The *Paramecium* circadian clock: a phase-plane analysis of entrainments by discrete changes in light intensity and continuous light involved in a light/dark cycle[1]

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Summary. Functional roles of four factors involved in ambient light/dark cycles on entraining the circadian behavioral rhythm in a population of *Paramecium* were explored by a phase-plane technique: discrete changes in light intensity (dawn and dusk) discretely phase-advance the rhythm, and continuing light conditions (day and night) predominantly decelerate the rhythm.

Key words. Circadian rhythm; limit cycle; locomotor activity rhythm; *Paramecium multimicronucleatum*; phase-plane analysis.

Abbreviations: CT, circadian time; DD, constant darkness; dTF, differential traverse frequency (cells/h^2); LD, light/dark cycle; t, duration of complete photoperiod or interpulse interval of skeleton photoperiod; TF, traverse frequency (cells/h).

All eukaryotic organisms are known to show circadian rhythms in their physiological functions (e.g., cycles of activity and rest, body temperature, evoked responses, etc.). These rhythms have a period of 'about a day', when all known periodic environmental cues associated with the solar day, the so-called 'zeitgeber', are excluded[2–5]. Under natural conditions, the rhythmic functions are entrained to the precise 24-h period by the zeitgeber (s)[2, 3]. The most ubiquitous zeitgeber is the light/dark cycle associated with the movement of the sun.

In a light/dark cycle, four components can be considered to be involved as daily repetitive factors to entrain the circadian rhythms: 1) a discrete increase in light intensity (dawn); 2) continuing light; 3) a discrete decrease in light intensity (dusk); and 4) continuing darkness. Our previous study[6] predicted that the continuing components of the 'complete photoperiod' acts so as to decelerate the angular velocity of the circadian behavioral rhythm in a population of a single-celled animal, *Paramecium multimicronucleatum*. It was also described that the pulses of a 'skeleton photoperiod'[5–8] (which involves two light pulses separated by t h per cycle, constituting the 'skeleton' of a 'complete' photoperiod of t-h duration[6]), as well as single pulses repeated at 24-h intervals[7], elementally cause only discrete phase-shifts in the *Paramecium* circadian behavioral rhythm.

The behavioral rhythm of the *Paramecium* population has been viewed as a model of the circadian behavioral rhythm in an animal, driven by circadian pacemakers found in discrete cells[6, 7, 9, 10], the mechanism of which has been considered to be basically common to all animals[3–5]. One of the most prominent characteristics of the *Paramecium* behavioral rhythm, documented with an index, 'traverse frequency' is that the rhythm exhibits a sinusoidal, free-running oscillation in constant darkness (DD)[9, 10]. Therefore, comparison of the rhythm in a light/dark (LD) cycle with the free-running rhythm allows us to demonstrate clearly the roles of the four factors involved in the LD cycle to entrain the *Paramecium* rhythm. Then the present study attempted to elaborate upon the functional roles of the four factors, by making use of a phase-plane technique[13–17] for the *Paramecium* behavioral rhythms in regimens of LD (12:12), DD and the skeleton photoperiod of 12 (t = 12 h).

Materials and methods. A suspension of *P. multimicronucleatum* (syngen 2), grown in a hay infusion bacterized with *Klebsiella pneumoniae* in a complete photoperiod of 12 (LD, 12:12; L, 1000 lux from a cool white fluorescent lamp), was kept in the same LD at 20 °C for several cycles in an experimental vessel and subsequently exposed to continuous darkness (DD), or to a skeleton photoperiod of two pulses (LDLD 1 · 5:10 · 5:1 · 5:10 · 5; L, 1000 lux). The circadian behavior of the population of *Paramecium* was indexed by measurement of 'traverse frequency' (= TF, hourly number of paramecia which traversed small marks in the experimental vessel), made with a 'microcomputerized close-up video photoamplifier system', details of which have been described elsewhere[9–11].

Generally, a value of a rhythmic system (e.g., $x = \sin\omega t$; where ω = frequency, and t = time) and its differential [$dx/dt = \omega \cdot \cos\omega t$ ($\equiv p$)] depict a closed elliptic trajectory ($x^2/C^2 + p^2/\omega^2C^2 = 1$, where C is constant), when plotted on a phase plane. Depending on the constant C, pairs of x and p (state variables) are uniformly spaced and form straight, radial isochrones[12–14]. When perturbed by a small external force, the system usually depicts a trajectory, with states distributed outside the straight, radial isochrones. If the system returns to the previous elliptic trajectory after the removal of the external force, the trajectory is called a limit cycle[12–14]. If the perturbed trajectory is intrinsically equivalent to the unperturbed limit cycle, the comparison of states within the limit cycle and the perturbed trajectory allows characteristics of the forced system relative to the unperturbed rhythmic system to be described qualitatively[12–16].

In the present study, rhythmic, hourly sequentials, TF_i (traverse frequency at ith hour) and differentials, dTF_i ($= \mathrm{TF}_i - \mathrm{TF}_{i-1}$) are chosen as state variables and plotted on a phase plane ($i = 0, 1, \ldots$), with TF_i along the abscissa and dTF_i along the ordinate (fig. 1). The TF sampling times, i, are assigned as the state number with $i = 0$ set to a circadian time (CT)[5] of 0 which corresponds to the beginning (dawn) of the complete photoperiod of LD and the 'subjective day'[5] in DD, and the first pulse of the skeleton photoperiod, because the rhythm of traverse frequency (TF rhythm) exhibits a circadian period close to 24 h even in DD[9].

State variables obtained in the LD prior to the DD appear to track a trajectory ($\equiv$ LD trajectory) which completely encloses a trajectory depicted by state variables in the DD ($\equiv$ DD trajectory) (fig. 1 a), which, in turn, approximates to an ellipse with rather uniformly distributed states. Therefore, as our working hypothesis for the present sutdy, the DD trajectory is assumed to be a limit cycle and the LD trajectory to be the perturbed form of the limit cycle induced by the complete photoperiod of 12. The effect of a 12-h light pulse can be considered to be superimposed on the basic DD free-run, for a qualitative description of characteristics of the LD trajectory relative to the DD trajectory[15, 16].

In practice, it is difficult to determine the exact topology of isochrones on the experimental DD trajectory, even if they are distributed uniformly, because its phaseless point, the origin of the straight and radial spacing of isochrones[12–14] is unpredictable or not apparent. However, the angle, 'R_i' contained by the line which connects the ith and i-1th states on the DD trajectory and the line which connects the

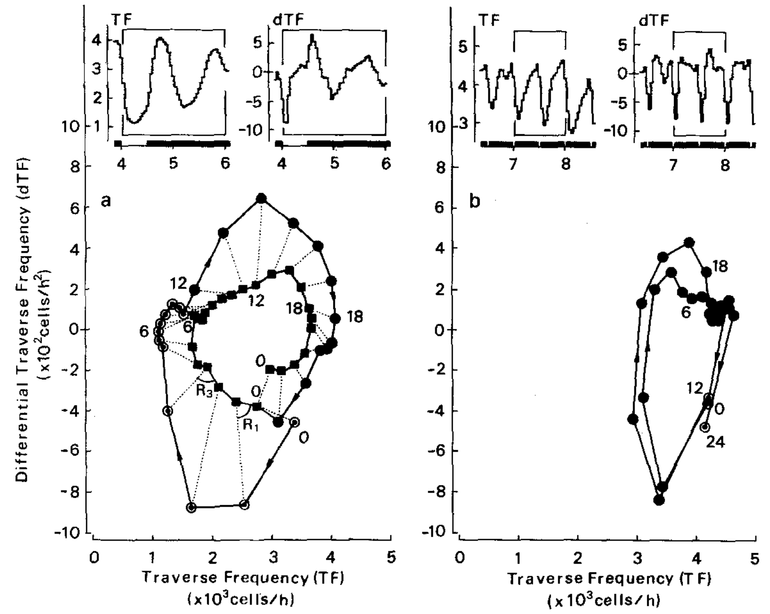

Figure 1. *a* Two-dimensional trajectories depicted by state variables, the traverse frequency (TF) and corresponding differential frequency (dTF, see text) of the *Paramecium* population in LD (12:12) and subsequent DD. Double circles (◉) indicate states of the photoperiod of the LD, black circles (●) those of the dark time, and black rectangles (■) those of the subsequent DD. Numbers in the figure denote phases of the trajectories, and R_1 and R_3 denote relative phase angles (see text). Dotted lines connect identical phases of the LD and DD trajectories. Insets: Fluctuations of TFs (left) and dTFs (right) in the LD and subsequent DD. Abscissae: days after the initiation of the experiment. Rectangles enclose TFs and dTFs depicted on the abscissa and ordinate of the figure, respectively. Black bars on abscissae indicate dark time.

b Two-dimensional trajectory of state variables, TFs and dTFs in one cycle of the skeleton photoperiod of 12 (LDLD 1 · 5:10 · 5:1 · 5:10 · 5). Double circles indicate states during which the light pulses (0 or 24 during the morning pulse and 12 during the evening pulse) were applied, and black circles indicate states during inter-pulse intervals. Insets: Fluctuations of TFs (left) and dTFs (right) recorded during the day in the skeleton photoperiod as indicated by numbers on the abscissae. Rectangles enclose TFs and dTFs depicted on the abscissa and ordinate of (b). Numbers in the figure denote phases of the rhythm.

isochronic states (*i*th states) on the DD trajectory and the LD trajectory (e.g., R_1 or R_3 shown in fig. 1 a), may provide a tool for the qualitative description of the change in angular velocity of the LD trajectory relative to that of the DD trajectory.

Results and discussion. Profiles of the LD trajectory relative to the DD trajectory may be visualized as follows (fig. 1a). For a few hours after the onset of photoperiod (CT0 – ~CT4), the LD trajectory plainly depicts a larger arc with a wider angle in the lower sector than does the corresponding portion of the DD trajectory. During the remainder of the photoperiod (~CT4 – CT12), the LD trajectory shrinks in terms of the arc and phase-angle more than does the DD trajectory of the corresponding period. For a few hours after the dusk (CT12 – ~CT18), the LD trajectory again depicts a larger arc with a wider angle in almost all of the upper sector than the corresponding portion of the DD trajectory. During the remainder of the dark period (~CT18 – CT24), the phase angles of the LD trajectory tends to diminish relative to the DD trajectory.

By contrast, the trajectory which corresponds to one cycle of the skeleton photoperiod of 12, portrays two almost closed loops in a 24-h day with phase states of CT0 and CT12 (and also CT24) distributed closely to each other, and these loops are apparently hemicircles lengthened downward (fig. 1b).

The relative phase angles, R_i shown in figure 1 a, allow us to describe the characteristic angular velocity changes of the LD trajectory relative to that of the DD trajectory, as they change in a bimodal manner (fig. 2). The angles abruptly increase after dawn to indicate an abrupt phase-advance, which is followed by a gradual decrease, evidence of phase delay. The angles abruptly increase again after dusk to demonstrate an abrupt phase-advance, which is followed by a gradual decrease, evidence of the phase delay.

These changes in R_i directly demonstrate that the continuous component of the complete photoperiod acts to decelerate the angular velocity of the TF rhythm of the *Paramecium* population, as predicted in our previous study[6]. This decelerating effect of the continuous action of light is also indicated in differences between TFs and dTFs in the complete photoperiod and the skeleton photoperiod shown in the insets in figure 1 a and b. In the skeleton photoperiod, the TFs are abruptly decreased in a similar manner by either the first or the second pulse, and thereafter increase gradually until they are abruptly decreased by the next pulse, which eventually results in 'bistability'[6]. However, TFs in the complete photoperiod are reduced during the photoperiod. The dTFs

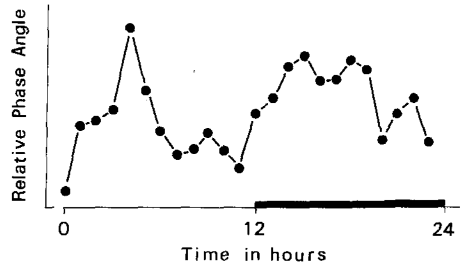

Figure 2. Profile of phase angles of the LD trajectory relative to the subsequent DD trajectory displayed as R_1 or R_3 in figure 1 a (see text). The angles were obtained from a geometry similar to that shown in figure 1 a, such that the DD trajectory was as close to a circle as possible. Black bar on the abscissa shows the dark time of LD prior to DD.

in the skeleton photoperiod appear to be decreased transiently by both pulses in a similar manner. By contrast, it is remarkable that dTFs in the complete photoperiod show opposite responses to the onset (dawn) and offset (dusk) of the photoperiod, i.e., a transient decrease immediately after the dawn and a transient increase immediately after the dusk.

These opposite responses of the dTFs to the dawn and the dusk may be attributed to the decelerating effect of the continuing complete photoperiod, abrupt removal of which may provoke a rebound reaction in TFs such that they increase transiently. By contrast, in the skeleton photoperiod of 12, the TF rhythm may be abruptly reset to its new CT 0 identically by both the first and the second pulse, thus depicting identical hemicircle loops. [Indeed, recent experiments in our laboratory reveal that the TF rhythm previously entrained to the skeleton photoperiod of 12, when scheduled into DD after either the first or the second pulse, initiates the free-running oscillation with the first maximum in the rhythm occurring around 12 h after the pulse, no matter which was the first or the second pulse].

The present results, therefore, describe the roles of the four factors involved in the LD cycles to entrain the circadian rhythm of traverse frequency in the *Paramecium* population as follows: 1) the discrete onset of light acts principally to reset the rhythm to a new phase, causing an abrupt phase-advance in the rhythm; 2) the subsequent continuing action of light functions to decelerate the rhythmic oscillation of traverse frequency, which prevents the rhythm from being completely reset by the discrete onset of light and eventually decelerates the rhythm during the remainder of the light period; 3) the abrupt removal of the continuing light releases the rhythm from the decelerating effect, provoking a rebound reaction which transiently accelerates the rhythm; and 4) the rhythmic oscillation of traverse frequency gradually slows down so that it gradually returns to the innate DD free-run.

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Infection of frog tadpoles (Amphibia) by insect parasitic nematodes (Rhabditida)

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Summary. Infective stage juveniles of *Neoaplectana carpocapsae* (Steinernematidae) and *Heterorhabditis heliothidis* (Heterorhabditidae) were able to penetrate through the alimentary tract of young tadpoles of *Hyla regilla* (Hylidae) and *Xenopus laevis* (Pipidae) and enter the body cavity. Some infectives of *N. carpocapsae* were able to release their symbiotic bacterium, *Xenorhabdus nematophilus* inside the host and in two cases, the nematodes developed into adult females before they perished. Tadpole mortality was associated with foreign bacteria entering the penetration holes made by the invading nematodes. The infective stage juveniles of both nematodes frequently encountered a host defense reaction upon reaching the tadpole's coelom.

Key words. Neoaplectana; *Heterorhabditis*; frog; Amphibia; Nematoda; pathology.

Rhabditoid insect parasitic nematodes belonging to the genera *Neoaplectana, Steinernema* and *Heterorhabditis* are unusual in their ability to kill and develop in a wide variety of insects[2] as well as being able to penetrate, kill and at least partially develop in Symphylans[7], Isopoda[5], Areneae[3], Pseudoscorpionida[6] and millipedes[4]. The ability to infect arthropods other than insects was unusual but suggested that all arthropods shared certain structural and biochemical features to which nematodes could recognize and respond. However, it was perplexing when Kermarrec and Mauleon[1] reported that tadpoles of *Bufo marinus* could be killed by penetrating infectives of the 'Agriotos' strain of *Neoaplectana carpocapsae.* Since neoaplectanids and other rhabditoid nematodes are potential candidates for use against pest insects it was important to determine if the above results were applicable only to *Bufo* tadpoles because of some unknown combination of characters, or whether tadpoles of other amphibian species were also susceptible.

The present study was designed to test the effect of both *Neoaplectana carpocapsae* and *Heterorhabditis heliothidis* on the tadpoles of two frog species, one native and one exotic.

Materials and methods. The All strain of *Neoaplectana carpocapsae* Weiser (Steinernematidae) and the NC strain of *Heterorhabditis heliothidis* were used in this study. Tadpoles of the western tree frog *Hyla regilla* (Hylidae), a native of western North America were obtained from large earthen outdoor basins at the Magic Gardens Nursery in Berkeley, California. They were collected with a fish net and main-