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ABSTRACT

The flesh fly *Sarcophaga similis* show a clear photoperiodic response; they develop into adults under long days, whereas they arrest their development at the pupal stage under short days. Although the involvement of a circadian clock in photoperiodic time measurement is suggested in this species, the anatomical location of the clock neurons responsible for the time measurement has been unknown. We detected two PERIOD-immunoreactive cell clusters in the larval brain; one cluster was located at the dorsoanterior region and the other at the medial region. We further investigated their temporal changes in PERIOD-immunoreactivity and compared their patterns under different photoperiods.

KEYWORDS

Circadian clock neuron, external coincidence model, diapause, insect, PERIOD, photoperiod, photoperiodism, *Sarcophaga similis*

Introduction

Most insects anticipate forthcoming seasons that might be inappropriate for development and reproduction by measuring night length (or day length). This biological ability to respond to photoperiod is called photoperiodism (Saunders, 2002). For example, the flesh fly *Sarcophaga similis* arrests its development at the pupal stage (pupal diapause) under short-day conditions, whereas it averts diapause and develops without interruption under long-day conditions (Tanaka et al., 2008). Photoperiodism in insects comprises a sequence of several events including a photoperiodic time measurement system, which measures the night or day length (Saunders, 2002). Bünning (1936) first proposed the idea that circadian rhythms regulating daily activities form the basis of the photoperiodic time measurement system. Currently, the involvement of the circadian clock in photoperiodic time measurement is accepted in various organisms (reviewed by Nelson et al., 2010).

Although the molecular machinery of the circadian clock involved in insect photoperiodism has long been unknown, recent studies with RNA interference (RNAi) experiments revealed that circadian clock elements governing circadian activity rhythmicity are also involved in photoperiodism (Goto, 2013; Meuti & Denlinger, 2013). In insects, several elements including *period* (*per*), *timeless* (*tim*), *mammalian-type cryptochrome* (*cry-m*), *cycle* (*cyc*), and *Clock* (*Clk*) compose the negative feedback loops to establish a circadian clock (Tomioka & Matsumoto, 2010). In the bean bug *Riptortus pedestris*, RNAi of *per* disrupts

photoperiodism and induces nondiapause even under diapause-inducing short-day photoperiod (Ikeno et al., 2010). Involvement of not only *per* gene but also other circadian clock genes in insect photoperiodism has been reported in various species (Sakamoto et al., 2009; Ikeno et al., 2010, 2011ab, 2013; Meuti et al., 2015; Mukai and Goto, 2016; Urbanová et al., 2016). These studies suggest that the circadian clock comprised of these circadian clock genes is involved in the photoperiodic time measurement.

One of the most influential models describing how the circadian clock measures night length is the external coincidence model (Vaz Nunes & Saunders, 1999). This model hypothesizes 2 points: (1) involvement of an endogenous circadian oscillator in the photoperiodic time measurement and (2) presence of the photoinducible phase, φi, in the late phase of the subjective night. In this model, light functions as the factor entraining an endogenous circadian oscillator to the photoperiod and as the factor inducing the photoperiodic response at the time of exposure of φi to light, i.e., short-day responses are produced when φi is not exposed to light, whereas long-day responses are produced when φi is exposed to light (Pittendrigh & Minis 1964; Fig. S1). The external coincidence model is sufficiently applicable to the photoperiodic induction of diapause in *S. similis* (Goto & Numata, 2009b; Tagaya et al., 2010); however, the anatomical location of the clock responsible for photoperiodic time measurement is largely unknown in larvae of this species, which are sensitive to photoperiods.

To approach this issue, we examined distribution of PER-immunoreactive

neurons and their temporal expression patterns of the PER protein under long-day and short-day conditions in the brain of *S. similis* larvae, which are sensitive to photoperiods (Goto & Numata, 2009ab). If there are some change in some cells under different photoperiods, it may indicate that the *per*-expressing cells are involved in encoding or measuring night length.

Materials and methods

Insects

A laboratory colony of *S. similis* was maintained under diapause-averting long-day conditions of 16-h light and 8-h darkness (LD 16:8 h) at 20°C. Newly emerged adults were reared under short-day (LD 12:12 h) or long-day conditions at 20°C. Adult flies were provisioned with water, sugar, a lactic acid drink (Ikiiki-nyusankin, Luna Bussan Co., Ltd., Japan), and a piece of beef liver. Female flies larviposited 15 days after the provision of the beef liver. Larvae were continuously reared under the same photoperiodic conditions. Five days after larviposition, the third instar larvae at the wandering stage immediately before pupariation were used for immunohistochemistry. We used fluorescent lights (FLR15W, FLR20SW/M, FLR30SW, or FL40SW; Matsushita Electric Works, 0.8-1.8 $W \cdot m^{-2}$) as a white light source.

Diapause incidence

Diapause incidence was assessed 15-20 days after larviposition according to Fraenkel & Hsiao (1968).

Immunohistochemistry

Anti-PERIOD (anti-PER) immunohistochemistry was performed on the brain of the larvae collected at various times of the day (zeitgeber time [ZT] 0, 4, 6, 8, 12, 16, 18, and 21; ZT0 = light-on) under long- and short-day conditions by the ABC technique (Vectastain ABC standard kit, Vector Laboratories, Burlingame, CA) (n = 16-24 for each sampling point). Sampling during the dark phase was performed under a red light (approximately 30 $W \cdot m^{-2}$, Asahi Hikari Electric Industrial, Tokyo, Japan) within a few minutes for each larva, according to Muguruma et al. (2010). The anterior parts of the larvae were cut off and fixed for 12 h in 4% paraformaldehyde in 0.1 M phosphate buffer at 4°C. After 4 rinses with 0.1 M phosphate-buffered saline containing 0.5% Triton X-100 (PBST), the brains with the thoracicoabdominal ganglion (TAG) were dissected out in 0.1 M phosphate-buffered saline (PBS; pH 7.4). The tissues were incubated in 0.3% H_2O_2 in PBS for 1 h to reduce endogenous peroxidase activity, blocked with 0.5% bovine serum albumin (BSA) in PBST for 1 h, and incubated with a primary antiserum, goat anti-*Drosophila melanogaster* PERIOD (sc-15720; Santa Cruz Biotechnology) diluted at 1:1000 in 0.5% BSA in PBST for 2 days at 4°C. After 4 rinses with PBST, the brains were blocked with 0.5% BSA in PBST for 1 h and incubated with a

secondary antiserum, biotinylated anti-goat IgG at a dilution of 1:400 in the blocking solution, for 1 day at 4°C. After 4 rinses with PBST, the brains were incubated in an avidin-biotin complex solution (biotinylated horseradish peroxidase molecules cross-linked by avidin into a dimensional array, working solutions in Vectastain ABC standard kit) at a dilution of 1:100 for 1 day at 4°C. After 4 rinses with PBST, the tissues were preincubated in 0.03% diaminobenzidine in Tris-HCl (pH 7.4) for 1 h at 4°C and subsequently incubated in 0.03% diaminobenzidine containing 0.01% H_2O_2 for 5-15 min until all brains were stained to similar intensity. After 3 rinses with Tris-HCl, the brains were dehydrated in an ethanol series and cleared in methyl salicylate for observation.

A specificity test was performed using a pre-absorption technique. The anti-PER antiserum at a dilution of 1:1000 was incubated in 200 $\mu g \cdot mL^{-1}$ of PER protein (sc-15720 P; Santa Cruz Biotechnology) overnight at 4°C. Immunohistochemistry was performed on the brains using the antigen-antiserum complex instead of the primary antiserum. No staining was observed in this control experiment (data not shown), confirming the specificity of the antibody to the PER protein.

Results

Diapause incidence

Sarcophaga similis showed a clear photoperiodic response; most flies (91.2%)

entered pupal diapause (n = 487) under short-day conditions, whereas under long-day conditions, all pupae averted diapause (n = 627).

Distribution of PERIOD-immunoreactive cells

Immunohistochemistry identified 2 PER-immunoreactive (PER-ir) cell clusters in the brain. One cluster, consisting of 4 cells, was located at the dorsoanterior region of each hemisphere. Hereafter, these neurons are named as dorsal neurons (DNs) (Fig.1, black arrowheads). The other cluster, consisting of 5 cells, was located at the medial region of each hemisphere. Hereafter, these neurons are named as lateral neurons (LNs) (Fig.1, white arrowheads).

Temporal changes in the number of PER-immunoreactive cells

Temporal changes in the number of PER-ir cells were observed in DNs and LNs under long-day and short-day conditions (Fig. 2; Kruskal-Wallis test, $P < 0.05$). Such temporal changes are considered to be caused by changes in PER abundance. When PER abundance decreases below the threshold for immunohistochemical detection, the cell is not stained, resulting in a decrease in the number of cells observed (Muguruma et al., 2010).

In DNs, the oscillation patterns under long-day and short-day conditions were similar (Fig. 2A1-3). The numbers of PER-ir DNs were the highest during early and mid photophase (light phase); however, they started to decrease at ZT12 and reached a trough at ZT16 under both photoperiods. Subsequently, the

numbers increased gradually. A significant difference in the number of PER-ir DNs was observed at ZT12 between the two photoperiods (Mann-Whitney *U* test, $P < 0.05$), although the difference was very small.

LNs also showed diel oscillations in the number of PER-ir cells under long- and short-day conditions (Fig. 2B1-3). Under short-day conditions, the number of PER-ir cells was the highest during early and mid photophase; however, it started to decrease at ZT8 and reached at a trough phase at ZT12. Subsequently, the number increased immediately and reached to the highest value at the end of scotophase (dark phase). The number of PER-ir LNs under long-day conditions showed a similar temporal pattern to that under short-day conditions. However, the trough was longer in long-day conditions than in short-day conditions; the number of PER-ir cells appeared to be the smallest even at ZT16 under long-day conditions, whereas it appeared to increase at the ZT under short-day conditions. Therefore, it might take longer on short days to move from trough to peak phase than on long days. Statistical difference in the number of PER-ir LNs was observed at ZT16 between the two photoperiods (Mann-Whitney *U* test, $P < 0.01$). Thus, photoperiods slightly but significantly affected the oscillation patterns of LNs, in contrast to DNs.

It is also noteworthy that DNs and LNs showed distinct phase relationships to photoperiods. DNs were in the descending phase of oscillation at ZT12, whereas LNs reached the trough of oscillation, irrespective of the photoperiods. At ZT16 under short-day conditions, DNs were in the trough phase; however, LNs were in

the ascending phase. At ZT 21 under both photoperiods, DNs were in the ascending phase, whereas LNs reached the peak. Thus, even under the same photoperiodic conditions, DNs and LNs behaved independently and showed distinct waveforms.

Discussion

The distribution of PER-ir cells in the brain has been reported in various insects (for example, Závodská et al., 2003). In the fly's larval brain, Kaneko et al. (1997) identified *per*-expressing cells in the larval brain of *D. melanogaster* using *per*-promoted reporter assay. Koštál et al. (2009) identified PER-ir cells in the larval brain of the flesh fly *Sarcophaga crassipalpis* using immunohistochemistry with anti-*Drosophila* PER antibody. Based on the cellular location and their numbers, DNs in *S. similis* might correspond to $DN1^{L}$s and $DN2^{L}$s in *D. melanogaster* (Kaneko et al., 1997). Similarly, LNs in *S. similis* might correspond to LNs in *D. melanogaster*. In the brain of *S. crassipalpis* first instar larvae, 4 types of cell clusters (frontal, fronto-dorsal, lateral, and medial groups) are found (Koštál et al., 2009). DNs in *S. similis* might correspond to the frontal and fronto-dorsal groups in *S. crassipalpis*. However, it is difficult to conclude if the LNs in *S. similis* correspond to the lateral and medial groups of *S. crassipalpis* because the former is located more centrally as compared to the latter. No PER-ir cells are found in the TAG in the third instar larvae of *S. similis*, in contrast to the first instar larvae of *S.*

crassipalpis, which has 12 PER-ir cell clusters in the ganglion (Koštál et al., 2009). We do not know whether such differences are derived from differences in the species or stage or technique.

The adult of the blow fly *Protophormia terraenovae* possesses 4 groups of circadian clock neurons in the brain, i.e., large ventral lateral neurons (l-LN$_v$s), small lateral ventral neurons (s-LN$_v$s), dorsal lateral neurons (LN$_d$s), and medial dorsal neurons (DN$_m$s). Muguruma et al. (2010) investigated the temporal changes in PER-immunoreactivity in these circadian clock neurons under diapause-inducing short-day conditions and diapause-averting long-day conditions. They found that different groups of clock neurons respond to photoperiods in different manners. Using the larval brain of *S. similis,* the present study also verified that DNs and LNs showed different phase relationships of PER-immunoreactivity and that photoperiodic conditions differentially affected the oscillation patterns of the number of PER-ir cells. These results indicate that photoperiodic information is encoded by LNs and DNs in different manners.

In adults of *P. terraenovae*, surgical removal of the brain region containing s-LN$_v$s disrupts not only behavioural rhythms but also photoperiodic induction of diapause, suggesting the significance of s-LN$_v$s, as circadian clock neurons, in photoperiodism. In the present study, LNs, in contrast to DNs, changed their oscillation pattern in response to photoperiods. In LNs, PER accumulation appears to start only after "light-off" in both photoperiods, producing small but significant difference in the number of PER-ir cells at least at ZT16 between

long-day and short-day conditions. Such "phase-set" of PER accumulation is also noted in other dipteran species (Shafer et al., 2004; Muguruma et al., 2010). This photoperiodic sensitivity of LNs implies their significance for encoding or measuring night length, but further studies are obviously needed to draw a conclusion. Analyses of the oscillation patterns of LNs and DNs under shorter and longer photoperiods (Shafer et al., 2004) may provide more insights about their involvement in the time measurement. Although we examined PER immunoreactivity only at 8 time points during a day in the present study, more time points are necessary to determine exact timing. It is also noteworthy that, in *S. similis*, a short (15 min) light pulse of white light and a 2-h light pulse of the short wavelength of light (< 470 nm) during the early scotophase sufficiently cause a phase delay of the circadian oscillator governing the position of φi (Goto & Numata, 2009b). It is of interest to investigate whether such light pulses could change the oscillation pattern of DNs or LNs. These results would clarify whether these clock neurons are indeed responsible for photoperiodic time measurement.

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Declaration of interest

The authors declare no conflicts of interest.

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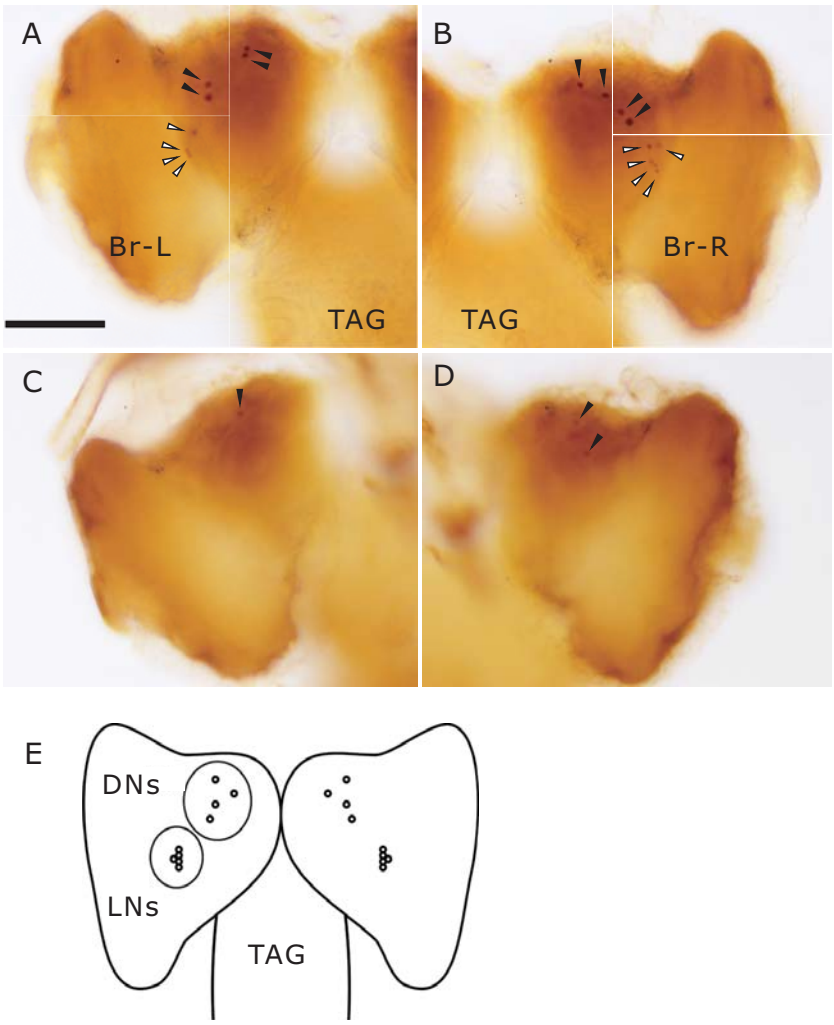
Figure legends

Fig. 1. Photomicrographs of PERIOD (PER)-immunoreactive dorsal neurons (DNs; black arrowheads) and lateral neurons (LNs; white arrowheads) in the left (A & C; Br-L) and right (B & D; Br-R) hemispheres of the brain of the third instar larvae of *Sarcophaga similis* (dorsal view). (A & B) The brains were collected at ZT6 (6 h after light-on) under short-day conditions (LD 12:12 h). In these specimens, 4 DNs in both hemispheres and 4 and 5 LNs in the right and left hemispheres, respectively, are detected. (C & D) The brains were collected at ZT12 (12 h after light-on) under short-day conditions. In these specimens, only a few DNs were detected faintly. Scale bar, 100 μm. (E) A schematic illustration of PER-immunoreactive cells (dorsal view). TAG, thoracicoabdominal ganglion. LD, Light:Dark.

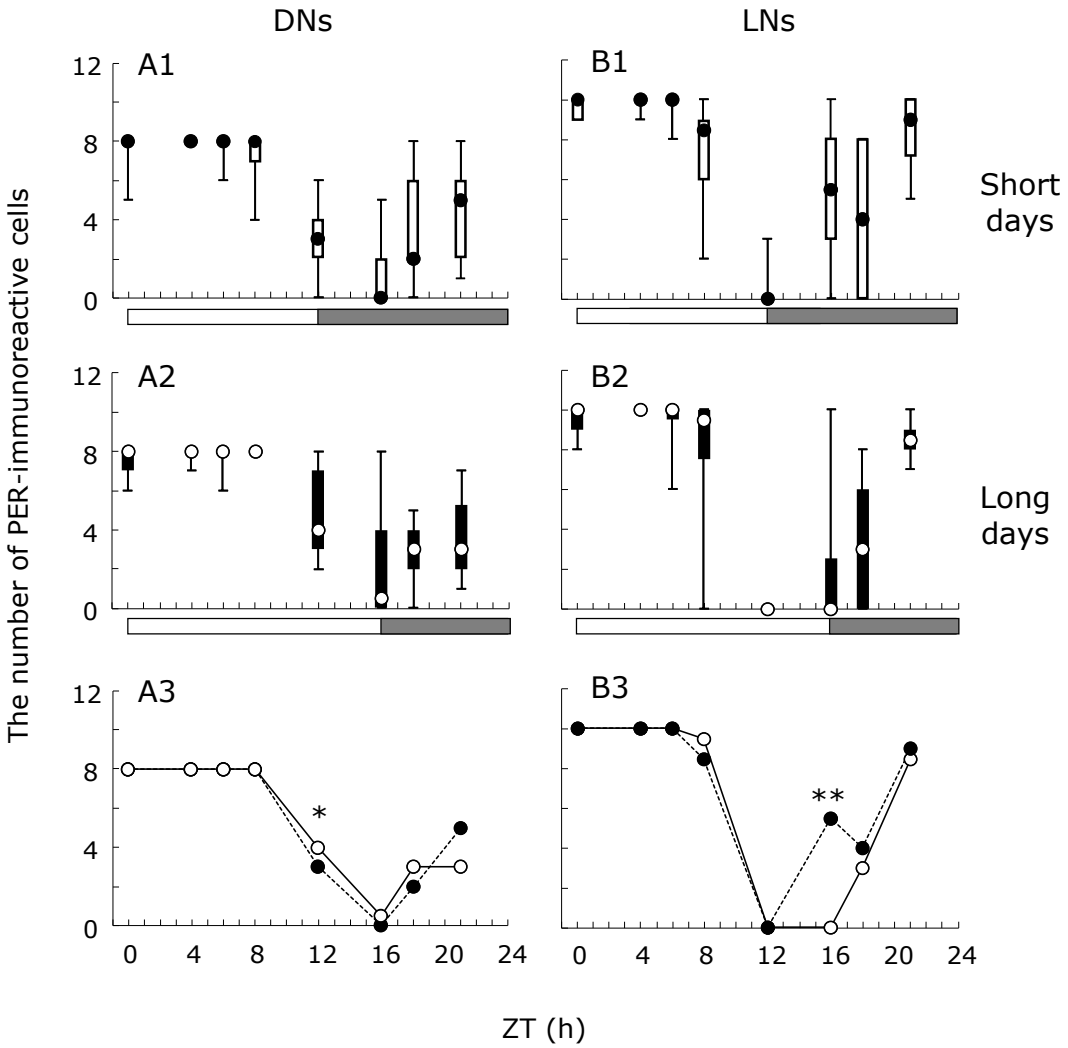
Fig. 2. The number of PERIOD (PER)-immunoreactive cells per brain in dorsal neurons (DNs; A) and lateral neurons (LNs; B) of the third instar larvae of *Sarcophaga similis* reared under short-day (LD 12:12 h; A1 and B1) and long-day (LD 16:8 h; A2 and B2) conditions. The circles indicate medians, the boxes the 25% and 75% quartiles, and the whiskers the maximum and minimum numbers. Horizontal open and closed boxes under X-axes in A1, A2, B1 and B2 indicate photophase and scotophase, respectively. Medians of DNs and those of LNs under long-day (open circles) and short-day (closed circles) conditions are superimposed in A3 and B3, respectively. * $P < 0.05$, ** $P < 0.01$ (Mann-Whitney U test). n = 16-24.

LD, Light:Dark.

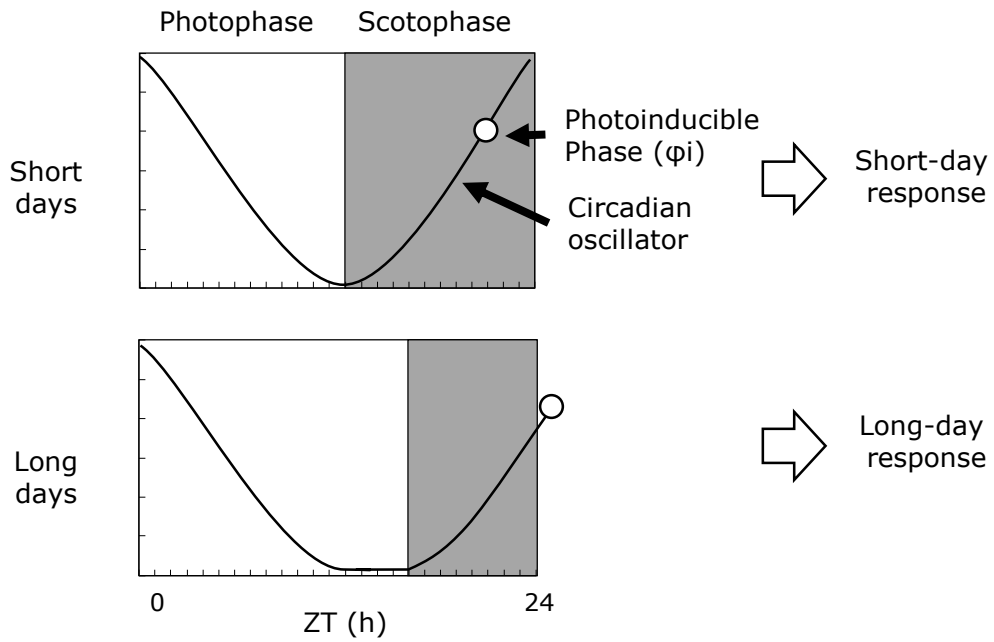
Fig. S1. Conceptual diagrams of the external coincidence model. Time measurement in the external coincidence model is based on a circadian clock, which sets its phase at dusk and defines the position of the photoinducible phase (φi) in the latter half of scotophase. Under short days (upper panel), φi is not exposed to light, eliciting a short-day response. Under long days (lower panel), long photophase causes a phase delay of the circadian oscillator without changing waveform during scotophase, and thus, φi is delayed sufficiently to coincide with the photophase exposed to light, eliciting a long-day response.



Yamamoto et al., Fig. 1



Yamamoto et al. Fig. 2.



Yamamoto et al. Fig. S1.