

# System Analysis of the Circadian Rhythm of *Euglena gracilis*, I. Linearities and Non-Linearities in the Response to Temperature Signals

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The approach of control theory is used to describe the structure of the circadian system of *Euglena gracilis*. As a first step we discriminate linear and non linear properties of the dynamics.

The cellular motility as measured via long time records of sedimentation parameters in cultures is defined as the system output; sinusoidal temperature signals are used as input. By means of non stationary signal processing procedures we estimate gain and phase of the output signal. The problem of defining an appropriate gain of a cell suspension with an indefinite number of cells is solved by using the superimposition of two different input signals and by keeping one of them fixed as a reference signal.

Linear properties are shown with a linear frequency transfer and with the validity of the superposition principle at least within distinct regions of amplitude and frequency. Non linear properties are the signal distortion, the restriction of linear amplification to a distinct range of input temperature and the ambiguity of phase coupling near the circadian eigenfrequency.

The apparent lack of a limit of entrainment – an unexpected linear property – is explained by the masking effect of thermokinesis. A model is proposed describing the simultaneous control of motility by thermokinesis and the circadian system. On the base of that model further experiments are outlined.

## 1. Introduction

Circadian oscillations are commonly known as rhythmicities in time series representing physiological data with periods near, but not exactly, by 24 h. They persist in a damped or undamped fashion under conditions excluding periodic signals (Zeitgeber) in the range of circadian frequencies. As compared with chemical oscillations (e.g. Zhabotinski reaction, glycolysis etc.) or biological rhythms with shorter periods (e.g. flagella beats, circumnutations etc.), they are characterized by an extraordinary stability and insensitivity to environmental fluctuations. The homeostasis of the oscillation is the background for its function as a “physiological clock” [1] which serves for a variety of adaptations within the daily and seasonal time scale.

Despite the many attempts that were started in the past two decades to analyse the biochemical mechanism or at least to identify some biochemical essentials for the circadian system, no clear picture has been evolved yet. Under proper conditions

almost all major parts of the cellular metabolism have been demonstrated to play an essential role, and have consequently been incorporated into models (Table I). Altogether, this diversity of results indicates that either a diversity of biochemical mechanisms exists in order to realize the same regulatory structure, or all biochemical subsystems are essentially involved. Without excluding biochemical strategies, the situation demands for completion by an approach adequate to regulatory properties. One approach is to consider mathematical models of self-sustained oscillations which are necessarily nonlinear models. A differential equation is implicitly or explicitly assumed (Table II) to control the observable parameters. Based on experimental data, the model parameters are generally adjusted by computer simulations [16, 28].

In order to verify the obtained model, the typical experiment consists of the search for a point of singularity. The existence of such a point would prove a limit cycle behaviour [26]. This analysis is a kind of identification of the model, but there rests one problem: One does not know whether the performed experiments are sufficient to describe the input-output behaviour of the model. Therefore, this analysis is in a certain sense incomplete. To close this gap, control theory can be applied. Such an ap-

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Table I. Examples which indicate the essential involvement of major cellular sub-systems in the mechanism of the circadian clock of plants.

Cellular sub-system	Experimental evidence	Corresponding general model	
Photosynthesis/plastids	LONERGAN and SARGENT [2]	SEL'KOV [5]	
Respiration/mitochondria	DIECKMANN and BRODY [3]		
Overall energy balance	WAGNER <i>et al.</i> [4]		
Plasma membrane transport	ADAMICH <i>et al.</i> [6]	NJUS <i>et al.</i> [7]	
Transsscription	SCHWEIGER and SCHWEIGER [8]	EHRET and TRUCCO [9]	
Translation	DUNLAP <i>et al.</i> [10]	SCHWEIGER and SCHWEIGER [11]	
Cell cycle	JARRETT and EDMUNDS [12]	EDMUNDS and ADAMS [13]	

Table II. Examples of proposed mathematical models of circadian rhythms and related experimental realization.

Mathematical model		Reference	Experimental realization	Reference
Single oscillator model	van der Pol-oscillator	WEVER [14] MEYER-GUICKING [15]	man	WEVER [16]
	Feedback oscillator	JOHNSSON-KARLSSON [17]	<i>Kalanchoe</i>	JOHNSSON <i>et al.</i> [18]
	General "limit-cycle"-oscillator	PETERSON [19]	mosquito	PETERSON [20]
Multi oscillator model	"clockshop" Two coupled van der Pol-oscillators Several coupled feedback oscillators Several coupled general "limit-cycle"-oscillators	LINKENS [21]		
		GANDER-LEWIS [22]	<i>Weta</i>	GANDER [23]
		KAWATO-SUZUKI [24]	<i>Tupaia</i>	HOFFMANN [25]
	Master slave oscillator model Pittendrigh's A- and B-oscillator	WINFREE [26]	<i>Euglena</i> (and lateron verified with rodents)	PITTENDRIGH-BRUCE [27]

proach consists in starting with a general model which can be considered to correspond to a class of model equations. Now, to each model, there belongs a class of experiments which are known to be sufficient to identify the explicit structure of the model and its parameters. It is surprising that hitherto no systematic application of control theory was used in analysing circadian rhythms as has been performed for the analysis of membrane transport functions [29], for instance.

As a first step towards a model identification of a circadian rhythm, we discriminate linear and non-

linear parts of the unknown structure. Such an experimental analysis has not yet been performed besides the attempt to identify only the linear part of the circadian system. This was done with the investigation of light responses of leaf movements of clover [30].

With the present contribution, we analyse the circadian responses of motility of the unicellular algae *Euglena gracilis* to temperature signals. The temperature signal was chosen because some information is available about the influence on cellular motility of *Euglena* with respect to circadian rhythm-

micity [31] as well as with emphasis of the mechanism of motility [32].

## 2. Material and Methods

### 2.1. Organism and culture technique

We used *Euglena gracilis*, strain 1224/5-9 from the Algensammlung Göttingen (FRG). This strain is fully equipped with pigments and known to exhibit well expressed circadian oscillations of motility. Since phase responses to temperature steps are more stressed in stationary photo-lithotropic populations [31], the cells were grown in the following mineral medium: KCl 2.8 mM,  $\text{NH}_4\text{NO}_3$  35.7 mM,  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$  0.77 mM,  $\text{KH}_2\text{PO}_4$  7.4 mM,  $\text{CaSO}_4 \cdot 2\text{H}_2\text{O}$  0.5 mM, Fe-EDTA-Komplex 10.0  $\mu\text{M}$ ,  $\text{H}_3\text{BO}_3$  0.2 mM,  $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$  36.6  $\mu\text{M}$ ,  $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$  3.0  $\mu\text{M}$ ,  $\text{MoO}_3$  0.6  $\mu\text{M}$ ,  $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$  1.2  $\mu\text{M}$  and a vitamine B mixture (Iloban, Merck) of 0.1 ml/l.

The cultures were grown in 1.5 l bottles at room temperature and at ca. 2000 lux continuous light (OSRAM "Fluora"). The bottles were aerated with  $\text{CO}_2$ -enriched air. At a density of approximately  $10^6$  cells/ml batches of 150 ml were transferred into the test cuvettes ( $5 \times 5 \times 10$  cm). The cuvettes were sealed with cotton stoppers to keep axenic conditions throughout the test.

### 2.2. Test for rhythmicity of the cellular motion

The test for motility is based on the sedimentation of cells in populations [33]. When kept in darkness the cells settle down to an equilibrium which is characterized by a vertical density gradient. The higher the motility of the cells, the less the sedimentation, the smoother the equilibrium density

gradient in the vertical axis of the cuvette. Thus the change of the optical density along a horizontal axis 15 mm above the bottom of the cuvette describes changes in the average motility in that way that an increase in the optical density indicates an increase of motility (Fig. 1).

The test light is offered in parallel beams from one side of the cuvette. It simultaneously serves for the energy supply of the cells. The test beam is selected by a photodiode, mounted 15 mm above the bottom on the opposite side on the cuvette. In order to prevent interactions of the test light with circadian parameters, the test light is offered in a regular rhythm of either 100 min darkness: 20 min light or 33.3 min darkness: 6.7 min light provided by slide projectors (ca. 4000 lux). Such a light program has already been used by Pohl when introducing *Euglena* in circadian rhythm research [34]. This short light-dark program is known not to alter the free-running period of *Euglena* [35]. Continuous light however, would vanish the observable circadian rhythm. The optical density is measured automatically at the beginning of each light pulse and digitally recorded. Since these values indicate the sedimentation equilibria reached at the end of each darkness, they represent the time history of motility in darkness. We could show that the dark interim is long enough to avoid long lasting influences of light stimuli on the equilibrium of cells at the end of the darkness.

### 2.3. Temperature programs

The test cuvettes are mounted in temperature controlled water bathes. Temperature is controlled by means of an optomotoric regulatory system with the outputs heating and cooling (Joens, Düsseldorf,

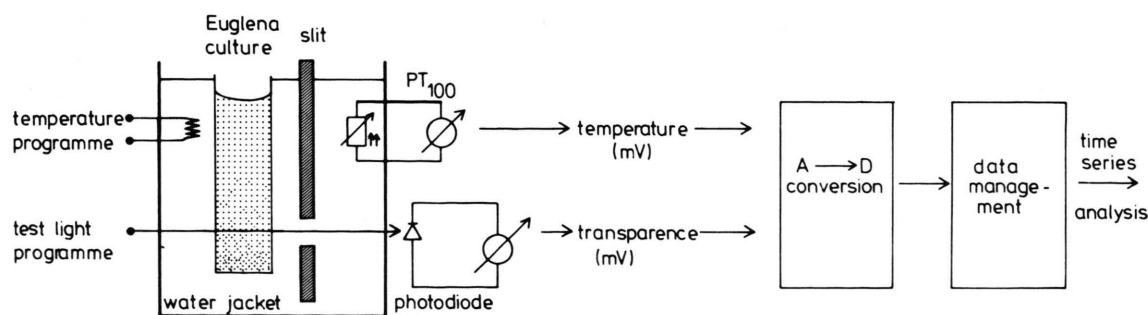


Fig. 1. Experimental device and data processing for the system analysis of the circadian rhythm of cellular motility in cultures of *Euglena gracilis*. Sinusoidal temperature signals are used as input.

FRG). Any temperature program with changes not exceeding ca.  $0.2^{\circ}\text{C}/\text{min}$  may be applied by that device. This is no restriction for our purpose. Since we want to perform a system identification approach on the circadian system, we are mainly interested in periodic temperature signals with period lengths from about 5 h to 60 h. The lower bound is dictated by the applied light-dark program (sect. 2.2.), whereas the upper bound is given by the natural requirement of finite time experiments. The good performance of the used regulatory system allows us even to use sinusoidal temperature signals which ease the evaluation of the experiments. In the language of circadian rhythm analysis, this reads as performing entrainment experiments using sinusoidally varying temperature as the input signal which is called a cue (Zeitgeber), if entrainment takes place.

The temperature programs are first plotted by means of a computer program, and then copied to a tape for the optomotoric processing.

#### 2.4. Data processing and analysis

Data was sampled using 24 channels, each channel representing the data of one cuvette. The 24 channels are grouped into 4 sets of 6 channels. Each set was treated by one temperature program. Applied temperature has also been sampled by using an additional channel for each set. Data analysis was performed in an off-line mode. Using the facilities of an IBM 370/168 at the "Regionales Hochschul-rechenzentrum" of the University of Bonn, we stored all the results in a data pool. This allowed us either to analyse each channel per se or to group the data being produced under the same experimental conditions.

Each data analysis of entrainment experiments in circadian rhythm analysis requires a decision whether a synchronization between input signal and system output has been achieved. This is a problem which can be solved by using non-stationary signal processing [36]. The key procedure is complex demodulation which allows to estimate time spans where input and output oscillation are locked on, and what is most important, to estimate the duration of the transient time before locking on takes place [37]. Thus, we determined the range of steady state entrainment, and at the same time, we were able to decide whether an entrainment was present or not.

Then, we had to estimate the parameters of the output signal as amplitudes and phases of the

Fourier representation of the observed periodicity. All this analysis was performed by using TIMES-DIA, an interactive computer program system especially equipped for the analysis of periodic time series [38, 39].

#### 2.5. Analysis of oscillators using control theory

The standard nonlinear feedback system to model self-sustained oscillations is given in Fig. 2. In the simplest case, this is a univariate system. The nonlinear element may depend on both, amplitude and frequency of the applied input signal. This structure is known to present various types of nonlinear differential equations depending on the choice of the nonlinearity and/or the linear part of the feedback loop [40].

In order to identify the linear and nonlinear part actual being present in the circadian system under investigation, we applied sinusoidal input signals (sect 2.3.) with varying frequency and amplitude. A variation of the amplitude of the input signal with a fixed frequency allows to estimate the nonlinear part, whereas a series of input signals with varying frequency, but constant amplitude is necessary to identify the linear part. The determination of the linear part consists in estimating its transfer function.

In our analysis, we use a technique proposed by Strobel [41], which we have implemented as a computer program.

Data necessary for such an analysis, are the amplitudes of the output signal and the phase difference between output and input signal. To determine phases and amplitudes, we used the first harmonic of the Fourier series representation (sect. 2.4).

#### 2.6. The problem of a meaningful amplitude of motility in darkness

Our approach of system identification requires a reliable description of phase and amplitude of the signal. Whereas the determination of a phase offers

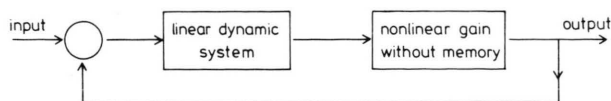


Fig. 2. The simplest nonlinear feedback loop which is capable to be a self-sustained oscillator. In this case, the input is a constant.

no major problem, there is some doubt, however, on the meaning of the amplitude of the observed biological signal. It depends on several factors such as electrical amplification, number of cells involved, etc. such that the read-off values cannot be easily related to the actual behaviour of a population of cells. To overcome this problem we intended the following "relative amplitude". Assuming an additive (linear) superimposition of responses to two simultaneously applied temperature signals: the amplitude of one of the responses may be taken as reference for the amplitude of the other one. By keeping one of the temperature cycles constant, *i.e.* fixing amplitude and frequency to a constant value, throughout a series of experiments, the amplitude of the varying one can always be expressed as related to the constant one, and thus, serve as a reliable measure of a relative amplitude. The assumption of a linear superimposition, however, has to be checked and will be discussed lateron.

As a reference period we used 24 h, since we assumed to achieve the most stable resonance at that input period.

### 3. Results

Analysing a nonlinear relationship by methods of control theory needs the linear behaviour of the system in certain ranges of period and amplitude.

#### 3.1. Frequency transformation

Most important is that the system does not change the period of the input signal. This is not evident for the circadian system, since tests with forcing signals showed that only in some period domain output signals are synchronized by the input period (range of entrainment), far from which either no periodic output is found or the system starts self-sustained oscillations with its eigen period [42]. While these effects exhibit at least a clearly defined output frequency, Wever [43] reported virtual synchronization (relative coordination or relative entrainment)

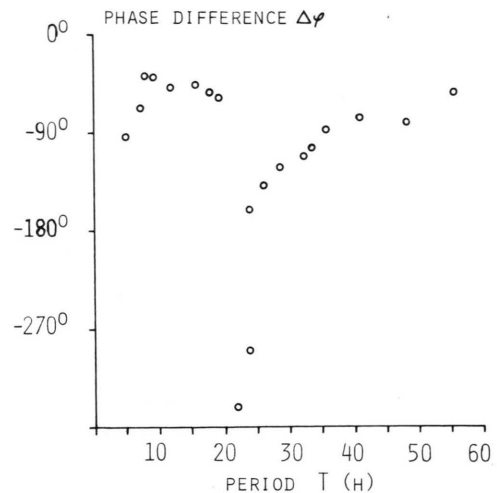


Fig. 3. Phase differences between the rhythm of motility and the input signal (temperature). Mean temperature was 23 °C, the amplitude  $A^{\text{in}} = 5$  °C. Excitement by one single temperature sinus was performed.

towards the limits of entrainment: Quasi-periodic output was found, but neither the input period nor the eigen period of the organism.

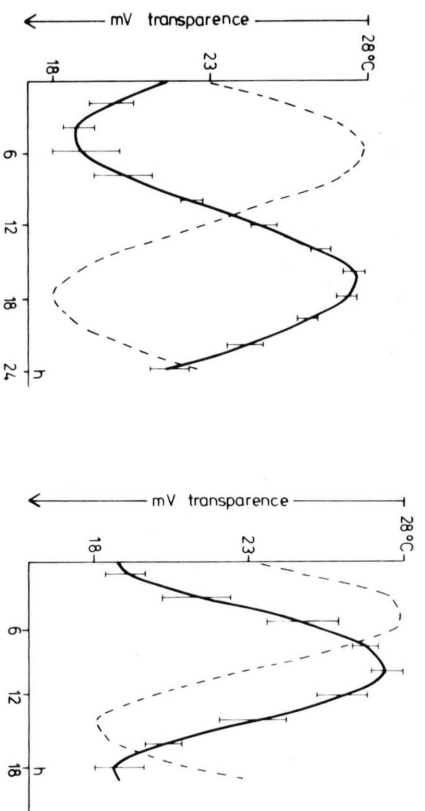
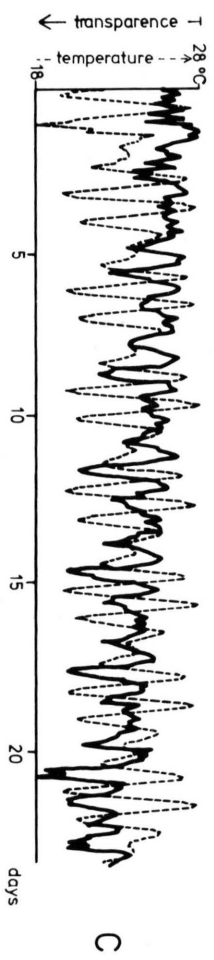
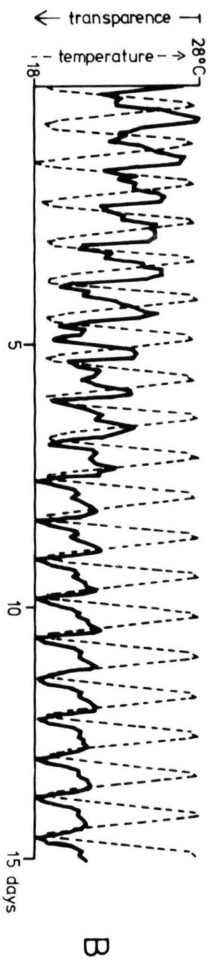
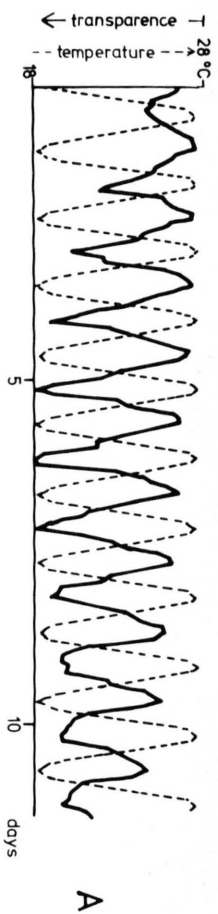
In *Euglena*, we excited the motility with sinusoidal temperature signals

$$\vartheta(t) = \vartheta_L + A^{\text{in}} \sin(2\pi t/T^{\text{in}})$$

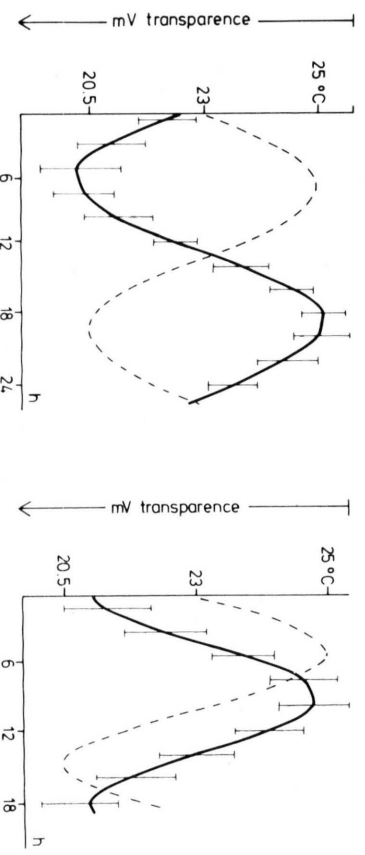
in the range  $4.8 \text{ h} \leq T^{\text{in}} \leq 55.7 \text{ h}$ ,  $\vartheta_L = 25$  °C,  $A^{\text{in}} = 5$  °C. Fig. 3 shows the phase response of the motility of *Euglena* to these exciting input signals.

It is essential that there is  $T^{\text{out}} = T^{\text{in}}$  over the whole range of applied input periods without detectable limits of entrainment. This strongly differs from results which have been obtained by using light as an input signal [35]. As we do not know whether this effect is a response of the circadian system alone – the temperature dependence of motility will certainly contribute to the overall output – we prefer the expression 'exciting' instead of 'entraining'. Possibly the range with remarkable phase changes (18 h to 30 h) characterizes the circadian response. This would be in correspondence with phase plots of oscillating linear systems. But because of the lack of

Fig. 4. Transparence of *Euglena* cultures (full line) excited by temperature sines (dotted line) with the corresponding average signals. Transparence is plotted on an inverted scale, *i.e.* maximal transparence gives minimal motility. A) Excitement by single temperature sine with  $T^{\text{in}} = 24 \text{ h}$ ,  $A_{24}$  the corresponding average signal; B) Excitement by single temperature sine with  $T^{\text{in}} = 18 \text{ h}$ ,  $B_{18}$  the corresponding average signal; C) Excitement by two additively superposed temperature sines with  $T_0^{\text{in}} = 24 \text{ h}$ ,  $T_1^{\text{in}} = 18 \text{ h}$ , and amplitude ratio equal to one;  $C_{18}$  the average signal of the 18 h component;  $C_{24}$  the average signal of the 24 h component. The average signals represent the first harmonic of the calculated Fourier-series. Error bars show 95%-*t*-confidence intervals.



**B<sub>18</sub>**



**C<sub>24</sub>**

amplitude behaviour, system identification is not yet possible with this data.

### 3.2. Superimposition

For obtaining amplitude information, we prefer to measure relative amplitudes (*cf.* sect. 2.6) by superimposing two sinusoidal temperature signals

$$\vartheta(t) = \vartheta_L + A^{\text{in}} \sin(2\pi t/T^{\text{in}}) + A_0^{\text{in}} \sin(2\pi t/T_0^{\text{in}}).$$

We have to prove whether the response of motility is the linear superimposition of the single excited responses. Hitherto we can check this only

by looking for the periods and phase responses, namely whether both periods appear in the output signal, and whether the phase differences change when the input periods are superimposed. Fig. 4 shows the identical processing of the single signals compared with the components of the superimposition. This experiment was made with  $T_0^{\text{in}} = 24$  h,  $T^{\text{in}} = 18$  h,  $\vartheta_L = 23^\circ\text{C}$  and  $A^{\text{in}} = A_0^{\text{in}} = 5^\circ\text{C}$ . Because of the experimental expense, we proved only one period  $T^{\text{in}}$  for the present. This further linear property encouraged us to look for more linearities in the system.

### 3.3. Variable input amplitudes

In pure linear relationships, amplification and phase shifting must be independent from the input amplitude. Varying temperature amplitude in a range from  $0.5^\circ\text{C}$  to  $6.0^\circ\text{C}$  (Fig. 5) with  $T_0^{\text{in}} = 24$  h,  $A_0^{\text{in}} = 1^\circ\text{C}$  and  $T^{\text{in}} = 17$  h for all applied amplitudes  $A^{\text{in}}$  does result in dramatic phase changes of the output signal.

But if one tests the slope of a regression line fitted to the measured phases of the output signals (Fig. 5), the hypothesis of a constant phase over the whole range of applied input amplitudes is to be rejected. Only if the ratio  $r := A^{\text{in}}/A_0^{\text{in}}$  is included in the interval  $0.5 \leq r \leq 2$ , the system's action can be adequately described by a linear model.

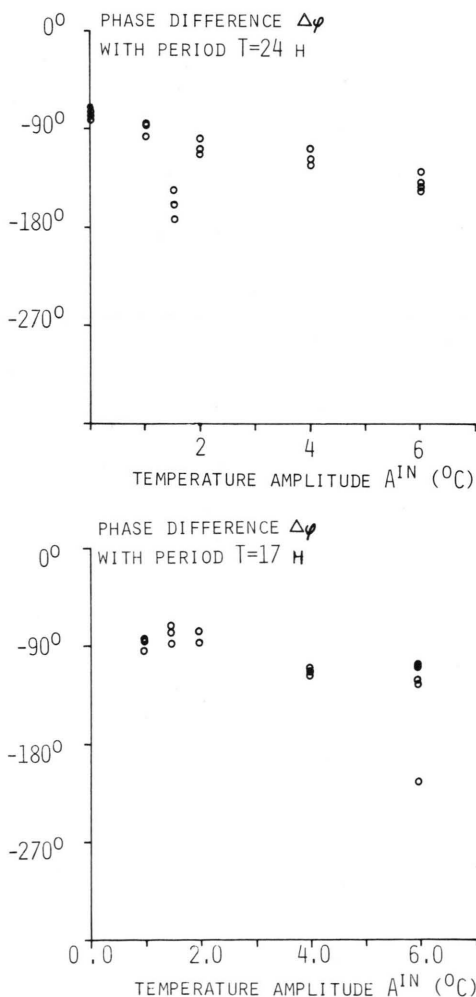


Fig. 5. Phase differences between the rhythm of motility and the input signal (temperature) in dependence of the amplitude of the temperature signal. Excitement was done by two additively superposed temperature sines with  $A_0^{\text{in}} = 1^\circ\text{C}$ ,  $T_0^{\text{in}} = 24$  h,  $T^{\text{in}} = 17$  h, varying  $A^{\text{in}}$  and  $\vartheta_L = 23^\circ\text{C}$ .

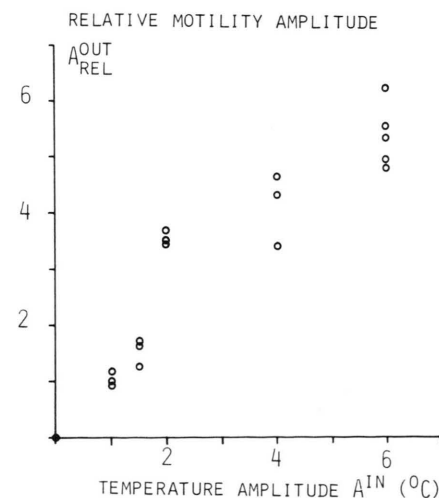


Fig. 6. Relative amplitude of motility in dependence of the amplitude  $A^{\text{in}}$  of one of two additively superposed temperature sines with  $A_0^{\text{in}} = 1^\circ\text{C}$ ,  $T_0^{\text{in}} = 24$  h,  $T^{\text{in}} = 17$  h and  $\vartheta_L = 23^\circ\text{C}$ .

### 3.4. Frequency spectrum

At this point, we could assume that it would be possible to measure the linear part of the clock's control system by using double exciting temperature signals with small amplitudes. So, we started with taking up amplification and phase responses over the whole range of periods (cf. Fig. 3 and sect. 2.3).

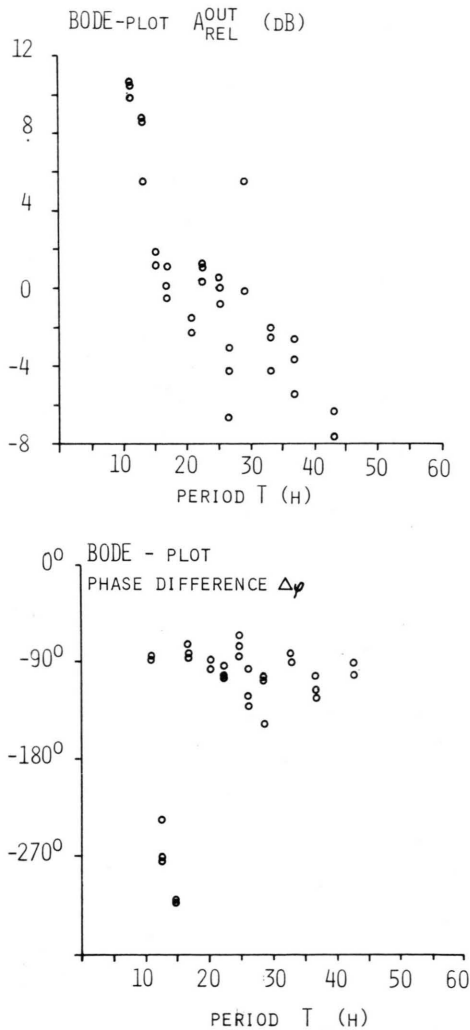


Fig. 7. Gain and phase of motility excited by two additively superposed temperature sines with  $\vartheta_L = 23^\circ\text{C}$ ,  $T_0^{\text{in}} = 24\text{ h}$ ,  $A_0^{\text{in}} = 1^\circ\text{C}$  and varying  $T^{\text{in}}$ . A) Relative output amplitude representing the motility rhythm on a dB-scale. B) Phase difference between the rhythm of motility and the temperature sine defined by  $A^{\text{in}}$  and  $T^{\text{in}}$ . Each point corresponds to one single experiment. Shifting the definition of phase  $0^\circ$  by  $180^\circ$  would allow to interpret the system as a differentiator. The physiological interpretation of such a phase definition will be discussed in a forthcoming paper.

We used amplitudes  $A^{\text{in}} = 1^\circ\text{C}$  for both periods  $T^{\text{in}}$  and  $T_0^{\text{in}} = 24\text{ h}$ . The test light rhythm was chosen to be 33.3 min D: 6.7 min L in order to obtain a better frequency resolution. The result is presented in Fig. 7. Even if we take into account certain technical artefacts, there is no hint for a comprehensible frequency behaviour. Also analysing the data by Strobel's procedure [41] for estimation of transfer function yielded no further clarification.

### 3.5. Ambiguity under double exciting conditions

As we could not find a dependence between nonlinear distortion and applied period (Fig. 8), we looked for a period dependence in the amplification and phase response of the 24 h reference  $T_0^{\text{in}}$  caused by the test temperature component  $T^{\text{in}}$ .

Thus, we examined the phase response of the  $T^{\text{in}}$ -component. If the assumption of linear behaviour under the chosen conditions is true, the phase differences of the 24 h period should be the same over the whole range of periods. Obviously, this is not the case (Fig. 9). This demands for further experiments in order to locate ranges of low ambiguity.

### 3.6. Obtained linear and nonlinear properties

We have the situation of some remarkable linearities:

- input period equal to output period,
- superimposition option,
- phase constance in spite of varying amplitude.

Unfortunately, these properties are not yet sufficient for a successful system identification. The nonlinear properties we could observe are:

- distortion,
- nonlinear amplification,
- ambiguity of two given periods.

Although nonlinearities of this kind are not surprising for a self-sustained oscillator, we did not expect the ambiguity property when starting the series of experiments. Thus, the presented results cannot be assembled to a proposable model mechanism as has been successfully done by Robinson [30] for the linear characteristics of their studied circadian system, but we have to demand for additional experiments.

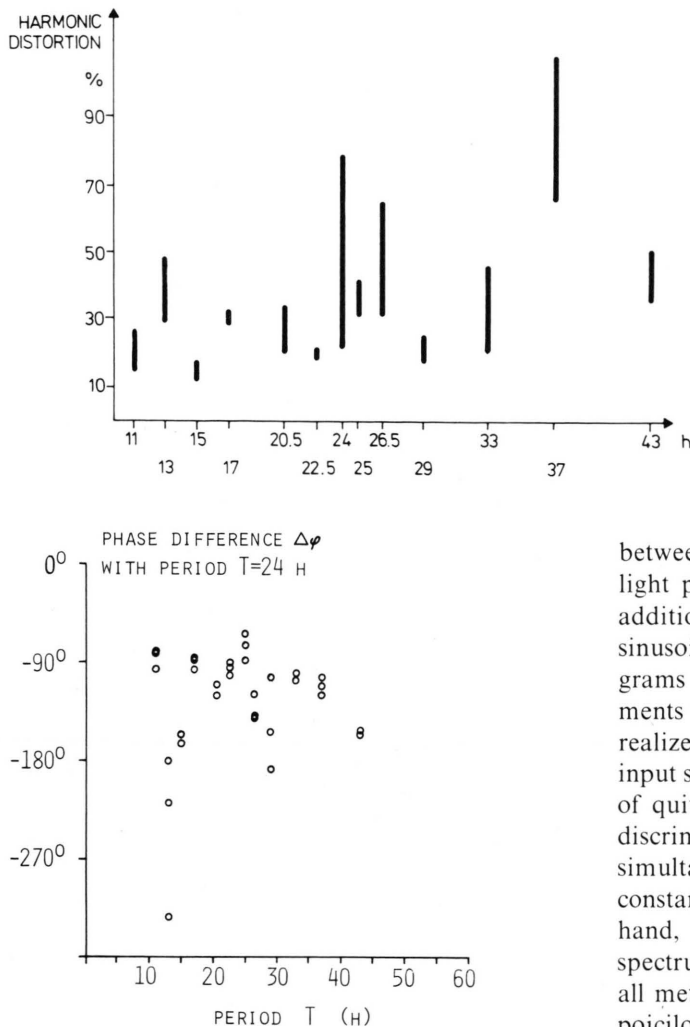


Fig. 9. Phase difference between the rhythm of motility and the temperature sine defined by  $A_0^n$  and  $T_0^n$ , but here in dependence of  $T^n$ . The represented data points correspond to the same experiments as shown in Fig. 7. The phase jump at  $T^n = 12$  h may be due to an interaction between both input signals, since  $2T^n = T_0^n$ . The same argument may be applied to the phases shown in Fig. 7. This has to be checked by further experiments.

#### 4. Discussion

##### 4.1. General responses to temperature

Our approach to analyze the circadian system of *Euglena gracilis* is based on responses of the cellular motility to temperature signals. Although light clearly entrains the circadian rhythm of *Euglena* [35] and therefore could be used as an input variable in a similar way, we favoured temperature for technical and analytical reasons. We feared severe interactions

Fig. 8. Harmonic distortion of the output signals representing motility of *Euglena*. The shown results correspond to the experiments exhibited by Fig. 7. Here, we have not indicated each experiment by one point, but we use a bar to represent the range of harmonic distortions we have observed considering all experiments.

between the light program as an input signal and the light program necessary for testing the motility. In addition, it was more convenient for us to apply sinusoidal temperature waves than sinusoidal programs of light intensity with constant color. Experiments with pulse or step-signals which can be easily realized with light must be excluded because such input signals would simultaneously stimulate systems of quite different time constants which we want to discriminate: In the circadian literature, the simultaneous response of systems with different time constants is known as "masking effect". On the other hand, we wanted an input signal with a broad spectrum of effects. Temperature enhances almost all metabolic fluxes and physiological processes of a poicilothermic organism and still has specific effects on the circadian clock.

Preceding experiments have shown that the circadian phase of *Euglena* strongly responds to temperature steps [31, 33]. The phase shift depends on the input phase of the step, but is independent of its heights (Fig. 10).

The lack of correlation between the responses of phase and amplitude can be interpreted by two different models: either by assuming a superposed additive response of two separate systems (differential response of the circadian phase with low threshold and the cellular motility due to thermokinesis acting like an integrator) or by assuming one limit cycle system the state variables of which are set to different concentric trajectories with identical time shifts ([26], pg. 133 ff.). Without a predecision on the model, we started our investigation with the working

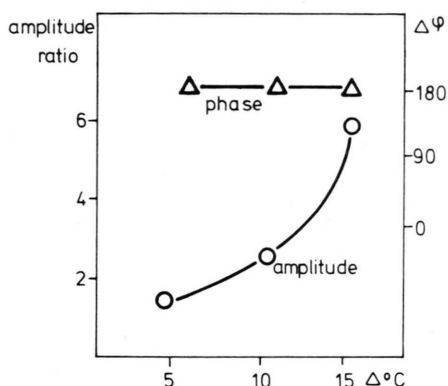


Fig. 10. Phase response and relative amplitude of the circadian rhythm of motility of *Euglena gracilis* after the input of temperature steps of different heights. All steps were applied at the minimum of the free running rhythm. The phase is inverted in all cases whereas the amplitude ratio depends on temperature. The lack of correlation of both parameters indicates two different response systems: a threshold response of the circadian phase and an analog response of the maximum motility of the cells due to thermokinesis. Data redrawn from [33], Fig. 4.

hypothesis that the circadian system transfers temperature signals mainly in a linear way, but expected some non-linearities, for instance a limit of entrainment towards shorter temperature periods.

We were astonished, however, that *Euglena gracilis* is fully excited by all applied temperature periods from 4.8 to 56 h (Fig. 3). There is no indication of a limit of entrainment. Moreover, the response amplitude increases monotonously with decreasing temperature periods (Fig. 7a). A resonance peak as expected at the eigen-frequency of 23.6 h (this is the averaged free running period length of motility of autotrophic *Euglena gracilis* [31]) cannot be identified. Those resonances, for instance, are demanded for van der Pol-type oscillators [44].

But, in contrast to this result, the experiments with a single sinusoidal input signal reveal a phase jump and even a phase uncertainty in the range of the eigen-frequency. These contradicting results rise the problem that the circadian responses may vigorously be masked by direct temperature influences on the motility ("thermokinesis"), as shown in the diagram

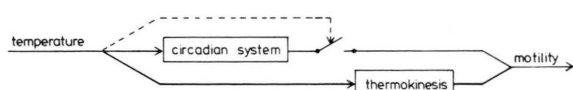


Fig. 11. Graphical representation presently modelling the dependence of motility of *Euglena gracilis* on temperature. The dashed connection indicates that the switch operates in dependence of the applied input period length.

(Fig. 11). Thus, our primordial idea of applying sinusoidal input signals in spite of pulse or step signals turns out to be not sufficient to solve the problem of masking.

The existence of thermokinesis has directly been verified by laser light scattering experiments [32]. In terms of our model, masking is to be interpreted in the following way: Either the circadian system is even suppressed at certain period ranges or it is disconnected from entering motility which does not exclude that a circadian organization still operates in other cellular subsystems.

At the present state, we cannot decide what type of model is correct. Additional information will come from experiments in which the temperature excitement is stopped. Non-appearance or re-appearance with changed parameters immediately after releasing from the temperature program will indicate whether there have been interactions with the circadian system. Those experiments will be discussed in comparison with light responses in a forthcoming paper.

To close this subsection, we should point out that suppression of the circadian system includes also the case that the output has an undetectable low amplitude. Since we have used very sophisticated signal processing procedures, we know that in this case, the contribution of the circadian system to the overall response can be neglected without committing a severe error.

#### 4.2. Linearities and non-linearities

An indicative test for linearity is the superposition of the input signals and checking whether the output is also a simple superposition of the single output signals. The response to a superposition of two temperature signals with  $T_0^{\text{in}} = 24$  h and  $T^{\text{in}} = 18$  h with equal amplitudes  $A_0^{\text{in}} = A^{\text{in}} = 5^\circ\text{C}$  undoubtedly indicates a linear signal transfer (Fig. 4): The two components of this superposition experiment as identified using time series analysis are statistically identical with the corresponding average signals of the single excited cases. From that experiment, we justified the use of a reference signal of 24 h, because we expected the resonance peak of the dynamic system at that frequency, but as shown in Fig. 7a, that suspicion turned out to be wrong.

A further test for linearity consists of looking for linear gain. This cannot hold for the whole range of physiological applicable amplitudes as indicated by

Fig. 10, but as shown in Figs. 5 and 6, the prediction holds up till approximately double the reference amplitude. Beyond that limit the data cannot be fitted by a linear model. The deviation from linearity in the amplitude gain does not contradict to the linearity property as revealed from the superimposition test (Fig. 4), because the amplitude ratio of the two components of the superimposition has been 1, and this value falls into the range of the system's linearity.

A comparison of both results reveals that the linear range seems not to be restricted by the absolute amplitude, but by the amplitude ratio (*cf.* sect. 3.3) of simultaneously applied temperature signals, because the absolute amplitude of the superimposition test with linear behaviour (Fig. 4) has been double the absolute amplitude beyond which non-linearities arise in the linear gain test (Fig. 7a). This suggests that the circadian system normally adapts to linear operations. The linear range is passed over in cases in which adaptation is demanded for simultaneously offered environmental signals which are too far apart from each other. It may well be that the threshold amplitude ratio for passing the linear range is correlated with the frequency difference of the simultaneously applied signals. In any case, to clarify this situation, the information of further experiments is necessary, and we do know that our experiments do only tell one aspect of the "truth" in restricting to the range of linear behaviour.

The core of our investigation should be an identification of the linear part of the circadian system by measuring the transfer function of this linear system (Bode-diagram) as represented in Fig. 7. But before having solved at first the masking problem, we are not able to identify poles and zeros of the linear part of the circadian system. Thus, at the present state, we restrict our discussion on the linear and non-linear properties which we were able to discriminate up to now. The measured frequency transfer function of Fig. 7b does not fit any linear model although an amplitude ratio of one has been used for the superimposition of both temperature input signals. Obviously, the deviation from linearity depends on the frequency difference of both superimposed input signals: There is a strange difference in the two phase plots of Fig. 3 and 7b, and the principal resonance peak seems to be shifted by about 10 h to shorter periods in Fig. 7b. Thus, the

action of the reference signal may have influenced the action of the other one. This again indicates a "strong" non-linearity and supports the adaptation hypothesis mentioned above.

One of the most surprising results is the corresponding amplitude plot of Fig. 7a. The lack of resonances and limits of entrainment in the amplitude response have already been discussed. The increase of gain with decreasing period suggests a differential signal transfer, but it does not contradict to a non-linear interpretation. From the observed frequency transfer properties, we conclude that the diagram of Fig. 11 describes the actual situation. Our working hypothesis is the following: temperature cycles stimulate the motility of *Euglena gracilis* via thermokinesis and via transfer through the circadian system. The thermokinesis system is supposed to act linearly in a broad range, the transfer by the circadian system is linear within adaptational limits, but it is easily pushed into non-linear ranges by passing to situations which do not allow the system to adapt. Due to this model, both transfer processes contribute to control the apparent motility. The type of combination is not yet clear, but since the contribution of the circadian system for certain input period lengths is not observable even if we have applied sophisticated signal processing procedures, we suppose the existence of a switch which disconnects the circadian control from motility. This does not exclude the possibility that the circadian organization might even be lost under such extreme environmental conditions; this will be discussed in a subsequent paper.

We must acknowledge that because of the present huge masking effect, the excitement by temperature signals might not be appropriate for a system analysis. On the other hand, we fear that similar problems arise when exciting by light because of masking by photokinesis. The analysis of the circadian system of clover exhibited masking by photonastic responses of the leaf movements [30]. Therefore, in the following subsection, we consider further strategies for proceeding in revealing the dynamic properties of the circadian system of *Euglena gracilis* by anticipating the results of this investigation.

#### 4.3. Further experiments

The best way to avoid masking would be to cut off the masking process. Unfortunately, this is not possible as far as the test for circadian rhythmicity is

based on cellular motility. Other available parameters are either not suited for an automatic long time record (metabolic activities, chemical composition etc.) or indirectly related with motility (sticking [45]).

Thus, the circadian system of *Euglena* cannot be tested without masking. It appears possible, however, that the masking process itself can be subjected to a system analysis without involvement of the circadian system. Continuous light disconnects motility from circadian control [31, 35]. This does not necessarily mean that the circadian system completely vanishes – there are hints that the circadian rhythm of sticking persists in continuous bright light [46]. A comparison of the results of a system analysis performed with temperature signals in continuous light with the system analysis presented in this paper should clarify how thermokinesis and circadian transfer contribute in controlling the apparent motility, and thereby help to interpret the Bode diagram of the complete system (Fig. 7).

Another line of investigation which we tend to follow is a combination of light and temperature signals. A step down in light similarly sets the circadian phase as a step up in temperature. Both

signals, however, have contrary effects on the motility. An appropriate combination of these signals as calculated from the individual step-phase-response curves should minimize the masking, but rises the new problem of how the two different signals enter the circadian system and interact. On the line of such an investigation, our next step will therefore be a comparative study of the excitation of *Euglena gracilis* by both light and temperature signals.

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