11 Ciracadian Rhythmicity

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11.1 *Clusia*'s Clock: The Background of Endogenous Rhythmicity of C_3- and C_4-Photosynthesis and Crassulacean Acid Metabolism (CAM)

Does *Clusia* have an endogenous clock? Does *Clusia* need a clock? In the prokaryotic cyanobacterium *Synechococcus* almost all genes and in the vascular higher plant *Arabidopsis thaliana* (L.)Heynh. very many genes are oscillating in their transcription with an endogenous circadian period close to 24 h (from the Latin words *circa* = approximately, *dies* = day), i.e. they are clock controlled (Liu et al. 1995; Michael and McClung 2003). Circadian rhythmicity is a basic property of living organisms. Thus, we must assume that also *Clusia* has circadian rhythmicity.

In the plant kingdom most of the overt circadian oscillations are related to light and directly or indirectly involve photosynthesis (for review see Lüttge 2002a). Rhythms of C_3-photosynthesis in leaves have been analysed at the levels of gas exchange (CO_2 and water vapour), leaf conductance and stomatal opening, respiration, and metabolism of CO_2 assimilation and for example starch accumulation (Hennessey and Field 1991; Freeden et al. 1991; Li et al. 1992; Hennessey et al. 1993; Geiger et al. 1995; Lu et al. 2005; Lu and Sharkey 2006). Also in the C_4-plant *Sorghum bicolor* net photosynthesis, soluble sugar and starch accumulation showed circadian oscillations (Britz et al. 1987). At the molecular level studies are most advanced in the C_3 vascular plant *A. thaliana*. (For reviews of this rapidly and broadly developing field see, e.g., Millar 1999; Somers 1999; Staiger and Heintzen 1999; McClung 2000; Staiger 2002; Michael and McClung 2003.)

Rhythmicity of CAM has been mainly studied in obligate CAM species of the genus *Kalanchoë*. (For reviews see, e.g., Wilkins 1992; Borland et al. 1999; Lüttge 2000, 2002a, b; Nimmo 2000). The major overt output oscillations assessed generally were CO_2 and water vapour gas exchange and leaf conductance and night/day organic acid oscillations (reviews as above), but also car-

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bon isotope discrimination, $\delta^{13}C$, and activities of the carboxylating enzymes phospho*enol*pyruvate carboxylase (PEPC) and ribulose-bis-phosphate carboxylase/oxygenase (RubisCO) (Grams et al. 1996) and quantum yield of photosystem II (Rascher et al. 2001; Rascher and Lüttge 2002). At the molecular level regulation of PEPC-activity via phosphorylation by PEPC-kinase (PEPCK) has been investigated, and it was seen that there was a feedback loop of regulation of PEPC by biochemical and biophysical processes involved in the metabolism and vacuolar compartmentation of malic acid during the CAM cycle (Carter et al. 1991; Hartwell et al. 1996, 1999, 2002; Borland et al. 1999; Borland and Taybi 2004; for details of the biochemistry of CAM see Sect. 8.3, Figs. 8.9, 8.12 and 8.14). However, at the molecular level insights are now more rapidly advanced using the C_3/CAM-intermediate annual species *Mesembryanthemum crystallinum* L. (Boxall et al. 2005). It has been recognized that its major upstream and central clock genes are the same as those of *A. thaliana*, namely *CCA1 (circadian clock associated), LHY (late elongated hypocotyl), TOC1 (timing of CAB expression;* "chlorophyll a, b binding protein"), *ELF 3* and *ELF 4 (early flowering 3* and *4), ZTL (zeitlupe), FKF1 (flavin-binding kelch repeat F-box 1).* Most interesting for a comparative evaluation of diurnal and circadian C_3- and CAM-oscillations are the genes *CCA1/LHY, TOC1* and *ELF 4.* TOC1 is mainly expressed in the late light and early dark period and its breakdown is regulated by CCA1 and LHY oscillating with a phase shifted by ca. 180° in relation to TOC1 (Kikis et al. 2005). *TOC1* is also thought to be involved in circadian oscillations of stomatal guard cell movements (Somers et al. 1998) which are a very important aspect of circadian oscillations of photosynthesis. Comparing C_3- and CAM-oscillations in *M. crystallinum* it is most noteworthy that some of the genes were oscillating only in either the C_3- or the CAM-mode, but *CCA1/LHY, TOC1* and *ELF 4* were oscillating in both modes of photosynthesis. The phases of *TOC1* oscillations in the C_3- and CAM-mode were strongly offset against each other and also the phases of *CAA1* and *LHY* but to a lesser extent.

While the investigations with the C_3/CAM-intermediate annual species *M. crystallinum* provide important comparative insights in C_3- and CAM-rhythmicity, it still appears essential to also study the C_3/CAM-intermediate species *Clusia minor* L. in this respect. Both species represent two highly different life forms. In contrast to the therophyte *M. crystallinum* where due to the short life time of the plants reversibility of C_3 to CAM switches is rather limited (Ratajczak et al. 1994) longevity of the leaves of the tropical trees of *Clusia* for two or more years (Olivares 1997) allows repeated shifts between the two modes of photosynthesis and *C. minor* is particularly flexible (Chaps. 8 and 9). However, considering endogenous circadian rhythmicity in *Clusia* at this stage we have to dwell much on the background given in this section, because so far the only work available on *Clusia*'s rhythmicity described in the following section (Sect. 11.2) is the dissertation of Duarte (2006).

11.2 *Clusia minor*'s Clock

11.2.1 Endogenous Oscillations of Gas Exchange and Effective Quantum Yield of Photosystem II in the C_3- and CAM-Modes of Photosynthesis

The C_3/CAM intermediate *C. minor* was adapted to perform C_3-photosynthesis and CAM, respectively, by growing plants at constant temperature day and night and at an irradiance of 120 μmol m^{-2} s^{-1} during the light period and keeping them well watered and withholding water for up to four days, respectively (see Sects. 8.5 and 8.8.1). Endogenous rhythmicity of net CO_2 exchange, J_{CO2}, and leaf conductivity for water vapour, g_{H2O}, was observed under continuous irradiance (LL) of 120 μmol m^{-2} s^{-1} at three different temperatures (Fig. 11.1). It was most pronounced at a medium temperature of 25 °C in the C_3-mode and at 25 °C but also at 30 °C in the CAM mode. This temperature dependence shows similarities with the behaviour of the obligate CAM plant *Kalanchoë daigremontiana* Hamet et Perrier where rhythmicity is only obtained within a temperature window above a lower and below an upper temperature threshold (Lüttge and Beck 1992; Grams et al. 1996). Due to homeoviscous adaptation of molecular tonoplast membrane structure governing malate compartmentation (Kluge et al. 1991; Kliemchen et al. 1993) the absolute temperatures marking the thresholds depend on growth temperature (Grams et al. 1995). As the *C. minor* plants were grown at temperatures between 20 °C (night) and 28 °C (day) rhythmicity in the CAM-mode still pertaining at 30 °C is consistent with the observations in *K. daigremontiana*.

It is noteworthy that both in the C_3- and in the CAM-mode rhythmicity is highly dampened and lost after only a few endogenous periods, which is in strong contrast to endogenous circadian rhythmicity in both obligate C_3- and obligate CAM-species (Sect. 11.1). In the C_3-mode loss of rhythmicity was more rapid than in the CAM-mode. A more detailed analysis is given for the temperature of 25 °C, where in both modes of photosynthesis rhythmicity was expressed particularly well, and where circadian oscillations of effective quantum yield of photosystem II (PS II), $\Delta F/F_m'$, measured every 20 min during the recording of gas exchange by pulse amplitude modulated chlorophyll fluorometry are also shown (Fig. 11.2). The dampening of oscillations is seen again. The oscillations of $\Delta F/F_m'$ in LL had a larger amplitude in the CAM-mode than in the C_3-mode and persisted longer. In both modes the oscillations of $\Delta F/F_m'$ dampened out more rapidly than those of J_{CO2} and g_{H2O} and the dampening out of endogenous rhythmicity was associated with an overall decline of $\Delta F/F_m'$. This was correlated with a general reduction of g_{H2O} in the arrhythmic stage attained after the loss of rhythmicity which must have been the reason for a similarly reduced J_{CO2} explaining the reduced $\Delta F/F_m'$ by sub-

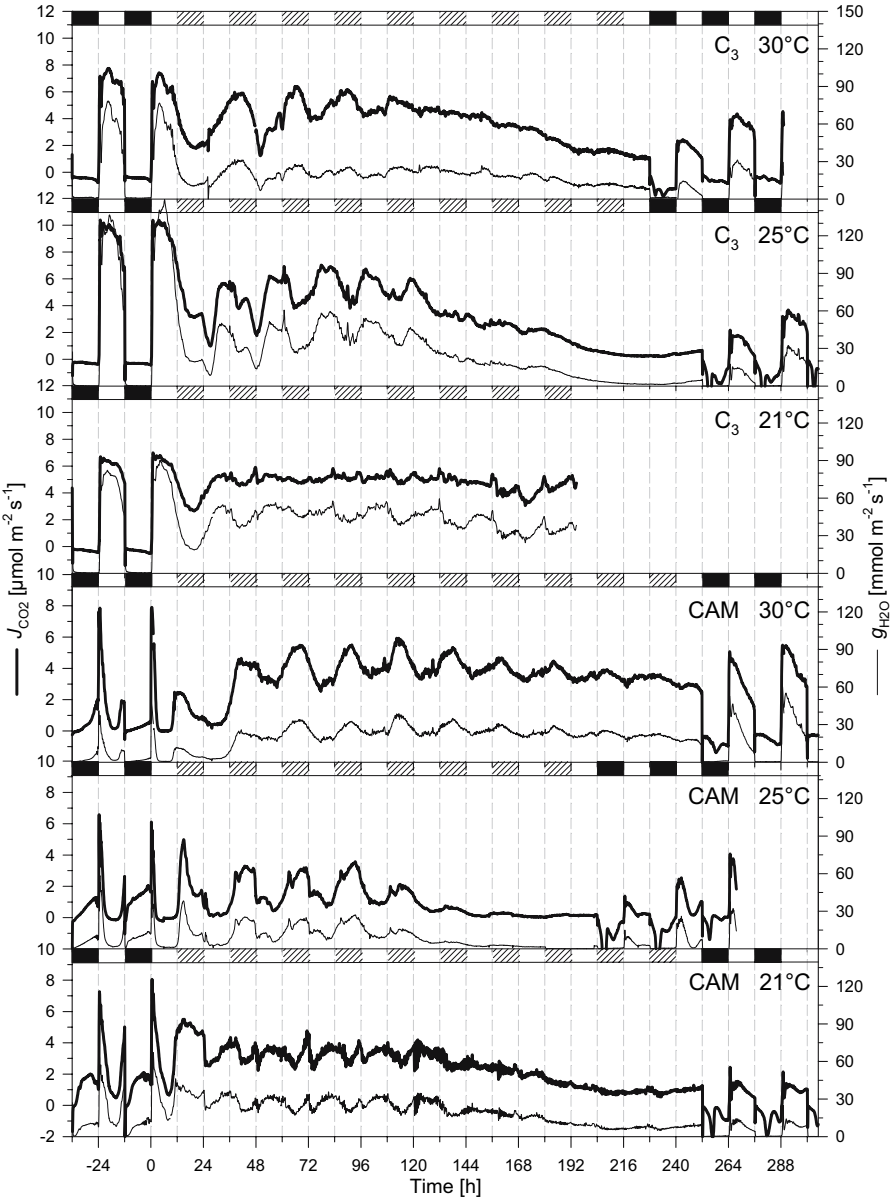

Fig. 11.1. Net exchange of CO_2, J_{CO2}, and leaf conductance for water vapour, g_{H2O}, of the leaves of *C. minor* plants adapted to perform C_3- and CAM-photosynthesis at three different temperatures as indicated. *Black bars* indicate darkness (D), *white bars* light (L) and *hatched bars* the subjective dark periods under constant illumination (LL)

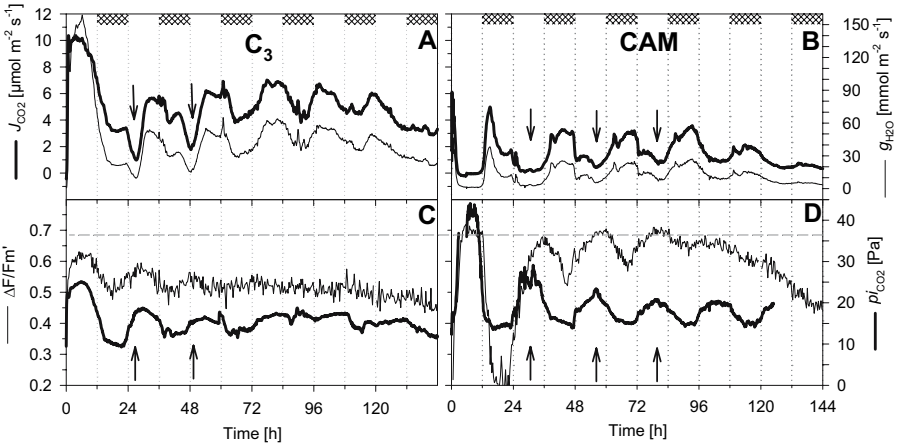

Fig. 11.2.A Gas exchange, J_{CO2} and g_{H2O}. **B** p^i_{CO2}, and effective quantum yield of PS II, $\Delta F/F_m'$, for leaves of *C. minor* plants adapted to the C_3-mode (*left*) and to the CAM-mode (*right*) at 25 °C. *Black bars* indicate darkness (D), *white bars* light (L) and *hatched bars* the subjective dark periods under continuous illumination (LL). **C** The first 60 h (C_3-mode) and **D** the first 72 h (CAM-mode), respectively, under LL are amplified. The *arrows* indicate high p^i_{CO2} and $\Delta F/F_m'$ at simultaneously low J_{CO2} and g_{H2O}. The *horizontal lines* in C and **D** mark the ambient CO_2 partial pressure, p^a_{CO2}.

strate limitation of the carboxylase activity of RubisCO, and hence energy use by PSII as indicated by $\Delta F/F_m'$. The amplification of the first periods of the oscillations (Fig. 11.2C) reveals that in the C_3-adapted plants changes of $\Delta F/F_m'$ and internal CO_2 partial pressure, p^i_{CO2}, followed each other and were inversely related to J_{CO2} and g_{H2O}. This confirms that energy use is related to the availability of internal CO_2. It is remarkable that two times in sequence in the C_3-adapted plants the increase in $\Delta F/F_m'$ and p^i_{CO2} was observed together with a reduction in J_{CO2} and g_{H2O} (arrows in Fig. 11.2C). This shows that the increase of p^i_{CO2} was not due to CO_2 uptake but rather to internal CO_2 sources. It suggests that there were internal sources of CO_2, possibly due to some organic acid mobilisation and decarboxylation, i.e. that the C_3-adapted plants had kept a residual CAM capacity possibly due to activity of the major leaf vein chlorenchyma (see Sect. 8.8.1). In the CAM-adapted leaves in the first 35 h under LL $\Delta F/F_m'$ was correlated with p^i_{CO2} and inversely correlated with J_{CO2} and g_{H2O}, corresponding to what one expects in CAM, i.e. that during organic acid mobilisation and decarboxylation p^i_{CO2} increases and J_{CO2} and g_{H2O} decrease. However, subsequently the coupling of $\Delta F/F_m'$ and p^i_{CO2} was not so tight anymore. The peaks of p^i_{CO2} showed a clear tendency to become smaller, which suggests a weakening of the CO_2 concentrating mechanism of CAM as time under LL moves on. This conclusion is very interesting in relation to the following observation. The C_3-adapted plants performing C_3-pho-

tosynthesis before application of LL as expected still performed C_3-photosynthesis when an external dark/light rhythm (DL) was given again after rhythmicity had dampened out in LL (Fig. 11.1). However, the CAM-adapted plants well expressing the four phases of gas exchange of CAM (Chap. 8.1) before LL showed C_3-type gas exchange under DL following LL (Fig. 11.1). This means that the plants had changed the mode of photosynthesis and shifted from CAM to C_3-photosynthesis during LL.

Although *K. daigremontiana* is an obligate CAM-species it shows a most noteworthy analogy to this behaviour of *C. minor*. In the circadian rhythm of *K. daigremontiana* only for the first endogenous periods night/day oscillations of malic acid levels are observed, which are highly dampened. This loss of organic acid oscillations is not reflected at all in the overt rhythmicity of gas exchange which – as noted above (Sect. 11.1) – continues for very many endogenous periods (Wyka and Lüttge 2003; Wyka et al. 2004; see also Borland and Taybi 2004). Thus, control of rhythmicity in *K. daigremontiana* is handed over from a CAM-type oscillator with a hysteresis switch based on malate metabolism and vacuolar compartmentation (Lüttge 2000) to a C_3-type oscillator. The nature of the latter is not known but could be RubisCO activation and activity or stomatal guard cell movements, which are also known to show circadian rhythmicity in C_3-plants (see Sect. 11.1).

11.2.2 Endogenous Oscillations of Oxygenase activity of RubisCO in the C_3- and CAM-Modes of Photosynthesis

Photorespiration was followed during endogenous oscillations by applying a gas mixture with only 1 % O_2 for 20 min at intervals during registration of gas exchange (J_{CO2}, g_{H2O}) and effective quantum yield of photosystem II, relΦ_{PSII}, measured via chlorophyll fluorescence imaging. This causes non-photorespiratory conditions and the difference between maximum possible CO_2 uptake obtained under 1 % O_2 (J_{CO2}max) and CO_2 uptake obtained under 21 % O_2 equals the oxygenation activity of RubisCO, J_{O2} (Figs. 11.3 and 11.4). The dampening of the endogenous oscillations again was seen to be faster in the C_3-mode than in the CAM-mode. In both modes J_{CO2}max and J_{O2} followed the curves of J_{CO2} and g_{H2O}. In the C_3-mode J_{O2} in per cent of total RubisCO-activity, %J_{O2}, was largely in phase with the other parameters. However, in the CAM-mode the pattern of %J_{O2} was much more complex. It was shifted in phase as it increased ahead of J_{CO2} and g_{H2O} when these were still low, and the highest values of %J_{O2} were reached when J_{CO2} and g_{H2O} changed from lower to higher values. %J_{O2} was also higher in the CAM-mode than in the C_3-mode, the maximum, values in the peaks were 50 and 35 %, respectively. When rhythmicity had dampened out the values of %J_{O2} of the CAM-adapted plants came close to those of the C_3-adapted plants which corresponds to the observed CAM to C_3 shift under LL.

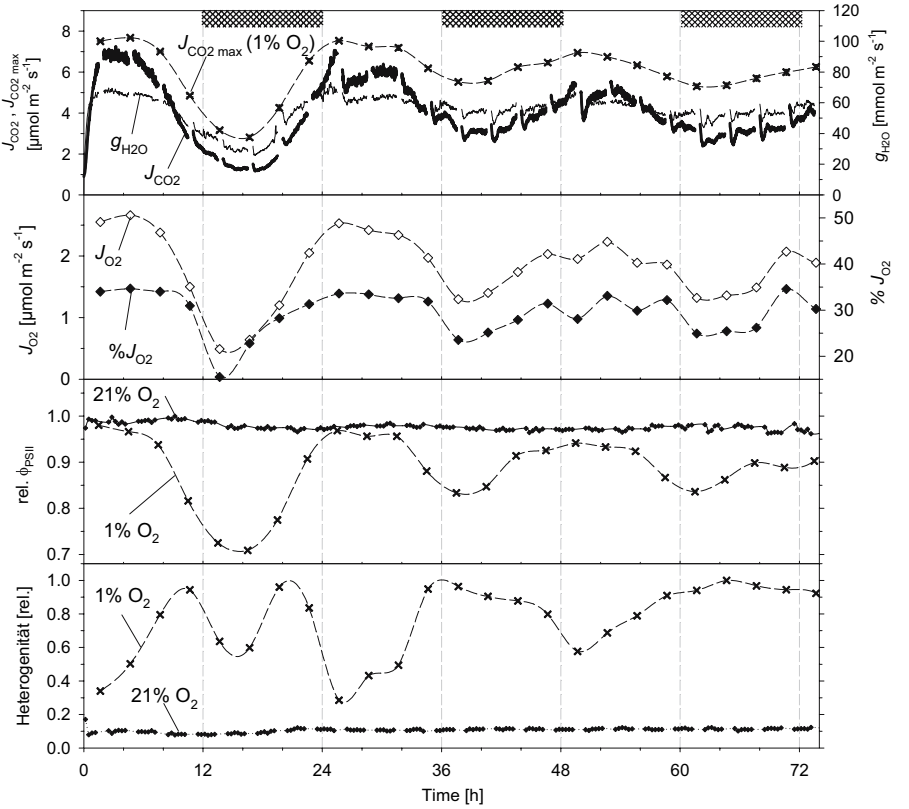

Fig. 11.3. Endogenous rhythm of photorespiratory activity of a leaf of a C_3-adapted *C. minor* plant at 25 °C with maximum carboxylation activity of RubisCO, J_{CO2}max, in the presence of 1 % CO_2, net CO_2-exchange, J_{CO2}, and g_{H2O} in the presence of 21 % O_2 (*uppermost panel*), J_{O2}, i.e. the difference between J_{CO2}max and J_{CO2} and %J_{O2} (J_{O2} in per cent of J_{CO2}max) (*second panel*), relΦ_{PSII} at 21 % and 1 % O_2 (*third panel*) and heterogeneity at 21 % and 1 % O_2 (*bottom panel*). Time 0 is the beginning of LL, *white bars* indicate light and *hatched bars* the subjective dark periods under LL

RelΦ_{PSII} under 21 % O_2 did not oscillate under LL in both modes of photosynthesis and there was no heterogeneity of relΦ_{PSII} over the leaves calculated by a nearest neighbour matrix algorithm from the images of relΦ_{PSII}. By contrast, under 1 % O_2 in both modes clear oscillations of relΦ_{PSII} were borne out. They were in phase with oscillations of J_{CO2} and g_{H2O} and also dampened out more rapidly in the C_3-mode than in the CAM-mode. RelΦ_{PSII} is a measure of the use of irradiance energy and excitation. Thus, by reducing external O_2 to 1 % it was seen that in the CAM-adapted plants the highest energy demand during an endogenous period was reached in the peaks of J_{CO2} and g_{H2O}, which was so strong that in the first peaks during LL there was almost no difference between relΦ_{PSII} at 21 % and 1 % O_2. This

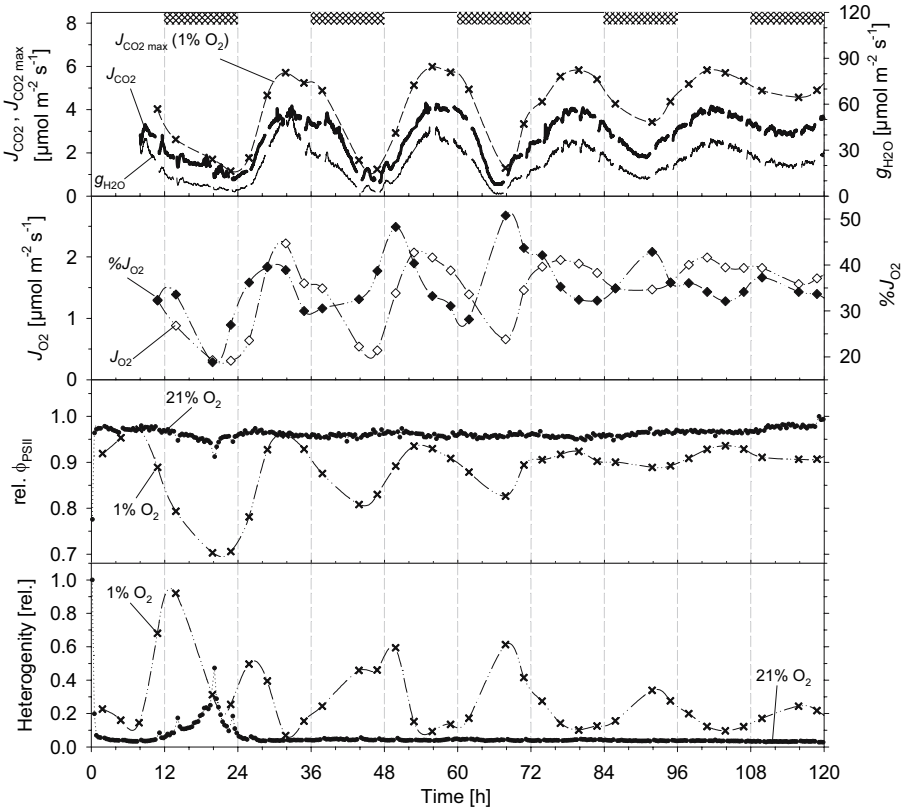

Fig. 11.4. Endogenous rhythm of photorespiratory activity of a leaf of a CAM-adapted plant at 25 °C. Further details as for Fig. 4

implies that when photorespiration was suppressed the energy consumed due to the oxygenation activity of RubisCO was deviated to other energy consuming processes. In the normal external dark/light rhythm of CAM organic acid synthesis and transport into the vacuoles mainly occur in the dark and use respiratory energy. In LL the energy for these processes can also be supplied by the light reactions of photosynthesis but now in direct competition with the Calvin cycle and photorespiration. This competition was largest in the peaks and lowest in the valleys of the rhythm. It shows that under varying energy demand photorespiration has a compensating effect on relΦ_{PSII} and confirms conclusions that photorespiration stabilizes and synchronises energy use in the whole leaf (Sect. 8.5).

Under 1 % O_2 also heterogeneity of relΦ_{PSII} was observed over the leaves in both modes and the spatial structure showed oscillations between homogenous and heterogeneous states, which were more pronounced and more regular in the CAM-mode than in the C_3-mode. Maximum values of heterogeneity

were reached while J_{CO2}, g_{H2O}, and $rel\Phi_{PSII}$ were in the phase of increasing from low to high values. The minimal values of heterogeneity in both modes were found at the peaks of J_{CO2}. In the CAM-mode maximum heterogeneity was attained at maximum $\%J_{O2}$.

11.3 Oscillator Elements and their Cryptic Network

Evidently the studies on *K. daigremontiana*, *M. crystallinum* and *C. minor* reveal oscillator elements at different hierarchical levels, such as the central oscillator genes, the metabolism related genes and the functional pacemakers of C_3 and CAM rhythms. In CAM we know of a feedback loop from the functional pacemaker malate accumulation/remobilisation to a metabolism gene, viz. *PEPCK* (Borland et al. 1999). However, largely it remains unknown how the various oscillator elements are connected in an operating network. *K. daigremontiana* and much more pronouncedly *C. minor* pose us the question of how such a network might function in mediating the CAM to C_3 shift during ongoing endogenous rhythmicity of gas exchange. Where are the feedback connections? Is the system feeding back to a central oscillator element like *TOC1* which is so strongly shifted in phase in oscillations in the CAM-mode as compared to the C_3-mode of photosynthesis in *M. crystallinum* (Boxall et al. 2005)? Unfortunately the so well advanced molecular studies in *M. crystallinum* are not accompanied by biochemical and physiological information to allow full assessment of the meaning of these observations for a functional network. We do not know if possibly *M. crystallinum* like *K. daigremontiana* and very conspicuously *C. minor* may also perform a CAM to C_3 shift during ongoing overt rhythmicity. The molecular study of Boxall et al. (2005) does not provide any information on concomitant malic acid oscillations and it only covers the first periods, when CAM type malate oscillations are still observed in *K. daigremontiana*. We do not know what happens later on and much more work is clearly needed. Conversely, molecular analyses like those of *M. crystallinum* are lacking for *K. daigremontiana* and *C. minor.* In particular the intriguing questions *C. minor* is asking us here would need to be addressed at the molecular level.

Evidently the answer to the first initial question (Sect. 11.1) "does *Clusia* have an endogenous clock?" is yes, albeit its oscillation is so strongly dampened. However, "does *Clusia* need a clock?" then, if it is so strongly dampened? It is widely assumed in the literature that endogenous circadian rhythmicity is important for fitness by anticipation of regularly changing environmental parameters in external night/day cycles. Hard evidence for this is not abundant, however. A competition experiment with *Synechococcus elongatus* strains of different endogenous period lengths reveals a selective advantage of circadian rhythmicity (Ouayang et al. 1998; Johnson and Golden 1999). Syn-

chronization of the clock to external cycles promotes photosynthetic activity, biomass increases, survival and competitive capacities (Dodd et al. 2005). The well known requirement of circadian time keeping for photoperiod sensing is a necessity in environmental adaptations. On the other hand, circadian rhythmicity may be just an inescapable side-product of evolution of life under the continuous entrainment by the natural environmental rhythm of days and nights (Lüttge 2002a). If circadian rhythmicity really provides preparedness for conditions to be regularly anticipated, it could be a hindrance for plasticity. Thus, it would make sense if strongly dampened rhythmicity as observed here were an intrinsic property of a plant as versatile and flexible ecophysiologically as *C. minor* (Chaps. 8 and 9).

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