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Oxygen isotope composition of CAM and C_3 *Clusia* species: non-steady-state dynamics control leaf water ^{18}O enrichment in succulent leaves

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ABSTRACT

Leaf gas exchange and leaf water ^{18}O enrichment ($\Delta^{18}O_L$) were measured in three *Clusia* species under field conditions during dry and wet seasons and in *Miconia argentea* during the dry season in the Republic of Panama. During the dry season, all three *Clusia* species used crassulacean acid metabolism (CAM); during the wet season *Clusia pratensis* operated in the C_3 mode, while *Clusia uvitana* and *Clusia rosea* used CAM. Large departures from isotopic steady state were observed in daytime $\Delta^{18}O_L$ of the *Clusia* species, especially during the dry season. In contrast, daytime $\Delta^{18}O_L$ was near isotopic steady state in the C_3 tree *M. argentea*. Across the full data set, non-steady-state predictions explained 49% of variation in observed $\Delta^{18}O_L$, whereas steady-state predictions explained only 14%. During the wet season, when $\Delta^{18}O_L$ could be compared with *Clusia* individuals operating in both C_3 and CAM modes, steady-state and non-steady-state models gave contrasting predictions with respect to interspecific variation in daytime $\Delta^{18}O_L$. The observed $\Delta^{18}O_L$ pattern matched that predicted for the non-steady state. The results provided a clear example of how non-steady-state control of leaf water ^{18}O dynamics can shift the slope of the relationship between transpiration rate and daytime $\Delta^{18}O_L$ from negative to positive.

Key-words: crassulacean acid metabolism; oxygen isotope ratio; tropical tree.

INTRODUCTION

Clusia is a neotropical genus comprising about 300 species of woody trees and shrubs. It is unique, in that it possesses tree species that utilize crassulacean acid metabolism (CAM) (Lüttge 2006). Strong CAM expression has been described in a small number of *Clusia* species, whereas weak and facultative CAM, where CAM expression is up-regulated in response to environmental stress, appears to be a more common option (Lüttge 1999; Holtum *et al.* 2004; Winter, Garcia & Holtum 2008). *Clusia rosea* and *Clusia uvitana* are two examples of *Clusia* species that can exhibit pronounced CAM (Ting *et al.* 1985; Winter *et al.* 1992; Zotz & Winter 1993, 1994; Winter *et al.* 2008).

The CAM is characterized by nocturnal CO_2 fixation by the enzyme phosphoenolpyruvate (PEP) carboxylase. This carboxylation reaction produces malic acid, which is then stored in the central vacuole until the following day, when it is decarboxylated to provide gaseous CO_2 for fixation by ribulose 1·5-bisphosphate carboxylase/oxygenase (Rubisco) in the photosynthetic carbon reduction cycle in illuminated leaves. CAM provides an advantage over C_3 photosynthesis in terms of water-use efficiency (Winter, Aranda & Holtum 2005) because the stomata are mainly open at night and closed during the warmest parts of the day, when evaporative demand is at a maximum. Additionally, CO_2 fixation by PEP carboxylase can proceed at a lower ratio of intercellular to ambient CO_2 partial pressures compared with C_3 photosynthesis (Holtum, O'Leary & Osmond 1983; Griffiths *et al.* 2007), further increasing the water-use efficiency of CAM. It has been suggested that CAM helps to overcome diffusion limitations on photosynthesis in succulent leaves, caused by low mesophyll conductance (Maxwell, von Caemmerer & Evans 1997; Griffiths *et al.* 2008), and that extended PEP carboxylase activity during the early light phase helps to prevent photoinhibition at midday (Roberts *et al.* 1998).

The analysis of stable carbon isotope ratios has made an important contribution to the study of CAM (Holtum *et al.* 1983; Griffiths 1992; Winter & Holtum 2002). The enzyme PEP carboxylase discriminates against ^{13}C to a lesser extent than Rubisco, with the former showing a net discrimination of about –5.7‰ relative to CO_2 in air, compared with about 29‰ for the latter (Farquhar, Ehleringer & Hubick 1989a). Given this difference, measurements of $\delta^{13}C$ in plant tissues can be used to estimate the relative contributions of nocturnal and daytime carbon fixation in CAM plants (Pierce, Winter & Griffiths 2002; Winter & Holtum 2002). Additionally, instantaneous measurements of carbon isotope discrimination have been used to quantify the low mesophyll conductance in *Clusia* and other CAM species (Griffiths *et al.* 2000).

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The analysis of stable oxygen isotope ratios in the tissues of CAM plants provides an additional tool for studying environmental and developmental regulation of CAM (Sternberg, Deniro & Johnson 1986; Helliker & Griffiths 2007; Reyes-Garcia *et al.* 2008). The ^{18}O enrichment of leaf water relative to source water taken up by plant roots varies in response to stomatal and environmental constraints on evaporative water loss from leaves (Craig & Gordon 1965; Dongmann *et al.* 1974; Farquhar & Lloyd 1993). The leaf water signal is then integrated into plant organic material (Farquhar, Barbour & Henry 1998; Barbour *et al.* 2000b; Roden, Lin & Ehleringer 2000; Helliker & Ehleringer 2002; Cernusak, Wong & Farquhar 2003). In order to meaningfully interpret $\delta^{18}O$ signals in CAM plants, however, a mechanistic understanding of the processes contributing to ^{18}O enrichment of leaf water and organic material is required (Helliker & Griffiths 2007; Reyes-Garcia *et al.* 2008).

Of particular interest in this context is the extent to which the leaf water system in CAM plants approaches isotopic steady state during photosynthetic gas exchange. Isotopic steady state means that the $^{18}O/^{16}O$ composition of transpired water vapour is equal to that of water entering the plant from the soil (Craig & Gordon 1965; Harwood *et al.* 1998; Farquhar & Cernusak 2005). If significant departure from isotopic steady state were to occur in photosynthesizing leaves, it could have consequences for the interpretation of $\delta^{18}O$ variation and its relationship with stomatal control over transpiration (Farquhar, Cernusak & Barnes 2007). CAM activity is typically associated with leaf succulence (Winter *et al.* 1983; Winter & Smith 1996), a condition which might cause the leaf water ^{18}O enrichment to respond relatively slowly to changes in its environmental and physiological drivers. Succulent leaves have large leaf water concentrations, and they could therefore be expected to show long time constants for the approach to isotopic steady state following a change in predicted steady-state ^{18}O enrichment.

In the steady state, leaf water ^{18}O enrichment is expected to correlate negatively with leaf transpiration rate for plants growing in the same environment (Farquhar & Lloyd 1993; Barbour & Farquhar 2000; Farquhar *et al.* 2007). We hypothesized that an increased departure from isotopic steady state in CAM compared with C_3 plants could cause the relationship between leaf water ^{18}O enrichment and transpiration rate to diverge from the steady-state prediction. The daily CAM cycle can be described by four phases (Osmond 1978): in Phase I, nocturnal CO_2 uptake proceeds via PEP carboxylase, with the stomata open to allow CO_2 to diffuse into the leaf; Phase II is characterized by further CO_2 uptake in the early morning by both PEP carboxylase and Rubisco with continued stomatal opening; Phase III takes place in the middle of the day, when the stomata close in response to high intercellular CO_2 partial pressures caused by malate decarboxylation; finally, Phase IV takes place in the afternoon, when malate decarboxylation approaches completion and the stomata open again to allow CO_2 uptake from the atmosphere by Rubisco. The pronounced midday stomatal closure during Phase III in CAM plants, combined with the tendency towards leaf succulence, could curtail the midday isotopic enrichment of leaf water by slowing the approach to steady state precisely at the time when the steady-state prediction would normally be at a daily maximum. On the other hand, because the stomata open again later in the afternoon and during the night in Phases IV and I, respectively, the daily depletion of ^{18}O from leaf water may proceed at a rate similar to that of a C_3 plant. According to this scenario, the CAM plant with a lesser daily transpiration rate than a neighbouring C_3 plant would also have a lesser daily leaf water ^{18}O enrichment; the relationship between leaf water ^{18}O enrichment and transpiration rate for the two plants would thus be positive, and opposite to the relationship expected for isotopic steady state.

In this study, we aimed to assess the controls over ^{18}O enrichment of leaf water and leaf organic material associated with CAM and C_3 photosynthesis. We judged *Clusia* to be a useful taxon for this comparative study, because of the existence of morphologically similar species that show a large range of variation in their expression of CAM (Lüttge 1999; Holtum *et al.* 2004; Winter *et al.* 2008). We limited our analysis to terrestrial phenotypes, in order to minimize the possible variation in source water $\delta^{18}O$ associated with an epiphytic lifestyle (Helliker & Griffiths 2007; Reyes-Garcia *et al.* 2008).

METHODS

Plant material and study site

The experiment was carried out at the Smithsonian Tropical Research Institute, Santa Cruz Experimental Field Facility, Gamboa, Republic of Panama (9°7′ N, 79°42′ W). The altitude at the study site is approximately 28 m above sea level. Leaf and twig materials were collected for isotopic analysis of dry matter in March 2005 from *Clusia* individuals growing outdoors individually in 200 L pots under full sunlight. Species identities are given in Table 1. A second collection was made in February 2006; at this time, leaf and twig materials were collected from individuals of *C. rosea*, *C. uvitana* and *Clusia pratensis* growing in the ground at the same site, as well as from an individual of *Miconia argentea* growing in the ground alongside them. These plants were growing approximately 10–20 m from the forest edge. Five to 10 outer-canopy leaves and a section of branch approximately 1 cm in diameter were collected from each plant. The samples were oven dried for several days at 70 °C before being ground to a fine powder for isotopic analysis. The species of the individuals sampled and their rooting environment are given in Table 1. All plants in Table 1 were approximately 5 years old at the time of sampling. The species are classified in Table 1 as C_3, weak CAM or strong CAM based on previous measurements of diel acid fluctuations, shoot gas exchange and carbon isotope ratios (Popp *et al.* 1987; Franco, Ball & Lüttge 1990; Winter *et al.* 1992; Roberts *et al.* 1998; Holtum *et al.* 2004).

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Table 1. Leaf traits and isotopic composition of leaf and twig dry matters for various *Clusia* individuals and an individual of *Miconia argentea*, growing in Gamboa, Panama

Species	Growth condition	Photosynthetic mode	Leaf size (cm^2)	Specific leaf area ($m^2\ kg^{-1}$)	Leaf water concentration ($mol\ m^{-2}$)	Leaf dry matter $\delta^{13}C$ (‰)	Twig dry matter $\delta^{13}C$ (‰)	Leaf dry matter $\delta^{18}O$ (‰)	Twig dry matter $\delta^{18}O$ (‰)
Clusia croatii	200 L pot	Weak CAM	34	7.0	17.7	−26.0	−26.2	22.0	19.9
Clusia cupulata	200 L pot	C_3	50	3.6	46.1	−26.2	−25.0	26.1	20.4
Clusia cylindrica	200 L pot	Weak CAM	40	7.8	25.1	−27.3	−26.8	24.7	19.5
Clusia divaricata	200 L pot	C_3	61	7.7	17.8	−24.6	−23.9	26.1	21.5
Clusia fructiangusta	Open soil	Weak CAM	77	4.9	29.6	−26.6	−25.8	23.3	20.3
Clusia liesneri	200 L pot	C_3	26	4.8	25.7	−25.7	−25.6	23.6	20.1
Clusia lineata	200 L pot	Weak CAM	25	6.7	20.1	−25.7	−24.4	25.3	20.5
Clusia longipetiolata	200 L pot	Weak CAM	260	3.8	26.8	−23.5	−22.5	25.8	20.9
Clusia minor	200 L pot	Weak CAM	32	6.8	27.2	−25.6	−25.6	22.9	19.0
Clusia odorata	200 L pot	Weak CAM	20	7.6	24.3	−26.8	−26.3	24.4	19.5
Clusia osaensis	200 L pot	Weak CAM	96	4.9	43.3	−24.3	−22.3	30.7	23.5
Clusia palmana	200 L pot	C_3	17	6.0	24.8	−24.9	−25.2	25.1	19.1
Clusia peninsulae	200 L pot	C_3	22	5.6	17.7	−27.2	−27.3	24.9	20.1
Clusia pratensis	200 L pot	Weak CAM	29	6.0	31.7	−25.5	−24.5	23.5	21.2
C. pratensis	Open soil	Weak CAM	46	6.9	34.0	−25.6	−26.9	24.3	22.7
Clusia quadrangula	200 L pot	Weak CAM	58	6.0	24.3	−26.7	−24.9	20.9	19.5
Clusia rosea	200 L pot	Strong CAM	98	2.6	43.7	−19.3	−14.8	24.1	20.2
C. rosea	Open soil	Strong CAM	119	3.3	45.5	−20.1	−17.5	25.1	20.6
Clusia stenophylla	200 L pot	C_3	31	4.7	19.9	−26.1	−25.3	22.0	19.1
Clusia uvitana	200 L pot	Strong CAM	59	4.3	42.3	−20.1	−17.7	21.5	18.5
C. uvitana	Open soil	Strong CAM	102	5.4	41.9	−20.8	−20.0	25.2	19.9
Clusia valerioi	200 L pot	Weak CAM	34	4.5	17.5	−23.9	−23.8	26.3	21.0
M. argentea	Open soil	C_3	267	7.8	9.5	−27.8	−28.1	23.4	22.0

All plants were approximately 5 years old at the time of sampling.
CAM, crassulacean acid metabolism.

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A third collection of leaf material for isotopic analysis was carried out in March 2007. On this occasion, leaves were collected from six individuals of *C. rosea*, four individuals of *Clusia cylindrica* and two individuals of *C. pratensis*. These plants were approximately 2 years old at the time of sampling. They were growing individually in 400 L pots in full sunlight. The pots were watered to saturation approximately every second day. For plants growing in pots, occasional water deficits can never be completely ruled out; however, we assumed that for most of the time, these plants did not experience water shortage. This contrasts with the potted 5-year-old plants shown in Table 1, which grew in pots half the size of the 2-year-old plants, and likely experienced drought stress, particularly during the dry season. The *Clusia* individuals shown in Table 1, which grew in the ground, also likely experienced drought stress during the dry season.

Leaf gas exchange measurements

We measured the diel patterns of leaf gas exchange and leaf water enrichment in the individuals of *C. rosea*, *C. uvitana* and *C. pratensis* growing side by side in the ground in February 2006, during the dry season, and again in September 2006, during the wet season. Additionally, we measured the diel pattern of leaf gas exchange and leaf water enrichment in the individual of *M. argentea* growing alongside these *Clusia* individuals in February 2006, to provide an example of leaf gas exchange and leaf water enrichment for a C_3 tree during the dry season. At this time, all three of the *Clusia* individuals sampled for leaf gas exchange and leaf water enrichment were operating in the CAM mode. The *Miconia* individual was approximately 10 m tall, and the canopy was accessed with scaffolding. The *Clusia* individuals were accessed from the ground.

Micrometeorological conditions at the site (air temperature, relative humidity, solar radiation, wind speed, and rainfall) were recorded every 15 min during the leaf gas exchange and leaf water sampling campaigns with an automated weather station (Campbell Scientific, Logan, UT, USA), as described previously (Winter *et al.* 2001, 2005). The micrometeorological sensors were positioned approximately 2 m above the ground.

Leaf gas exchange was measured on five leaves of each plant using a Li-Cor 6400 portable photosynthesis system (Li-Cor Inc., Lincoln, NE, USA). Measurements were made on the same set of leaves at intervals ranging from approximately 1 to 4 h during a complete diel cycle for each sampling campaign. The leaf cuvette enclosed 6 cm^2 of leaf area and had a transparent cover that allowed natural sunlight to enter from one side. The other side of the cuvette was metal and excluded sunlight. Air was drawn through the cuvette from a 19 L buffer volume that was insulated with reflective material and maintained in the shade to prevent excessive heating. Leaf temperatures of the five pre-selected leaves on each plant were measured at regular intervals with a hand-held infrared thermometer (Raytek MT Minitemp, Forestry Suppliers Inc., Jackson, MS, USA). These measurements were made to determine native leaf temperatures outside the influence of the leaf cuvette. During night-time, the cuvette temperature was maintained a few degrees above air temperature to prevent condensation inside the cuvette and to enable accurate measurements of transpiration and stomatal conductance. For our purposes of interpreting leaf water ^{18}O dynamics, we reasoned that the benefit of having accurate measurements of night-time stomatal conductance (Cuntz *et al.* 2007) outweighed the impact of any potential bias in nocturnal CO_2 exchange estimates caused by a gentle heating of the leaves in the cuvette.

Leaf water, stem water and atmospheric water vapour collections

Leaf material was collected for the determination of the $\delta^{18}O$ of leaf water from each of the individual plants sampled for gas exchange. At approximately 4 h intervals, leaf sections were collected from randomly selected leaves and placed in screw-cap, glass vials sealed with rubber septa. The leaf sections were cut with scissors from near the middle of the leaf and excluded the leaf mid-vein. Approximately 10 cm^2 of leaf area was collected from each of the three leaves on each individual plant; material from each of the three leaves was analysed separately. At the conclusion of gas exchange and leaf water sampling, branch sections were collected from each plant for the determination of xylem water $\delta^{18}O$. Three branch sections of approximately 1 cm diameter were collected from each plant; bark was removed and branch xylem material was sealed in glass vials. During the February sampling, atmospheric water vapour was collected for the determination of $\delta^{18}O$ at approximately 4 h intervals for periods of about 30 min. Air was drawn from a height of about 1 m through a glass trap submerged in a mixture of ethanol and dry ice (Cernusak *et al.* 2003; Cernusak, Farquhar & Pate 2005). Water was extracted from the leaf and xylem samples by cryogenic distillation in the Department of Plant Sciences, University of Cambridge, Cambridge, UK.

At the conclusion of the gas exchange measurements, the studied leaves were harvested for the determination of leaf water contents. Leaf fresh weight was measured immediately after severing the leaf from the plant. Projected areas of fresh leaves were then determined with an LI-3100 leaf area meter (Li-Cor Inc.), after which, the leaves were dried to constant weight at 70 °C for the determination of the leaf dry weight. Leaf water concentration of the 5-year-old *Clusia* individuals growing in 200 L pots was similarly determined in January 2007.

Stable isotope analyses

Carbon and oxygen stable isotope ratios of leaf and branch dry matters were measured at the Idaho Stable Isotopes Laboratory, Department of Forest Resources, University of Idaho, Moscow, ID, USA. For the $\delta^{13}C$ analyses, powdered samples of approximately 3 mg were combusted in an

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NC2500 elemental analyser (CE Instruments, Milan, Italy), coupled to a Delta Plus isotope ratio mass spectrometer (Finnigan MAT, Bremen, Germany) operating in continuous-flow mode. For the $\delta^{18}O$ analyses, approximately 1 mg of powdered sample was pyrolysed in a high-temperature furnace (Thermoquest TC/EA, Finnigan MAT), coupled to a Delta XP isotope ratio mass spectrometer (Finnigan MAT) operating in continuous-flow mode. The $\delta^{18}O$ of leaf and xylem water was determined by CO_2 equilibration (Scrimgeour 1995). Equilibrated CO_2 samples were analysed on a dual-inlet isotope ratio mass spectrometer (VG SIRA 10, modified by Provac Services, Crewe, UK) at the Department of Plant Sciences, University of Cambridge. The $\delta^{13}C$ and $\delta^{18}O$ values were calculated with respect to standards of Pee Dee Belemnite and Vienna Standard Mean Ocean Water, respectively.

Leaf water and organic material $\Delta^{18}O$ modelling

We used a model of leaf water and organic material ^{18}O enrichment to aid the interpretation of the ^{18}O dynamics in CAM and C_3 *Clusia* species. Leaf water ^{18}O enrichment at the evaporative sites in leaves was predicted according to the model of Craig & Gordon (1965), following modifications by subsequent authors (Dongmann *et al.* 1974; Flanagan, Comstock & Ehleringer 1991; Farquhar & Lloyd 1993):

$$\Delta^{18}O_e = \varepsilon^+ + \varepsilon_k + (\Delta^{18}O_v - \varepsilon_k)\frac{w_a}{w_i}, \quad (1)$$

where $\Delta^{18}O_e$ is the ^{18}O enrichment above source water of water at the evaporating sites in the leaf, ε^+ is the equilibrium fractionation that occurs during the phase change from liquid water to vapour, ε_k is the kinetic fractionation that occurs during water vapour diffusion through stomatal pores and the leaf boundary layer, $\Delta^{18}O_v$ is the ^{18}O enrichment of atmospheric water vapour with respect to water taken up by the roots (source water) and w_a and w_i are water vapour mole fractions in the atmosphere and leaf intercellular air spaces, respectively. The ^{18}O enrichment ($\Delta^{18}O$) is defined with respect to source water as $\Delta^{18}O = R/R_s - 1$, where R is the $^{18}O/^{16}O$ of the leaf water or organic material and R_s is that of source water. The equilibrium fractionation, ε^+, was calculated as a function of leaf temperature (Bottinga & Craig 1969):

$$\varepsilon^+(‰) = 2.644 - 3.206\left(\frac{10^3}{T}\right) + 1.534\left(\frac{10^6}{T^2}\right), \quad (2)$$

where T is the leaf temperature in K. The kinetic fractionation, ε_k, was calculated as (Farquhar *et al.* 1989b):

$$\varepsilon_k(‰) = \frac{32r_s + 21r_b}{r_s + r_b}, \quad (3)$$

where r_s and r_b are the stomatal and boundary layer resistances to water vapour diffusion (m^2 s mol^{-1}) and 32 and 21 are associated fractionation factors scaled to per mil (Cappa *et al.* 2003).

In the case where atmospheric water vapour is in isotopic equilibrium with source water, such that $\Delta^{18}O_v$ is equal to $-\varepsilon^+$, Eqn 1 is simplified to (Farquhar *et al.* 2007):

$$\Delta^{18}O_e = (\varepsilon^+ + \varepsilon_k)\left(1 - \frac{w_a}{w_i}\right). \quad (4)$$

Equation 4 demonstrates that the relative humidity term that drives steady-state ^{18}O enrichment of evaporative sites is $1 - w_a/w_i$, that is, the deviation from unity of the water vapour mole fraction of ambient air relative to that inside the leaf.

It has been observed that the leaf water ^{18}O signal most relevant to plant organic material is that of average lamina leaf water, $\Delta^{18}O_L$, rather than that of the evaporative sites (Cernusak *et al.* 2003). Farquhar & Lloyd (1993) and Farquhar & Gan (2003) suggested that the $\Delta^{18}O_L$ in the steady state ($\Delta^{18}O_{Ls}$) can be predicted from $\Delta^{18}O_e$ according to the relationship:

$$\Delta^{18}O_{Ls} = \frac{\Delta^{18}O_e(1 - e^{-\wp})}{\wp}, \quad (5)$$

where $\wp$ is a Péclet number, defined as $EL/(CD)$, where E is the transpiration rate (mol m^{-2} s^{-1}), L is a scaled effective path length (m), C is the molar concentration of water (mol m^{-3}) and D is the diffusivity of $H_2{}^{18}O$ in water (m^2 s^{-1}). The D can be calculated as (Cuntz *et al.* 2007):

$$D = 119 \times 10^{-9} e^{\left(-\frac{637}{T-137}\right)}, \quad (6)$$

where T is the leaf temperature in K.

Equation 5 predicts $\Delta^{18}O_L$ under steady-state conditions. For leaves with high water contents and low stomatal conductance, such as would be the case for many *Clusia* species, leaf water enrichment may not be at steady state. Non-steady-state variation in $\Delta^{18}O_L$ can be calculated as follows (Farquhar & Cernusak 2005):

$$\Delta^{18}O_L = \Delta^{18}O_{Ls} - \frac{\alpha^+\alpha_k}{gw_i} \cdot \frac{1 - e^{-\wp}}{\wp} \cdot \frac{d(W\Delta^{18}O_L)}{dt}, \quad (7)$$

where $\Delta^{18}O_{Ls}$ is the steady-state prediction of $\Delta^{18}O_L$ from Eqn 5, α^+ is defined as $1 + (\varepsilon^+(‰)/1000)$, α_k is defined as $1 + (\varepsilon_k(‰)/1000)$, W is the lamina leaf water concentration (mol m^{-2}), t is time (s) and g is the total conductance to water vapour of the stomata plus the boundary layer (mol m^{-2} s^{-1}).

Barbour & Farquhar (2000) proposed that the ^{18}O enrichment of plant cellulose ($\Delta^{18}O_c$) can be predicted from $\Delta^{18}O_L$ as follows:

$$\Delta^{18}O_c = \Delta^{18}O_L(1 - p_{ex}p_x) + \varepsilon_{wc}, \quad (8)$$

where p_{ex} is the proportion of oxygen atoms that exchange with local water during cellulose synthesis in the developing plant tissue, p_x is the proportion of source water (i.e. water not subject to evaporative ^{18}O enrichment) in the

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developing tissue and ε_{wc} is an equilibrium fractionation between oxygen in organic molecules and medium water (~27‰). Finally, the ^{18}O enrichment of plant dry matter ($\Delta^{18}O_p$) can be related to that of plant cellulose by adding a term (ε_{cp}) to describe the difference between the two (Barbour & Farquhar 2000):

$$\Delta^{18}O_p = \Delta^{18}O_c + \varepsilon_{cp}. \quad (9)$$

Equations 8 and 9 suggest that variation in $\Delta^{18}O_p$ should reflect variation in $\Delta^{18}O_L$, so long as $p_{ex}p_x$, ε_{wc} and ε_{cp} are relatively constant among the samples being compared (Barbour 2007).

The leaf water enrichment model was parameterized using measured gas exchange data, leaf temperatures measured with the infrared thermometer and data collected by the automated weather station. The $\Delta^{18}O_L$ was predicted at time steps of 15 min, the frequency at which measurements of air temperature, relative humidity and wind speed were logged by the weather station. Estimates of native leaf temperature, measured outside the gas-exchange cuvette, and stomatal conductance were calculated by linear interpolation for time steps in which they were not measured. Native transpiration rates were calculated as the product of total conductance (stomata plus boundary layer) and leaf to air vapour mole fraction difference, based on native leaf temperatures. Boundary layer conductance was calculated from measured wind speed and an assumed leaf characteristic dimension (Campbell & Norman 1998), which was calculated as the square root of the mean individual leaf area for each species. The scaled effective path length, L, used to calculate $\wp$ for Eqn 5, was assumed to be 50 mm for all species. This is similar to the value estimated for several woody tree species (Cernusak *et al.* 2005, 2008). Leaf water concentration was taken as the mean value measured for each species and assumed to be constant through time. The $\Delta^{18}O_v$ for the dry season sampling was calculated using the mean of the measurements of $\delta^{18}O$ for atmospheric vapour and the xylem water $\delta^{18}O$ for each plant. For the wet season sampling, $\Delta^{18}O_v$ was assumed equal to $-\varepsilon^+$, where ε^+ was calculated from the air temperature. Leaf water enrichments above source water were calculated as $\Delta^{18}O_L = (\delta^{18}O_L - \delta^{18}O_s)/(1 + \delta^{18}O_s/1000)$, where $\delta^{18}O_L$ is the observed leaf water $\delta^{18}O$ in per mil, and $\delta^{18}O_s$ is the $\delta^{18}O$ of source water in per mil, which we assumed equal to that measured for xylem water.

For the application of the non-steady-state leaf water model, it is necessary to define an initial condition of $\Delta^{18}O_L$. We initialized the model to predict $\Delta^{18}O_L$ on the days during which $\Delta^{18}O_L$ was measured by 'spinning up' the model over the preceding week. In these simulations, measured air temperature, relative humidity and wind speed from the weather station were used. The diel profile of stomatal conductance for each plant was assumed the same as was determined on the day of measurement. The difference between leaf temperature and air temperature, $T_L - T_a$, was predicted from the empirical relationships between $T_L - T_a$ and photon flux density measured at the weather station. The $T_L - T_a$ was then added to the air temperature for each time step to predict leaf temperature. All other calculations were as described previously. In addition to these 'spin-up' periods, we ran the non-steady-state $\Delta^{18}O_L$ model in this way for the wet-season month of September 2006, to examine the predicted variation in $\Delta^{18}O_L$ between C_3 and CAM *Clusia* species over a time period long enough to smooth out day to day variation in interspecific enrichment patterns.

RESULTS

Leaf gas exchange

Dry season measurements of net CO_2 exchange, stomatal conductance and transpiration are shown in Figs 1 and 2. All three *Clusia* species were operating in the CAM mode during the dry season sampling. This can be seen by the positive nocturnal values of net CO_2 exchange, indicating CO_2 uptake at night for all three species (Fig. 1a). *C. pratensis* and *C. uvitana* showed net CO_2 uptake in the afternoon on day 1, whereas *C. rosea* maintained a net CO_2 efflux. All three species continued net CO_2 uptake after sunrise, until about midmorning on day 2, at which point, net CO_2 exchange shifted from uptake to efflux. *C. rosea* showed a pronounced 'burst' of CO_2 efflux at about 1000 h on day 2, at which point, we recorded a mean net CO_2 exchange of -22.8 μmol CO_2 m^{-2} s^{-1} for this species (Fig. 1a).

In contrast to the *Clusia* species, the individual of *M. argentea* showed a normal pattern of CO_2 exchange for a C_3 tree species, with CO_2 efflux at night and photosynthetic CO_2 uptake during the day (Fig. 2a). There was a peak in net CO_2 uptake at about 0900 h and another at about 1200 h, with mean values near 8 μmol CO_2 m^{-2} s^{-1}.

Stomatal conductance and transpiration were generally low for all three *Clusia* species during the dry season sampling (Fig. 1b,c). Stomatal conductance to water vapour remained below 80 mmol m^{-2} s^{-1}, and transpiration did not exceed 1.8 mmol m^{-2} s^{-1} (Fig. 1b,c). For the *M. argentea* individual, on the other hand, stomatal conductance peaked near 300 mmol m^{-2} s^{-1}, and transpiration reached 3.5 mmol m^{-2} s^{-1} (Fig. 2b,c).

Wet season gas exchange measurements for the three *Clusia* species are shown in Fig. 3. At this time, *C. pratensis* was operating in the C_3 photosynthetic mode, while *C. uvitana* and *C. rosea* operated in the CAM mode. *C. pratensis* showed net CO_2 uptake throughout day 1, followed by nocturnal CO_2 efflux and renewed photosynthetic CO_2 uptake starting shortly after sunrise on day 2 (Fig. 3a). *C. uvitana* and *C. rosea* shifted from CO_2 uptake to CO_2 efflux at about 0900 h on day 1. *C. uvitana* then showed a small CO_2 uptake in the afternoon on day 1, whereas *C. rosea* maintained a net CO_2 efflux until sunset. Both showed nocturnal CO_2 uptake (Fig. 3a). *C. rosea* again showed a pronounced 'burst' of CO_2 efflux at about 1000 h on day 2 (Fig. 3a).

Daytime stomatal conductance was higher for all three *Clusia* species in the wet season than in the dry season. We

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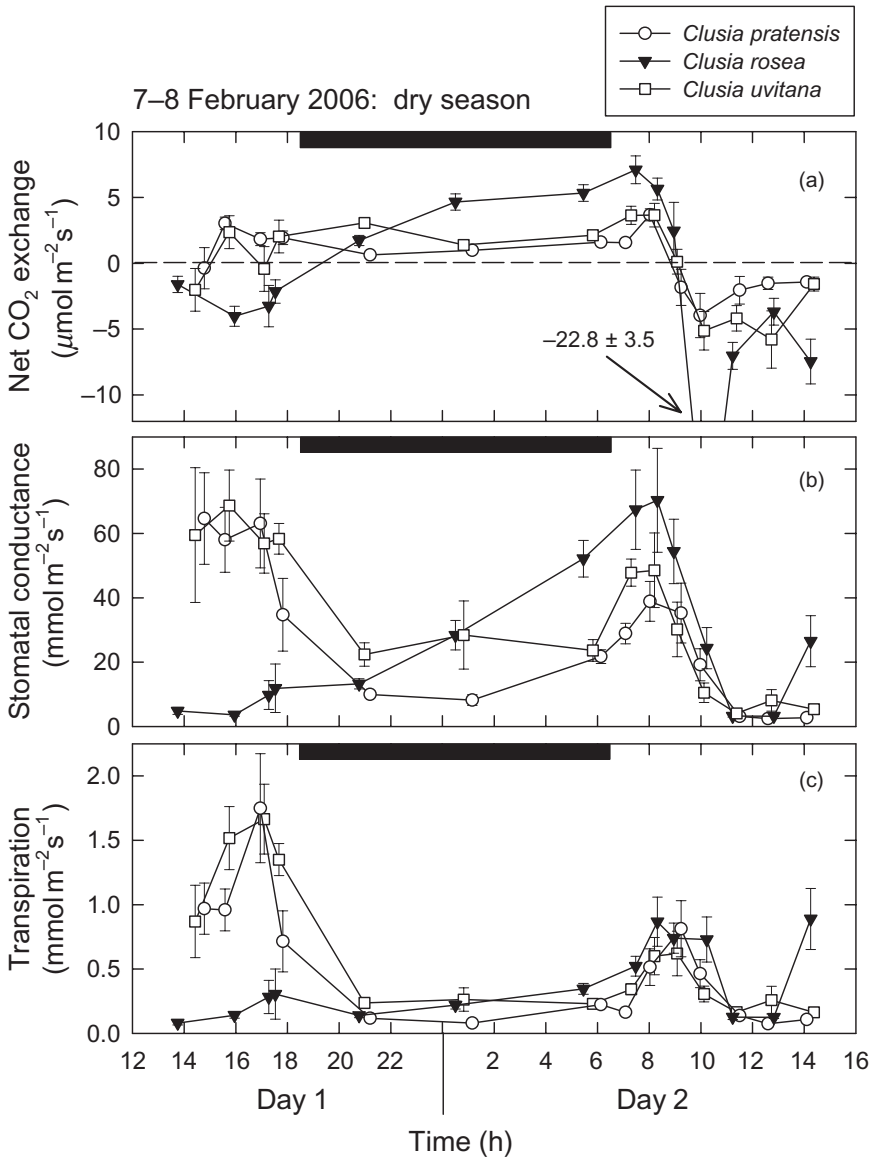

Figure 1. Net CO_2 exchange (a), stomatal conductance (b) and transpiration (c) for three species of *Clusia* growing side by side in Gamboa, Panama. Measurements were made during the dry season. Positive values of net CO_2 exchange indicate CO_2 uptake, and the negative values indicate CO_2 efflux. Data points are the mean of five leaves; error bars represent 1 SE. Solid horizontal bars indicate night-time.

observed maxima between 200 and 250 mmol m^{-2} s^{-1} in the morning on day 1 (Fig. 3b). *C. uvitana* and *C. rosea* showed a very pronounced stomatal closure during the middle of the day, whereas *C. pratensis* showed a more gradual decline in stomatal conductance throughout the day (Fig. 3b). The three species had similarly low nocturnal stomatal conductance to water vapour, each exhibiting a nocturnal maximum of about 25 mmol m^{-2} s^{-1}. Transpiration was higher for *C. pratensis* than for *C. rosea* or *C. uvitana*, with a maximum of about 4 mmol m^{-2} s^{-1} for *C. pratensis*, compared with about 2 mmol m^{-2} s^{-1} for *C. rosea* and *C. uvitana* (Fig. 3c).

Figures 4 and 5 show dry season measurements of leaf temperature, irradiance and the deviation from unity of the water vapour mole fraction of ambient air relative to that inside the leaf, $1 - w_a/w_i$. Leaf temperatures inside the cuvette during gas exchange measurements for the *Clusia* species were generally higher than ambient air temperature by about 3–5 °C (Fig. 4a). Native leaf temperatures, measured outside the leaf cuvette, were similar to air temperature at night, but higher than air temperature by as much as 10 °C during the day (Fig. 4a). A similar pattern was observed for *M. argentea* (Fig. 5a). The wet season pattern of leaf temperature for the *Clusia* species was similar to that for the dry season (Fig. 6a); however, daytime temperatures outside the leaf cuvette were not elevated above air temperature to the same extent as during the dry season measurements.

Photon flux density recorded in the open showed typical diurnal variations during the three sampling campaigns, with intermittent reductions as a result of variable cloud cover (Figs 4b, 5b & 6b). Incident photon flux density inside the leaf cuvette during gas exchange measurements was typically less than that recorded in the open (Figs 4b, 5b & 6b).

Figures 4c, 5c and 6c show the departure from unity of the water vapour mole fraction of ambient air relative to that

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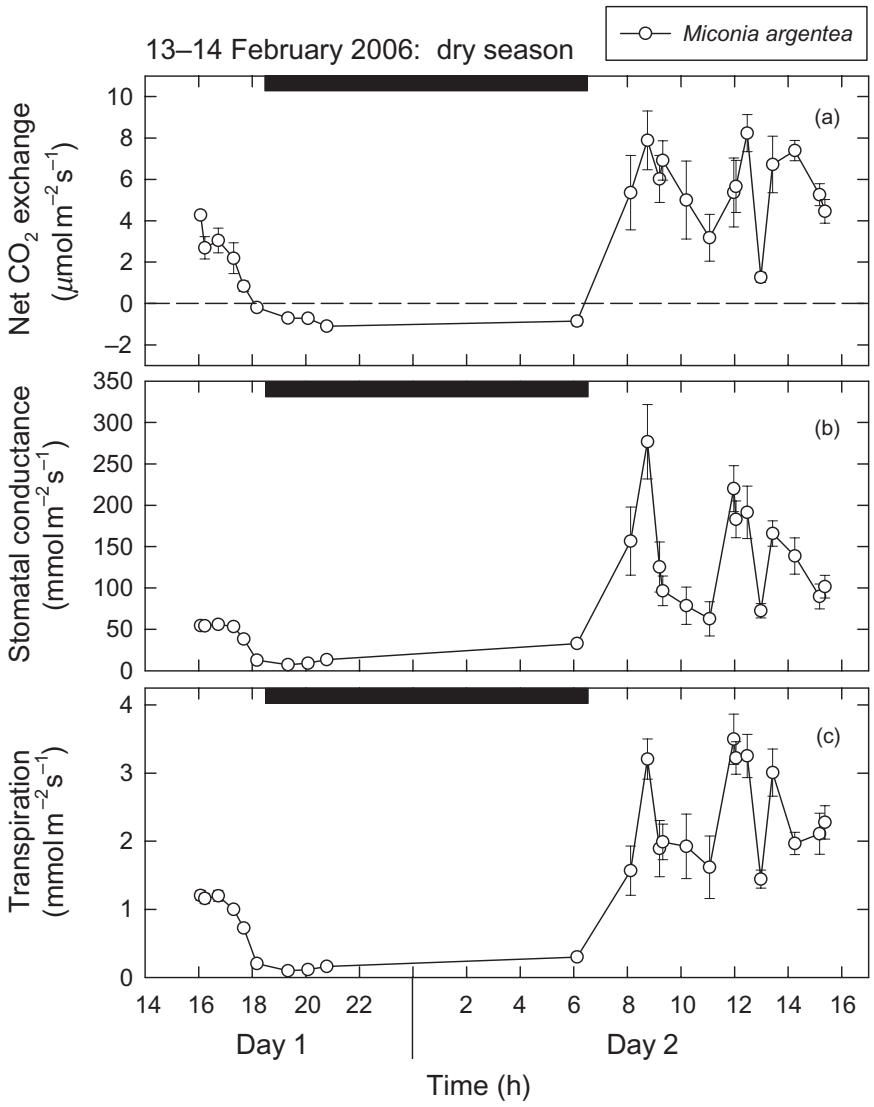

Figure 2. Net CO_2 exchange (a), stomatal conductance (b) and transpiration (c) of an individual of *Miconia argentea* growing alongside the *Clusia* individuals in Gamboa, Panama. Measurements were made during the dry season. The mean of measurements on each of the five leaves is shown; error bars represent 1 SE. Solid horizontal bars indicate night-time.

inside the leaf, $1 - w_a/w_i$, for the three sampling campaigns. This is the term that drives the estimate of steady-state leaf water enrichment at the evaporating sites, as shown in Eqn 4. The $1 - w_a/w_i$ for the *Clusia* species during the dry season reached maximum values of about 0.7 (Fig. 4c), whereas maximum values during the wet season were near 0.4 (Fig. 6c). The maximum for *M. argentea* during the dry season was about 0.6 (Fig. 6b).

Leaf water, stem water and atmospheric water vapour $\delta^{18}O$

We observed little variation in xylem water $\delta^{18}O$. Dry season observations were –3.2, –3.4, –3.4 and –3.7‰ for *C. pratensis*, *C. uvitana*, *C. rosea* and *M. argentea*, respectively; wet season observations were –3.5, –4.2 and –3.2‰ for *C. pratensis*, *C. uvitana* and *C. rosea*, respectively. The $\delta^{18}O$ of atmospheric water vapour observed during the dry season had a mean value of –11.0‰, with individual observations ranging from –9.2 to –13.0‰; these values are reasonably close to that predicted for equilibrium with xylem water, which would be about –12.4‰.

Leaf water ^{18}O enrichments with respect to xylem water are shown in Fig. 7. The $\Delta^{18}O_L$ observed in the *Clusia* species in the dry season ranged between about 5 and 15‰ (Fig. 7a), whereas values observed in the wet season ranged between about 0 and 10‰ (Fig. 7c). The $\Delta^{18}O_L$ of *M. argentea* in the dry season ranged between about 5 and 15‰ (Fig. 7b). The $\Delta^{18}O_L$ of *M. argentea* showed a pronounced diel variation, with an early morning minimum and afternoon maximum, whereas $\Delta^{18}O_L$ of the *Clusia* species showed only slight diel trends (Fig. 7a,c). In general, there appeared to be considerably more heterogeneity among leaves sampled at the same time for the *Clusia* species compared with *M. argentea* (Fig. 7).

Steady-state and non-steady-state predictions of $\Delta^{18}O_L$ are shown in Fig. 7. There was a strong divergence between the two sets of predictions for the *Clusia* species in the dry season (Fig. 7a). The steady-state scenario considerably over-predicted observed $\Delta^{18}O_L$ during the day, and

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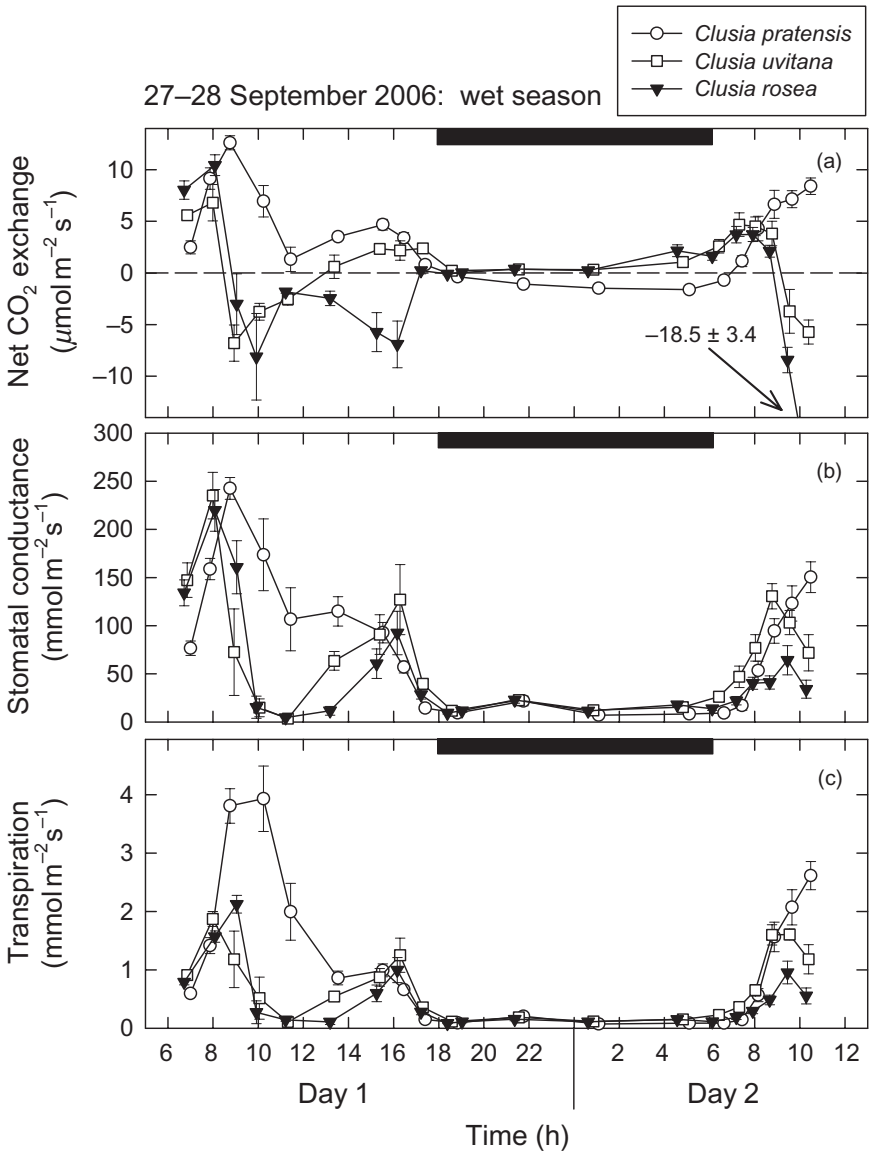

Figure 3. Net CO_2 exchange (a), stomatal conductance (b) and transpiration (c) for three species of *Clusia* during the wet season. The mean of measurements on each of the five leaves is shown; error bars represent 1 SE. Solid horizontal bars indicate night-time.

under-predicted observed $\Delta^{18}O_L$ during the night. In contrast, the non-steady-state prediction showed a significantly damped diel trend that matched the observed $\Delta^{18}O_L$ trend reasonably well (Fig. 7a). Modelling results for *M. argentea* during the dry season differed from those for the *Clusia* species. In the case of *M. argentea*, the steady-state and non-steady-state predictions diverged during the night, but were very similar during the day (Fig. 7b). Observed $\Delta^{18}O_L$ was close to daytime predictions, but fell in between the steady-state and non-steady-state predictions during the night.

Steady-state and non-steady-state predictions of $\Delta^{18}O_L$ for the *Clusia* species during the wet season were generally lower than corresponding predictions during the dry season (Fig. 7c), but showed similar patterns of divergence during the day and night. Importantly, the steady-state and non-steady-state predictions for the wet season differed with regard to interspecific patterns of variation in daytime $\Delta^{18}O_L$. The steady-state scenario predicted that *C. rosea* and *C. uvitana* would have a higher daytime $\Delta^{18}O_L$ than *C. pratensis*. In contrast, the non-steady-state scenario predicted that *C. pratensis* and *C. uvitana* would have higher daytime $\Delta^{18}O_L$ than *C. rosea*. Figure 7d shows an expanded view of the daytime hours of day 1 from the wet season sampling, in which the contrasting predictions of interspecific variation in daytime $\Delta^{18}O_L$ among the *Clusia* species can be more easily discerned.

To further clarify the interspecific pattern in $\Delta^{18}O_L$ predicted for the non-steady-state, Fig. 8 shows average non-steady-state predictions for the wet-season month of September for the three *Clusia* species. Thus, each 15 min time step in Fig. 8 represents the mean of the predictions for that time of day over the 30 d in September 2006. In this presentation, one can clearly see the predicted diel patterns of interspecific variation in daytime $\Delta^{18}O_L$ for the non-steady-state scenario. *C. pratensis*, operating in the C_3 mode, is predicted to have the highest daytime $\Delta^{18}O_L$; *C. rosea*,

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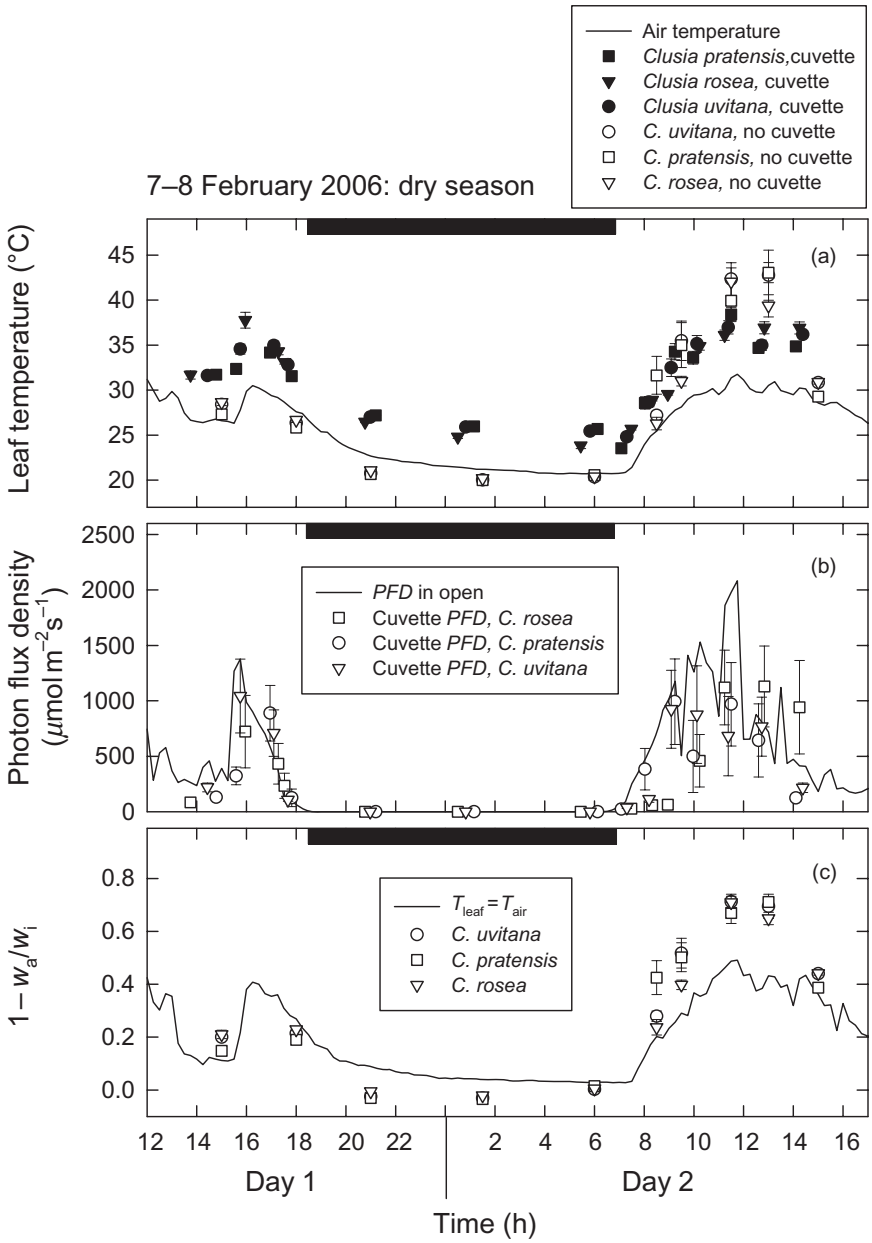

Figure 4. Leaf temperature inside and outside the gas exchange cuvette, along with air temperature (a), photosynthetic photon flux density inside and outside the cuvette (b) and the deviation from unity of the water vapour mole fraction of ambient air relative to that inside the leaf (c) for three *Clusia* species during the dry season. The line in (c) is the value that would be calculated if leaf temperature was assumed equal to air temperature. The term $1 - w_a/w_i$ drives the predicted steady state ^{18}O enrichment of evaporative sites in leaves. The calculations of w_i are based on leaf temperatures measured outside the leaf cuvette. The mean of the measurements on each of the five leaves is shown; error bars represent 1 SE. Solid horizontal bars indicate night-time. PFD, photon flux density.

operating in the CAM mode, is predicted to have the lowest daytime $\Delta^{18}O_L$; and *C. uvitana*, also operating in the CAM mode, is predicted to have daytime $\Delta^{18}O_L$ intermediate between those of *C. pratensis* and *C. rosea*.

Mean observed leaf water ^{18}O enrichments for the *Clusia* species for daytime and night-time, and for the dry and wet season samplings, are shown in Fig. 9. Analysis of variance, taking species, season and day/night as independent factors, indicated that season ($P < 0.0001$, $n = 112$) and species ($P = 0.004$, $n = 112$) were significant sources of variation in observed $\Delta^{18}O_L$, whereas day/night and the various interaction terms among independent factors were not statistically significant. Importantly, the interspecific pattern of observed $\Delta^{18}O_L$ for daytime during the wet season matched the pattern predicted by the non-steady-state scenario, where *C. pratensis* showed the highest mean daytime $\Delta^{18}O_L$, *C. rosea* the lowest and *C. uvitana* an intermediate value between *C. pratensis* and *C. rosea* (Fig. 8a, solid bars).

Across the full data set, the non-steady-state model explained 49% of variation in observed $\Delta^{18}O_L$ ($R^2 = 0.49$,

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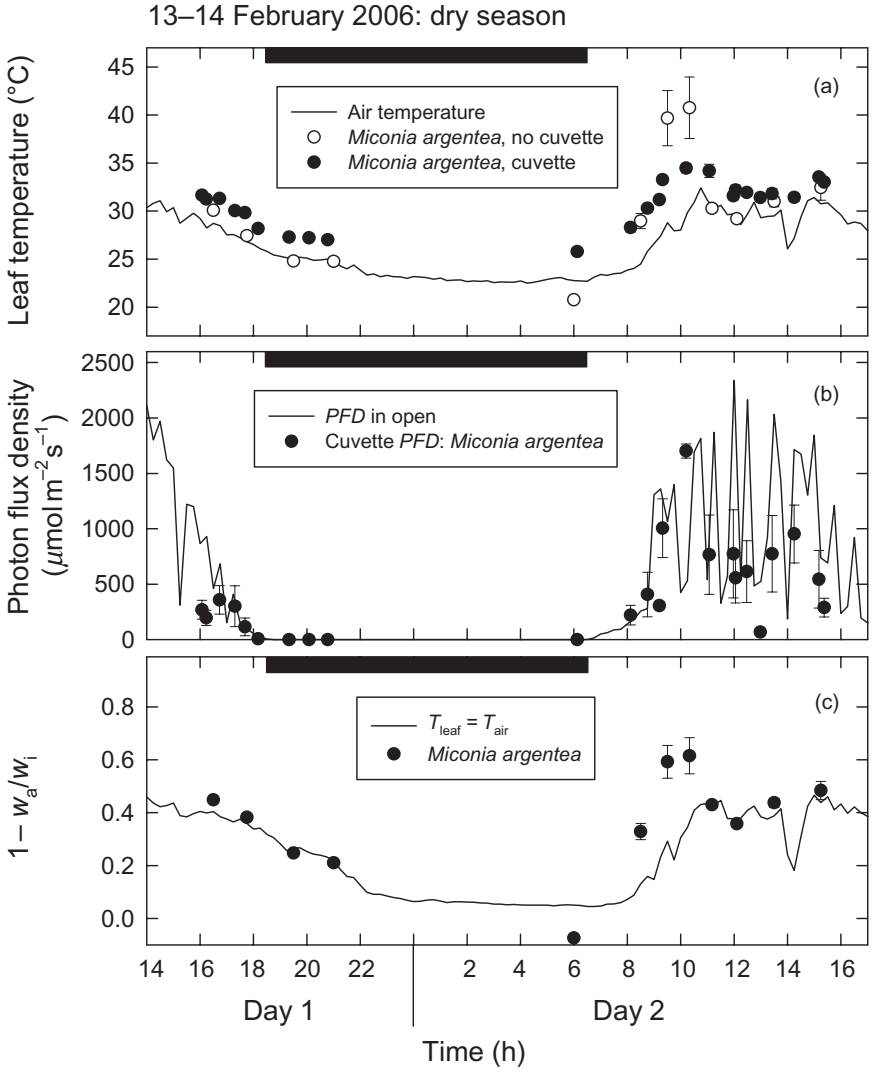

Figure 5. Leaf temperature inside and outside the gas exchange cuvette (a), photosynthetic photon flux density inside and outside the cuvette (b) and the deviation from unity of the water vapour mole fraction of ambient air relative to that inside the leaf (c) for *Miconia argentea* during the dry season. The mean of measurements on each of the five leaves is shown; error bars represent 1 SE. Solid horizontal bars indicate night-time. PFD, photon flux density.

$P < 0.0001$, $n = 127$). In contrast, assuming isotopic steady state caused the model to explain only 14% of variation in observed $\Delta^{18}O_L$ ($R^2 = 0.14$, $P < 0.0001$, $n = 127$). For the non-steady-state model, the slope of the relationship between the observed and predicted $\Delta^{18}O_L$ did not differ from unity ($P = 0.80$). However, the intercept differed from zero by –1.9‰ ($P = 0.03$), suggesting a slight offset between the observed and predicted values.

Stable isotope composition of dry matter

Results for the stable isotope composition of leaf and twig dry matters from the 5-year-old *Clusia* plants are shown in Table 1, along with the results for the individual of *M. argentea*. The strong CAM species, *C. rosea* and *C. uvitana*, could be clearly distinguished from the other *Clusia* species based on $\delta^{13}C$ of either leaf or twig dry matters (Table 1). On the other hand, species classified as C_3 and weak CAM were indistinguishable from each other based on dry matter $\delta^{13}C$. Mean values for the leaf dry matter $\delta^{13}C$ for the strong CAM, weak CAM and C_3 species were –20.1, –25.6 and –25.8‰, respectively; mean values for the twig dry matter $\delta^{13}C$ were –17.5, –25.0 and –25.4‰, respectively. Leaf and twig dry matters $\delta^{13}C$ were closely correlated with each other ($r = 0.96$, $P < 0.0001$, $n = 22$). There was no significant variation among strong CAM, weak CAM and C_3 *Clusia* species for the 5-year-old plants in the $\delta^{18}O$ of leaf dry matter ($P = 0.88$, $n = 22$) or in the $\delta^{18}O$ of twig dry matter ($P = 0.42$, $n = 22$). Leaf and twig dry matters $\delta^{18}O$ were correlated with each other ($r = 0.70$, $P = 0.0003$, $n = 22$).

In contrast to the 5-year-old *Clusia* plants, the 2-year-old *Clusia* plants showed significant variation in the leaf dry matter $\delta^{18}O$ between weak CAM and strong CAM species (Fig. 10). Because these plants were well watered over the course of their lives, *C. pratensis* and *C. cylindrica* were assumed to have operated predominantly in the C_3 mode, whereas *C. rosea* operates in the CAM mode even when well watered (Fig. 3). These differences can be clearly seen in the $\delta^{13}C$ of the leaf dry matter (Fig. 10a). The mean leaf $\delta^{13}C$ for the 2-year-old *C. pratensis* and *C. cylindrica* was –27.0‰, about 1.4‰ more negative than the average leaf dry matter $\delta^{13}C$ for the 5-year-old weak CAM species. The

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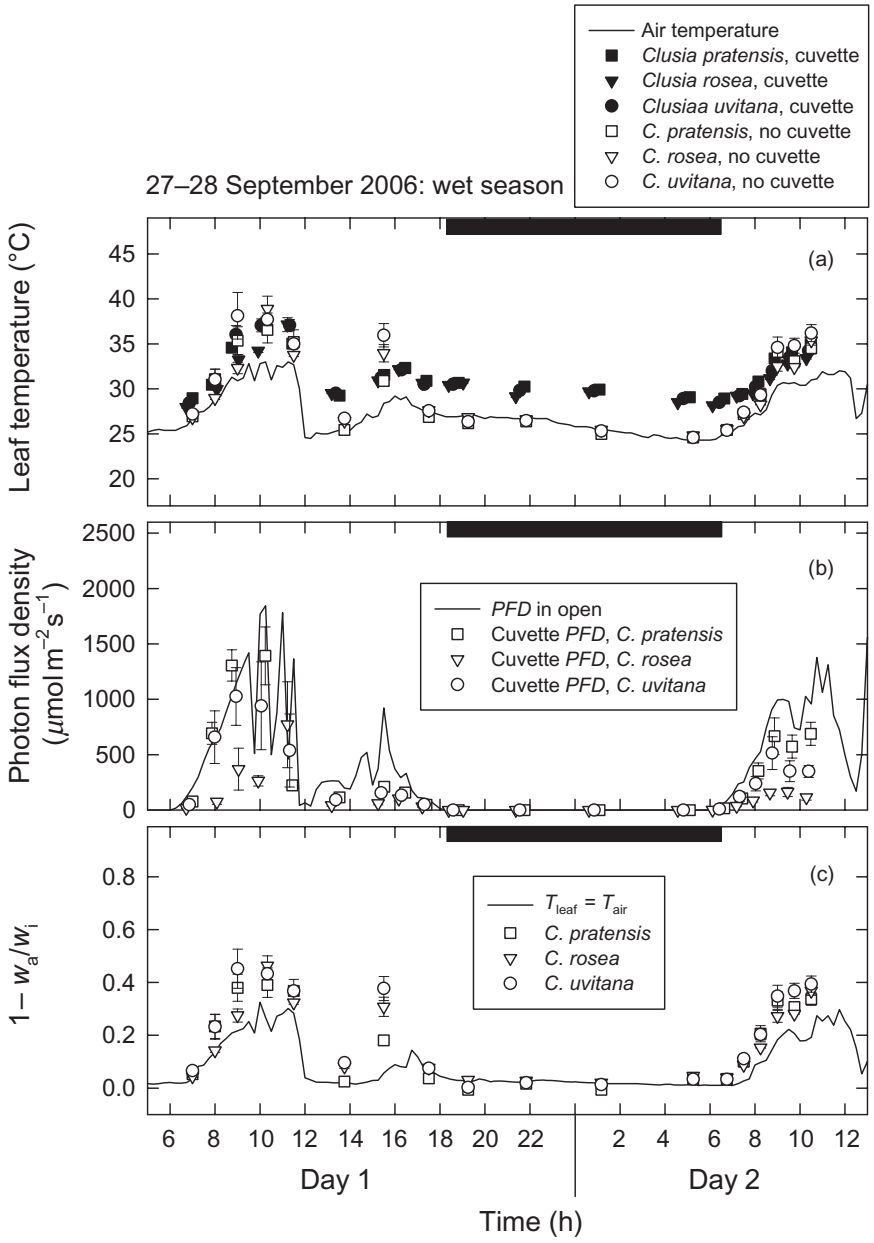

Figure 6. Leaf temperature inside and outside the gas exchange cuvette (a), photosynthetic photon flux density inside and outside the cuvette (b) and the deviation from unity of the water vapour mole fraction of ambient air relative to that inside the leaf (c) for three *Clusia* species during the wet season. The mean of measurements on each of the five leaves is shown; error bars represent 1 SE. Solid horizontal bars indicate night-time. PFD, photon flux density.

strong CAM species *C. rosea* was less enriched in the leaf dry matter $\delta^{18}O$ by about 2‰ compared with the weak CAM species *C. pratensis* and *C. cylindrica* (Fig. 10b).

DISCUSSION

We measured leaf water ^{18}O enrichment concurrently with leaf gas exchange in individuals of three *Clusia* species growing side by side under dry and wet season conditions. Two of the species, *C. rosea* and *C. uvitana*, operated in the CAM mode during both the dry and wet seasons. The third, *C. pratensis*, was a weak CAM species, for which CAM likely makes only a small contribution to total carbon gain, as judged by the $\delta^{13}C$ of the leaf and twig dry matters (Table 1, Fig. 10a). During the dry season, *C. pratensis*, shifted to CAM, in response to seasonal drought. However, during the wet season, *C. pratensis* operated in the C_3 mode, and this is presumably when most of its growth took place. In order to have an example of the pattern of leaf water ^{18}O enrichment for a C_3 tree during the dry season, we also made measurements on an individual of *M. argentea*, growing alongside the *Clusia* individuals. In addition to the gas exchange and leaf water analyses, we measured $\delta^{13}C$ and $\delta^{18}O$ of leaf and twig dry matters in these, and various other *Clusia* individuals growing at the study site. This suite of measurements allowed us to analyse the controls over the leaf water and dry matters ^{18}O enrichment in *Clusia* individuals operating in CAM and C_3 modes.

Control of daytime $\Delta^{18}O_L$ in the *Clusia* individuals was dominated by non-steady-state dynamics, and this was

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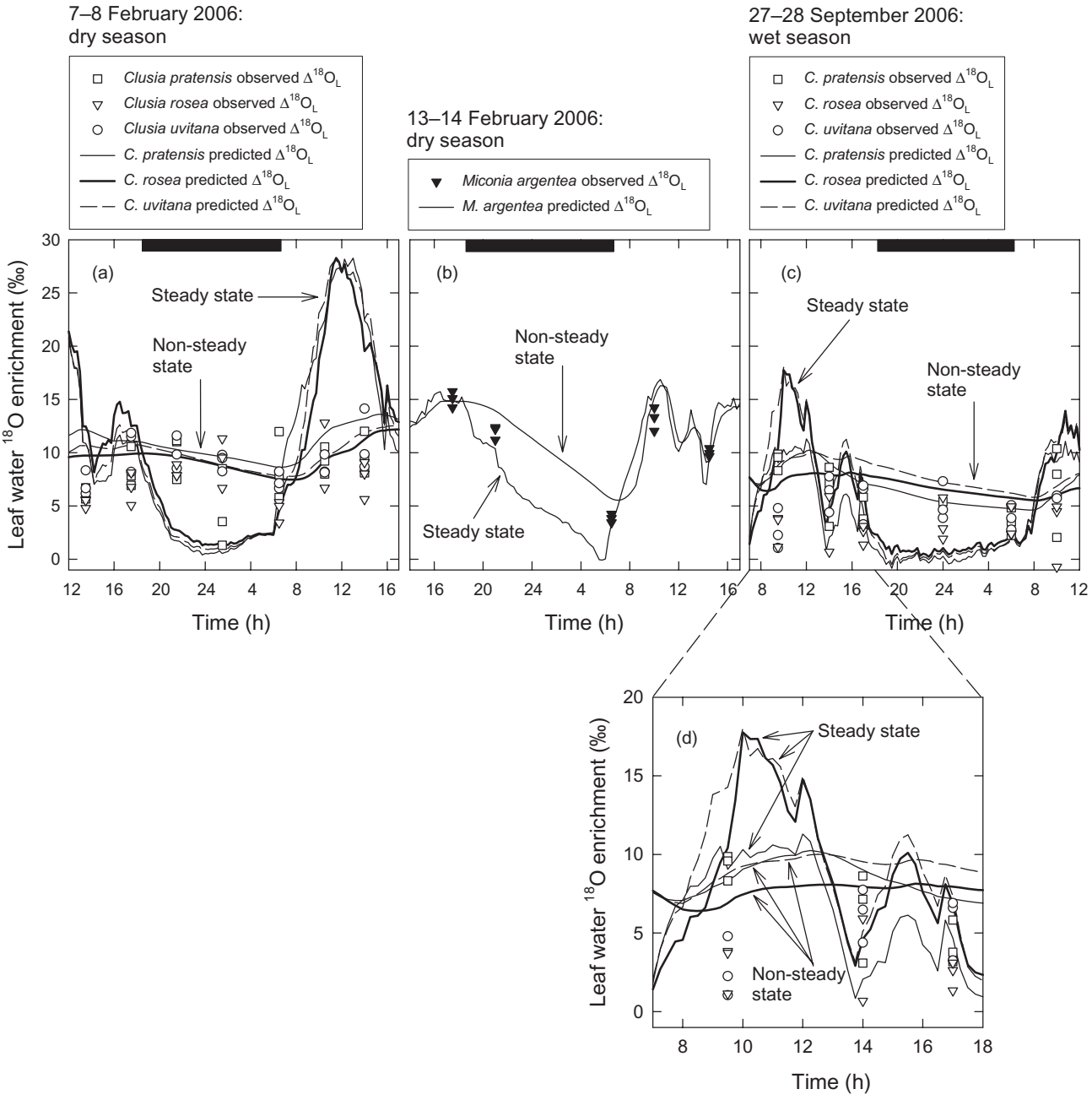

Figure 7. Predicted and observed leaf water ^{18}O enrichment for three *Clusia* species during the dry season (a), for *Miconia argentea* during the dry season (b) and for the *Clusia* species during the wet season (c). Panel (d) shows an expanded view of the daytime hours on day 1 of the wet-season sampling. The expanded view allows the steady-state and non-steady-state predictions for each species to be more easily contrasted. Solid horizontal bars indicate night-time.

especially apparent during the dry season (Fig. 7a). The same was true for night-time $\Delta^{18}O_L$, but we focused on daytime $\Delta^{18}O_L$ because this is presumably when the leaf water signal is transferred to organic molecules through the photosynthetic carbon reduction cycle (Farquhar *et al.* 1998; Barbour, Cernusak & Farquhar 2005; Barbour 2007). The non-steady-state control of daytime $\Delta^{18}O_L$ in *Clusia* was in sharp contrast to *M. argentea*, for which steady-state and non-steady-state predictions converged during the day in the dry season, and both were close to the observed daytime $\Delta^{18}O_L$ (Fig. 7b). These observations for *M. argentea* are similar to those recorded previously for other plant species, both in terms of the diel pattern of $\Delta^{18}O_L$ and the proximity of observed daytime $\Delta^{18}O_L$ to the steady-state prediction (Dongmann *et al.* 1974; Zundel *et al.* 1978; Allison, Gat & Leaney 1985; Flanagan & Ehleringer 1991; Walker & Lance 1991; Flanagan, Marshall & Ehleringer 1993; Cernusak, Pate & Farquhar 2002; Cernusak *et al.* 2005; Ometto *et al.* 2005; Barnard *et al.* 2007). In contrast, there are far fewer examples of observed daytime $\Delta^{18}O_L$ showing

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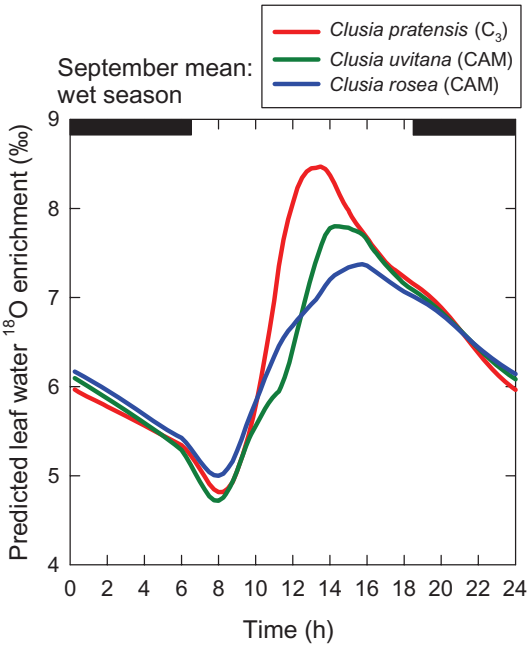

Figure 8. Average predicted non-steady-state leaf water ^{18}O enrichment for the wet-season month of September 2006. The calculations were performed in 15 min time steps and the mean taken across the full 30 d for each time step. Solid horizontal bars indicate night-time. CAM, crassulacean acid metabolism.

large departures from isotopic steady state (Harwood *et al.* 1998; Pendall, Williams & Leavitt 2005; Seibt *et al.* 2006).

For a given set of environmental conditions, the steady-state model predicts that a leaf with a higher stomatal conductance, and therefore a higher transpiration rate, will have a lower $\Delta^{18}O_L$ than a leaf with a lower stomatal conductance (Farquhar *et al.* 2007). This is because a higher stomatal conductance decreases ε_k, while a higher transpiration rate cools the leaf, decreasing $1 - w_a/w_i$, and increases the Péclet number, $\wp$. All three processes contribute to a decrease in $\Delta^{18}O_L$, and this leads to a positive correlation between the term $1 - \Delta^{18}O_L/\Delta^{18}O_e$ and transpiration rate under steady-state conditions (Ripullone *et al.* 2008). These considerations have led to the suggestion that the measurements of $\Delta^{18}O$ in organic material can be used to infer variation in stomatal conductance for plants grown in a common environment (Farquhar, Condon & Masle 1994; Yakir & Israeli 1995; Scheidegger *et al.* 2000). Thus, $\Delta^{18}O$ of leaf organic material would be expected to correlate negatively with stomatal conductance, and this has been observed experimentally (Barbour & Farquhar 2000; Barbour *et al.* 2000a; Grams *et al.* 2007; Sullivan & Welker 2007).

The predominance of the non-steady-state dynamic in controlling $\Delta^{18}O_L$ in the *Clusia* leaf water system leads to the opposite outcome. This is best seen in Fig. 7d, which shows an expanded view of the daytime hours of day 1 for the wet season sampling, and in Fig. 8, which shows average predictions of $\Delta^{18}O_L$ over the wet-season month of September. Here, *C. pratensis*, operating in the C_3 mode, clearly had a higher stomatal conductance and transpiration rate than *C. rosea*, operating in the CAM mode, when averaged throughout the day (Fig. 3b,c). We estimated the total daytime transpiration to be 40 mol m^{-2} for *C. pratensis*, compared with 15 mol m^{-2} for *C. rosea* (Table 2). The steady-state model accordingly predicted higher $\Delta^{18}O_L$ in *C. rosea* than in *C. pratensis* over the course of the day (Fig. 7d). In contrast, the non-steady-state model predicted the opposite pattern, that *C. pratensis* would have higher $\Delta^{18}O_L$ than *C. rosea* over the same time period (Fig. 7d), and averaged over the month (Fig. 8). The non-steady-state prediction matched the interspecific pattern observed for daytime $\Delta^{18}O_L$ (Fig. 9a, solid bars). Moreover, this is consistent with the results for the leaf dry matter $\delta^{18}O$ observed in the 2-year-old plants, in which *C. pratensis* and *C. cylindrica* operated in the C_3 mode, whereas *C. rosea* operated in the CAM mode (Fig. 10).

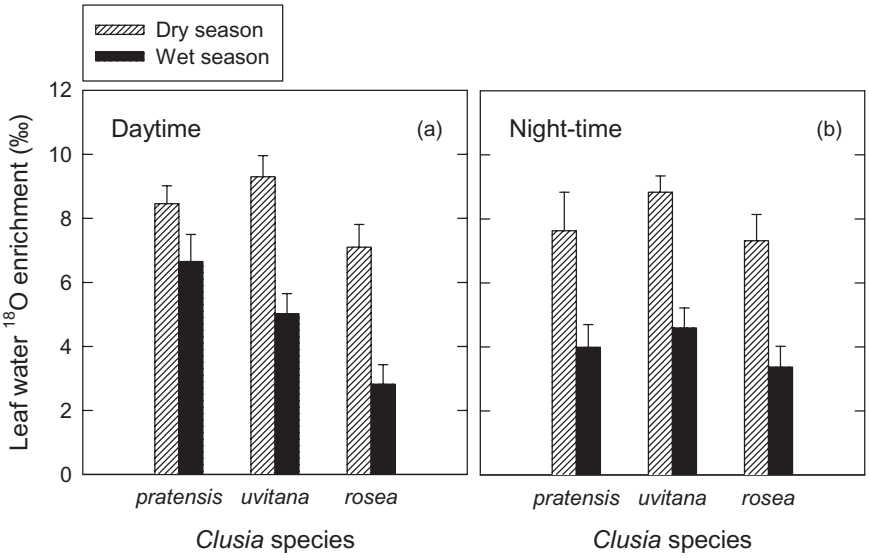

Figure 9. Mean values for observed leaf water ^{18}O enrichment during daytime (a) and night-time (b) for three *Clusia* species during the dry and wet seasons. Error bars represent 1 SE.

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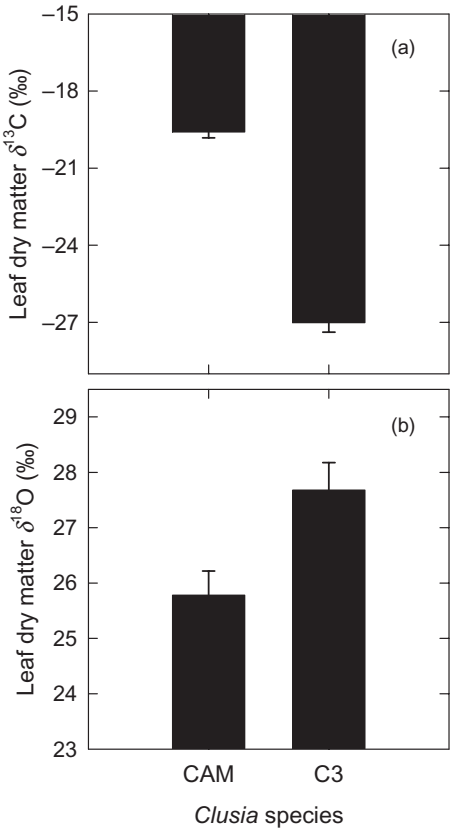

Figure 10. Carbon (a) and oxygen (b) isotope compositions of leaf dry matter from 2-year-old *Clusia* plants. The crassulacean acid metabolism sample comprises six individuals of *Clusia rosea*, whereas the C_3 sample comprises four individuals of *Clusia cylindrica* and two individuals of *Clusia pratensis*. The plants were grown in 400 L pots and were well watered throughout their lives. Thus *C. cylindrica* and *C. pratensis* were assumed to have operated in the C_3 mode. Error bars represent 1 SE.

Positive relationships between transpiration and $\Delta^{18}O$ of leaf water or organic material, opposite to the relationship predicted for the steady state, have also been observed previously (Sheshshayee *et al.* 2005; Cernusak *et al.* 2007). In the present example, the positive relationship between daytime $\Delta^{18}O_L$ and transpiration rate emerged both because of high leaf water concentrations in the *Clusia* leaves, and because of the midday stomatal closure associated with Phase III of the daily CAM cycle. For the CAM *Clusia* species, the large W and very low midday g slowed the midday accumulation of leaf water ^{18}O enrichment. Subsequently, the reopening of the stomata in the afternoon for CAM Phase IV allowed leaf water ^{18}O depletion to proceed at a rate similar to that of the C_3 *Clusia* species. Thus, the reversal of the relationship between $\Delta^{18}O_L$ and transpiration rate relative to the steady-state expectation resulted from the combination of large W and the characteristic diel pattern of stomatal conductance associated with CAM. Further research into the control of daytime $\Delta^{18}O_L$ by W and midday stomatal closure would be helpful to determine whether such phenomena might provide an explanation for

Table 2. Leaf water concentrations (W), efflux rates (E and gw_i) and residence times (W/E and W/gw_i) for *Clusia* individuals during the dry and wet seasons and an individual of *Miconia argentea* during the dry season in Gamboa, Panama

		E (mol m^{-2})		W/E (h)		gw_i (mol m^{-2})		W/gw_i (h)	
	W (mol m^{-2})	24 h total	Daytime total	24 h average	Daytime average	24 h total	Daytime total	24 h average	Daytime average
Dry season									
Clusia pratensis	34.4	15.1	14.6	54.7	28.3	51.3	38.9	16.1	10.6
Clusia rosea	44.1	12.6	12.2	84.0	43.4	47.7	29.5	22.2	17.9
Clusia uvitana	41.7	15.9	15.1	62.9	33.1	60.2	40.4	16.6	12.4
M. argentea	9.5	62.3	60.0	3.7	1.9	147.6	131.0	1.5	0.9
Wet season									
C. pratensis	33.6	40.6	40.5	19.9	10.0	148.2	134.5	5.4	3.0
C. rosea	46.9	15.6	15.1	72.2	37.3	87.2	70.2	12.9	8.0
C. uvitana	42.2	24.7	24.5	41.0	20.7	103.0	85.8	9.8	5.9

The net efflux of water vapour from the leaf is given by E, whereas the one-way efflux of water vapour is given by gw_i, where g is the total conductance to water vapour of the stomata plus the boundary layer and w_i is the intercellular water vapour mole fraction. The E is relevant from a hydration perspective, whereas gw_i is relevant from an isotopic perspective. Both were calculated either on a 24 h basis or as a total for daytime hours only. Residence times are given in hours. Each value is the mean of measurements on five leaves.

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the interesting results of Sheshshayee *et al.* (2005) showing positive relationships between $\Delta^{18}O$ and transpiration in C_3 plants.

The time constant for the approach of $\Delta^{18}O_L$ to a new steady state following a step change in one of its environmental or physiological determinants can be approximated as W/gw_i (Dongmann *et al.* 1974; Farquhar & Cernusak 2005). A brief examination of this term reveals why $\Delta^{18}O_L$ of *Clusia* leaves, and especially of the strong CAM species, is typically not at steady state. Table 2 shows the leaf water concentration, W, along with 24 h and daytime estimates of gw_i, and transpiration, E. The leaf water residence time from a water relations perspective can be described as W/E, whereas the relevant term from an isotopic perspective is W/gw_i. The former takes account of the net flux of water vapour out of the leaf, whereas the latter takes account of the one-way efflux. From an isotopic perspective, the one-way fluxes of water vapour into and out of the leaf must be differentiated, because they carry different isotopic signatures (Farquhar & Cernusak 2005). Whereas W/gw_i was less than 1 h in the C_3 tree *M. argentea* during the dry season, values for the *Clusia* species ranged from 11 to 18 h in the dry season and from 3 to 8 h in the wet season (Table 2). The W/gw_i for *M. argentea* is similar to that calculated previously for another C_3 tree, *Eucalyptus globulus* (Cernusak *et al.* 2005). Estimates for W/E showed that *C. rosea* transpired only about one-third of its leaf water content over a 24 h period (Table 2), a trait that likely contributes to its ability to survive epiphytically.

In general, large values of W and small values of g caused $\Delta^{18}O_L$ in the *Clusia* leaves to change relatively slowly over the diel time course (Figs 7 & 9). This result is similar to a previous report of $\Delta^{18}O_L$ in CAM versus C_3 plants, in which the CAM species showed considerably damped diurnal variation in $\Delta^{18}O_L$ compared with the C_3 species, and had lower afternoon maxima (Sternberg *et al.* 1986). This pattern likely holds for succulent plants in general, although an interesting exception to it has also been reported (Cooper & DeNiro 1989). The regular movement of water into and out of storage tissues in succulent plants (Smith & Nobel 1986; Smith, Schulte & Nobel 1987) may also have implications for $\Delta^{18}O_L$. Smith & Nobel (1986) estimated that most of the water exchanged between xylem and storage tissues in *Agave deserti* occurred across cell membranes, rather than through cell walls. This could have implications for the effective path length, L, which partly defines the Péclet number, $\wp$ (Barbour & Farquhar 2004).

We observed that $\delta^{18}O$ of leaf and twig dry matters did not differ between strong CAM and weak CAM or C_3 *Clusia* species for the 5-year-old plants (Table 1). Our measurements and modelling of $\Delta^{18}O_L$ for *C. pratensis*, *C. uvitana* and *C. rosea* suggested that under wet-season conditions, the weak CAM species *C. pratensis* should have a higher daytime $\Delta^{18}O_L$ than the strong CAM species, *C. rosea* and *C. uvitana* (Figs 7–9). A general prediction, based on this analysis, is that $\delta^{18}O$ of organic material should be lower in strong CAM *Clusia* species than in weak CAM or C_3 *Clusia* species when grown in the same environment. This is what we observed for the leaf dry matter $\delta^{18}O$ in the 2-year-old plants (Fig. 10). We suggest that the $\delta^{18}O$ of organic material in the 2-year-old plants best represented the leaf water ^{18}O dynamics that we observed in the wet season, because the 2-year-old plants were maintained under well-watered conditions. In contrast, the 5-year-old C_3 and weak CAM plants experienced occasional drought stress, which would have caused their $\Delta^{18}O_L$ to become more CAM-like, as a result of decreased stomatal conductance during the day. The 1.4‰ increase in the leaf dry matter $\delta^{13}C$ in the 5-year-old weak CAM species compared with the 2-year-old weak CAM species supports this interpretation.

We observed pronounced 'bursts' of CO_2 efflux from the strong CAM species *C. rosea* during midmorning in both the dry and wet seasons (Figs 1a & 3a). We considered that these very high apparent CO_2 efflux rates could have been caused by the enclosure of the leaves in the gas exchange cuvette. However, there would not appear to be an artefact associated with an increase in leaf temperature caused by cuvette enclosure, because leaf temperatures inside the cuvette were similar to those measured outside the cuvette at these times (Figs 4a & 6a). Another possibility is that the leaf was partly shaded, because the lower surface of the cuvette did not transmit sunlight, and that this caused an overestimate of CO_2 efflux compared with that which would have occurred outside the cuvette. However, if in fact these very high CO_2 efflux rates are a real phenomenon, they may play an important role in signalling rapid stomatal closure at the initiation of Phase III of the daily CAM cycle, by causing a sudden spike in intercellular CO_2 concentrations (Lüttge 2007). The delay in stomatal closure until midmorning might then relate to the extension of PEP carboxylase activity into the light period in *Clusia*, with decarboxylation delayed to maximize CO_2 regeneration and prevent photoinhibition at midday (Roberts *et al.* 1998). We note, however, that our interpretation of ^{18}O dynamics in *Clusia* is not dependent on the authenticity, or otherwise, of the apparent midmorning burst of CO_2 efflux in *C. rosea*.

We found that *Clusia* species that had been classified previously as either C_3 or weak CAM (based on diel acid fluctuations) were generally indistinguishable from each other based on measurements of the leaf and twig dry matters $\delta^{13}C$ (Table 1). This is consistent with previous observations for *Clusia* (Holtum *et al.* 2004). More generally, it has been observed that CAM plants can typically be categorized as strong or weak CAM phenotypes, with few intermediate observations when CAM activity is estimated from dry matter $\delta^{13}C$ (Pierce *et al.* 2002; Winter & Holtum 2002; Silvera, Santiago & Winter 2005). In plants classified as weak CAM based on tissue $\delta^{13}C$, strong CAM activity might occur for a short time but make little contribution to overall carbon gain (Holtum *et al.* 2004; Silvera *et al.* 2005). Interestingly, in *C. rosea*, generally considered a strong CAM plant, fruit tissues were observed to carry a C_3-like $\delta^{13}C$ signal, suggesting that carbohydrate exported from leaves for fruit production may have been primarily derived from that fixed during Phases II or IV of the daily CAM cycle (Borland & Dodd 2002).

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In conclusion, we observed strong departures from isotopic steady state in leaf water ^{18}O enrichment of three *Clusia* species during both the dry and wet seasons at a field site in the Republic of Panama. During the wet season, when C_3 and CAM photosynthetic modes could be compared among the *Clusia* species, we observed that *C. pratensis*, operating in the C_3 mode, had a higher daytime $\Delta^{18}O_L$ than *C. rosea*, operating in the CAM mode. *C. uvitana*, also operating in the CAM mode, had a daytime $\Delta^{18}O_L$ intermediate between *C. pratensis* and *C. rosea*. The observed interspecific pattern in $\Delta^{18}O_L$ matched that predicted when non-steady-state effects were taken into account. In contrast, the observed interspecific pattern was opposite to that which would have been predicted if daytime $\Delta^{18}O_L$ was at an isotopic steady state. As a consequence, the observed relationship between daytime transpiration rate and daytime $\Delta^{18}O_L$ was opposite to that which would be predicted under steady-state conditions. These observations provided a clear example of how non-steady-state leaf water dynamics can shift the relationship between transpiration rate and daytime $\Delta^{18}O_L$ from negative to positive for succulent-leaved plants showing varying degrees of midday stomatal closure.

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