Period-2 Cycles and 2:1 Phase Locking in a Biological Clock Driven by Temperature Pulses

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Crassulacean acid metabolism (CAM) serves as a botanical model system for the investigation of circadian rhythmicity. In a new set of experiments with the obligatory CAM plant Kalanchoë daigremontiana the response to periodic stimulations with temperature pulses has been studied. On the basis of an experimental phase–response curve of net CO₂-gas exchange the effect of periodic stimulation has been simulated using a finite-difference equation. These simulations revealed the locations of two period-2 cycles in the CO₂ uptake of the CAM plant. In subsequent experiments based upon the simulated bifurcation diagram the position and amplitude of one of these cycles were confirmed, while experimental evidence for the second cycle could be found. Possible roles of such dynamics for the functioning of the biological clock are discussed.

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1. Introduction

The crassulacean acid metabolism (CAM, see e.g. Winter & Smith, 1996) plant Kalanchoë daigremontiana Hamet et Perrier de la Bâthie shows an endogenous circadian rhythm of net CO₂ exchange (JCO₂) under constant external conditions in continuous light (Lüttge & Ball, 1978). Previous studies have demonstrated that above a certain threshold, temperature changes from rhythmic to arrhythmic behaviour of JCO₂ occur (Lüttge & Beck, 1992) and that this is reversible when the temperature is lowered again (Grams et al., 1996). It has been shown that these findings are well reproduced by a system of four-coupled nonlinear differential equations with temperature, light intensity and external CO₂ concentration as external parameters (Blasius et al., 1997, 1999). Due to the combination of a highly controllable experimental set-up and a consistent theoretical representation we may regard the endogenous circadian CAM rhythm of K. daigremontiana as an excellent model system for the study of the biological clock.

In the present paper, we discuss the results of a series of theoretical investigations and experiments focusing on the periodic stimulation of the CAM plant with temperature pulses. In the first experiment, the phase–response curve \( \Psi(\Phi) \) of the CAM plant has been measured, which gives the position \( \Psi \) of the response peak as a function of the position \( \Phi \) of the temperature pulse (in units of the endogenous period of the plant). On the basis of this experimental function, the periodic stimulation of the CAM plant could be simulated using the mathematical methods first studied by (Guevara et al., 1981) for the
case of chick heart cells. The results of these simulations can be represented in terms of a bifurcation diagram, in which the locations of higher-order dynamical effects, such as bifurcations, cycles or deterministic chaos, are easily identified. The experiments performed on the basis of these theoretical simulations confirmed the existence of, at least, one of the period-2 cycles in the metabolism of the CAM plant.

2. Material and Methods

2.1. PLANTS

Plants of *K. daigremontiana* were raised from adventitious plantlets obtained from leaves of the plant collection of the Botanical Garden, Darmstadt University of Technology. They were grown in soil culture in the glasshouse until they had produced six to seven pairs of fully developed leaves, and were about 0.4–0.5 m tall. During winter additional light (HQI-T, 400 W, Philips) was provided to extend the daylight period up to 12 hr. Prior to the experiments the plants were adapted for at least 3 days to 12 hr dark and 12 hr light. During adaptation the temperature was set to 21 and 28°C in the dark and the light period, respectively.

2.2. GAS EXCHANGE MEASUREMENTS

The measurements of net CO₂ exchange were performed in a climate-regulated chamber of the phytotron in the Department of Biology in Darmstadt, as previously described (Lüttege and Beck, 1992). Net CO₂ exchange was recorded using the minicuvette system of H. Walz (Effeltrich, Germany). A mature leaf of a plant was enclosed in the gas exchange cuvette while the remaining was attached to the plant. For a better control of the leaf temperature, the thermistor usually used for measuring the temperature inside the cuvette was carefully attached to the lower side of the leaf. Thus, leaf temperature rather than air temperature was exactly regulated. The leaves were subjected to temperature changes of 7°C (from 21 to 28°C and vice versa), which were performed within 10 min. Considering the time-scale of 24 hr of the plant’s endogenous rhythm it is clear that this corresponds to an almost instantaneous temperature change. Gas exchange data were recorded every 5 min using a PC and a datalog program. The relative humidity of the air inside the cuvette was set at 60±5% and was held constant. Irradiance (photon flux density, PFD) was measured in the range of 400–700 nm using a LICOR quantum sensor (LICOR) and was set to 110±10 μmol m⁻² s⁻¹. The conditions inside the phytochamber were adapted to the conditions inside the gas exchange cuvette. Net CO₂ exchange rate was calculated according to Farquhar & Sharkey (1982).

3. Results

3.1. THEORETICAL BACKGROUND

Phase-locking phenomena are of high interest in nonlinear dynamics, as it is known that nonlinear oscillators produce extremely rich patterns of synchronization to an external periodic stimulus, where the main control parameters are the external frequency and the strength (or amplitude) of the external signal (see e.g. Pikovsky *et al.*, 2001; Tass, 1999). In the present paper, we will apply the following scheme, which we believe is closest to the current theoretical usage. The synchronization between gas exchange and external signal observed in the amplitude of the plant’s response will be classified as phase locking, while all responses involving the peak position will be discussed using the language of finite-difference equations. For the classification of phase locking one basically counts, how many periods (say *n*) of the external driving force (i.e. the temperature pulses) pass, before the cycle of the internal oscillator (seen in the gas exchange curve) repeats. If such a relation between the external and internal oscillators exists, it is called an *n*:1 phase locking. Bifurcations are classified with respect to the branching from one dynamical behaviour to another. The sudden change from a fixed point (steady state) to a cyclic behaviour with a period of 2 (i.e. needing two peaks of the gas exchange curve before the same structure repeats) is called a (forward) bifurcation. At a change of the control parameter the fixed point becomes unstable and a stable period-2 cycle takes over as the attractor of the system. In this way, we
may speak of a 2:1 phase locking accompanied by a period-2 cycle (with the latter seen as an oscillatory pattern in the distance between neighbouring peaks). Later, in Fig. 5, an example of such a behaviour will be seen.

3.2. EXTRACTION OF THE PEAK POSITIONS IN THE CO₂ EXCHANGE CURVE

From the remarks in the previous section it is clear that the peak extraction, together with an unambiguous quantification of the relative positions of the peaks is an important pre-requisite of a successful interpretation of the experimental results in the language of phase locking and bifurcations. However, the peaks in the CO₂ gas exchange curves of the CAM plant show significant differences in shape and size. In order to avoid systematic errors in the extraction of the peak positions due to such differences, we used a very flexible mathematical function for a least-squares fit of each of the peaks:

\[ F(t) = a_0 \frac{\theta_1^m}{\theta_1^n + \theta_2^m} \left( 1 - \frac{\theta_2^m}{\theta_2^n + \theta_2^m} \right). \]

This function is the product of two Hill-type functions, leading to an independent description of the ascending and descending part of each peak, respectively. The sum of squares of deviations of the curve from the data points is minimized by variation of the five parameters present in eqn (1), namely the peak height \( a_0 \), the position of the right \( (\theta_1) \) and left \( (\theta_2) \) flanks and the slopes \( n \) and \( m \) of the two flanks. It is assumed that in CAM the regimes of rising and falling \( J_{CO_2} \) curves can be regarded as independent processes. Rising net CO₂-uptake occurs as internal CO₂-concentration is lowered due to CO₂-fixation for synthesis and vacuolar accumulation of malic acid. Falling net CO₂-uptake occurs as internal CO₂-concentration is increased due to malic acid remobilization and decarboxylation. These are characteristic elements of CAM, but note that there is no additional physiological interpretation attempted with the choice of the function given in eqn (1). The location of the peaks was then determined by extracting the position \( \theta_2 \) of the left flank of each of the gas exchange peaks, which seemed to be the most reliable parameter, i.e. the one based on the largest number of independent data points. The corresponding analysis has also been performed by defining the peak position as \( (\theta_1 + (\theta_2 - \theta_1)/2) \), leading qualitatively to the same results. Typical results of this fit procedure are shown in Fig. 1.

3.3. PHASE RESPONSE CURVE (PRC) AND THEORETICAL RESULTS FOR A PERIODIC STIMULATION WITH TEMPERATURE PULSES

The first of the temperature-pulse experiments reported here has been designed to measure the phase response curve \( \Psi(\Phi) \). To this end, after three day–night cycles and two further days in continuous light, the CAM plant has been exposed to a temperature pulse in a previously selected phase with respect to the preceding peak. A temperature pulse is a 7°C increase of temperature for a period of 2 hr, starting from a baseline temperature of 21°C. Then the following peak in the gas exchange curve (response peak) has been observed. After one endogenous period, which was included in order to minimize memory effects, the procedure was repeated in a different phase. In this way, the PRC has been measured at 12 different points with four repetitions. The order of the phases \( \Phi \) has been randomized, again to minimize long-term memory effects. An example of this procedure of condensing two peaks around a temperature pulse into a point of the PRC is shown in Fig. 2.

By “phase” we simply mean the time difference (e.g. between two peaks or between a temperature pulse and a peak) divided by the endogenous period \( \gamma \). Thus, such a phase denotes the time between subsequent peaks of the perturbed rhythm related to the phase of the endogenous oscillation. Note that our definition of the PRC in Fig. 2 differs by an additive \( \Psi \) from the one used e.g. in Guevara et al. (1981), i.e. the PRC in Guevara et al. (1981) is obtained from our curve shown in Fig. 2 by subtracting \( \Psi \) at each point. The phase–response curve obtained here is in good agreement with the results of Wilkins (1992). In Fig. 3 two consecutive temperature pulses from a periodic treatment with a period \( \tau \) are shown including the surrounding gas exchange peaks. It is immediately seen that the quantities \( \Psi', \Phi_i \) and \( \Phi_{i+1} \) are not independent,
\[
F_i + 1 = \frac{F_i}{C_0} C_i (F_i) + t.
\]

Equation (2) is valid only in the case where the period \( \tau \) is smaller than twice the endogenous period \( \gamma \) of the CAM plant, otherwise two or more gas exchange peaks are found between the temperature pulses. In that case the right-hand side of eqn (2) has to be replaced by its remainder on subtraction of the largest possible integer keeping a nonnegative result (i.e. modulo 1). Mathematically, eqn (2) is a nonlinear finite-difference equation of first order. The nonlinearity enters with the function \( \psi_i(\Phi_i) \) which has been measured experimentally. Equation (2)
serves as an efficient basis for a simulation of the plant’s response to a periodic stimulus. By varying the control parameter $\tau$ and registering the occurring values of $\Phi_i$ in the time series simulated via eqn (2), one obtains a bifurcation diagram with respect to $\tau$, which is shown in Fig. 4. It gives the possible distances of two consecutive peaks in the CO$_2$ gas exchange curve under the influence of an external temperature pattern with a period of $\tau$. One immediately sees regions, where two such distances appear in the time series, i.e. where a short and a long interval between two peaks alternate (period-2 cycle). A major ambiguity in generating the bifurcation diagram arises due to the experimental function $C(F)$. Here, different methods of including this function have been used (e.g. different smoothing algorithms on the data points as well as different analytical functions interpolating the points). A selection of typical results is presented in Fig. 4 as different grey scales. Obviously, some important features of the bifurcation diagram are robust against variation of such a mechanism, e.g. the regions of a period-2 cycle. Other properties of the diagram are less robust with respect to the different ways of including $\Psi(\Phi)$, namely the sizes and positions of windows with an irregular behaviour (cf. the corresponding remarks in Section 4). For convenience the bifurcation parameter $\tau$ in Fig. 4 is measured in units of the endogenous period $\gamma$. It is clear from Fig. 3 that in order to obtain explicit values for the peak-to-peak distance $\Psi$ the (experimental) function $\Psi(\Phi)$ has to be applied again to the iteration result obtained from eqn (2). This step has been included in Fig. 4. Note that the bifurcation diagram shown in Fig. 4 does not contain any information on the height of the peaks. Any pattern observed there as resulting from the external periodic stimulus has to be interpreted in the language of phase locking.

3.4. EXPERIMENTAL RESULTS FOR PERIODIC STIMULATION

On the basis of the bifurcation diagrams shown in Fig. 4, two different periods of stimulation have been selected in order to verify experimentally the dynamical behaviour found theoretically. The vertical dashed lines in Fig. 4 indicate the corresponding values of the bifurcation parameter $\tau$. The CO$_2$ gas exchange curves are given in Fig. 5. In both cases the plant has been adapted to two day-night cycles of 24 hr and was then transferred into continuous light ($t = 0$ hr). Subsequently, four consecutive peaks have been measured at a constant temperature in order to extract the endogenous period $\gamma$ of the plant. It is known (Lütte & Beck, 1992) that the endogenous period $\gamma$ varies significantly from plant to plant. Therefore, $\gamma$ has to be measured for each plant individually. Next, at a point of low CO$_2$ exchange the pattern of temperature pulses has been started. For the lower value of $\tau$ (at $\tau \approx 0.46$, i.e. in the center of the first calculated period-2 cycle, Fig. 5a) the gas exchange curve shows an obvious pattern in the peak heights: peaks with a small amplitude alternate with high-amplitude peaks. As discussed in Section 3.1, this is the signature of a 2:1 phase locking. At the higher value of $\tau$ [at $\tau \approx 0.73$, i.e. slightly below the second period-2 cycle, Fig. 5(b)] apart from a small global dampening there is no discernable modulation of peak heights.

The important dynamical variable for bifurcation phenomena is the position of the peaks in the CO$_2$ gas exchange curve, rather than their height. The analysis of the gas exchange time series with the curve-fitting method introduced in Section 2.2 allows one to precisely extract the peak positions. In Fig. 5(c) and (d) the differences in the positions of consecutive peaks are shown as a function of the peak number. While the first case shows a clear oscillatory pattern in
the peak-to-peak distance \([\text{Fig. 5(c)}]\), a relaxation into a fixed point is seen for the larger value of \(\tau\) [Fig. 5(d)]. The amplitude of oscillation in Fig. 5(c) compares favourably with the one predicted by the simulated bifurcation diagram (i.e. with the diameter of the region displaying the period-2 cycle). At a higher value of \(\tau\) one again finds evidence of an oscillatory behaviour (data not shown). Due to technical constraints in this latter case the quality of the data is rather low and only a short time series (about eight driver periods) could be measured.
4. Discussion

In this work it could be established experimentally that with temperature pulses the metabolism of *Kalanchoë daigremontiana* displays a period-2 cycle at certain periods of external stimulation. The periods leading to these cycles have been theoretically predicted on the basis of a finite-difference equation containing an experimental phase–response curve, which has also been reported here. The size of the peak shifts in the corresponding gas exchange patterns agrees well with the values found in the theoretical simulation. In addition, at the lower value of $\tau$ a 2:1 phase locking of the gas exchange curve to the external periodic stimulus is observed.

While the low-$\tau$ cycle could be well established with the experimental result reported here, in the case of the fixed-point behaviour between the two main cycles, we only had some evidence for the second period-2 cycle (at higher $\tau$) seen in Fig. 4. An attempt to repeat the experiment at this value of $\tau$ failed, i.e. no oscillation in the peak-to-peak distance has been found. We believe that the unavoidable error in the extraction of the endogenous period can easily result in missing the oscillatory window.

The current accuracy of the phase–response curve still leaves room for some ambiguities in the full dynamics of the periodically driven system. The existence and location of smaller and/or higher-order bifurcations cannot be reliably predicted. The possibility of a chaotic regime cannot be excluded on the basis of the experiments reported here, although a careful theoretical analysis of the experimental PRC data suggests that such a regime, if it exists, would be rather small.

In principle, it should be also possible to find some more complex (higher-order) phase-locking phenomena. Experimentally, however, this is a difficult task, as it involves a two-dimensional parameter space with period and strength of the external stimulus to be varied independently. Here more model simulations would provide an important tool, where, e.g. Arnold-tongue structures in the phase-locking diagram can first be suggested by the model and then confirmed at a small number of characteristic points in the parameter space. An important next step would be to study the mathematical model of CAM, e.g. as described in (Blasius *et al.*, 1999), under temperature pulses, as this would help compare model and experiment on a much higher level, namely in terms of a bifurcation diagram like Fig. 4. Within the model one can then study the transitions between different phase-locking regions in more detail to see, whether, e.g. a bistable behaviour is found.

From experiments (Lüttge & Beck, 1992) and model simulations (Blasius *et al.*, 1997) we know that the CAM plant reacts similarly to changes in temperature and light intensity. The former is easier to control experimentally. The latter is frequent ecologically, e.g. with fluctuating or periodically varying light intensities of light flecks in the canopy of tropical rain forests (see, e.g. Lüttge, 1997) or rapidly moving clouds. Varying light intensities may imply temperature changes. Thus, we see our results also as an indication of what effect a periodic variation of light intensity could have on the plant.

Physiologically, it would be interesting to understand, to what extent the height and position of a peak in the gas exchange curve are independent quantities. Here the language of phase locking and bifurcations is a useful starting point. The knowledge of the bifurcation structure is an important prerequisite for the discussion of the universality of the biological clock (when different organisms are compared) and for an evaluation of the effect of external influences on the functioning of a circadian oscillator. The results shown here clearly indicate that a periodic stimulus in one of the control parameters may severely disrupt the circadian rhythm of this biological clock.

REFERENCES


