



Research Article

Intraspecific Plasticity in Circadian Rhythms Within *Euglena gracilis* Strain Z

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Abstract

Little is known about how natural plasticity in overt circadian rhythms help *Euglena gracilis* adapt to environmental stimuli. We used four groups of strain Z. Two groups were from our laboratory, Z_{Obihiro1} and Z_{Obihiro2}; Third group was from the National Institute for Environmental Studies, Japan (Z_{NIES-48}) and the other was from Osaka Prefecture University (Z_{Osaka}). The latter two were grown photoautotrophically as were ours, for two months prior to experiments. Results showed that Z_{Obihiro2} and Z_{Osaka} grew faster than Z_{Obihiro1} and Z_{NIES-48}. Upon transferring from light to darkness, population growth ceased within 8-10 h with the cell number increase in the dark of 41% in Z_{Obihiro1} and Z_{Obihiro2}, 35% in Z_{Osaka} and remarkably low 22% in Z_{NIES-48}. Magnitudes of the circadian rhythm of cell population growth in 24 h light-dark cycles were different: both Z_{Obihiro1} and Z_{Obihiro2} completely doubled, but Z_{NIES-48} multiplied by 1.9, and Z_{Osaka} multiplied feebly by 1.7. The photoinduction of commitment to cell division in DD followed a circadian rhythm. All four showed the same peak at subjective dusk, but the amplitudes differed in the order, Z_{Obihiro2} > Z_{Osaka} > Z_{Obihiro1} >> Z_{NIES-48}. The resistance to photosensitization against Rose-Bengal follows a clear circadian rhythm in all substrains except in Z_{NIES-48}. Z_{Obihiro1} and Z_{Osaka} showed the phasing similar to UV resistance rhythm, but Z_{Obihiro2} did not. These results suggest the plasticity of circadian rhythms within a species, if not within a strain. Moreover, it is also apparent that different substrains/ecotypes present within the same Z strain.

Keywords: *Euglena gracilis*; Circadian rhythms; Intraspecific Plasticity

Introduction

Circadian rhythms are a ubiquitous occurrence in the natural world. Most of the physiological processes and behavioral functions in many diverse organisms are expressed rhythmically according to the day and night cycles. These circadian rhythms are controlled and maintained by self-sustaining biological oscillators, and provide the organisms with survival advantages by

optimizing the organisms' responses to its environment and enhancing its fitness (Green *et al.*, 2002).

Studies on circadian rhythms in diverse organisms have led to an appreciation of the similarities and differences that exist between the organization of clocks in these organisms. Circadian rhythms, when present, can be quite plastic even among individuals of the same species (Cohen *et al.*, 2009),

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thus allowing the individuals to adapt and anticipate the variable environments. Such plasticity in overt circadian rhythms might be observed because the clock is masked by the influence of external factors, is uncoupled from behavioral outputs, or results from genuine plasticity in the clock machinery (Shemesh *et al.*, 2007).

Photosynthetic unicellular algal flagellate *Euglena gracilis* Z (Klebs) has widely been used in chronobiology for decades (e.g. Edmunds., 1965; Lonergan and Sargent, 1978; Goto and Beneragama, 2010). This strain (Z) is certainly the most studied laboratory strain among all the *Euglenoids* (Zakrys, 1997). Although various circadian rhythms in *E. gracilis* have been elucidated to date, the nature of the cellular machinery responsible for those overt and mostly robust rhythms is yet one of the central puzzles. Apparently none of the studies have dealt with plasticity of circadian rhythms. It might be doubtful as to why a motile organism like *Euglena* requires plasticity in circadian rhythms. On the other hand, those who worked with strain Z, have never paid attention on the existence of substrains within Z.

In the present study, we attempted to show that there could be several substrains or ecotypes within the same Z strain of *E. gracilis*, which show remarkably different plastic responses in various circadian rhythms. We hypothesized that *E. gracilis* Z can evolve (perhaps genetic materials have changed) under the laboratory conditions, thus show plasticity in circadian rhythms. To test this hypothesis, we obtained four Z strain collections that had been stocked completely independent from each other. In the present study, we examined the circadian rhythms related to cell population growth, photo-inductive capacity of commitment to cell division and resistance to oxidative stress in four different Z strain collections.

Material and Methods

Organisms and Culture Conditions

We used four groups of strain Z. Two groups were from our laboratory; Z_{Obihiro1} and Z_{Obihiro2}. Third group was from the National Institute for Environmental Studies, Japan (Z_{NIES-48}) and the other was from Osaka Prefecture University, Japan (Z_{Osaka}). Hereinafter we use the term “substrains” to these four groups for clarity, although we are not yet certain about the taxonomic nomenclature of these.

For all the experiments, the algae were cultured axenically at 25°C and photoautotrophically under LL with cool-white fluorescent lamps (National FL20SS-N18, Tokyo: see Bolige and Goto 2007, for the lamp irradiance spectrum) at 84 $\mu\text{mol m}^{-2} \text{s}^{-1}$ in modified Cramer-Meyer medium according to Bolige *et al.* (2005). Z_{NIES-48} and Z_{Osaka} were maintained under the same conditions for two months prior to experiments.

Cell Population Growth During LL

Cell population growth was monitored by progressively counting the cell number every 2-h starting from two days after inoculation. 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., Hialeah, FL, USA). The time taken to double the cell population (generation time - GT) was estimated by linear regression of the semi-logarithmic plot for the log-linear growth curve in LL using the formula; $GT = [\ln 2 \times t] / [\ln(N/N_0)]$ where the cell number increases from N_0 to N in t hours.

When the cell titer reached $2-3 \times 10^4$ cells/ml, the algae were transferred to DD to arrest the cell-cycle progression. The percent of number of cells that are committed to cell division by the time of transferring to DD was calculated as the ratio between the cell number at the time of DD transferring to the final constant cell number in DD.

Cell Population Growth During LD: 12,12 Cycles

The alga was first grown under 84 $\mu\text{mol m}^{-2} \text{s}^{-1}$ (LL) for two days after inoculation. When the cell titer reached $4-5 \times 10^3$ cells/ml, the cultures were then synchronized by LD:12,12 cycles. Light intensity during the light period was essentially the same as that of LL. Cell number was counted every 2-h as described above. Factorial increase in cell number (step size) during the dark period was calculated as the ratio of number of cells/ml after a division burst to the number of cells/ml just before fission burst.

Circadian Rhythms of Photo Induction in The Commitment of G2-Phase Cells to Cell Division

Exponentially growing cultures in LL (84 $\mu\text{mol m}^{-2} \text{s}^{-1}$) were transferred to DD when the cell titer reached $1-1.5 \times 10^4$ cells/ml. Cell suspensions (7 ml) from cultures that had been transferred to DD were withdrawn every 2 h starting from 16th h in DD and were exposed to a 4-h light pulse of 84 $\mu\text{mol m}^{-2} \text{s}^{-1}$ and placed them back in DD. The number of cells was counted after >12 h and compared with an unirradiated control, and the percent increase in the number of cells caused by the light pulse was expressed as the percent photoinduction.

Circadian Rhythms of Resistance to Photosensitization Against Rose-Bengal (RB)

Experiment protocol was essentially the same as that of UV experiments. Cell suspensions (5 ml) from cultures that had been transferred to DD were withdrawn every 2 h starting from 18th h in DD and exposed to a white light (EFD25ED/22, National, Tokyo) pulse of 640 $\mu\text{mol m}^{-2} \text{s}^{-1}$ for 25 min in the presence of 40 μM RB, and only for Z_{Obihiro1}, a white light pulse of 126 $\mu\text{mol m}^{-2} \text{s}^{-1}$ for 30 min in the presence of 15 μM RB has been used. Viability test was carried out based on RB staining.

Results

Cell Population Growth

All four substrains grew log-linearly under LL (Fig.1). The GT was remarkably different among substrains; *Z_{Obihiro1}* and *Z_{Osaka}* grew with a GT of 15 and 15.6 h respectively, whereas the cell populations of both *Z_{Obihiro1}* and *Z_{NIES-48}* doubled every 16.6 h. Upon transferring from LL to DD, the cells that are already committed to cell division undergo cell-cycle transitions in DD until they are dark-arrested. The percent of number of cells that were committed to cell division in DD was also significantly different among substrains. In this case, both *Z_{Obihiro1}* and *Z_{Obihiro2}* achieved the highest percentage among four, i.e. ca. 41% (Fig. 1a and b) while *Z_{Osaka}* and *Z_{NIES-48}* resulted in 35 and 22 % respectively (Fig. 1c and d).

Entrainment of circadian rhythm underlying the timing of cell division in all four substrains was achieved by the light-dark cycles (LD: 12,12) employed in the present study in such a way that the cell population growth took place only in the dark intervals (Fig. 2). Indeed, the pattern of synchrony was almost identical in all four substrains, the phase reference point (ϕ_r), i.e. the onset of division burst, occurring 2 h before the onset of dark period. The factorial increase in cell number during one cycle (step size) was however differed among substrains; perfect division synchrony (step size =2) was achieved by both *Z_{Obihiro1}* and *Z_{Obihiro2}* whereas *Z_{Osaka}* and *Z_{NIES-48}* multiplied by a factor of 1.7 and 1.9 respectively. The data are from at least 15 cycles of LD: 12,12.

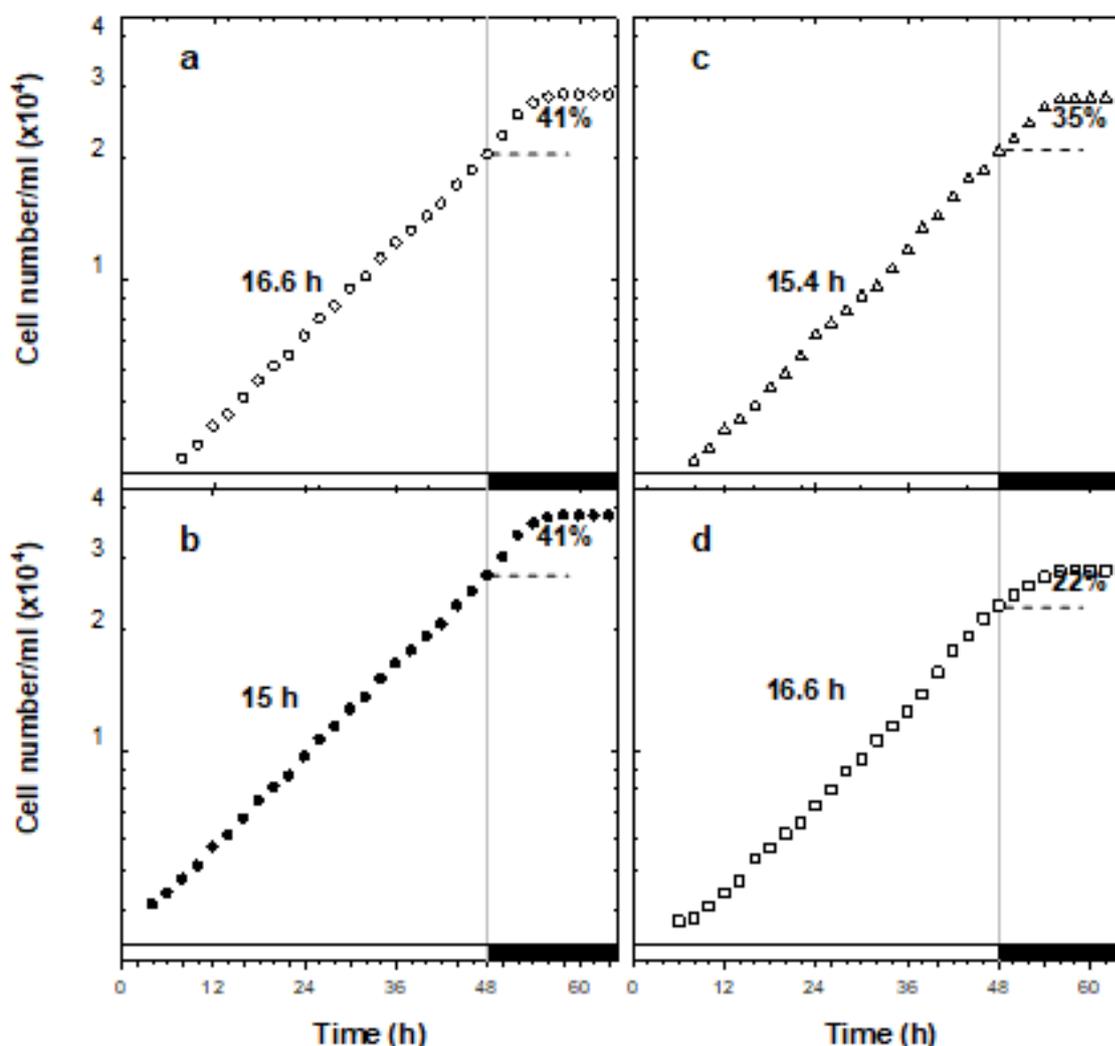


Fig. 1: Cell population growth of four substrains of *Euglena gracilis* Z during the log-linear growth mode at 25°C in continuous light (LL) followed by continuous darkness (DD). Shaded area along the abscissa indicates the darkness. The values denoted in the figure are the average GT (h) and the percent of numbers of cells that were committed to cell division by the time of transferring to DD. a) *Z_{Obihiro1}*, b) *Z_{Obihiro2}*, c) *Z_{Osaka}*, d) *Z_{NIES48}*. The abscissa indicates the time (h) elapsed two days after inoculation.

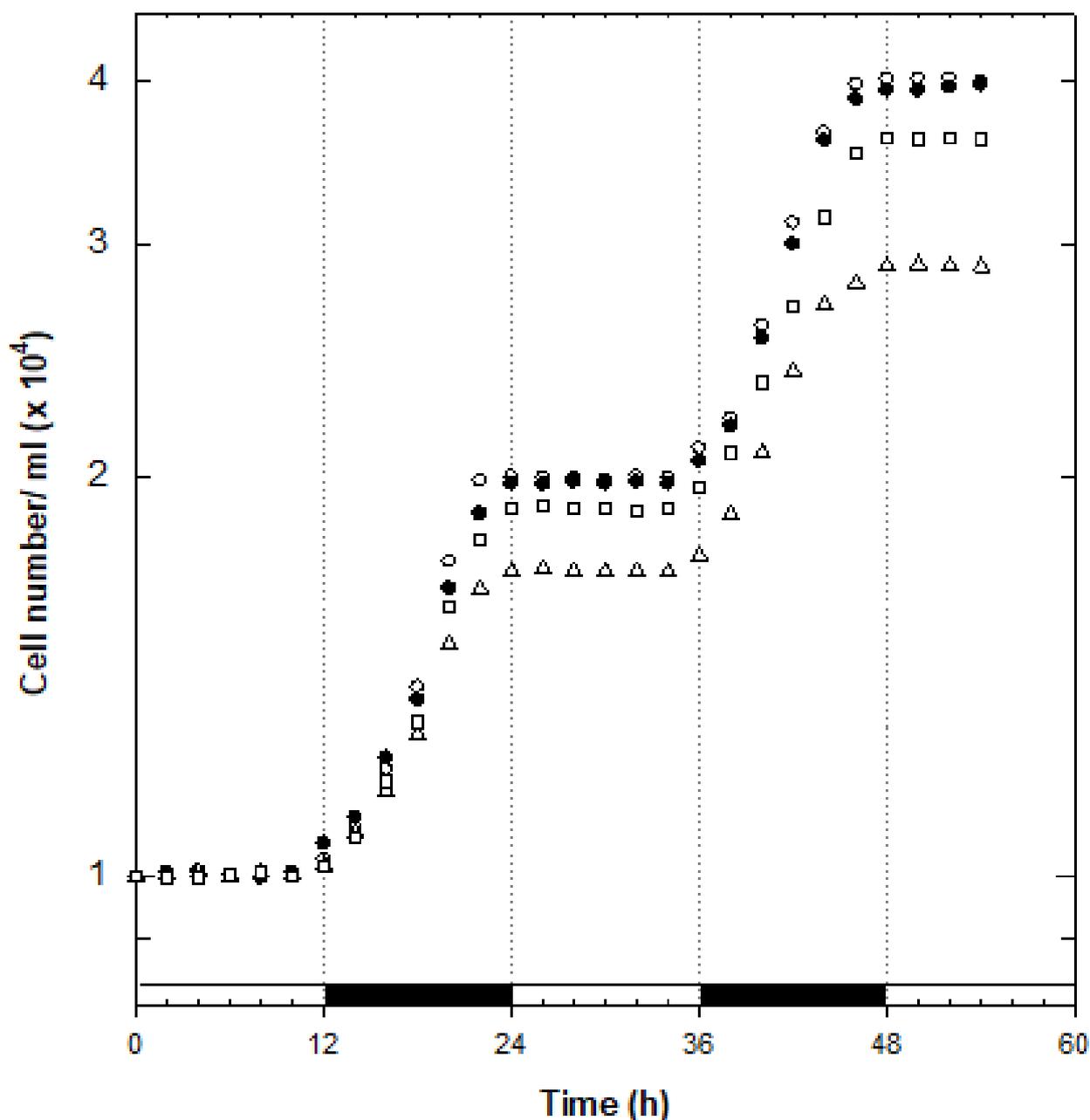


Fig. 2: Entrainment of the circadian rhythm of cell division in four substrains of *Euglena gracilis* Z by LD: 12,12 cycles at 25°C during the log-linear growth mode. Light intensity during the light period was $84 \mu\text{mol m}^{-2} \text{s}^{-1}$. Shaded areas along the abscissa indicate the dark periods. Step sizes (ratio of number of cells/ml after a division burst to the number of cells/ml just before fission) are, $Z_{\text{Obiihiro1}} = \sim 2$ (open circles), $Z_{\text{Obiihiro2}} = \sim 2$ (closed circles), $Z_{\text{Osaka}} = \sim 1.7$ (open triangles) and $Z_{\text{NIES48}} = \sim 1.9$. The period of the rhythm (τ) is essentially the same for all four substrains, ~ 24 h. The abscissa indicates the time (h) elapsed after the end of second dark period. Cell number at the beginning of the third light period in each substrain has been normalized to 1×10^4 cells/ml for easy comparison.

Circadian Rhythm of Photoinduction of Cell Division

As shown in Fig. 3, 4-h light pulse of $84 \mu\text{mol m}^{-2} \text{s}^{-1}$ afforded some of the dark-arrested G2 cells with the capability of progressing through cell division in the subsequent darkness. The maximum dark capability was induced when irradiated at around 26th h (around subjective dusk) in all four substrains, whereas it was not induced at all when pulsed at around 40th h (around subjective dawn). The amplitudes of the peak dark capability differed in the

order, $Z_{\text{Obiihiro2}} > Z_{\text{Osaka}} > Z_{\text{Obiihiro1}} > Z_{\text{NIES-48}}$. A second peak was manifested by the light pulse only in $Z_{\text{Obiihiro2}}$ (around 54th h) and Z_{Osaka} (around 52nd h), indicating the circadian nature of the photoinduction regulation. The period of the rhythm (τ) was approximately 26 h.

Circadian Rhythm of Resistance to Photosensitization in The Presence of Rose-Bengal (RB)

Here we investigated the cellular resistance to oxidative damage specifically by singlet oxygen ($^1\text{O}_2$) and its

circadian rhythmicity, if any. Photoexcitation of Rosebengal (RB) generates mostly 1O_2 hence, RB was used as the photosensitizer. Preliminary studies were carried out to identify the LD₅₀ of the strength of oxidation and it was found that 50% of the cell population was killed in substrains *Z_{Obihiro2}*, *Z_{Osaka}* and *Z_{NIES-48}* when cell suspensions were irradiated with 640 $\mu\text{mol m}^{-2} \text{s}^{-1}$ for 25 min in the presence of 40 μM RB. For *Z_{Obihiro1}*, a light pulse of 126 $\mu\text{mol m}^{-2} \text{s}^{-1}$ for 30 min was used in the presence of 15 μM RB.

As shown in Fig. 4, a clear circadian rhythm of resistance to singlet oxygen damage persisted in DD with a period (τ) of about 24 h in *Z_{Obihiro1}*, *Z_{Obihiro2}* and *Z_{Osaka}*, and with a τ of ca. 18 h in *Z_{NIES-48}*. Once again, the phasing of minima and maxima in all four substrains ran anti-parallel to each other's. Apparently, the waveform of the rhythm was sinusoidal in all four substrains. It was noticeable that, although the phase maxima of circadian rhythm of resistance to singlet oxygen damage in *Z_{Obihiro1}*, *Z_{Obihiro2}* and *Z_{Osaka}* were in-phase with those of UV-C and UV-B resistance rhythms, the phase minima were out-of-phase except in *Z_{Osaka}*.

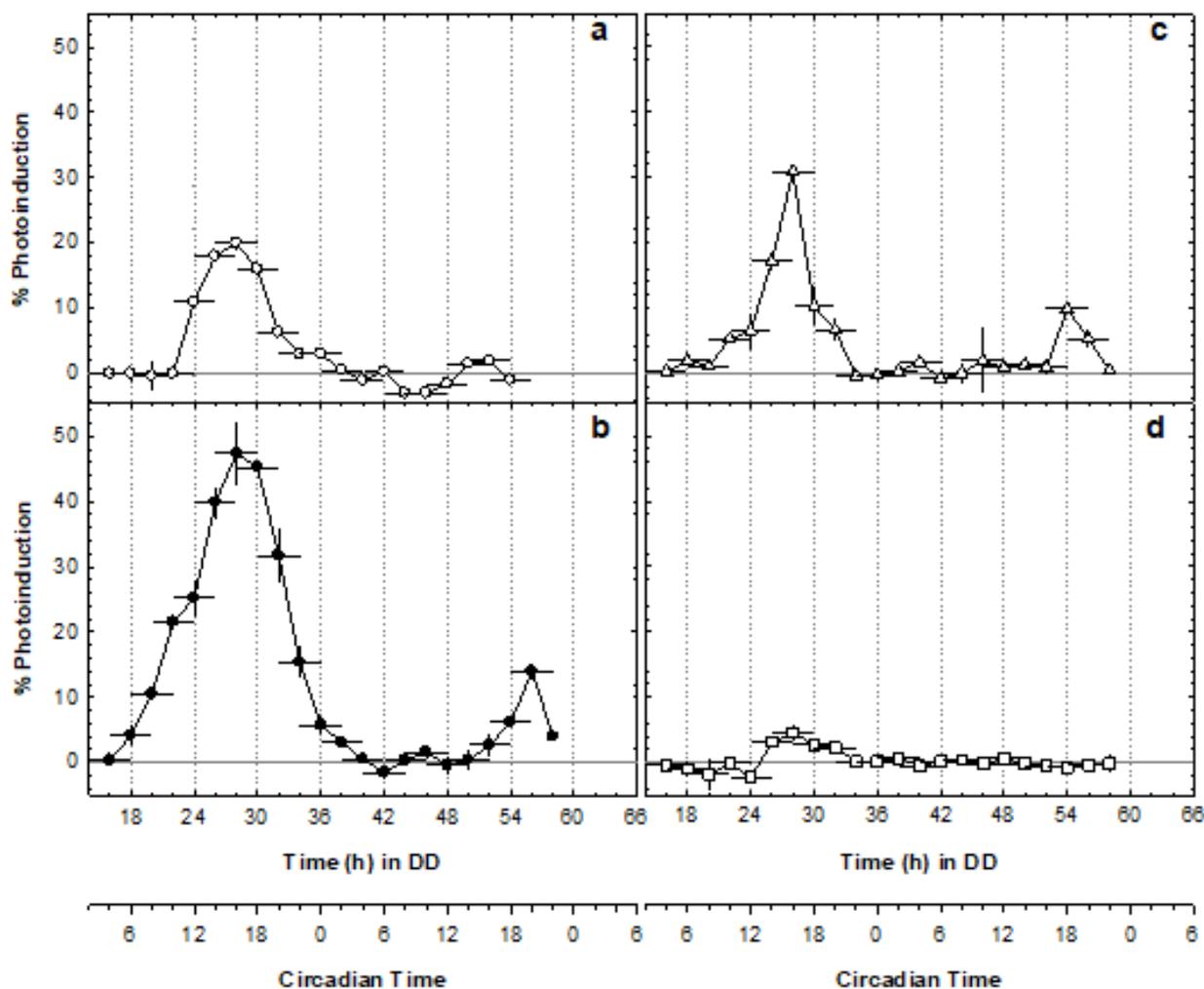


Fig. 3. Circadian rhythms of photoinduction in the commitment of G2-phase cells to cell division in four substrains of *Euglena gracilis* Z in DD. a) *Z_{Obihiro1}*, b) *Z_{Obihiro2}*, c) *Z_{Osaka}*, d) *Z_{NIES48}*. The vertical bars crossing each symbol represent the SEM (n=3) and the horizontal bars represent the duration of light pulse (4 h).

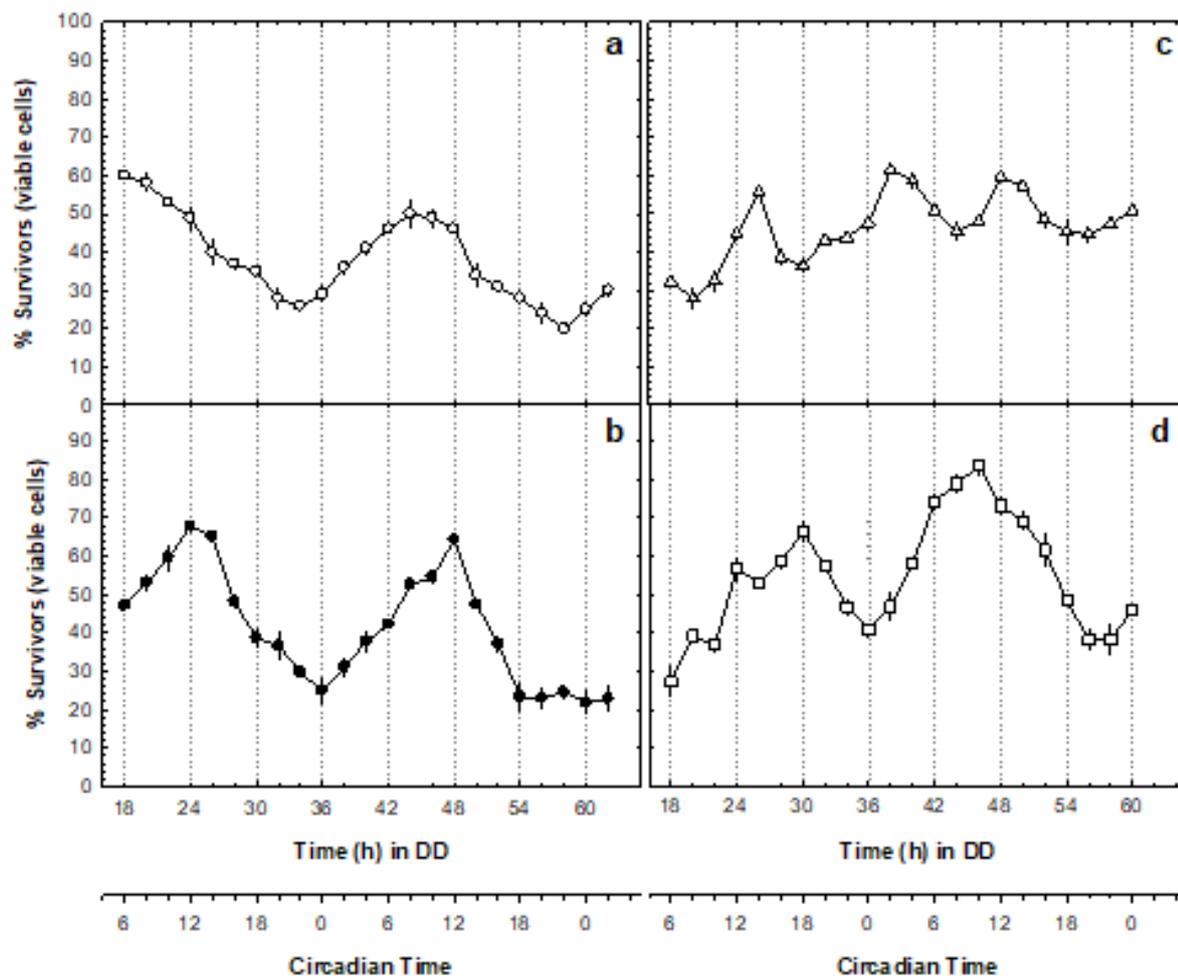


Fig. 4. Circadian rhythms of resistance to photosensitization against Rose-bengal in four substrains of *Euglena gracilis* Z in DD. a) ZObihiro1, b) ZObihiro2, c) ZOsaka, d) ZNIES48. Exponentially growing cells in LL ($84 \mu\text{mol m}^{-2} \text{s}^{-1}$) were transferred to DD. At the times indicated on the abscissa, cell suspensions were withdrawn and exposed to a white light pulse (see Materials and Methods for details). The vertical bars crossing each symbol represent the SEM ($n=3$).

Discussion

This study revealed that, although we used the same strain of *E. gracilis* Z, the characteristic features in relation to circadian rhythmicity of four different “substrains” were remarkably different. Consequently, we found that the circadian rhythmicity is plastic even within a species, if not within a strain.

At the very first instance, we thought the differences among substrains, particularly among ZObihiro2, ZOsaka and ZNIES-48 were due largely to the microenvironmental differences in the culture history of these substrains, as we started experiments allowing ZOsaka and ZNIES-48 to acclimate to our culture conditions only for two months. Nevertheless, even after two years of acclimation to our conditions, both these substrains did not change any of their characteristics, thus disproves the possibility of microenvironmental differences in culture history.

Same strain, but different growth characteristics

E. gracilis Z has been used in our laboratory since 1986. Since then, the cell population growth in either LL or in LD cycles has been monitored for more than 100 times. As summarized in Hagiwara *et al.* (2001), this strain has shown a GT of ca. 14-18 h when cultured at $84 \mu\text{mol m}^{-2} \text{s}^{-1}$ under our experimental conditions. In the present study, the GT of all substrains fell within that range although ZObihiro2 showed the fastest rate (GT=15 h) among all four. On the other hand, when cells are transferred from LL to DD, the number of cells that are committed to cell division in DD was observed to depend on the light intensity in LL (Beneragama and Goto, 2010; Hagiwara *et al.*, 2001; Bolige and Goto, 2007). Based on our results, it appears that even when the same intensity was employed, the number of cells committing to cell division, is different depending on the substrain that we use. Moreover, the relations between the GT in LL and the numbers of cells that are committed to cell division in DD, i.e. lower the GT, higher will be the cells committed to cell division (Hagiwara *et al.*, 2001), did not obey in the present study (Fig. 1).

When entrained to LD: 12,12 cycles, *E. gracilis* Z shows a perfect division synchrony, i.e. a step size of two (Edmunds and Funch, 1969; Hagiwara et al., 2002). In this respect, only Z_{Obihiro1} and Z_{Obihiro2} showed the characteristic typical to the Z strain. Most probably the responsiveness to light intensity in Z_{NIES-48} for growth and to acquire dark capability is lower compared to other three substrains as was evident from Fig. 1 and Fig. 3.

Plasticity of circadian rhythms

We observed remarkable differences in circadian rhythms among the substrains (Figs. 3 and 4), which suggest the plasticity of circadian rhythms within the Z strain. It is known that circadian rhythms can be quite plastic in some species and vary from one individual to another within a species (Cohen et al., 2009). Studies conducted with different strains of mice (Uz et al., 2002; Henricks et al., 1997) and different cultivars (taxonomically equivalent to strains) of *Pharbitis* (Hayama et al., 2007), *Arabidopsis* (Green et al., 2002) and rice (Lee and An, 2007) revealed different circadian rhythms among strains (or cultivars) within the same species. As all these organisms reproduce by sexual means too, the differences in the genetic materials, hence the differences in rhythms among strains/cultivars can easily be predicted. When the organisms, those reproduce purely by asexual means (so-called true clones), are considered, to our knowledge, *E. gracilis* Z is the first organism to show intra-strain differences in circadian rhythmicity. Similar results have been reported previously (Beneragama et al., 2014).

Three major mechanisms may account for plasticity in overt circadian rhythms (Shemesh et al., 2007). First, the influence of environmental factors on the motor controlling centers may be stronger than that of the circadian system ("masking"). This possibility can easily be negated as we cultured all four substrains under the same conditions. Second, there can be an uncoupling of motor controlling centers. It has been reported that circadian rhythms are generated by coupling two or more ultradian rhythms (Barrio et al., 1997; Pavlidis, 1978; Paetkau et al., 2006). This appears to be a possible mechanism because we found there is a bimodal rhythm in Z_{Osaka} (Fig. 4c), thus suggesting the bimodal rhythm was a result of uncoupling of circadian oscillators, although there is no evidence of having more than one oscillator in *Euglena* as yet. Mohabir and Edmunds (1999) also found a bimodal rhythm in the Z strain in cyclic AMP levels. Third, there could be a genuine reorganization of the internal circadian system, a modification in the genome for example. Although we do not have scientific evidences, one possibility could be the DNA methylation, which is a major epigenetic enzyme-catalyzed chemical modification of the DNA structure without causing coding change (Shih et al., 2006). It is known that DNA methylation can abolish the expression of circadian genes

and alter the circadian rhythms (Bnsch et al., 2007; Cheng et al., 2008, Shih et al., 2006).

In the present study, not all the circadian rhythms were plastic among substrains; circadian rhythms of cell division in LD:12,12 and the circadian rhythm of photoinduction capacity of cells in DD were found to be rigid. These robust rhythms seem to be crucial for the survival and growth of populations in all four substrains, hence tightly held.

Ecological significance of 'plastic' nature of rhythms

Plasticity is defined in eco-physiology as the ability of a genotype (a single set of genes) to generate a range of different phenotypes, depending on the environment that the organism must endure (see Chambel et al., 2005). It has been shown that these 'plastic' responses are either due to the environment or to the genotype, or sometimes due to random developmental irregularities (Bradshaw, 1965), thus plasticity seems not always inherently adaptive. Although the plasticity in response to the environment is said to increase the ecological fitness of organisms, in some cases it might represent inevitable responses of the organisms that do not imply enhanced fitness (Meyers and Bull, 2002). Although plasticity plays a crucial role in heterogeneous and variable environments for sessile organisms, motile organisms like *Euglena* also might benefit with the plasticity, particularly when they form water-blooms. However, we are yet uncertain as to why all our four substrains displayed the plasticity in rhythms in the same environmental conditions employed in the present study.

Does *E. gracilis* strain "Z" comprise of many ecotypes or substrains?

Among all the Euglenoids, *E. gracilis* "Z" is the most studied laboratory strain that has been an excellent experimental material for almost a century in physiological (e.g., Cook, 1963), cytological (e.g., Buetow, 1968), biochemical (e.g., Saida and Schiff, 1994), and in genetic (e.g., Greenwood et al., 1996) analyses. To our knowledge, those who have worked with the "Z" strain up until now have never paid attention on the existence of substrains or ecotypes, although some authors have occasionally mentioned the accession number of the culture collection where they have obtained the organism (e.g. Takahashi et al., 2006). However, our findings suggest that strain "Z" is not just a single uniform strain, but could be comprised of a few (or several) different substrains.

The four substrains that we examined are perhaps different ecotypes (or actually "substrains" in taxonomical jargon) of the Z strain. In definition, an ecotype is a group of population that has acquired a small number of extra genetic elements or mutations, enabling the population to exploit a slightly different ecological niche but preserving the genetic signature and the full ecological potential that characterizes its species (Konstantinos and Tiedje, 2005). Although the

emergence of new ecotypes is common in nature, we believe, it is still possible to appear new ecotypes/substrains even under the laboratory conditions for some unknown reasons. Our Z_{Obihiro1} and Z_{Obihiro2} provide a classical example for this; the culture conditions and the medium were essentially the same. Although these four substrains are morphologically not different from each other when observed through a microscope ($\times 100$ objective), it is highly probable that these four substrains are different genetically. *E. agilis* (= *E. pisciformis*), a close relative of *E. gracilis* (Zakrys et al., 2002) has also shown a similar phenomenon of genetic polymorphism, although in natural populations, which is said to be the result of intense microevolutionary processes at the intraspecific level (Zakrys and Kucharski, 1996). On the other hand, no correlation was found between the size or shape of cells and the genetic characteristics of clones of *E. agilis* (Zakrys et al., 1996). Although the genetic polymorphism in clonal populations has also been noted in freshwater zooplanktons (Mort, 1991; Okamura et al., 1993), parasitic protozoans (Tibayrenc et al., 1990) and fungi (Zimard et al., 1994), these are not true uniparental organisms to compare with *Euglena*, because these organisms can also reproduce sexually, with a lesser or greater frequency (Zakrys, 1997).

Although it is very unlikely, but still there is a possibility that these four substrains can be four different species, because genus *Euglena* is notorious of having morphologically similar species (so-called "critical species": e.g., *E. stellata*, *E. viridis*, *E. pseudoviridis*), which are nearly impossible to distinguish from each other (Zakrys, 1997). Moreover, Zakrys et al. (2002) showed that two different species of *Euglena*, *E. geniculata* and *E. myxocylindracea* are virtually genetically and morphologically identical, a finding, which makes the scenario more complicated. However, there is no doubt about the genetic identity of the species that we used, as these clonal organisms are maintained at well-reputed and accredited scientific institutions.

Is *E. gracilis* Z frequently evolving?

The most recent studies of *Prochlorococcus marinus*, a highly successful photoautotrophic cyanobacterium, which is expected to be stable in evolutionary terms, showed that, *P. marinus* is evolving in to new niches now by the process of genome reduction (Mullineaux and Stanewsky, 2009). As the genome of *E. gracilis* is expected to be a hybrid of four main gene classes: i) *Euglena*-specific genes, ii) Kinetoplastida-specific genes, iii) eukaryotic genes, and iv) genes acquired during the secondary endosymbiosis (Ahmadinejad et al., 2007), during the evolutionary process, they acquired new genes, and at the same time they lost some. Therefore, this organism is highly likely to evolve to be ecologically successful. With our results, it is possible to hypothesize that *E. gracilis* is subjected to frequent changes, of which most are remained un-noticed.

Conclusion

The circadian rhythmicity in resistance to oxidative damage caused by photosensitized RB was highly plastic among substrains we used, however, it was apparent that some cell-division related rhythmicity, i.e. cell division rhythm in LD:12,12 cycles and photoinductive capacity rhythm of commitment to cell division in DD were kept almost equally robust among substrains. Additionally, our findings suggest that there could be different substrains or ecotypes present within the same Z strain, thus one has to be particular of the strain is to be used. Although these four substrains are morphologically similar, biochemical analysis might help identify some differences among those. Moreover, until we perform a detailed genetic analysis of these substrains, we will not be able to draw a concrete conclusion about the reasons for the observed differences among substrains.

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