System Analysis of the Circadian Rhythm of *Euglena gracilis*, II: Masking Effects and Mutual Interactions of Light and Temperature Responses

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Motility of *Euglena gracilis* shows free running circadian rhythms. The circadian system is sensitive to light and temperature signals, but it is always masked by direct responses of motility to light (photokinesis) and temperature (thermokinesis). By means of a compartimental model which defines the interrelations between the pathways of thermokinesis, photokinesis and the circadian system a unifying view of effects of temperature and light input signals is outlined. According to the model, and using double sine input signals the dynamics of thermokinesis is described by a differential amplifier with constant gain.

Although thermokinesis heavily masks circadian responses to temperature signals, the limited range of circadian entrainment is indirectly demonstrated by a limited reappearance of free running circadian oscillations after stopping the temperature program. Free running circadian oscillations do reappear only after pretreatment with temperature periods near the circadian eigentperiode.

A white mutant lacking photosynthesis is used to investigate the role of photosynthesis in the signal processing. Although light synchronizes the circadian rhythms of the white mutant if applied as single input, it does not affect the motility if applied together with temperature inputs near the circadian eigentperiode. These results indicate frequency dependent mutual interactions between the model compartiments.

1. Introduction

Our analysis of the circadian system of *Euglena gracilis* was started with the discrimination of linear and non-linear properties in the response of motility to temperature signals [1]. In that approach, an unexpected broad range of linearity was identified suggesting the involvement of thermokinesis responses. In the present contribution we manifest this suggestion to be true: Our special method of using two superimposed temperature signals in order to achieve reliable measurements of amplitudes turns out to suppress the appearance of non-linear properties of the circadian system in favour of linear properties of a "masking" by thermokinesis. According to Wever (2, chapt. 4.3.2), we denote by "masking" the direct influence of an external stimulus on a variable without any relation to the circadian system. Masking effects of light are well known since Aschoff's warning [3], and when studying circadian rhythms by means of entrainment experiments, the advice has always been to avoid masking by an appropriate experimental setup if realizable; but nobody paid attention to the masking process itself.

In our study, masking is inherent to the experimental procedure, *i.e.* it cannot be avoided. However, the systems "thermokinesis" and "circadian oscillator" may be separated, if they are interpreted as compartments of a functional network. In order to characterize these compartiments, we compare the results obtained with two superimposed sinusoidal temperature inputs with those of single temperature inputs. The network structure can be extended to the entrainment by light signals by adding a compartiment "photokinesis" (Fig. 6).

In contrast to the response to temperature signals, light-dark periods entrain the circadian system of *Euglena* only within the limits of roughly 16 \( \leq T \leq 48 \) h [4]. At shorter periods the circadian oscillation jumps back into its free running period which then is superimposed by the higher frequent photokinesis responses. Unfortunately, the circadian system cannot be identified by using these old data;
it will be subjected to a detailed investigation of light responses in a forthcoming project.

The proposed structure of interrelations between light and temperature effects on the motility of *Euglena* principally offers the opportunity of investigating mixed stimulation by both light and temperature. As an important assumption for those experiments we show in the present paper that photostimulated motility (*i.e.*, motility measured under a constant level of light instead of darkness) responds to temperature signals in the same way as motility in darkness does. Thus, light does not directly affect the response to temperature. Mixed excitement by light and temperature signals should help to identify substructures of the circadian compartment especially in cases of interactions between both signal transfer lines. In a set of additional experiments we therefore demonstrate that mutual influences exist indeed.

Further steps of investigation will include the manipulation of the biological system (*i.e.* by mutation), in order to identify the physiological meaning of the structures. In that sense, a white mutant of *Euglena* is demonstrated as an appropriate tool to illuminate the role of photosynthesis dependent steps in the response to light and temperature.

2. Definition of motility responses of *Euglena*

Our main biological parameter measured via the sedimentation behaviour of cell populations is called random motility in darkness [5–9]. Since the circadian control of random motility in darkness represents a scalar control of speed it files under kinesis responses [10]. Because of the endogenous character of the circadian control we will therefore define that kind of motility as "AUTOKINESIS" according to the general rule that the controlling factor is used as a prefix (photo-, thermo-, chemo-) to the type of motility response (-kinesis for scalar, -taxis for vectorial responses). In principle we follow the terminology of Dien [11], which is based on the first definitions introduced by Strasburger [12].

3. Materials and Methods

We used *Euglena gracilis*, strain 1224/5–9 (Algensammlung Göttingen, FRG), which is fully equipped with pigments and known to exhibit well expressed circadian rhythms of motility [5]. Details of culture technique, the method to measure motility by means of the sedimentation behaviour of the cells and the data processing and analysis are given in [1]. In order to include the measurement of light influences on motility the method was supplemented in the following two ways (experimental set up shown in Fig. 1):

1) In addition to the already presented measurement of optical densities at the beginning of each test light pulse (= OD I), optical densities were also taken at the end of the test light pulses (= OD II). The signal of OD I represents autokinesis, the signal of OD II represents motility influenced by the preceding test light pulse. Since directional influences of light on cellular movements (swimming towards or off the light source) cannot be discriminated by the recording system, only changes of the vertical gradient of population density are recorded. These changes are due to scalar effects of light on the cellular speed and therefore represent a level of motility stimulated by a response of photokinesis. The chosen length of the test light pulses in all cases exceeded the transition time of photokinesis responses [13, 9]; thus stationarity of photokinesis responses can be assumed in the present data. Fig. 2 shows the example of two simultaneously taken free running circadian oscillations of motility in darkness and light. The frequency analysis indicates no significant differences.

2) Independent of the test light rhythm (*i.e.* halogen projector lights PHILIPS 150 Watt, ca. 6000 lux at the inrance into the cuvettes, applied mostly in a light: dark period of 20:100 min) a second light program has been added in order to excite the circadian system and to test the transformation of light. The exciting light program (PHILIPS-TL, 2 x 40 Watt, ca. 1000 lux at the inrance of the test cuvettes) was also applied as a periodic flip flop of light and darkness with periods as indicated but always exceeding 12 h. For the combined excitement by light and temperature signals we used input periods *T* of 18/24 and 31.75/31.75 h for light/temperature. These periods range within the limits of circadian entrainment by light of *Euglena* [4].

4. Masking by thermokinesis and effects of temperature on the circadian system

The motility of *Euglena gracilis* clearly exhibits selfsustained circadian rhythms. The usual model of those rhythms is a non-linear oscillator. Normally
non-linear oscillators synchronize with external signals ("entrainment") only within a restricted frequency band centered around their eigen-frequency and indicated by resonance phenomena [14, 15]. However, as we have shown in the preceding paper [1], motility of Euglena transforms temperature sine waves in an one-to-one-mode over the total range of applied periods between ca. 5 and 55 h with no indication of resonance. Besides a slightly non-linear amplification beyond a threshold amplitude and a distortion of the shape in the output signal, the only hint for an involvement of non-linearity was shown to be an ambiguity in the phase response at the expected resonance near the circadian eigenfrequency.

In this situation one might conclude that the circadian system is not at all entrained and that motility is excited without any observable involvement of the circadian compartment. But this cannot be true, since if releasing motility from excitation by temperature signals, the free running circadian rhythm immediately reappears if the frequency of the input signal is near to the circadian eigen-frequency (Fig. 3a), and seems to be destroyed if the input frequency is far from the eigenfrequency (Fig. 3b, c). Applying these results to the experiments with two superimposed sinusoidal temperature inputs, we conclude, that the input period of 24 h which we used as amplitude reference in the superimposed double sine input entrains the circadian system in that way that it is always phase locked with the 24 h reference signal. Under this hypothesis and respecting the range of linear amplitude transfer, the second input signal which is superimposed to the phase locking reference signal bypasses the circadian system and identifies the masking process "thermokinesis".

The proposed structure of the processing of temperature signals via the circadian compartment and the thermokinetic one (Fig. 6) implies a revision of the previously published data with the following consequences:

(i) The data points near \( T_{m} = 12 \) h have to be skipped when identifying thermokinesis by means of two superimposed sinusoidal temperature signals (\( T_{m}^{1} = 24 \) h; \( T_{m}^{2} = 12 \) h).

In this case the differences between the components of the output signal (optical density indicating motility) and the corresponding components of the input signal (temperature) are significantly different from phase differences observed using other \( T_{m}^{1} \) values (1, Fig. 7b). \( T_{m}^{2} = 12 \) h is nearly double the eigenfrequency of the circadian system. It is known that non-linear oscillators can possess secondary ranges of entrainment at the subharmonics of the eigenfrequency [14]. Fig. 4 demonstrates this to be true for the circadian system of motility: stimulation...
Fig. 3. Motility before, during and after excitement by periodic temperature signals varying between 18 °C and 28 °C. Begin and end of periodic temperature are indicated by the bars. a) Period length of temperature is 33.62 h. After stopping temperature cycles a free running circadian rhythm is observed. b) Period length of temperature is 8 h. After stopping temperature cycles only noise is present (cf. Fig. 7). c) Period length of temperature is 55.7 h. No periodicities of motility are observed after stopping temperature cycles.

Fig. 4. Entrainment of motility of *Euglena* by temperature with a period length $T_0 = 11.95$ h. a) Autoregressive model spectrum. $\lambda_0$ is the frequency corresponding to $T_0$. The spectrum indicates frequency demultiplication (peak at $\lambda_0/2$). b) Time-History-Plot of motility output (thick line) and temperature input (thin line).

By a single sinusoidal temperature signal with $T^m = 11.95$ h shows frequency demultiplication, i.e. a bimodal periodic output with $T^\text{out} = 23.9$ h.

Hence, by a superimposition of two such input components, we have to expect non-linear interactions inside the circadian compartment and the fixed phase locking of the circadian system to the $T^m = 24$ h component cannot be assumed.

(ii) The definition of zero phase has to be changed.

In [1] we have calculated the phase difference between optical density of the populations (output)
and temperature (input), and by zero phase we have denoted the in-phase relation between both signals. But if we want to describe the dynamics of the masking of thermokinesis by a feasible physical system, it would be more appropriate to denote by zero phase the inverse phase relation. This allows to identify the corrected data of Fig. 5 (open dots) as a differential amplifier. In further physiological interpretations we have to note, however, that the original phase had been inverted.

After having revised the data with respect to circadian subharmonics and the interpretation of phase, we now compare the double excited set (Fig. 5, open dots) with the single excited set (Fig. 5, full dots). Clear deviations from the 90° phase level of the differentiator are shown in the single excited set indicating a kind of pole at the expected resonance near 24 h. In summary our interpretation results in the signal transfer structure shown in Fig. 6. This scheme involves a corresponding masking by photokinesis which we have to consider taking into account previous data of Schnabel [4], but which is not relevant for the above considerations.

In case of double sine temperature inputs with a 24 h reference input the circadian compartment is phase-locked to the reference input near to its eigenfrequency and thereby separated from the bypass thermokinesis. After substracting the fixed circadian resonance response (24 h reference) from the overall output, the residue describes thermokinesis. The corresponding phase and amplitude diagrams are shown in Fig. 5. These results are most appropriately fitted by a differentiator with constant gain. Using linear regression the gain has been calculated to be 3.64 relative to the 24 h reference output.

If single temperature inputs are applied, the overall output is dominated by the circadian dynamics — at least within the range of circadian entrainment — and superimposed by or interacting with thermokinesis. However, under single sine input the range of frequency transformation is still not limited. This fact can be interpreted either by an increasing superimposition of thermokinesis onto the circadian response when leaving the circadian frequency range or by an increasing loss of self-sustainment of the circadian system itself when enforced by temperature cycles too far from the circadian range.

The two alternative suggestions can be discriminated by investigating the free running motility after releasing from single sine temperature excitation. Experiments of this type are shown in Fig. 3 and 7. There are two facts which strongly support the hypothesis that the non-linear circadian dynamics gets blocked by temperature signals unfavourably exceeding the circadian frequency range: 1) free running circadian oscillations immediately reappear after entrainment by 33.6 h temperature periods (this is within the limit of circadian entrain-
Fig. 7. Analysis of motility output with a temperature input which is first constant (A), then periodic (B, $T_0 = 8$ h) and finally constant again (C). a) Input signal. b) Motility output signal corresponding to the section of the input signal indicated by the bars. The output signal is the trend removed signal of Figure 3b. c) Estimated Wigner-Ville spectrum of motility. A pseudo-Wigner estimator has been used with an observation window length of 63.5 h and a rectangular smoothing window with a duration of 12.5 h (cf. [24]). Frequency $\lambda_0$ corresponds to the input period length $T_0$. Sections A, B and C correspond to the same sections of part a) and b) of this figure.
...ent by light signals [4]) and 2) there is an entire lack of circadian oscillations after excitement by 55.7 or 8 h temperature periods (this is beyond the limit of light entrainment [4]).

The mode of blocking is shown in Fig. 7 which reveals a detailed time history of the case “8 h excitement” of Fig. 3. Beginning with the onset of 8 h entrainment the energy of the circadian signal immediately vanishes whereas the 8 h response does not appear with the beginning but slowly develops up to a stationary state after approximately 6 days. This stationary response does not involve any non-linear component, since only white noise is indicated after the stop of excitement (Fig. 7 b, part C). A non-linearity of the excited system is only exhibited by the appearance of subharmonics, for instance the $2/0$ component in part B. But the depicted non-linearity is time dependent and the energy of the subharmonics damps out. Hence one might explain the absence of circadian oscillations in part C by having suppressed the non-linear structure responsible for selfsustained oscillations. In order to prove this conjection further experiments are required.

It is striking that the circadian system is not blocked when passing its frequency range with light signals. In that case the circadian oscillation jumps back into its eigenfrequency and will simply be superimposed to the photokinesis response [4]. Obviously, the filter which stops the transformation of too short or too long light signals is unable to operate for temperature signals. We suggest thermodynamic reasons for this difference taking into account that temperature strongly acts on all reaction rates of all involved chemical processes. Whatever model will be developed for the chemical mechanism of the circadian clock it is feasible that stability conditions exist which may easily be violated by special temperature enforcements.

5. Combined effects of light and temperature on motility

Light acts on the motility of unicellular algae in a dual mode: vectorially by attracting or repelling the cells (taxis) and scalarly by stimulating or inhibiting speed (kinesis). As discussed above, we exclude vectorial effects from our measurements by means of the special set up of the experiments. Direct scalar effects (photokinesis), however, are inevitable when investigating the transformation of light signals into motility responses. In addition we have to face influences of light on the circadian eigen-frequency and effects of light on the transformation of temperature signals by the circadian system or light effects on thermokinesis. On the other hand competition and mutual interactions between the signal transformation of light and temperature will certainly help to identify substructures of the circadian component.

As an appropriate way to discriminate and evaluate the many ways of possible interrelations we now follow the scheme given in Fig. 6 and discuss the options of input signals.

5.1. Constant inputs

This class includes all cases of an endogenous circadian control of motility, i.e. autokinesis.

5.1.1. Different constant inputs of temperature at one constant light input

This option describing the temperature dependency of the circadian eigenfrequency has previously been investigated [5]. The circadian eigen-frequency of litho-autotrophic cells is independent of temperature whereas in mixotrophic cells it slightly decreases (!) with increasing temperature. This phenomenon seems to be typical for algae [16, 17] and has been denoted “hypercompensation”.

5.1.2. Different constant light inputs at one constant temperature

Since free running circadian oscillations of motility do not persist in constant continuous light we have to apply test light rhythms with short periods that do not entrain the circadian system [18, 4]. Taking into account the periodic structure of the test light, several modes of constant light inputs are possible.

* Even with the entrainment by light signals, Schnabel showed that the free running circadian oscillations after releasing from entrainment approach a minimum of amplitudes when the preceding entrainment approached the frequency limit for entrainment ([4], Fig. 4).

* In other parameters such as sticking on solid surfaces or cell division Euglena fairly well exhibits circadian oscillations in continuous light [19, 20]. These parameters have as yet not been investigated with respect to influence of the intensity of constant light on the circadian period.
5.1.2.1. Different light intensities at a fixed test light period and fixed light/dark ratio within test cycle. It has been shown that the circadian period is independent on that light intensity [5].

5.1.2.2. Different light dark ratios within fixed test light periods using fixed light intensities. Despite the ineffectivities of options 5.1.2.1 and 5.1.2.2, some cases were observed indicating an increasing circadian period with increasing light dark ratio of the test light cycle. Since a project designed to prove option 5.1.2.2 at different culture conditions did not confirm that observation [9] the effect of light dark ratios might depend on physiological conditions of the cells and needs further investigation.

5.1.2.4. Measuring motility either in darkness or in light

Optical densities can be taken either at the beginning of the test light pulses (= OD I) or at the end of the test light pulses (= OD II). As discussed in section 3, the parameter OD I represents motility in darkness, OD II represents photostimulated motility. In so far as the overall light dark program is concerned the parameters OD I and OD II describe circadian oscillations under identical conditions, however, taken at different levels of motility. In all cases hitherto investigated both parameters exhibit in phase circadian oscillations, differing only in its level (Fig. 2). This still remains true even if time varying temperature inputs are applied as shown in 5.2.1.

5.2. Time varying inputs with respect to the signal detection by the circadian system

This class includes all cases of exogenous control of circadian oscillations including masking effects by kinesis responses.

5.2.1. Variable temperature at a constant light test program

A detailed analysis of this option has been presented in [1] and revised in section 4. It results in discriminating a linear masking component operating as a differentiator (thermokinesis) and the circadian compartment transforming temperature signals in a non-linear manner. Leaving the range of circadian frequency, however, temperature signals heavily attack the system up to extinction of self-sustainment. Because of this phenomenon the usage of temperature signals is less suitable to identify the circadian system. On the other hand these attacks indicate the importance of temperature dependent kinetic constants in the unknown circadian mechanism.

We also tested whether photostimulated motility transforms temperature signals in the same way as motility in darkness. As shown in Table I and II temperature periods are transformed identically via OD I and OD II regardless whether they are applied as single inputs or as superimposed double input. From these examples we draw the conclusion that OD II does not provide new information on the circadian dynamics. Since OD I is always less noisy than OD II, we will further use only OD I, i.e. motility in darkness.

Table I. Phase responses of motility in darkness and light under stimulation by sinusoidal temperature cycles: superposition of two temperature signals vs. single temperature signals.

<table>
<thead>
<tr>
<th>Input signal</th>
<th>T_I</th>
<th>T_II</th>
<th>Δφ_1</th>
<th>Δφ_2</th>
</tr>
</thead>
<tbody>
<tr>
<td>( \delta_I + A_I^p \cdot \sin(2 \pi t/T_I^p) + A_I^{s}\cdot \sin(2 \pi t/T_I^{s}) )</td>
<td>18</td>
<td>24</td>
<td>-0.22</td>
<td>(27.14)</td>
</tr>
<tr>
<td>( \delta_I + A_I^p \cdot \sin(2 \pi t/T_I^m) )</td>
<td>18</td>
<td></td>
<td>-9.19</td>
<td>(1.52)</td>
</tr>
<tr>
<td>( - + 24 )</td>
<td></td>
<td></td>
<td></td>
<td>(-11.22, 24.11)</td>
</tr>
</tbody>
</table>

Upper panel: superposition of two temperature signals.
Lower panel: single temperature signals.
Δφ phase angle difference between motility in darkness and motility in light (OD I - OD II); in brackets 95%-confidence-limits. T_I temperature period in hours. \( T_I = 23^\circ C; A_I^p = A_I^{s} = A_I^{m} = 5^\circ C \).
Table D. Phase responses of motility in darkness and light under stimulation by single sinusoidal temperature inputs.

<table>
<thead>
<tr>
<th>$T_{in}$</th>
<th>$\Delta \varphi$</th>
</tr>
</thead>
<tbody>
<tr>
<td>18</td>
<td>$-9.19 (-18.96; 1.52)$</td>
</tr>
<tr>
<td>24</td>
<td>$6.28 (-11.22; 24.11)$</td>
</tr>
<tr>
<td>26.17</td>
<td>$11.78 (1.57; 21.86)$</td>
</tr>
<tr>
<td>36</td>
<td>$17.17 (-1.03; 34.78)$</td>
</tr>
<tr>
<td>55.7</td>
<td>$-7.01 (-13.29; 2.21)$</td>
</tr>
</tbody>
</table>

Symbols and units as in Table I.

5.2.2. Varying light inputs at constant temperature

As shown previously the circadian compartment only responds in a restricted frequency band [4], well known from other organisms as “range of circadian entrainment” [21]. The reappearance of free running circadian oscillations under light signals passing the range of circadian entrainment suggests that the circadian system is not attacked by light signals. On this basis and using input-output-analysis it should be possible to identify both light transforming systems. Since the previously published data are not available for computer analysis, however, this case needs reinvestigation.

5.2.3. Varying inputs of light and temperature

Providing the successful identification of both masking processes and the circadian transformation of light and temperature signals a combined input of light and temperature might come up with deviations from the predicted responses. Such deviations would indicate competitions or mutual interactions between the transferlines for light and temperature signals within the circadian compartment. In a pilot experiment we have chosen a sinusoidal temperature signal and a flip flop light dark rhythm, both with an identical period of 31.75 h, but with two different phase differences between each other. As shown in Fig. 8 the average signals of both outputs differ from each other; furthermore neither of the two signals coincides with the average output signal obtained with a single temperature input (Fig. 5b).

Although the average response to a corresponding single light input has not yet been analyzed, this example undoubtedly demonstrates that mutual influences between the transformation of light and temperature signals do exist.

Using a similar combination of light and temperature inputs but with phototactic response as biological parameter and with 24 h input period of each, Bruce revealed a typical non-linear phase behaviour [22]. In that case the phase difference between the two inputs had been varied over a broad range, resulting in an “intermediate” phase response including a phase jump. These data, however, still include a masking by direct temperature and light responses. By means of our model (Scheme Fig. 6) we interpret the phase of the output signal as the sum of the outputs of all 3 components. Hence, if the phase responses of thermokinesis and photokinesis are known, we can directly calculate the non-linear interactions of the two competing zeitgeber reactions. In further projects we will follow that line of investigation.

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Fig. 8. Two records with combined excitation by light and temperature with equal period lengths $T = 31.75$ h but different phase relations between light and temperature. a) Average signals of the input. Dotted line: temperature (this signal is the same for both experiments), dashed line: LD₁, full line: LD₂. b) Average signals of motility. Full line corresponds to excitation with LD₂, dashed line to LD₁.
5.3. External manipulation of the compartments

The following options will help to denote the physiological meaning of the items identified by system analysis.

5.3.1. Chemical inhibitors of known action

One example has been shown as yet: the inhibition of circadian phase responses to temperature steps in *Euglena* by cycloheximide [8]. Control experiments with proteinsynthesis did not confirm, however, the essential involvement of cytosol proteinsynthesis in that case.

5.3.2. Isolation of mutants

Most promising is the usage of a white mutant lacking photosynthesis. It transforms a combined input of temperature and light signals of identical periods in the same way as the green wild strain. The average signals of motility for two examples of this type are shown in Fig. 9. By the way these examples confirm the conclusion drawn in 5.2.1 that photostimulated motility does not contribute new information. Identical processing of light and temperature inputs by both strains using identical input periods, however, is not convincing since the temperature response may entirely mask differences in the light response caused by mutation. In order to discriminate between the two input signals, different input periods of the light and the temperature signal have to be applied. Such an example is shown in Fig. 10. The periodograms obtained after a combined input of an 18 h light and 24 h temperature period exhibit both periods in the output of the wild strain (Fig. 10a) but only the 24 h temperature response in the output of the white mutant (Fig. 10b). Since the energy of the 18 h signal is rather low, the periodograms have also been calculated after subtracting the 24 h component. Now only in the wild strain the residue clearly exhibits the 18 h light response (Fig. 10, right side).

The shown lack of entrainment by light in the white mutant could indicate that photosynthesis plays a keyrole in the light signal transformation. But this cannot hold, since circadian entrainment of motility in a white mutant by single flip-flop light dark inputs has well been established [23]. Thus photosynthesis is not essential for the existence of light signal transformation but affects this transformation especially in cases of combined inputs. We plan to include comparative studies with white mutant when further investigating the fine structure of the circadian compartment.