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Thalassomyxa australis Rhythmicity III. Entrainment by Combination of Different Zeitgeber

by

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ABSTRACT

The rhythmic change between an actively moving phase and a rounded up resting phase of the marine plasmodial rhizopod *Thalassomyxa australis*, sustained by the diatom *Amphiprora*, can be synchronized (1) By a combination of light-dark-cycles and temperature cycles. This method was successful in 1/3 of the cases (2) By shaking the cultures every 12 hours for 15 or 30 minutes. This method was successful in half of the cases (3) By combining light-dark-cycles, temperature cycles and periodic shaking. This method is synchronizing in all cases, but the phase relationship of the shaking to the other Zeitgeber is important for successful entrainment.

INTRODUCTION

Thalassomyxa australis is a marine amoeba discovered by Grell in sand samples on the south coast of the island Rottnest, 20 km of Perth, WA. Australia (Grell 1985). It exhibits a rhythmic change in its activity, and the period is in the circadian range at temperatures around 22°C. However, at higher and lower temperatures the period deviates considerably from the circadian range. Thus it violates the rule of temperature compensation usually found in circadian rhythms (Silyn-Roberts et al., 1986). This has been discussed as representing an early evolutionary stage of circadian rhythmicity (Silyn-Roberts and Engelmann, 1986). Another distinct difference to normal circadian rhythms is the lack of entrainability by light-dark- and temperature cycles (A. Smietanko et al., 1988). A synchronized culture is necessary for a more detailed study of the morphological, functional and biochemical differences in the two stages of this amoeba.

We tried to synchronize *Thalassomyxa australis* by combining different "Zeitgeber" conditions, because under natural circumstances the amoebae are

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under the influence of a number of environmental time cues.

Thalassomyxa australis was found in rockpools in the intertidal zone of a rocky shoreline. During low tide these cavities retain water, preventing the amoebae from drying out. *Thalassomyxa australis* is exposed there to the natural light-dark-cycle, to daily temperature fluctuations and to turbulences during the tidal changes.

At Perth the mean temperature of the sea water is 25.5° from April to May, and 18°C in August. Daily temperature changes are about 5° from September to March, and 3 to 4° from June to August (E.P. Hodgkin and B.F. Phillips, 1969). In the particular rockpools where these amobae were found the fluctuations might be larger due to the small volume of water retained during low tide, which can be heated up easily by the sun.

Tidal changes in this part of the world occur every 6.2 or 12.4 h, depending on the lunar cycles (simulations from Geodätisches Institut München). The amoebae are exposed to the turbulences in the rockpools for about 30 minutes (Grell, personal communication). To simulate the natural conditions we have used the following Zeitgeber conditions:

1. 12:12 h light-dark-cycles.
2. 6:18 h temperature cycles with 6 h high temperature (23°C) during low tide and light period, and 18 h low temperature (16°C) during high tide at light period and low and high tide during the dark period (see fig. 2 and 3).
3. To simulate the tides, the cultures were shaken every 6 or 12 h for 15 or 30 minutes. We did not take into account the 12.4 h period of the tides.

When only one or two of the above mentioned conditions were applied, complete synchronization of the cultures dit not take place. However, one hundred percent synchronization was achieved when all three time cues were combined, provided the phase relationship of the shaking cycle to the light-dark-cycle and the temperature cycle was adequate.

MATERIAL AND METHODS

Thalassomyxa australis was reared in the laboratory in Petri dishes of 9 cm diameter containing sea water and the diatom *Amphiprora* as a food organism. New Petri dishes were inoculated with *Thalassomyxa australis* by transferring single cells in the active stage with a fine brush. Or synchronous cultures were obtained by directing a jet of seawater from a 10 ml pipette into the culture dish. Cells in the active stage lost contact to the surface of the dish by this treatment and were poured off with sea water into new Petri dishes, whereas the inactive stages remained. This is also the easiest and most efficient way to obtain cultures in which the individual amoebae are synchronized among each other.

Every 5 days the sea water was replaced by pipetting fresh sea water gently into

the dish to a height of 4 mm. The handling did not displace active cells and the rhythm was not affected.

During our experiments, we did not use the method of observing the behaviour of individual cells by time lapse video recording as was done before (Silyn-Roberts et al., 1986), because the number of cells would have been too small. Instead we observed the behaviour of entire populations of cells. We determined the number of active and resting animals by visual observation under the microscope at a magnification of 100 fold at approximately 3 h intervals except at night. This magnification allowed quick and reliable determination of the state of the cells. The individual culture dishes were turned between the heads of two screws on the microscope stage in such a way that a circular band could be scanned. This band contained between 30 and 400 individuals depending on the density of the cultures. The results are expressed in percent active animals and plotted in a diagram as a function of time (Fig. 1a). The individual value points were connected by curved lines. Maxima (α_M) of activity are indicated by ▲.

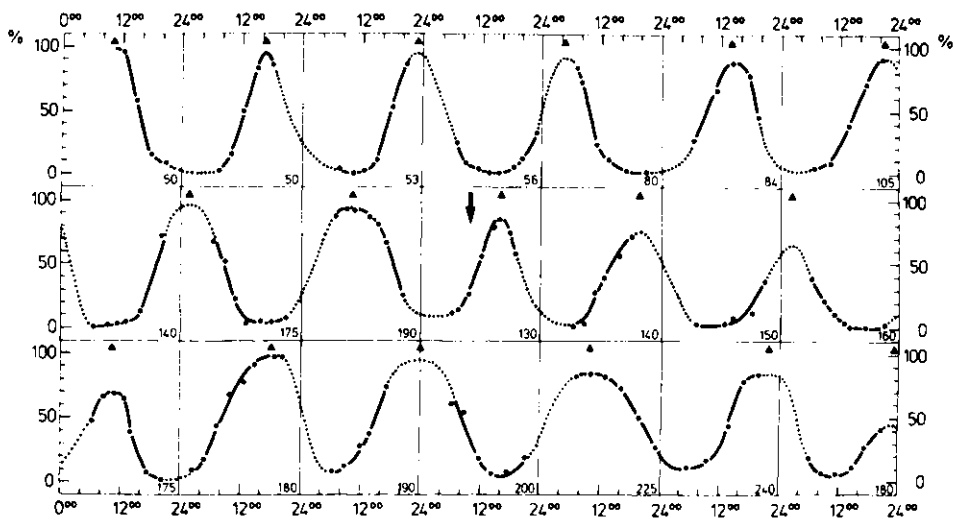

Figure 1a: A culture of *Thalassomyxa australis* under continuous light conditions (1800 lux) and constant temperature (18°C) was internally synchronized by collecting cells in the active stage (α) as described in the text. Percentage of cells in α as a function of time shown for three consecutive weeks (three rows). Time of α_M shown by triangles. The number of cells in the observation area, shown for each day in the lower right corner, increases until day 10 and the curves become more squarewave like with increasing cell density. On the 11th day (arrow) the culture density was reduced and it can be seen that the curve changes to a more sinusoidal shape and the amplitude is reduced. With increasing cell density the amplitude increases again (day 16, second day in last row) and again becomes more squarewave like. Cell density has increased to a maximum of 240 (day 20) and then declines (day 21). The cells in the culture soon died.

A second type of diagram (Fig. 1b) shows only the occurrence of maximal activity on consecutive days, allowing one to determine whether the cultures were synchronized by the 24 hour Zeitgeber (maximal activity in a vertical row) or not (compare with Fig. 1a).

Experiments were undertaken in temperature controlled chambers with light-dark-cycles from 10 Osram white fluorescence tubes (L65W/25), which produced a light intensity of 1800 lux at the surface of the dishes. Temperature was controlled by a thermostat. Tides were simulated by moving the culture dishes back and forth by a shaker with about one Hertz shaking frequency, and the time and duration of shaking was controlled by a timer.

RESULTS

1. Internal synchronization and general observations

Prior to the actual synchronization experiments, we tried to synchronize cultures internally using the method described in the *Material and Methods* section. The most reliable synchronization among the cells was achieved by the following method: All animals in the active stage were removed from a culture dish with high cell density. Several hours later, the amoebae which had changed in the meantime from the resting stage into the active stage, were poured into a new Petri dish using the same procedure as before. The resulting culture was highly synchronous and the individual amoebae stayed synchronized among each other under free run conditions for 5 to 60 days, depending on cell density, density of the diatoms, and perhaps physiological or environmental factors. Cultures with a high cell density and a low density of diatoms showed better internal synchronization than cultures with low cell density and a high density of diatoms. This can be seen from the form of the activity curve. At low or intermediate cell density (30 to 150 amoebae counted), the graph curve was sinusoidal (Figure 1a, days 1 to 8),

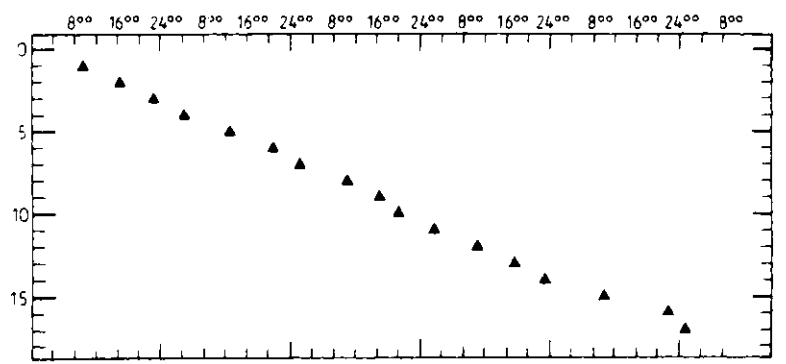

Figure 1b: Only the maxima (▲) of Fig. 1a are shown as a function of time (abscissa, time of day). Ordinate: consecutive days. Time of day (abscissa) has been duplicated five times for better display of the free run period.

whereas at higher densities of amoebae (200 to 400 cells) and lower density of diatoms, the curve was more rectangular (Figure 1a, days 9, 10 and 17 to 20). The rectangular form was the result of a change of most of the cells from the resting stage into the active stage within a few hours, and the change back to the resting stage was again very sudden. This rectangular waveform was especially clear in cultures in which the density of the amoeba was so high that a net of interconnected plasmodial cell branches was formed (Figure 2, days 6 to 11). In this syncytium, the active stage was especially long if the amount of diatoms available was low. Supplying diatoms to the cultures shortened the active stage (Figure 2). Cultures with interconnected cell branches seem to be beyond their "stationary phase" and die within a few days if not diluted and supplied with food organisms (Figure 2, day 11).

For synchronization experiments, we used cultures with an intermediate density of amoeba, exhibiting a sinusoidal curved activity pattern.

2. Combination of light-dark and temperature cycles

Nine cultures were kept in a 12:12 h light-dark cycle, with the light on from 9:00 to 21:00 h. Temperature was 23°C from 9:00 to 17:00 and 16° from 17:00 to 9:00. The light and temperature program is shown on top of Figure 3. The figure shows results of a culture which was kept for the first four days under these conditions. The percentage of active animals was plotted against time in a way that displays four consecutive days in a row and the second day plus three consecutive days below, the third day plus three consecutive days in the next row and so on. Active phase was 80% during the first maximum and dropped to 10% in the following minimum with signs of damping of the rhythm in the following

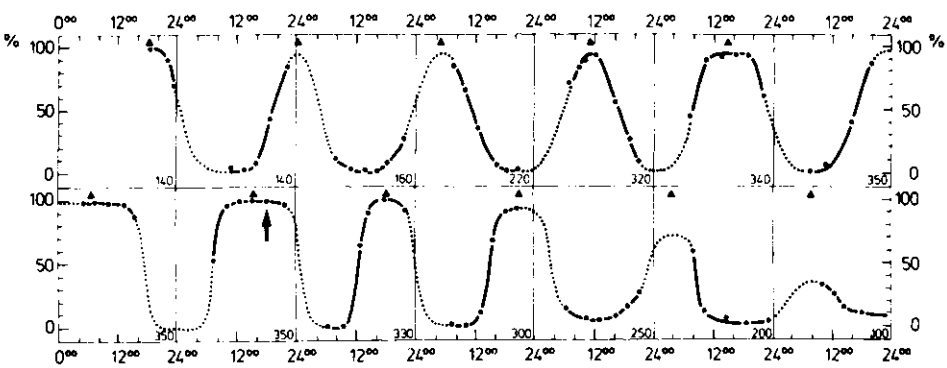

Figure 2: Percentage of cells in active stage α in an internally synchronized culture of *Thalassomyxa australis* under continuous light (1800 lux) and constant temperature (18°C). The culture reached a high density of *Thalassomyxa* cells on the 6th day forming an interconnected plasmodial net. The shape of the curve became more rectangular and due to a decrease in cell density of the food organism the amoebae spent more time in active phase α. Adding *Amphiprora* diatoms as food (arrow) shortened the active phase again, but the culture did not survive.

two days. It is, however, clearly visible in this particular case that the rhythm is not entrained by the light-dark-cycle in combination with a temperature cycle, but shows a free run with a period length of about 32 h (compare e.g. Figure 3b). Five further cultures were also free running despite the combined light-dark- and

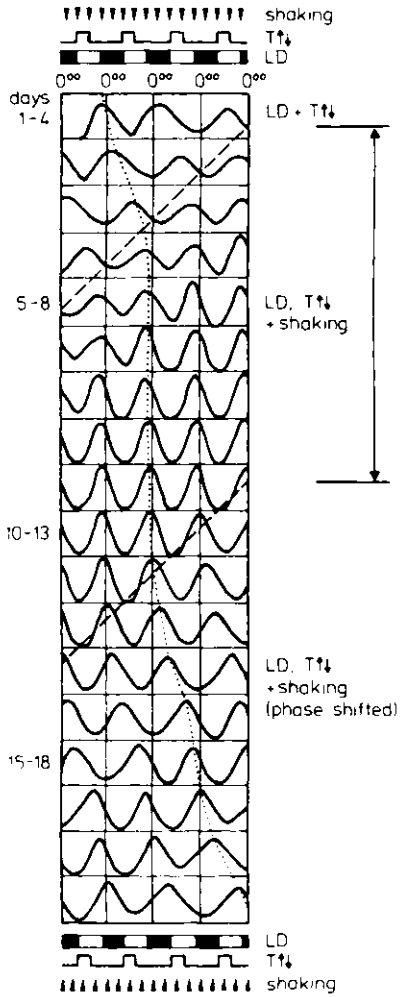

Figure 3a: Percentage of cells in active stage α in a combination of a 12:12 h light-dark-cycle and 8:16 h temperature cycle (23:16°C) during days 1 to 4, in a combination of a 12:12 h light-dark-cycle and 8:16 h temperature cycle (23:26°C) and 15 minute shaking every 6 hrs (see top of the figure) from day 5 to 12. Whereas under the light-dark-cycle and temperature cycle this particular culture showed a free running period, as indicated by the dotted line, the light-dark-, temperature- and shaking cycle synchronized the culture. However, a shift of the phase of the shaking cycle by three hours induced free run of the period again (days 13 to 21). In this figure consecutive days are shown below each other but also quadruple plotted horizontally, to facilitate recognition of free run and synchronization. See also Fig. 3b.

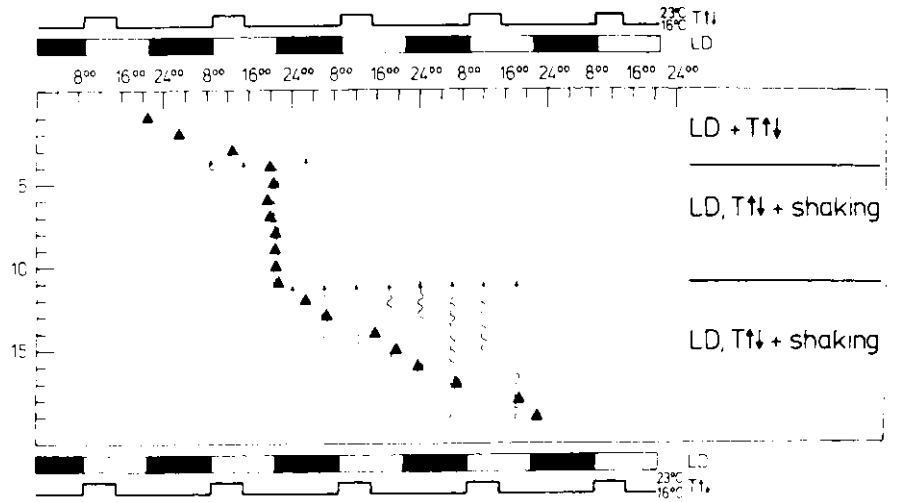

Figure 3b: Same as Fig. 3a, however only time of α_M indicated instead of the percentage curve. Light-dark- and temperature cycle conditions shown on top and bottom of figure. Shaking indicated by vertical wavy lines in the figure.

temperature cycles, whereas three of the nine cultures were synchronized. In these cases the maxima occurred at about 17:00 in the first, 21:00 in the second, and jumped from about 10:00 hrs to about 20:00 hrs in the third culture (Figure 4).

3. Combination of light-dark-cycles and temperature cycles with shaking

From the fourth day onward in Figure 3a, the light-dark cycle and temperature cycle was supplemented with 15 minutes of gentle shaking every 6 h. These times of shaking occurred at the beginning and end of the light period and in the middle of the light and dark period. This is indicated by arrows in Figure 3a and by wavy lines in Figure 3b. The amplitude of the curve representing percentage of animals in the active phase increases from day to day reaching maximal values from the fourth day of shaking onward. The time of the maxima is in this particular case at the end of the light period and coincides well with the shaking at 21:00. All 12 cultures which were exposed to shaking this way were synchronized to the external 24 h periodicity. Time of maximal activity occurred in nine cultures during the light period, coinciding more or less with one of the shaking periods (Figure 3a, 3b, 4). Occassionally the maxima jumped from one shaking period to another (Figure 4, last days under light-dark-, temperature- and shaking cycles). In three cultures, two or three activity peaks occurred coinciding with different shaking periods (Figure 5). Individual cells change, however, only once per day from rest to activity, i.e. the peaks are the expression of subgroups of the cell population and represent the behaviour of different individual cells.

For successful entrainment the phase relationship of the shaking cycle to the other Zeitgeber is important. After eight days of entrainment, the time of shaking

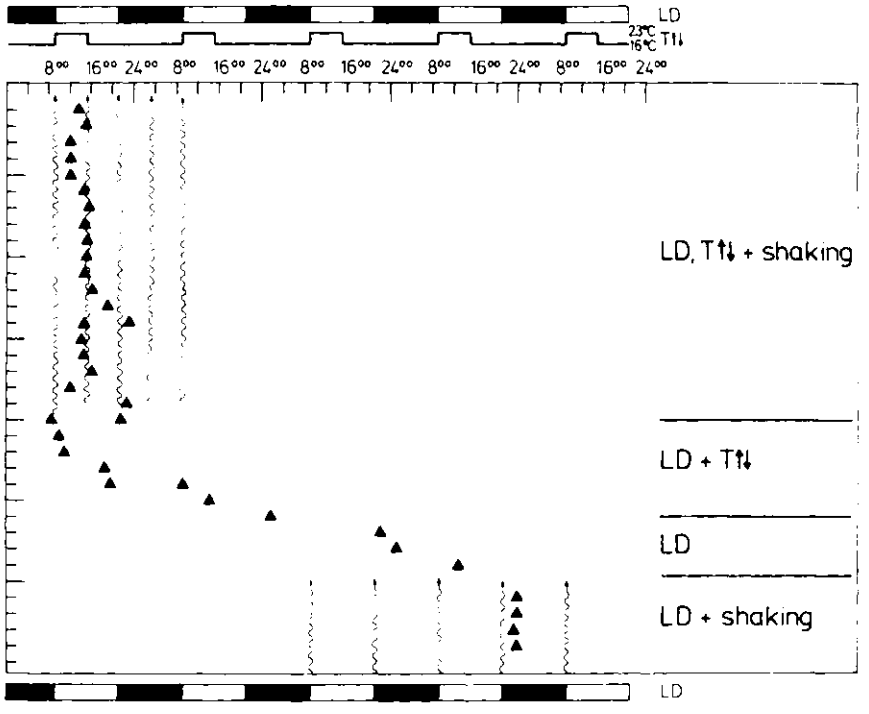

Figure 4: Time of α_M under different Zeitgeber conditions. Light-dark- and temperature cycle conditions shown on top of figure for days 1 to 26 and on bottom of figure for days 27 to 34. Shaking during the first 20 days (every 6 hrs for 15 minutes) and during the last 4 days (every 12 hrs for 30 minutes) indicated by vertical wavy lines in the figure. Entrainment to the light-dark-, temperature- and shaking cycle not perfect, as indicated on day 13 and 18, and by the occurrence of a double peak on day 14. During the first days (20-22) under light-dark- and temperature cycles the culture was more or less synchronized, but maxima jumped from about 10:00 h to about 21:00 h or two maxima occurred. From day 23 onward the culture was almost free running. A light-dark-cycle in combination with shaking from day 30 onward synchronized the culture again.

was shifted by 3 h in four of the cultures, being out of phase with both the light-dark cycle and the temperature cycle (see Figure 3b). The shifting of the shaking process led to a loss of synchronization, as illustrated in Figure 3a and more clearly in Figure 3b. There is, however, a tendency for maxima to occur close to, or at the time of shaking.

4. Combination of light-dark-cycles and shaking

In 10 out of 12 cultures, a combination of a 12:12 h light-dark-cycle with shaking for 15 minutes every 12 h (at light-on and light-off) entrained the rhythm (day 30 to 34 in Figure 4). Time of maxima was always around the end or beginning of the light period, i.e. coinciding with the time of shaking.

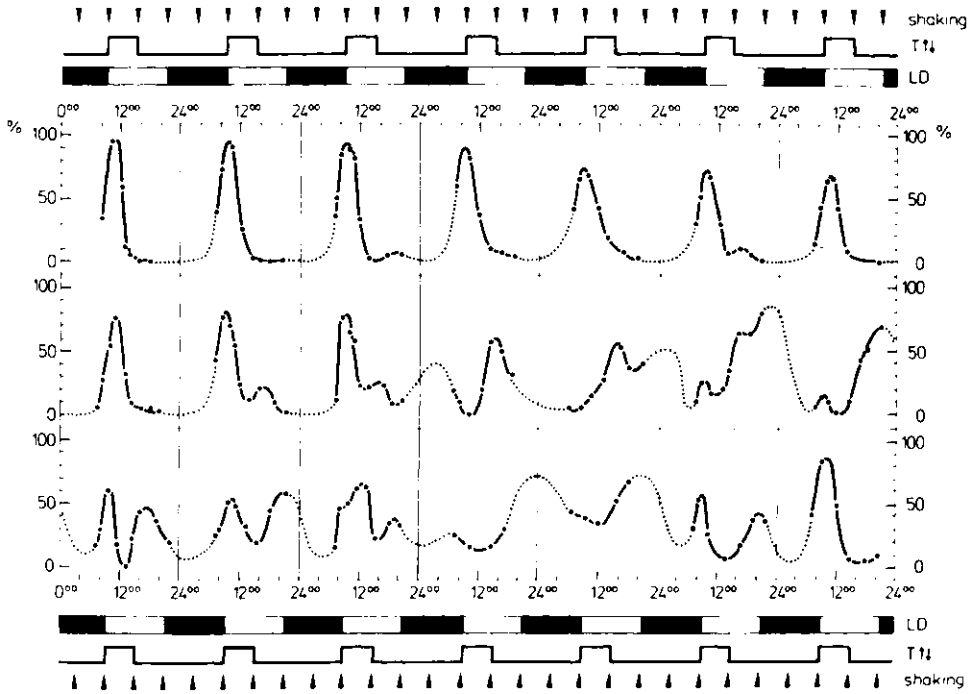

Figure 5: Percentage of *Thalassomyxa australis* as a function of time under a light-dark-, temperature- and shaking cycle timed as shown on top of figure. Shaking time was 15 minutes. This figure shows the occurrence of two peaks per cycle from day 9 onward which are due to subgroups of the pupulation (see text).

5. Shaking

Twenty-five cultures were exposed to shaking for 15 or 30 minutes every 12 h without other additional Zeitgeber. In about half of the cultures the rhythm was synchronized (Figure 6a, 6b, 7b). Maxima occurred at the time of shaking, jumping occassionally from one shaking period to the next (figure 7b). In the other half of the cultures, free run was observed (Figure 6c, 7a).

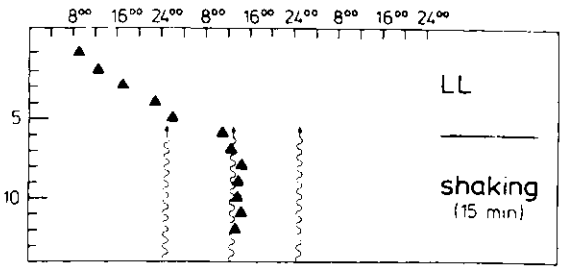

Figure 6a: Shaking every 12 hours for 15 minutes after five days of free run under continuous light conditions. Synchronization to the shaking period at noon.

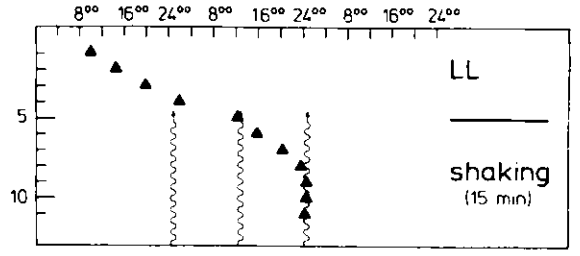

Figure 6b: Same as Fig. 6a. However, synchronization to the shaking period at midnight on the 9th day.

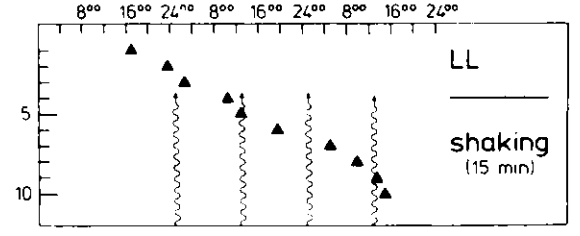

Figure 6c: Same as Fig. 6a. No synchronization by the shaking cycle.

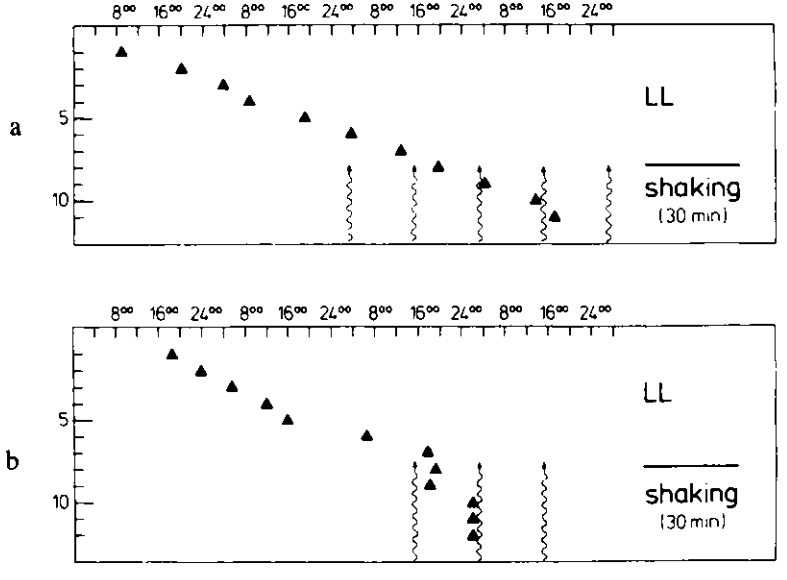

Figure 7: Same as in figure 6. However, shaking lasted 30 minutes instead of 15 minutes. a) example for a culture which was not synchronized by the shaking cycle. b) example for a culture which was synchronized by the shaking cycle, locking in the first instance to shaking at 15:00 hours and afterward to the shaking at 3:00 hours.

6. Phase relationship of the rhythm to the Zeitgeber

As already mentioned, the activity rhythm of synchronized cultures of *Thalassomyxa australis* has a specific phase relationship to the Zeitgeber. The three cultures synchronized by a combination of light-dark-cycles and temperature cycles exhibited maximal activity close to light-on or light-off. Cultures which were treated with shaking tend to be active close to one of the shaking periods. Activity of cultures which were shaken every 12 h and submitted to a light-dark-cycle occurs also close to the shaking periods which were identical with light-on and light-off. Cultures shaken every 6 h and treated with light-dark-cycles *and* temperature cycles showed activity peaks close to the shaking period at light-on in three cultures, close to the shaking period at light-off in five cultures, and close to noon in four cultures. Figure 7 shows the distribution of maxima of all synchronized cultures regardless of the kind of additional treatment. It can readily be seen that maximal activity tends to occur around the times of shaking.

DISCUSSION

Unlike typical circadian rhythms, the period length of the rhythmic change of *Thalassomyxa australis* cells between an active stage and a resting stage depends on the environmental temperature (Silyn-Roberts et al., 1986); light-dark-cycles and temperature cycles do not synchronize this rhythm (Smietanko et al., 1988). However, as shown here, gentle shaking of the cultures every 6 or 12 h for 15 or 30 minutes does synchronize the rhythm in about half of the culture dishes. Combining different potential rhythmic time cues such as light-dark- and temperature cycles, light-dark-cycles and shaking, or light-dark-, temperature- and shaking cycles synchronized parts of the cultures to different degrees (see Table 1). The combination of light-dark, temperature- and shaking cycles was the most effective entrainment. One hundred percent of the cultures were synchronized under these conditions. However, the phase relationship of the shaking cycle to the other Zeitgeber plays an important role. A shift of the shaking cycle by only

Zeitgeber	% cultures synchronized	nr of cultures
LD + $T_{\cdot 1}$	33	9
Shaking 15 min	50	10
Shaking 30 min	47	15
LD + shaking 15 min	83	12
LD + shaking + $T_{\cdot 1}$	100	12

Table 1: Percentage of cultures synchronized under different Zeitgeber combinations. Number of cultures in parenthesis.

three hours prevented entrainment, and the rhythm showed free run.

This indicates that *Thalassomyxa australis* is able to perceive all three time cues and that they all contribute to the synchronization, though to a different degree. Temperature- and light-dark-cycles seem to be rather weak Zeitgeber. Only three out of nine cultures were synchronized by a combination of both. For the timing of the activity maxima the changes from light to dark and vice versa seem to be more important than the temperature changes. Maxima occurred close to light-on and temperature increase or at light-off without a temperature change. No maxima were found at noon when the temperature decreased without a change in light.

Shaking seems to be the most important time cue, because shaking alone can be sufficient for synchronization. Cultures tend to show maximal activity close to the times of shaking regardless of whether shaking was applied alone or in combination with light-dark- and temperature cycles. However, when shaking was combined with the other time cues, they seem to contribute to the timing, since a three hour shift of the shaking rhythm leads to free run or relative coordination. Apparently, the *Thalassomyxa australis* rhythm tries to lock onto light-on and light-off as well as onto the shaking times. The conflicting time cues prevent synchronization.

The reasons, why some culture dishes under the different Zeitgeber conditions are synchronized while others are not, are unclear. The density of the amoeba and/or of the food organism might play a role. A dense *Thalassomyxa australis* culture with strongly interacting cells might strengthen synchronization to the

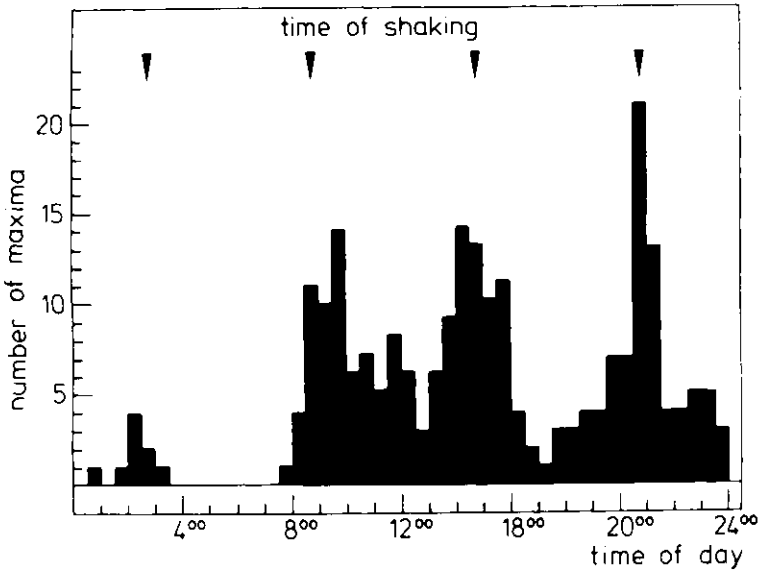

Figure 8: Frequency of occurrence of maximum of activity in respect to time of shaking, in 30 minute intervals. Maxima tend to occur around the time of shaking.

external Zeitgeber, whereas a high density of the *food organism* is less favourable.

The density of the amoebae and the food organism is at least important for internal synchronization of the cells. Substances produced by the amoebae might play a role for short-distance interactions in this mutual synchronization; groups of cells were found to be in e.g. the active stage α, whereas at the same time other groups further apart were in the resting stage ρ. Alternatively, direct cell contact might be necessary for mutual synchronization. We noticed frequently, for instance, that at higher and intermediate cell density contacts and fusions occurred between *Thalassomyxa australis*.

There might be other time cues, more suitable phase relationships of the different conditions or more appropriate light intensities or temperatures which could synchronize the rhythm. The kind of food organism, for instance, plays a role for synchronization to external rhythmic factors: Whereas the diatom *Amphiprora* as a food organism does not allow entrainment to a light-dark-cycle, *Dunaliella (Volvocales)* does (Silyn-Roberts 1988).

The time cues which might synchronize *Thalassomyxa australis* in its natural habitat are unknown. The results presented here show that it is possible to entrain the rhythm of this organism, but they do not exclude the effectiveness of other time cues nor do they exclude the possibility that the rhythm is not entrained to the 24 hour time structure of the environment. The behaviour under field conditions has not yet been studied.

For instance it is unknown, whether *Thalassomyxa australis* has adapted to the tidal changes and/or the diurnal differences in the coastal biotop. We used 6 or 12 h periods for the shaking cycle instead of 6.2 respectively 12.4 h, as found under natural conditions. The tidal rhythm, represented by the shaking periods, would thus slowly drift away from the 24 h rhythm of the light-dark cycle and this could, for a certain span of time, prevent synchronisation to external time cues as we showed under experimental conditions when the shaking rhythm was delayed by three hours relative to the light-dark-cycle.

Internal synchronization was facilitated in denser cultures, as mentioned before. Entrainment to external Zeitgeber might also be facilitated in denser cultures of *Thalassomyxa australis*, and this could be of ecological significance. We have shown that agitation detaches only amoebae in the active stage from the substrate, whereas cells in the resting stage stay attached to the ground. A dense culture would be synchronized by the tides more strongly than a thin culture, and thus a higher percentage of cells would thus be diluted by the tidal waves, because a high percentage of the cells would be swept away in their active stage. In a thin culture, on the other hand, the tides would loosen only a fraction of the cells, since they are not mutually and externally synchronized. The population size could be regulated in this way to a certain degree.

There could, furthermore, be beat phenomena of stronger and lesser synchronization due to the tides which shift in the course of the lunar cycle. Accordingly, more or fewer *Thalassomyxa* cells would be removed from the substrate. Whether

this is found under natural conditions and has any ecological significance can only be answered by observations under field conditions.

We hope to have shown that *Thalassomyxa australis* offers a number of intriguing possibilities to study the timing of the activity cycles of this fascinating organism (Grell 1987), and its inter- and intraspecific interaction perhaps by chemical means.

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