCN almost completely

re-entrained to the phase-shifted LD cycle with or without exposures to the novel environment. Recently, prokineticin 2 (PK2) and brain-derived neurotrophic factor (BDNF) were suggested to be released from the SCN and involved in the expression of behavioral rhythm (Cheng *et al.* 2002; Naert *et al.* 2006). Exposures to the novel environment could stimulate the release of these substances in the SCN and thereby strengthen the output signals to which the peripheral clock entrains. Alternatively, exposures to the novel environment may directly stimulate the peripheral clock(s) responsible for behavioral rhythms. This possibility has been suggested by the so-called prefeeding enhancement of wheel-running or other physiological measures under restricted daily feeding (Stephan 2002). The prefeeding peaks were detected even in the SCN lesioned animals (Krieger *et al.* 1977; Stephan 1983). In addition, the *Per1* expression rhythm in the SCN did not response to restricted feeding but those in the peripheral tissues phase-shifted differentially by restricted feeding (Stokkan *et al.* 2001). The feeding schedule is likely to affect the peripheral clocks directly. The scheduled exposures to the novel environment may directly reset the peripheral clocks in the lung and skeletal muscle similar to restricted daily feeding.

In the control mice subjected to the phase-advanced shift, the peripheral clocks in the lung and skeletal muscle seemed to phase-delay instead of phase-advance, despite that the behavioral rhythm showed phase-advanced shifts (Fig. 3, Fig. 6). Such re-entrainment by partition has been observed in humans under temporal isolation (Aschoff & Wever 1981), and in passengers who crossed many time zones rapidly (Takahashi *et al.* 1999). The mechanism for re-entrainment by partition is not known. One possible explanation for this phenomenon is that the peripheral clocks in different tissues have different sensitivities to the time cue and show different PRC for it. Even if the same time cue simultaneously hits the circadian oscillators, the direction of response would be different depending on the shape of each PRC.

The mediator of time cue(s) in the novel environment is not known. A mouse sniffs, walks around and runs a wheel in a new cage. The number of wheel-revolution in individual animals did not correlate with the amount of phase-shift with a statistically significant level. Some mice ran a wheel substantially but did not

show an accelerated phase-shift. Therefore, it is not clear whether or not the wheel-running is responsible for the acceleration of phase-shift. The endocrine systems such as the hypothalamic-pituitary-adrenocortical axis are known to be stimulated by novel environment. The plasma corticosterone level increased significantly in mice and rats after transferred to a new cage (Hennessy 1991; Yamazaki *et al.* 2005). Corticosterone was reported to reset the peripheral clocks and could be a mediator of the time cue (Balsalobre *et al.* 2000; Le Minh *et al.* 2001). The autonomic nervous system was also activated by novel environment, which may either enhance the output signal from the SCN pacemaker or directly reset the peripheral clocks. Timed and repeated injection of methamphetamine, a stimulant of the sympathetic nervous system, was reported to shift the circadian rhythms in clock gene expression in the peripheral tissues (Iijima *et al.* 2002). Catecholamines are the possible mediators of the time cue.

It is concluded that the scheduled exposures to the novel environment with a running-wheel differentially accelerate re-entrainment of the peripheral clocks in mice subjected to 8 h phase-advanced LD cycles.

Experimental procedures

Animals

Forty-nine male mice of C57BL6 strain and 50 males of *Per1*-luc transgenic mice of C57BL6 background (Inagaki *et al*. 2007) were used in the present *in vivo* and *in vitro* experiments, respectively. The mice were bred and reared in our animal quarter and maintained under an LD cycle of 12 hours light and 12 hours darkness (lights-on from 0600 to 1800) and with a light intensity of ca. 300 lx. The animal rooms were air-conditioned at constant temperature (22 - 24 °C) and humidity (50 - 60 %). They had free-access to food and water throughout the day. The animals were cared for according to the Guidelines for the Care and Use of Laboratory Animals in Hokkaido University.

Phase-shifts of LD cycle

At the age of 6 to 8 weeks, the mice were transferred to individual cages (15 x 25 x 15 cm) equipped with a running-wheel (10 cm diameter) to accustom them to the wheel for 10 days. A running-wheel was attached to the wire cover of the cage. Then, the animals were transferred individually to a light-proof chamber (40 x 50 x 30 cm) and housed in a cage without a running-wheel. The LD cycle in the chamber was the same as in the animal room. Locomotor activity was measured for at least 10 days to confirm entrainment of the circadian rhythm to the LD cycle, which was followed by an 8 hour phase-shift of LD cycle by shortening (phase-advance) or lengthening (phase-delay) the light phase. The new light phase was from 2200 to 1000 in the phase-advance shift, and from 1400 to 0200 in the phase-delay shift, respectively (Fig. 1). The animals were kept under the new LD conditions for one or three cycles and then released into DD for 14 days.

Two experiments were performed with the phase-advance protocol. In one experiment, the animals were subjected to the new LD for one cycle with 2 exposures to the novel environment and then released into DD. In the other, they were subjected to the new LD for 3 cycles with 4 exposures and released into DD. In the phase-delay

protocol, the mice were subjected to the new LD for 3 cycles with 4 exposures and released into DD. Animals were divided into two groups in each experiment. The mice in the exposed group were transferred to new individual cages with a running-wheel for 3 hours from the dark onset in the new LD cycle and DD by exchanging cages outside the chamber under dim red light (ca. <0.01 lx) within 30 sec (Fig. 1). The mice in the control group were treated with the same procedure except for cage exchange.

Measurement of locomotor activity

The spontaneous locomotor activity was recorded continuously by a thermal sensor and the number of movement was stored every 1 min by a computer system (The Chronobiology Kit, Stanford Software Systems, Stanford, CA, USA). The frequency of movement (counts/5min) was plotted in a standard double-plotted actogram for the visual estimation of daily activity onsets (CLOCKLAB software, Actimetrics, Evanston, IL). The amount of phase-shift of circadian locomotor rhythm was defined as the phase difference between the activity onset before the phase-shifts (baseline) and that on the second day of DD. The baseline phase in individual mice was defined as the mean of 10 successive activity onsets. The activity onset on the second day of DD was obtained individually from the regression line fitted to the first 10 successive activity onsets in DD. The number of wheel-revolution was counted with a micro-switch every 1 min.

Measurement of *Per1* gene expression in the SCN and peripheral tissues

Per1-luc mice were used for monitoring the circadian rhythms in clock gene, *Per1*, expression in the SCN, liver, lung, and skeletal muscle (Inagaki *et al*. 2007). The tissues were obtained from 1200 to 1700 in the baseline, and from 16 to 21 hours after the start of DD in the shifted groups. The brain and peripheral tissues were rapidly removed under dim red light (ca. <0.01 lx) and immersed in ice-cold Hanks' balanced salt solution (pH 7.4, Sigma). The coronal brain slices of 300 μm thick were prepared with Microslicer (Dosaka, Osaka, Japan), and a pair of SCN was dissected

with a surgical knife. The liver and lung were prepared in slices of 300 μm thick with Tissue Chopper (McIlwain Tissue chopper, The Mickle Laboratory, Surrey, UK). The rectus abdominis muscle was cut into fragments with a surgical knife. Each tissue was explanted on a culture membrane (Millicell CM, pore size 0.4 μm, Millipore) in a 35 mm Petridish sealed with Parafilm (American Can Co., Greenwich) and incubated at 37 °C with 1.3 ml DMEM (Gibco-Invitrogen) supplemented with $NaHCO_3$ (2.7 mM), HEPES (10 mM), Kanamycin (20 mg/L, Gibco), Insulin (5 μg/ml, Sigma), Putrescine (100 mM, Sigma), human Transferrin (100 mg/ml, Sigma), Progesterone (20 nM, Sigma), Sodium Selenite 30 nM (Gibco) and 0.1 mM D-Luciferin K salt (DOJINDO). Bioluminescence was monitored for 1 min at 10 min intervals with a dish type luminometer (AB2500 Kronos, ATTO; Lumicycle, Actimetrics) for 5 days.

Circadian phases of bioluminescence were analyzed with a modified method of Inagaki *et al.* (2007). Briefly, the original records were detrended by subtracting the 24 hour moving average from them and smoothed by a five-point moving average method. The peak observed within the next day of explantation was defined as the first circadian peak.

Statistics

Significant difference in the phase of activity onset between the baseline and shifted LD cycle was evaluated by paired t-test. The difference in the amount of phase-shift and that in free-running period between the control and exposed groups were compared by unpaired t-test. Differences in the first peak of circadian bioluminescence rhythm among the baseline, control and exposed groups were compared by one-way analysis of variance (ANOVA) with the post hoc Bonferroni test. Statistical analyses were performed with StatView 5.0 software (SAS Institute, Cary, NC).

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Figure legends

Fig.1 Experimental protocols of an 8 hour phase-advance and delay of LD cycle.
Open and grey areas in the horizontal columns indicate the light and dark periods, respectively. Dark areas indicate the period of 3 hour exposure. Arrows indicate the time of tissues sampling. Broken vertical lines indicate the onset of dark period in the baseline LD.

Fig.2 Actograms and onset phases of activity rhythm in mice exposed to the phase-advance shift with 2 exposures.
(*A*-*C*) Double-plotted actograms of individual mice in the phase-advance shift with (*B, C*) and without (*A*) 2 exposures to the novel environment. Arrows indicate the day of phase-shift of LD cycle. The dark phase is shaded in the right side of actogram. A solid line in the actograms indicates a regression line fitted to the successive activity onsets under DD.
(*D*) The activity onsets in individual mice of the control (open circles) and exposed (closed circles) groups before (B) and after the phase-shift of LD cycle (S). Open and grey areas indicate the light and dark periods.

Fig.3 Actograms and onset phases of activity rhythm in mice exposed to the phase-shifts with 4 exposures.
(*A*-*D*) Double-plotted actograms of individual mice in the phase-advance (left) and delay (right), with (*C, D*) and without (*A, B*) 4 exposures.
(*E, F)* The activity onsets in individual mice before and after the phase-advance (*E*) and phase-delay (*F*) shifts of LD cycle. See also the legend of Fig.2.

Fig.4 Circadian bioluminescence rhythms in different tissues of the mice in the phase-advance shift.
Representative circadian bioluminescence rhythms in the SCN, liver, lung and skeletal muscle from the mice before (Baseline) and after the phase-advance shifts, with

(Exposed) and without (Control) 4 exposures to the novel environment. The ordinate indicates the intensity of bioluminescence (relative light unit, RLU; $\times 10^3$ counts/min). The abscissa indicates the days in culture. White and Black bars on the abscissa indicate the subjective light and dark periods, and broken lines indicate the time of lights-on where the tissues were sampled. Arrows indicate the time of tissues sampling. Triangles indicate the first circadian peak.

Fig.5 Circadian bioluminescence rhythms in different tissues of the mice in the phase-delay shift.

Representative circadian bioluminescence rhythms in the SCN, liver, lung and skeletal muscle from animals before (Baseline) and after the phase-delay shifts, with (Exposed) and without (Control) 4 exposures to the novel environment. See also the legend of Fig.4.

Fig.6 Circadian peaks in *Per1* expression and activity onsets.

The first circadian peaks in *Per1* expression in the cultured SCN, liver, lung and skeletal muscle are plotted against the baseline and shifted LD cycle. Left and right panels indicate the results in the phase-advance and phase-delay shifts of LD cycle. Filled and open squares indicate the observed circadian peaks in the baseline and expected peaks in full re-entrainment. Open and filled circles indicate the circadian peaks of the control and exposed groups in the shifted LD. The activity onsets in the control and exposed groups are indicated by the open and filled circles, respectively. Open and gray areas indicate the light and dark periods. Values are expressed with the mean ± SD in local time. **P*<0.0167 vs. expected circadian peak; †*P*<0.0167 vs. control (Bonferroni test).

Table 1 Activity onset, phase-shift and free-running period of circadian rhythms in locomotor activity.

Values are expressed with the mean ± SD in local time. **P*<0.05; ***P*<0.01 vs. baseline (paired t-test); †*P*<0.01 vs. control (unpaired t-test); ¶*P*<0.01 advance vs. delay (two-way ANOVA with post hoc t-test).

Phase-shifts		Activity onset (h)	Phase-shifts (h)	Period (h)	
Advance with 2 exposures					
Control (n = 6)	Baseline	17.9 ± 0.3			
	DD 2nd	17.4 ± 1.5	0.5 ± 1.3	23.49 ± 0.11	
Exposed (n = 10)	Baseline	18.0 ± 0.2			
	DD 2nd	14.5 ± 4.3 *	3.5 ± 4.4	23.53 ± 0.14	
Advance with 4 exposures					
Control (n = 8)	Baseline	17.9 ± 0.2			
	DD 2nd	15.2 ± 1.4 **	2.7 ± 1.3	23.49 ± 0.10	
Exposed (n = 8)	Baseline	17.9 ± 0.2			
	DD 2nd	10.2 ± 3.7 **,†	7.8 ± 3.6 †	23.42 ± 0.16	
Delay with 4 exposures					¶
Control (n = 10)	Baseline	17.9 ± 0.2			
	DD 2nd	1.8 ± 0.3 **	-8.0 ± 0.3	23.81 ± 0.08	
Exposed (n = 10)	Baseline	17.9 ± 0.1			
	DD 2nd	1.7 ±0.3 **	-7.8 ± 0.3	23.77 ± 0.04	

Table 2　Circadian peaks of bioluminescence rhythms in the SCN, liver, lung and skeletal muscle.

Values are expressed with the mean ± SD in local time.　Numbers in the parentheses are the amount of phase-shift in hours (+, phase-advance; -, phase-delay).　*P<0.0167 vs. baseline; †P<0.0167 vs. control (Bonferroni test).

Phase-shifts	SCN	Liver	Lung	Skeletal muscle
Baseline *(n = 10)*	12.5 ± 0.9	21.1 ± 1.4	17.8 ± 1.3	21.8 ± 1.2
Advance with 4 exposures				
Control (n = 10)	6.2 ± 1.2 *	21.4 ± 2.4	1.6 ± 1.2 *	1.0 ± 1.2 *
	(+6.3)	(-0.3)	(-7.8)	(-3.2)
Exposed (n = 10)	6.2 ± 1.2 *	20.2 ± 3.8	9.7 ± 2.9 * †	14.5 ± 2.3 * †
	(+6.3)	(+0.9)	(+8.1)	(+7.3)
Delay with 4 exposures				
Control (n = 10)	19.4 ± 1.0 *	6.7 ± 3.4 *	1.6 ± 0.9 *	2.4 ± 1.5 *
	(-6.9)	(-9.6)	(-7.9)	(-4.6)
Exposed (n = 10)	19.5 ± 0.9 *	4.2 ± 1.6 *	1.3 ± 0.9 *	3.5 ± 1.9 *
	(-7.0)	(-7.1)	(-7.5)	(-5.8)

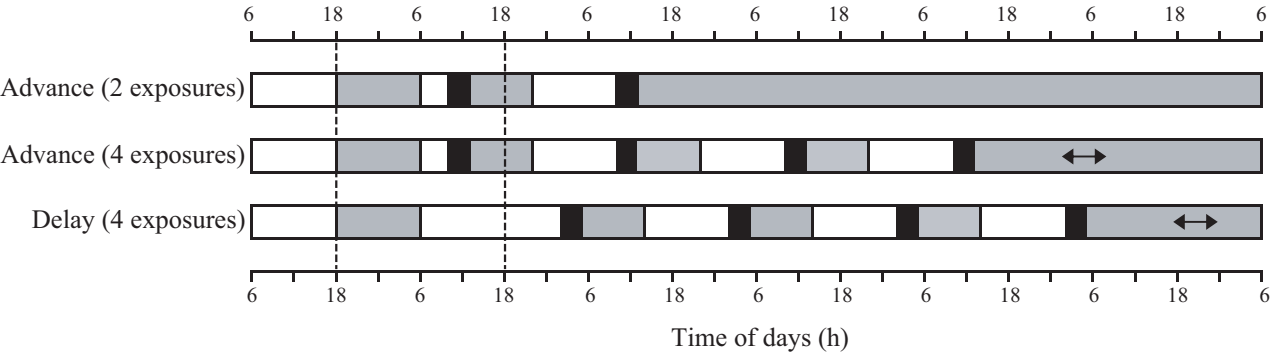
6 18 6 18 6 18 6 18 6 18 6 18 6
Advance (2 exposures)
Advance (4 exposures)
Delay (4 exposures)
6 18 6 18 6 18 6 18 6 18 6 18 6
Time of days (h)

Figure 1

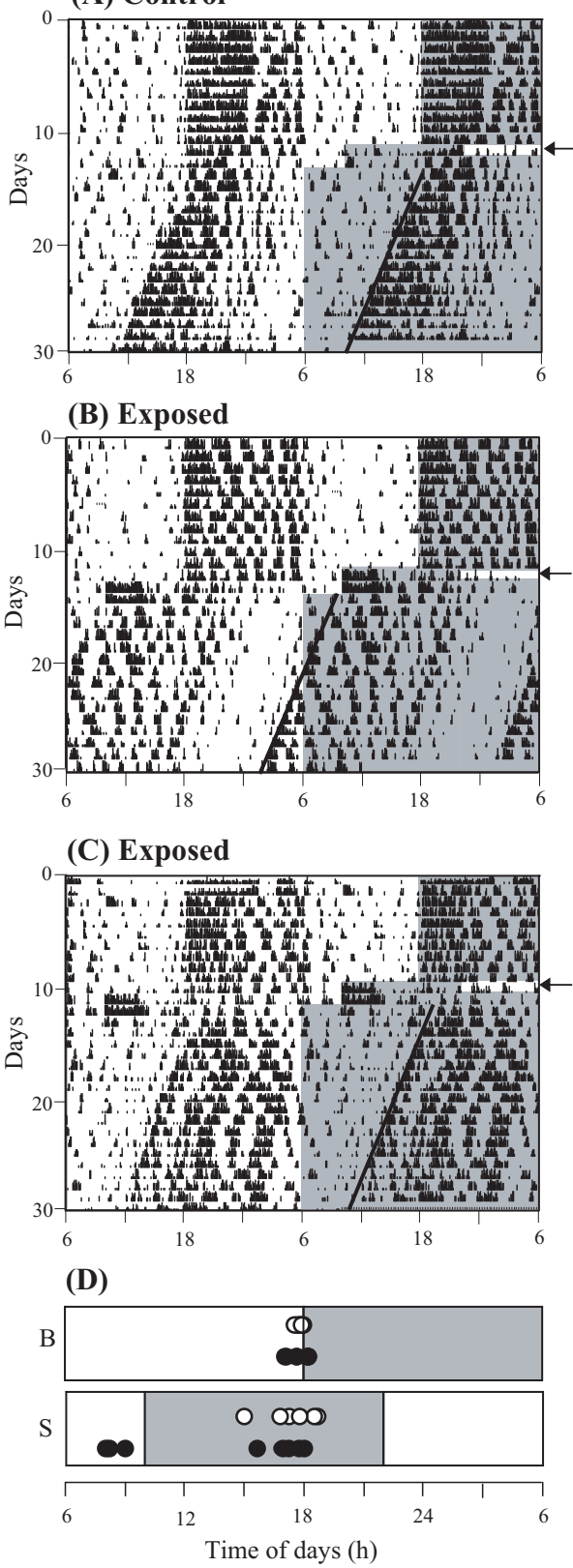
(A) Control
(B) Exposed
(C) Exposed
(D)
Days
0
10
20
30
6
18
6
18
6
B
S
12
24
Time of days (h)

Figure 2

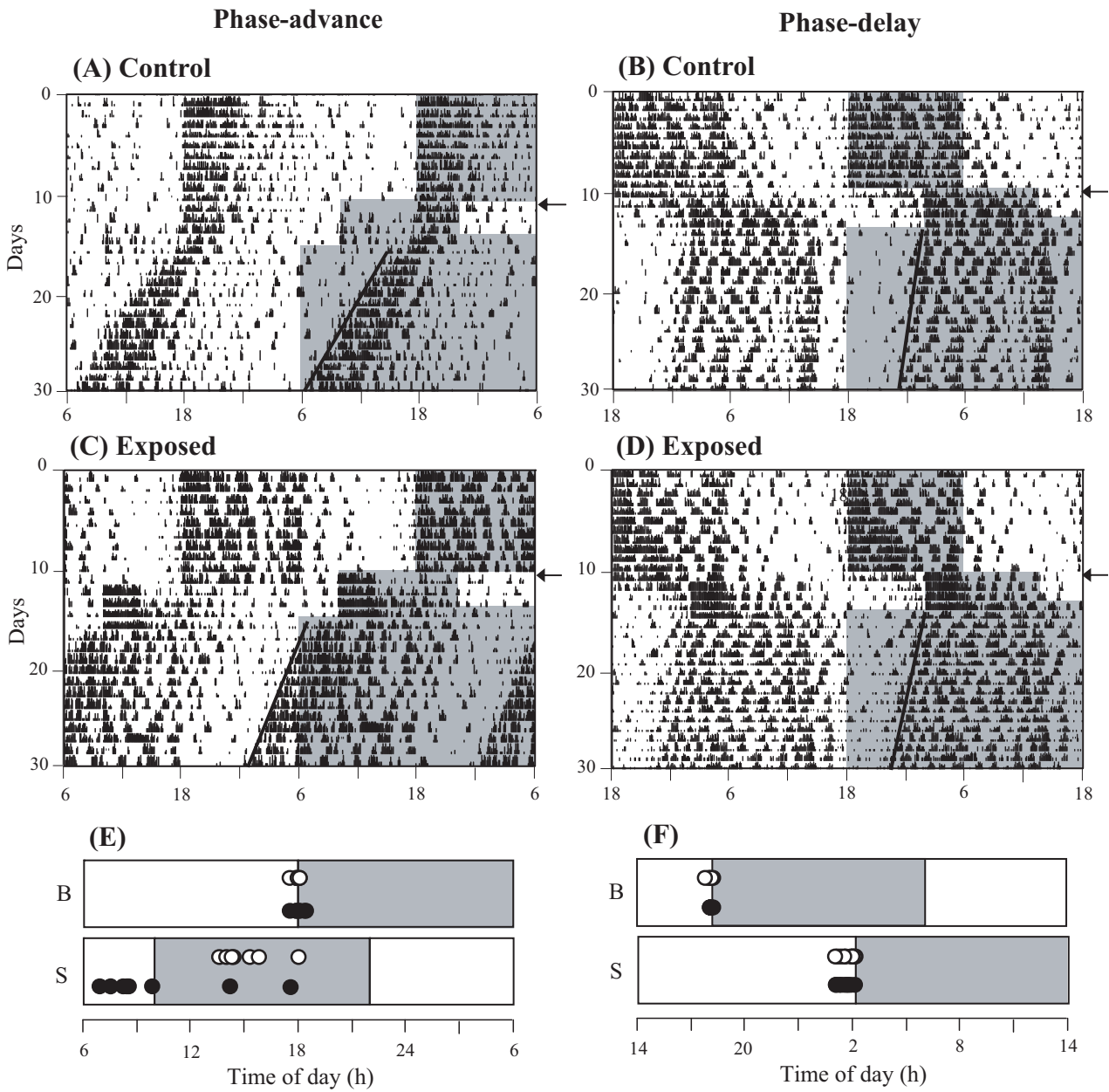
Phase-advance
Phase-delay
(A) Control
(B) Control
(C) Exposed
(D) Exposed
Days
0
10
20
30
6
18
(E)
(F)
B
S
12
24
14
20
2
8
Time of day (h)

Figure 3

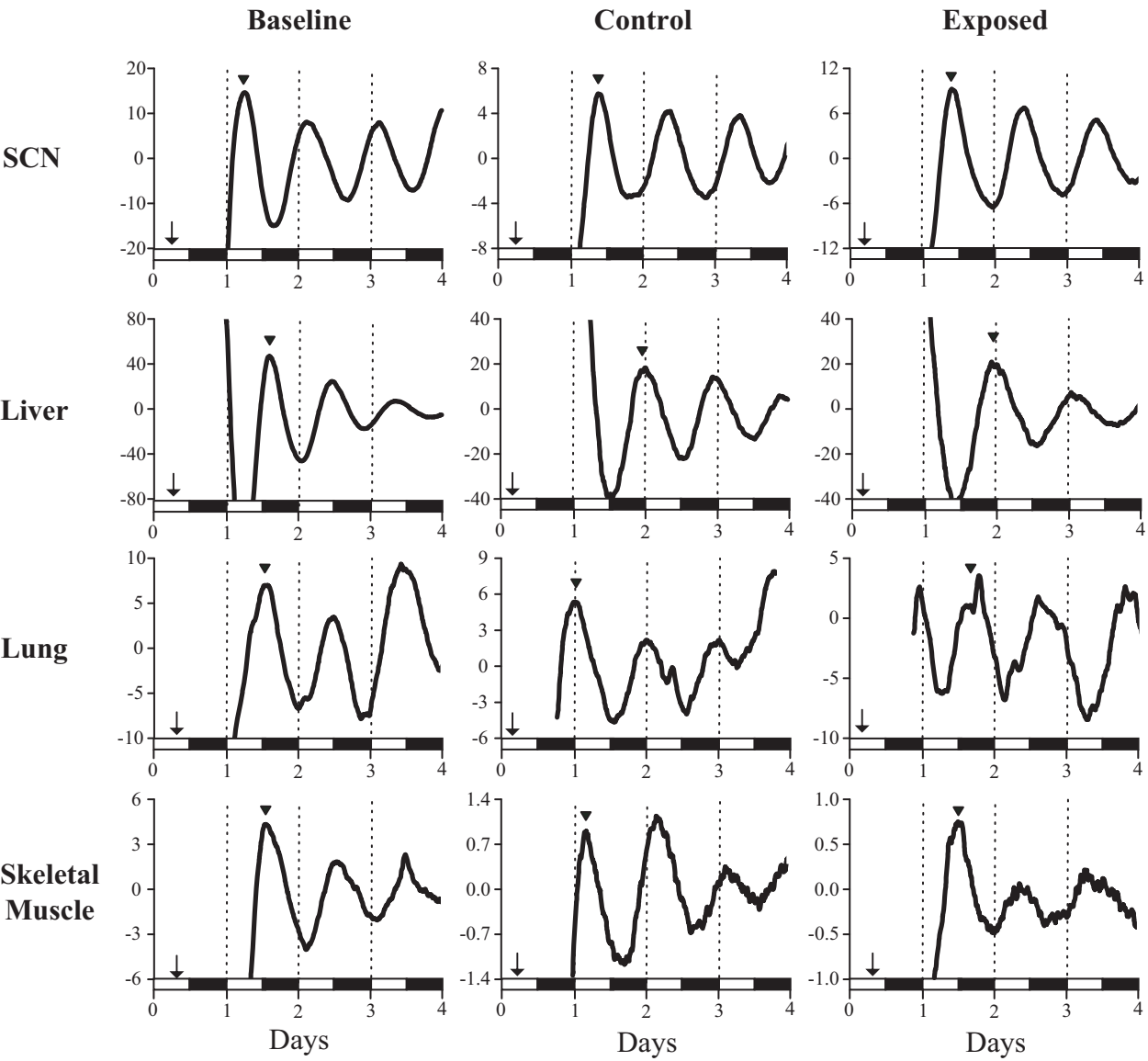
Baseline
Control
Exposed
SCN
Liver
Lung
Skeletal Muscle
Days
Days
Days

Figure 4

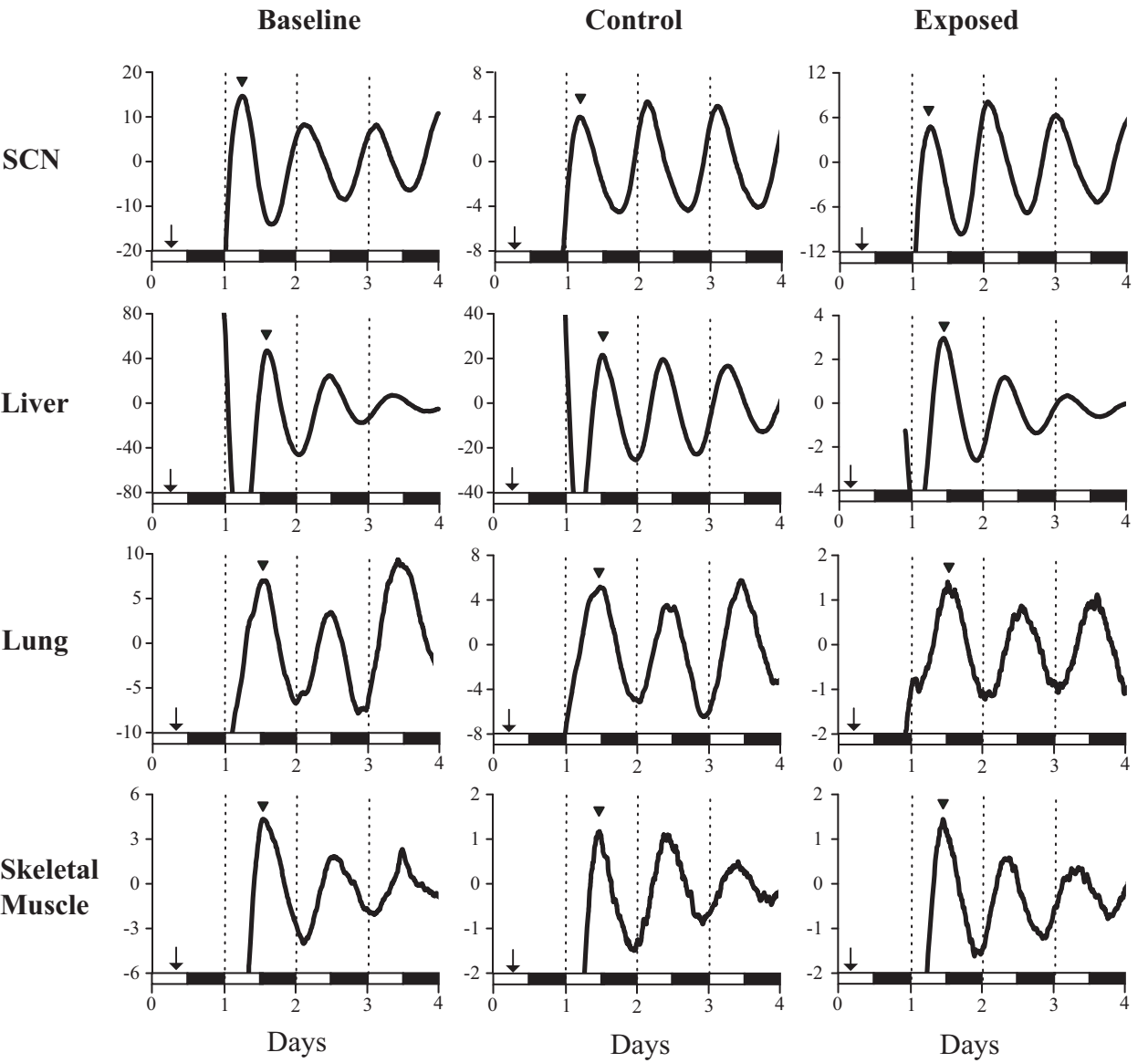
Baseline
Control
Exposed
SCN
Liver
Lung
Skeletal Muscle
Days
Days
Days

Figure 5

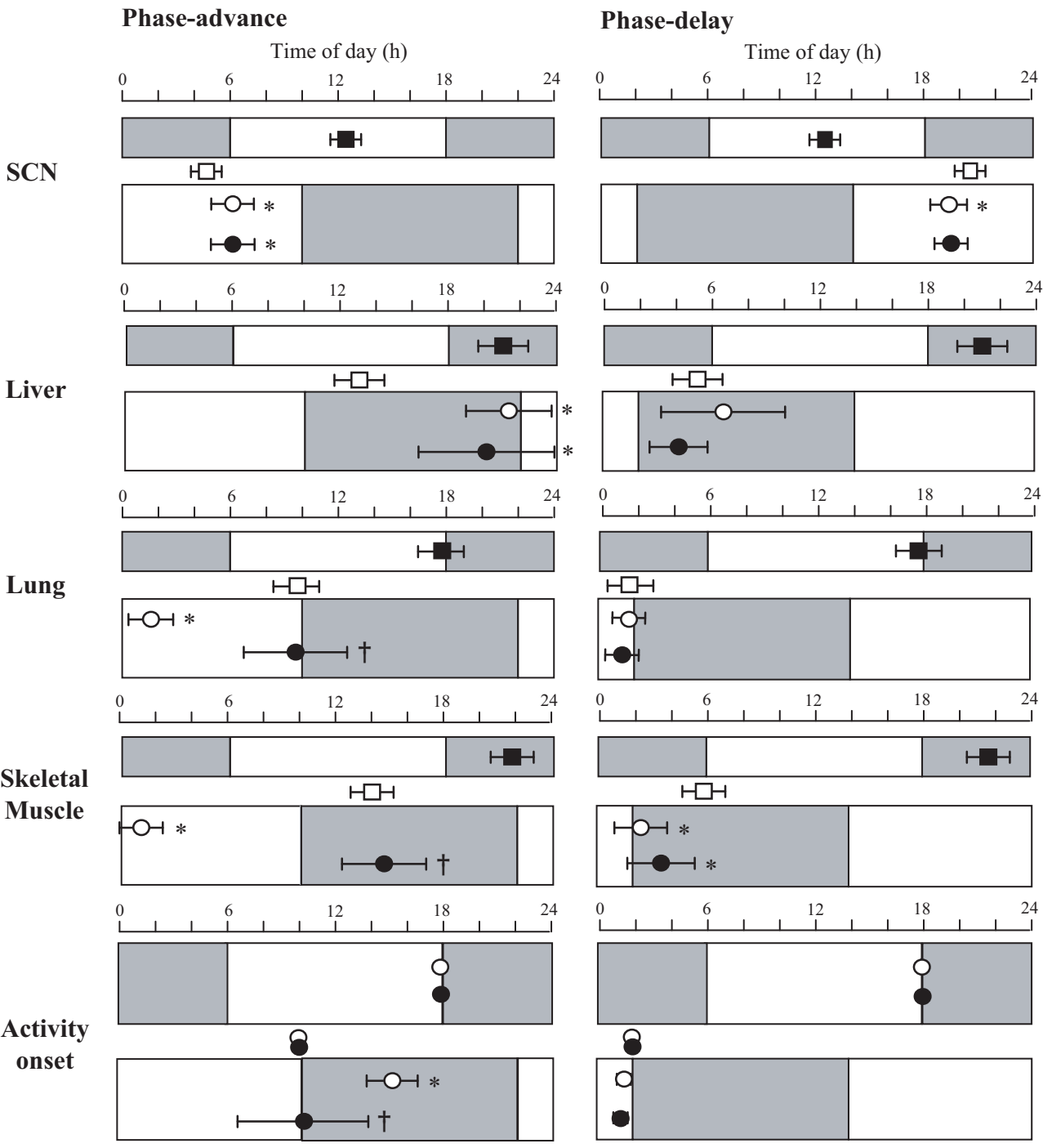
Phase-advance
Phase-delay
Time of day (h)
0
6
12
18
24
SCN
Liver
Lung
Skeletal Muscle
Activity onset
*
†

Figure 6