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When Does Subjective Day Come Under 24-h Light/Dark Cycles?: The Case of Circadian Rhythms of UV-C Resistance and Timing of Cell Division in *Euglena gracilis*

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Abstract: Circadian rhythms are generally believed to help organisms to adapt to the environments that fluctuate daily. To achieve this adaptation, they should entrain to the external cycles and establish a stable phase-relationship between internal and external cycles. However, no consensus has been obtained as to when each internal phase comes under the entrainment to 24 h Light/Dark (LD) cycles with various day-lengths. In the flagellated alga *Euglena gracilis* Z grown photoautotrophically, we report here that subjective (or internal) dawn, noon and dusk in the circadian rhythms of UV-C resistance comes exactly at the external dawn, noon and dusk, respectively, regardless of the day-lengths under 24-h LD cycles; circadian rhythm of the timing of cell division reveals consistent phase behavior. We conclude therefore that the entire subjective day (dawn to dusk) may exactly match the external day. Since, the circadian phototaxis rhythm in this alga behaves differently, we also suggest that the alga possesses at least two circadian clocks per cell that respond differently to external cycles. We moreover discuss about the fact that our conclusion may contradict the widely-held view that circadian clocks stop and stay at subjective dusk just after day-lengths exceed 12 h under 24 h LD cycles.

Key words: Circadian, entrainment, *Euglena*, UV-C, cell division

INTRODUCTION

Circadian rhythms are a biological mechanism that helps organisms to adapt in anticipation to environments that change daily and seasonally in the field (Sweeney, 1987; Edmunds, 1988; Goto and Beneragama, 2009). Freed of the natural 24 h timing cues, they persist with a period close to but not exactly of 24 h or a free-running period usually designated by τ. In the field, however, they entrain to the external cycles such as 24 h light/dark (LD) cycles to establish a stable and consistent phase-relations to and thus adapt to environmental cycles (Sweeney, 1987; Edmunds, 1988; Johnson *et al*., 2003; Roenneberg *et al*., 2003).

Circadian Time (CT) represents an endogenous state and is clearly defined when circadian rhythms persist in free-running conditions such as continuous light (LL) or continuous darkness (DD): for example CT12 or subjective dusk is defined as the endogenous state at which organisms are released from LL to DD and CT00 (subjective dawn) comes in half a free-running period later; each comes repeatedly with an interval of the free-running period. Subjective day is from CT00 to CT12 and subjective night is from CT12 to CT00 (= CT24; modulo 24).

Nevertheless, CT definition is usually difficult when the circadian rhythms entrain to external cycles, as exemplified by the fact that in almost all the literatures, Zeitgeber Time (ZT) or time (hours) in LD (LDT) have been used, instead of CT; Zeitgeber refers to a timing cue such as LD cycles (Johnson *et al*., 2003). In order to understand how well the organisms adapt through circadian rhythms to natural fields, an important question arises as to when the subjective day and night come under such environments. However, relatively little attention has been paid to this issue, particularly in recent years (Johnson *et al*., 2003; Roenneberg *et al*., 2003). Since, ZT at which a phase reference point (ϕ_r) of a rhythm comes, changes systematically with different day-lengths, the question is also related to a seasonal

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adaptation through photoperiodism, although this kind of photoperiodism (Pittendrigh, 1988), or photoperiodic adjustment of ϕ_r, has attracted relatively little attention, either.

On the other hand, much more studies have been carried out regarding another kind of photoperiodism, i.e., photoperiodic induction as seen in flowering and dormancy (Thomas, 2006; Hotta *et al*., 2007; Jackson, 2009). Still, little is known about at which ZT, an important ϕ_r, i.e., photoinductive phase comes in various day-lengths; the prevailing opinion, external coincidence model posits that, a long-day response may be invoked only when light falls at the ϕ_r (Pittendrigh, 1988; Carre, 2001; Kobayashi and Weigel, 2007; Sawa *et al*., 2008).

Empirical data generally reveal that, in day-active animals that usually possess $\tau > 24$ h, ϕ_r such as activity onset comes earlier (with reference to noon, the midpoint of the photoperiod) as photoperiods become longer under 24 h LD cycles, which may enable the animals to become active with dawn in all seasons, whereas, the reverse holds for night-active animals usually having $\tau < 24$ h and thus, the animals can start activities around dusk; dawn and dusk come earlier or later with reference to the noon, as the photoperiods become longer (Aschoff, 1981; Pittendrigh, 1988).

A prevailing, non-parametric entrainment theory (Pittendrigh, 1988; Johnson *et al*., 2003) may partly explain this photoperiodic adjustment of ϕ_r. It assumes that circadian clocks run at the same tempo both under the entrainment to 24 h LD cycles and free-running conditions (LL or DD), although the latter speed depends on the intensity of light and the presence or absence of light (Aschoff, 1979; Moore-Ede *et al*., 1982). It moreover, requires an instantaneous (or abrupt) phase-advance or delay in each cycle shortly before the subjective dawn (or after the subjective dusk) for the clocks with τ longer (or shorter) than 24 h, respectively, in order for the clock to cycle with the period of exactly 24 h; light signals around the subjective dawn (or dusk) generally induce advancing (or delaying) phase-shifts. The theory therefore, also requires the same magnitudes of phase-shifts for the entrainment, regardless of the day-lengths. Interestingly, stronger light signals (e.g., longer day-lengths) fallen at advanced phases in the subjective late-night (closer to and before the subjective dawn) cause the same magnitude of phase-advance as weaker signals; this means that ϕ_r of the clock with $\tau>24$ h advances in CT with increased day-lengths, as far as the entrainment is achieved; the similar discussion may explain the photoperiodic adjustment of ϕ_r as achieved in night-active animals.

Attractive though it may be, it is to be noted that it is based on several assumptions as described above. Particularly germane to the present study is the assumption of a constant progression of CT in each cycle except for the occasion of instantaneous phase-shift around subjective dawn or dusk.

The present study investigated photoperiodic adjustment of ϕ_r's of the flagellated alga *Euglena gracilis* (a unicellular eukaryote), in which many circadian rhythms have been documented and well characterized in its biochemical and physiological variables (Edmunds, 1988, 2005; Goto and Beneragama, 2009). Nevertheless, little is known about the photoperiodic adjustment of ϕ_r except for the circadian rhythm of phototaxis, whose ϕ_r (the onset of its increasing phase) are re-plotted in Fig. 1; it takes almost parallel to, but often far away from, the external dusk (Bruce, 1960). The dependence on the day-lengths of the phase-relationship between the phototactic ϕ_r and the 24 h LD cycles may cast doubt about an adaptational role of the phototaxis rhythm. Does it hold for the other circadian rhythms in this alga?

Therefore, the present study was undertaken to find out when each internal phase of *Euglena* comes under the

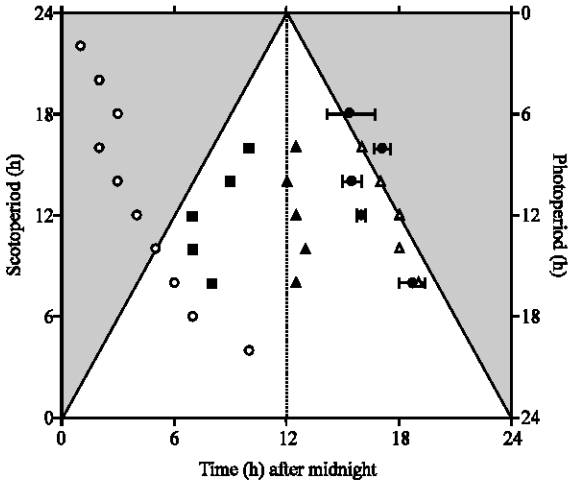

Fig. 1: Phase-relationships between circadian rhythms and 24 h LD cycles. From the top to down, the day-lengths become longer. The abscissa takes origin at the midnight. Shaded areas represent dark periods. Five distinct ϕ_r' s in *Euglena gracilis* are plotted: onset of increasing phototaxis (open circles), the data adapted from Bruce (1960), onset of cell population growth (closed circles), onset of declining phase (closed squares), trough (closed triangles) and peak (open triangles) of circadian UV-C resistance rhythm

entrainment to 24-h light/dark (LD) cycles with various day-lengths, in order to answer how well the algal circadian clock(s) may be helpful for the adaptation in anticipation to daily changes in environments. For this purpose, we examined photoperiodic adjustment of ϕ_r's of two different circadian rhythms in UV-C resistance (Bolige *et al*., 2006) and timing of cell division (Bolige *et al*., 2005). See Goto and Beneragama (2009) for a recent review for the physiological functions of various circadian rhythms in general and in *Euglena gracilis*.

MATERIALS AND METHODS

Euglena gracilis Z was originally donated by Dr. L. N. Edmunds, Jr. (SUNY, Stony Brook, NY, USA) in 1986. Until the present day, it has been stocked in photoautotrophic growth mode in media described in Hagiwara *et al*. (2002) at 25°C or room temperature. Laboratory relocation in the Spring of 2007 resulted in stock contamination. Repeated washing by filtration and centrifugation followed by plating ~100 cells of the final wash on 1.5% agar containing the medium in Petri dishes (Φ = 90 mm) led us to isolate a pure colony, which was used in this study. Since, the Z strain thus, obtained behaved differently from our previous strain in some aspects, we coined the previous and current Z's as $Z_{Obihiro1}$ and $Z_{Obihiro2}$, respectively; this statement is necessary only for comparing the papers from this laboratory. The experiments in this study were carried out from January, 2008 to August, 2009.

The algae were cultured axenically at 25°C and photoautotrophically under LL with cool-white fluorescent lamps at 84 μmol m^{-2} sec^{-1} (6 klx) according to Hagiwara *et al*. (2002) with the lamp irradiance spectrum as detailed in Bolige and Goto (2007). Approximately 8 mL of *Euglena* culture were withdrawn automatically every 2 h and fixed with 0.5 mL of 20% neutral formalin containing 20% NaCl. The cell number was counted with an electronic particle counter (Coulter Electronics, Inc., Hileah, FL, USA). When the cell titer reached 2-3×10^3 cells mL^{-1}, the algae were transferred to 24 h LD cycles with various day-lengths of 6-16 h; LD: x,y represents LD cycles with x-h photoperiod and y-h scotoperiod. This protocol allowed us to examine the circadian rhythm of the timing of cell population growth.

The same cultures (the cell titer of 5-7×10^4 in exponential and rhythmical growth mode) were also used for evaluating the circadian rhythms of UV-C resistance under 24 h LD cycles. For free-running rhythm in DD, cultures exponentially and arhythmically growing under LL was released into DD at the similar cell titer. Cell suspensions (5 mL) were withdrawn every 2 h and placed in a Petri dish (Φ = 37 mm), which was then placed on a turntable automatically rotating at 15 rpm and was exposed to UV-C from above. The procedure followed was essentially the same as in Bolige *et al*. (2006) except for higher UV doses. UV-C radiation from a germicidal lamp (peak = 254 nm; GL-15, Panasonic, Tokyo, Japan) had an intensity of 2.2 W m^{-2} to administer a dose of 1.1 kJ m^{-2} (See Bolige *et al*. (2006) for UV lamp irradiance spectra). The UV-C dose was administered at the median lethal dose (LD_{50}) as previously determined (unpublished data). Immediately after exposure to UV-C irradiation, viable cells were counted after staining with 0.03% Neutral Red. When the irradiation was near LD_{50}, the evaluation of cell viability was not as easy as in Bolige *et al*. (2006). Cells with both cytoplasm and chloroplasts stained red and brown-red, respectively, were considered dead. However, cells having a clear, unstained cytoplasm and chloroplasts with few or no red particles were considered alive.

RESULTS

Cultured photoautotrophically, the alga revealed a circadian rhythm of the timing of cell population growth. Bolige *et al*. (2005) found that the timing is determined by circadian gating of G2-to-M transition; once mitosis started, cytokinesis ends ~4 h later. Under the various day-lengths, the onset of the cell population growth occurred parallel to and around the external dusk: hours 6.4±1.3 (SEM; n = 5 cycles) under LD: 6, 18; hours 9.1±0.4 (n = 7 cycles) under LD: 8, 16; hours 8.5±0.5 (n = 17 cycles) under LD: 10, 14; hours 10.0±0.2 (n = 54 cycles) under LD: 12, 12 and hours 14.7±0.7 (n = 3 cycles) under LD: 16, 8, as schematically shown in Fig. 1 (closed circles). Thus, the alga behaved like night-active animals in which subjective dusk may come at around the external dusk independently of the day-lengths, although the rhythm revealed τ>24 h in various free-running conditions (Edmunds, 1988; Bolige *et al*, 2005; our data not shown) as in day-active animals (Aschoff, 1979; Moore-Ede *et al*., 1982). We could not examine when the subjective dawn and midday came in this rhythm.

On the other hand, the circadian rhythm of UV-C resistance manifested its entire waveform and we could use any of its peak, trough, mid-levels from peak to trough or from trough to peak as ϕ_r. As shown in Fig. 2, it not only free-ran in DD where cell cycle progression was ceased but also entrained to 24 h LD cycles with various day-lengths. With increasing day-lengths, the waveform widened. The trough and peak looked reliable ϕ_r's of this rhythm and came at around noon and dusk, respectively. We took another ϕ_r, the phase just about to decline in the UV-C resistance, although it looked less reliable. These

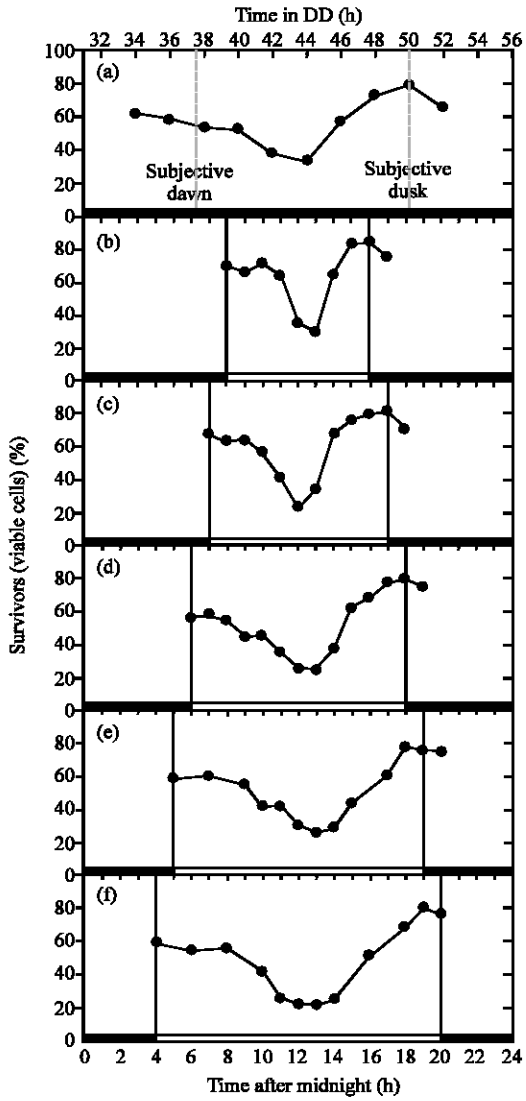

Fig. 2: Circadian UV-C resistance rhythm under DD or 24-h LD cycles. (a) DD, (b) LD: 8, 16, (c) LD: 10, 14, (d) LD: 12, 12, (e) LD: 14, 10 and (f) LD: 16, 8. Since, the UV-C resistance rhythm in DD revealed a period of a little longer than 24 h (closer to 25 h than 24 h) (data not shown) and since LL to DD is defined to be reset at CT12 (subjective dusk), we plotted the top panel as CT00 and CT12 come at hours ~37.5 and ~50, respectively (vertical dashed lines)

relations were plotted also in Fig. 1 to get a clearer view. It was obvious that the trough and peak were confined at around the noon and dusk, respectively, independently of the day-lengths of 8-16 h, whereas, the same was seen for the onset of declining resistance under the day-lengths of 8-14 h but not of 16 h.

DISCUSSION

We found in *E. gracilis* that the ϕ_r of the circadian UV-C resistance took a photoperiodic adjustment, such that the entire subjective day may almost exactly match the external day of 8 to 14 and perhaps 16 h under 24 h LD cycles; the ϕ_r of the circadian timing of cell division behaved consistently. To our best knowledge, a similar finding has scarcely been documented in other organisms, perhaps because, while it requires an entire waveform of circadian rhythms, those rhythms have hardly been subjected to the study on photoperiodic ϕ_r adjustment (Aschoff, 1981).

One might suppose that the photoperiodic ϕ_r adjustment we currently observed reflects a masking effect (Moore-Ede *et al*., 1982; Mrosovsky, 1999; Johnson *et al*., 2003), i.e., a direct and passive effect of light on the observable. However, we could not suppose but that, if it doesn't involve clock-mechanisms, there should be a mechanism for evaluating a midpoint of each photoperiod of various lengths with a constant light intensity for the timing of the minimum UV-C resistance. Moreover, the timing of the maximum resistance should require a mechanism for evaluating a few hours before dawn (for the day-lengths of 14-16 h in Fig. 1, 2); the timing of the onset of the cell population growth (and thus that of G2-to-M transition ~4 h earlier) should also require the similar mechanism (for the day-lengths of 10-16 h in Fig. 1). Not only these mechanisms have not been reported so far, but also they are not likely to occur; in any case, it should involve a time-measurement mechanism. We conclude therefore, the photoperiodic ϕ_r adjustment found in this study reflects the 24 h entrainment of the circadian rhythms.

It is to be noted that the UV-C resistance rhythms under 24 h LD cycles (Fig. 2) were obtained from cultures in exponential growth mode and it is therefore, possible that they may have reflected cell-cycle-dependent UV-C resistance. However, they were very similar to the free-running rhythm in DD (top panel of Fig. 2) where the cell-cycle did not progress, in all the aspects, waveform, phasing and amplitudes, strongly suggesting that UV-C resistance does not depend on cell-cycle phases in *E. gracilis* as in yeast and mammals (Bolige *et al*., 2006). Interestingly, mean levels of the UV-C resistance were also invariable whether, the alga was starved in DD or supplied with a rich source of light energy for photosynthesis in 24 h LD cycles; a functional role of the constant mean level is discussed in Goto and Beneragama (2009).

Functionally, the photoperiodic ϕ_r adjustment that shows the independence on the day-lengths of the

phase-relationship between ϕ_r and the 24 h LD cycles supports the widely-held view that the circadian rhythms are a biological mechanism for environmental adaptation (Sweeney, 1987; Edmunds, 1988; Goto and Beneragama, 2009) because unless otherwise the one-to-one matching would not be required. Nevertheless, it remains in the future studies why the minimum and the maximum occurred at around noon and dusk, respectively, because these phase-relationships may contradict the resistance to light theory proposed by Bolige *et al.* (2006).

The photoperiodic ϕ_r adjustment of the circadian UV-C resistance rhythm showed more than a complex of that of the activity onset rhythms of day- and night-active animals. Thus, with increasing day-lengths, the onset of the decrease in UV-C resistance advanced (relative to the noon) to always come at around the dawn as the activity onset of day-active animals (Pittendrigh, 1988, 1993), whereas, both the maximum UV-C resistance and the onset of cell population growth delayed to come at around the dusk as that of night-active animals. Still, the minimum UV-C resistance always came around the noon. Careful reading of the activity offset records of these animals might reveal photoperiodic ϕ_r adjustment similar to the UV-C resistance rhythm.

On the other hand, this behavior was remarkably different from the photoperiodic adjustment of ϕ_r (the onset of increasing phase of phototaxis rhythm) reported half a century ago (Bruce, 1960). The latter seems determined almost solely by light-off (or dusk) signal (Fig. 1). In this respect, the behavior resembles that of the activity onset of night-active animals; note that the algal phototaxis rhythm reveals τ>24 h as typical in day-active animals, whereas, the night-active animals reveal the contrary. Moreover, the phototactic ϕ_r is not confined around dusk as the activity onset rhythms of the night-active animals.

The day-length-dependent variability of the relationship between the phototactic ϕ_r and 24 h LD cycles suggests that the phototaxis rhythm may change its adaptational role, if any, depending on the seasons; ϕ_r occurs in the dark (or light) under shorter (or longer) photoperiods (Fig. 1). Alternatively, the phototaxis rhythm may serve as an environmental adaptation under limited day-lengths; it looks ineffective for the adaptation in the day-lengths shorter than 10-12 h (Fig. 1). This unusual behavior might be related to the fact that the preferred level of irradiance at which the alga tends to orient is unexpectedly low (4-20 μmol m^{-2} sec^{-1}), compared with that supporting maximal photosynthesis and growth (140 μmol m^{-2} sec^{-1}) (Clegg *et al.*, 2003).

In either case, the different photoperiodic ϕ_r adjustments between the circadian UV-C resistance and phototaxis rhythms may lead to another conclusion that both rhythms are controlled by two distinct circadian clocks that respond differently to 24-h LD cycles. However, the possibility remains that the difference may have resulted from the differences in culture conditions and/or the strains between Bruce (1960) and us. The dinoflagellate *Gonyaulax polyedra* (now renamed *Lingulodinium polyedrum*) is known to possess at least two clocks per cell (Roenneberg and Morse, 1993) which has been so far the only case of the existence of (more than) two circadian clocks per cell.

Mechanistically, the photoperiodic ϕ_r adjustment of the circadian UV-C resistance rhythm may contradict some of the basic assumptions of the prevailing, non-parametric entrainment theory (Pittendrigh, 1988, 1993; Johnson *et al.*, 2003; Roenneberg *et al.*, 2003). First, the fact that the entire subjective day of the UV-C resistance rhythm may exactly match the external day suggests that the progression of the entire subjective day may take the same tempo as that of external day. In other words, that progression may run at a constant tempo under the entrainment to 24 h LD cycles with a definite day-length, but the constant tempo may be lower at longer day-lengths. By contrast, the non-parametric theory is based on the assumption that the entire cycle, both the subjective day and night, may progress at a constant pace, whether, the circadian clock entrains or free-runs; thus, the day-length doesn't matter for the clock speed at all (Pittendrigh, 1988, 1993; Johnson *et al.*, 2003).

Secondly, the non-parametric entrainment theory predicts that photoperiodic ϕ_r adjustment depends on τ as described in introduction; with increasing day-lengths, activity onset of day-active animals (τ>24 h) tracks the advancement of dawn, whereas that of night-active animals (τ<24 h) does so the delay of dusk (Pittendrigh, 1988, 1993; Johnson *et al.*, 2003). Thus, the algal circadian UV-C resistance rhythm should behave like the activity onset of day-active animals, because it reveals τ>24 h in DD (Bolige *et al.*, 2006). However, it behaved like more than a complex of day- and night-active animals as discussed above.

Thirdly, the non-parametric entrainment theory assumes that circadian clocks may stop and stay at CT12 when the day-lengths last longer than 12 h (Pittendrigh, 1988,1993; Johnson *et al.*, 2003); progression through CT00 to CT12 takes 12 h and CT12 continues thereafter, as long as the day lasts. Instead, our result suggests that the progression through CT00 to CT12 may exactly cope with the external day progression; its speed

may become slower with increasing day-lengths. Note that the end of the photoperiod is CT12 in either case.

Taken together, our current study may contradict the non-parametric entrainment theory (Pittendrigh, 1988, 1993; Johnson *et al*., 2003) and may necessitate parametric effect of light on the entrainment. This does not necessarily mean that non-parametric effect may have nothing to do with the entrainment to 24-h LD cycles; it may play an important role in ensuring the exact 24-h period through both dawn and dusk signals. Finally, our discussion should hold for the 8- to16-h day-lengths with the constant irradiance of 84 μmol m^{-2} sec^{-1} (6 klx) in which the circadian UV-C resistance rhythm was examined in this study.

CONCLUSION

The ϕ_r of the circadian UV-C resistance and circadian timing of cell division took a photoperiodic adjustment in *E. gracilis* in such a way that the entire subjective day almost exactly matches the external day of 8 to 14 and perhaps 16 h under 24 h LD cycles. This one-to-one matching supports the widely-held view that the circadian rhythms are a biological mechanism for environmental adaptation. However, our findings contradict the non-parametric entrainment theory (Pittendrigh, 1988, 1993; Johnson *et al*., 2003). It would be interesting to see in future studies what happens to photoperiodic ϕ_r adjustment of the UV-C resistance rhythm under other entraining cycles including natural fields, as well as that of other circadian rhythms reported in *E. gracilis* (Edmunds, 1988, 2005; Goto and Beneragama, 2009).

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