Oxygen isotope composition of CAM and C₃ 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 C₃ 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 C₃ 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 C₃ 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 model. 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; Zott & Winter 1993, 1994; Winter et al. 2008).

The CAM is characterized by nocturnal CO₂ 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 CO₂ for fixation by ribulose 1,5-bisphosphate carboxylase/oxygenase (Rubisco) in the photosynthetic carbon reduction cycle in illuminated leaves. CAM provides an advantage over C₃ 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, CO₂ fixation by PEP carboxylase can proceed at a lower ratio of intercellular to ambient CO₂ partial pressures compared with C₃ 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 CO₂ 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).
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 (Sterbeng, Denio & 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 vapor 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 C3 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 C3 plant. According to this scenario, the CAM plant with a lesser daily transpiration rate than a neighbouring C3 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 C3 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. ivitana 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 C3, 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

<table>
<thead>
<tr>
<th>Species</th>
<th>Growth condition</th>
<th>Photosynthetic mode</th>
<th>Leaf size (cm²)</th>
<th>Specific leaf area (m² kg⁻¹)</th>
<th>Leaf water concentration (mol m⁻²)</th>
<th>Leaf dry matter δ¹³C (%)</th>
<th>Twig dry matter δ¹³C (%)</th>
<th>Leaf dry matter δ¹⁸O (%)</th>
<th>Twig dry matter δ¹⁸O (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Clusia croatii</td>
<td>200 L pot</td>
<td>Weak CAM</td>
<td>34</td>
<td>7.0</td>
<td>17.7</td>
<td>-26.0</td>
<td>-26.2</td>
<td>22.0</td>
<td>19.9</td>
</tr>
<tr>
<td>Clusia cupulata</td>
<td>200 L pot</td>
<td>C₃</td>
<td>50</td>
<td>3.6</td>
<td>46.1</td>
<td>-26.2</td>
<td>-25.0</td>
<td>26.1</td>
<td>20.4</td>
</tr>
<tr>
<td>Clusia cylindrica</td>
<td>200 L pot</td>
<td>Weak CAM</td>
<td>40</td>
<td>7.8</td>
<td>25.1</td>
<td>-27.3</td>
<td>-26.8</td>
<td>24.7</td>
<td>19.5</td>
</tr>
<tr>
<td>Clusia divaricata</td>
<td>200 L pot</td>
<td>C₃</td>
<td>61</td>
<td>7.7</td>
<td>17.8</td>
<td>-24.6</td>
<td>-23.9</td>
<td>26.1</td>
<td>21.5</td>
</tr>
<tr>
<td>Clusia fructiangusta</td>
<td>Open soil</td>
<td>Weak CAM</td>
<td>77</td>
<td>4.9</td>
<td>29.6</td>
<td>-26.6</td>
<td>-25.8</td>
<td>23.3</td>
<td>20.3</td>
</tr>
<tr>
<td>Clusia liesneri</td>
<td>200 L pot</td>
<td>C₃</td>
<td>26</td>
<td>4.8</td>
<td>25.7</td>
<td>-25.7</td>
<td>-25.6</td>
<td>23.6</td>
<td>20.1</td>
</tr>
<tr>
<td>Clusia lineata</td>
<td>200 L pot</td>
<td>Weak CAM</td>
<td>25</td>
<td>6.7</td>
<td>20.1</td>
<td>-25.7</td>
<td>-24.4</td>
<td>25.3</td>
<td>20.5</td>
</tr>
<tr>
<td>Clusia longipetiolata</td>
<td>200 L pot</td>
<td>Weak CAM</td>
<td>260</td>
<td>3.8</td>
<td>26.8</td>
<td>-23.5</td>
<td>-22.5</td>
<td>25.8</td>
<td>20.9</td>
</tr>
<tr>
<td>Clusia minor</td>
<td>200 L pot</td>
<td>Weak CAM</td>
<td>32</td>
<td>6.8</td>
<td>27.2</td>
<td>-25.6</td>
<td>-25.6</td>
<td>22.9</td>
<td>19.0</td>
</tr>
<tr>
<td>Clusia odorata</td>
<td>200 L pot</td>
<td>Weak CAM</td>
<td>20</td>
<td>7.6</td>
<td>24.3</td>
<td>-26.8</td>
<td>-26.3</td>
<td>24.4</td>
<td>19.5</td>
</tr>
<tr>
<td>Clusia osaensis</td>
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<td>Weak CAM</td>
<td>96</td>
<td>4.9</td>
<td>43.3</td>
<td>-24.3</td>
<td>-22.3</td>
<td>30.7</td>
<td>23.5</td>
</tr>
<tr>
<td>Clusia palmana</td>
<td>200 L pot</td>
<td>C₃</td>
<td>17</td>
<td>6.0</td>
<td>24.8</td>
<td>-24.9</td>
<td>-25.2</td>
<td>25.1</td>
<td>19.1</td>
</tr>
<tr>
<td>Clusia peninsulae</td>
<td>200 L pot</td>
<td>C₃</td>
<td>22</td>
<td>5.6</td>
<td>17.7</td>
<td>-27.2</td>
<td>-27.3</td>
<td>24.9</td>
<td>20.1</td>
</tr>
<tr>
<td>Clusia pratensis</td>
<td>200 L pot</td>
<td>Weak CAM</td>
<td>29</td>
<td>6.0</td>
<td>31.7</td>
<td>-25.5</td>
<td>-24.5</td>
<td>23.5</td>
<td>21.2</td>
</tr>
<tr>
<td>C. pratensis</td>
<td>Open soil</td>
<td>Weak CAM</td>
<td>46</td>
<td>6.9</td>
<td>34.0</td>
<td>-25.6</td>
<td>-26.9</td>
<td>24.3</td>
<td>22.7</td>
</tr>
<tr>
<td>Clusia quadrangula</td>
<td>200 L pot</td>
<td>Weak CAM</td>
<td>58</td>
<td>6.0</td>
<td>24.3</td>
<td>-26.7</td>
<td>-24.9</td>
<td>20.9</td>
<td>19.5</td>
</tr>
<tr>
<td>Clusia rosea</td>
<td>200 L pot</td>
<td>Strong CAM</td>
<td>98</td>
<td>2.6</td>
<td>43.7</td>
<td>-19.3</td>
<td>-14.8</td>
<td>24.1</td>
<td>20.2</td>
</tr>
<tr>
<td>C. rosea</td>
<td>Open soil</td>
<td>Strong CAM</td>
<td>119</td>
<td>3.3</td>
<td>45.5</td>
<td>-20.1</td>
<td>-17.5</td>
<td>25.1</td>
<td>20.6</td>
</tr>
<tr>
<td>Clusia stenophylla</td>
<td>200 L pot</td>
<td>C₃</td>
<td>31</td>
<td>4.7</td>
<td>19.9</td>
<td>-26.1</td>
<td>-25.3</td>
<td>22.0</td>
<td>19.1</td>
</tr>
<tr>
<td>Clusia uvitana</td>
<td>200 L pot</td>
<td>Strong CAM</td>
<td>59</td>
<td>4.3</td>
<td>42.3</td>
<td>-20.1</td>
<td>-17.7</td>
<td>21.5</td>
<td>18.5</td>
</tr>
<tr>
<td>C. uvitana</td>
<td>Open soil</td>
<td>Strong CAM</td>
<td>102</td>
<td>5.4</td>
<td>41.9</td>
<td>-20.8</td>
<td>-20.0</td>
<td>25.2</td>
<td>19.9</td>
</tr>
<tr>
<td>Clusia valerioi</td>
<td>200 L pot</td>
<td>Weak CAM</td>
<td>34</td>
<td>4.5</td>
<td>17.5</td>
<td>-23.9</td>
<td>-23.8</td>
<td>26.3</td>
<td>21.0</td>
</tr>
<tr>
<td>M. argentea</td>
<td>Open soil</td>
<td>C₃</td>
<td>267</td>
<td>7.8</td>
<td>9.5</td>
<td>-27.8</td>
<td>-28.1</td>
<td>23.4</td>
<td>22.0</td>
</tr>
</tbody>
</table>

All plants were approximately 5 years old at the time of sampling.

CAM, crassulacean acid metabolism.
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 C₃ 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 cm² 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 handheld 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 ⁸¹⁸O 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 CO₂ 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 ⁸¹⁸O 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 1 cm² 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 ⁸¹⁸O. 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 ⁸¹⁸O 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 ⁸¹³C analyses, powdered samples of approximately 3 mg were combusted in an
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 δ18O 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 δ13C of leaf and xylem water was determined by CO2 equilibration (Scrimgeour 1995). Equilibrated CO2 samples were analysed on a dual-inlet isotope ratio mass spectrometer (Finnigan MAT) operating in continuous-flow mode.

The δ18O enrichment to aid the interpretation of the 18O dynamics in CAM and C3 Clusia species. Leaf water 18O 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):

\[
\Delta^{18}O_e = \varepsilon^e + \varepsilon_k + \left( \Delta^{18}O_L - \varepsilon_k \right) \frac{w_i}{w_a},
\]

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

\[
\varepsilon^e (\%o) = 2.644 - 3.206 \left( \frac{10^3}{T} \right) + 1.534 \left( \frac{10^6}{T^2} \right),
\]

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

\[
\varepsilon_k (\%o) = \frac{32r_b + 21r_s}{r_s + r_b},
\]

where \(r_s\) and \(r_b\) are the stomatal and boundary layer resistances to water vapour diffusion (m2 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 \(\Delta^{18}O_s\) is equal to \(-\varepsilon^e\), Eqn 1 is simplified to (Farquhar et al. 2007):

\[
\Delta^{18}O_e = \varepsilon_k \left( \frac{w_i}{w_a} \right).
\]

Equation 4 demonstrates that the relative humidity term that drives steady-state \(\Delta^{18}O\) enrichment of evaporative sites is \(-w_i/w_a\), 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 18O 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:

\[
\Delta^{18}O_{LS} = \frac{\Delta^{18}O_e (1 - e^{-\varphi})}{\varphi},
\]

where \(\varphi\) is a Peclét 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 H218O in water (m2 s-1). The \(D\) can be calculated as (Cuntz et al. 2007):

\[
D = 119 \times 10^{-9} \left( \frac{\text{St}}{T - 123} \right),
\]

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):

\[
\Delta^{18}O_L = \Delta^{18}O_{LS} - \frac{\alpha_s \varepsilon_k w_i}{gW} 1 - e^{-\varphi} dt dW^{18}O_L,}
\]

where \(\Delta^{18}O_{LS}\) is the steady-state prediction of \(\Delta^{18}O_L\) from Eqn 5, \(\alpha_s\) is defined as \(1 + (\varepsilon^e (\%o)/1000)\), \(\varepsilon_k\) is defined as \(1 + (\varepsilon_k (\%o)/1000)\), \(W\) is the lamina leaf water concentration (mol m-3), \(t\) 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 18O enrichment of plant cellulose (\(\Delta^{18}O_c\)) can be predicted from \(\Delta^{18}O_L\) as follows:

\[
\Delta^{18}O_c = \Delta^{18}O_L (1 - p_sp_g) + \varepsilon_{wc},
\]

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

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developing tissue and $\epsilon_{wc}$ 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 ($\epsilon_{qp}$) to describe the difference between the two (Barbour & Farquhar 2000):

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

Equations 8 and 9 suggest that variation in $\Delta^{18}O_p$ should reflect variation in $\Delta^{18}O_c$, so long as $\rho_{co}, \epsilon_{wc}$ and $\epsilon_{qp}$ 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 $\Delta^{18}O_c$ 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 $\phi$ 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 $\Delta^{18}O_c$ 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, $\Delta^{18}O_c$ was assumed equal to $-\epsilon$, where $\epsilon$ was calculated from the air temperature. Leaf water enrichments above source water were calculated as $\Delta^{18}O_c = (\delta^{18}O_L - \delta^{18}O_x)/(1 + \delta^{18}O/1000)$, where $\delta^{18}O_L$ is the observed leaf water $\delta^{18}O$ in per mil, and $\delta^{18}O_x$ 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 $\Delta^{18}O_c$. We initialized the model to predict $\Delta^{18}O_c$ on the days during which $\Delta^{18}O_c$ 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 $\Delta^{18}O_c$ model in this way for the wet-season month of September 2006, to examine the predicted variation in $\Delta^{18}O_c$ 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 $-22.8$ mmol 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 $8$ mmol 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
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$-\frac{w_{a}}{w_{i}}$. 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 inside the leaf.
inside the leaf, \(1 - w_i/w_a\), 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 \(1 - w_i/w_a\) 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 \(-3.7\%o\) for \(C. pratensis, C. uvitana, C. rosea\) and \(M. argentea\), respectively; wet season observations were \(-3.5, -4.2\) and \(-3.2\%o\) 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 \(-11.0\%o\), with individual observations ranging from \(-9.2\) to \(-13.0\%o\); these values are reasonably close to that predicted for equilibrium with xylem water, which would be about \(-12.4\%o\).

Leaf water \(\delta^{18}O\) 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\%o (Fig. 7a), whereas values observed in the wet season ranged between about 0 and 10\%o (Fig. 7c). The \(\Delta^{18}O_l\) of \(M. argentea\) in the dry season ranged between about 5 and 15\%o (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
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*,
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$_L$ ($R^2 = 0.49$,
P < 0.0001, n = 127). In contrast, assuming isotopic steady state caused the model to explain only 14% of variation in observed D_{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 D_{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 δ^{13}C of either leaf or twig dry matters (Table 1). On the other hand, species classified as C3 and weak CAM were indistinguishable from each other based on dry matter δ^{13}C. Mean values for the leaf dry matter δ^{13}C for the strong CAM, weak CAM and C3 species were -20.1, -25.6 and -25.8‰, respectively; mean values for the twig dry matter δ^{13}C were -17.5, -25.0 and -25.4‰, respectively. Leaf and twig dry matters δ^{18}O 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 C3 Clusia species for the 5-year-old plants in the δ^{18}O of leaf dry matter (P = 0.88, n = 22) or in the δ^{18}O of twig dry matter (P = 0.42, n = 22). Leaf and twig dry matters δ^{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 δ^{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 C3 mode, whereas C. rosea operates in the CAM mode even when well watered (Fig. 3). These differences can be clearly seen in the δ^{13}C of the leaf dry matter (Fig. 10a). The mean leaf δ^{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 δ^{13}C for the 5-year-old weak CAM species. The
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
especially apparent during the dry season (Fig. 7a). The same was true for nighttime $\Delta^{18}O_L$, 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 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.
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 $D^{18}O_L$ than a leaf with a lower stomatal conductance (Farquhar et al. 2007). This is because a higher stomatal conductance decreases $e_k$, while a higher transpiration rate cools the leaf, decreasing $1-w_i/w_o$, and increases the Péclet number, $\varphi$. 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 C3 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 C3 mode, whereas $C. rosea$ operated in the CAM mode (Fig. 10).

![Figure 8](image.png)

**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.

![Figure 9](image.png)

**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.
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

**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.

| Table 2. Leaf water concentrations ($W$, mmol m$^{-2}$), efflux rates ($E$, mmol m$^{-2}$) and residence times ($W/E$ and $W/gw$) for Clusia individuals during the dry and wet seasons and an individual of Miconia argentea during the dry season in Gamboa, Panama. |
|---|---|---|---|---|
| | W (mmol m$^{-2}$) | $E$ (mol m$^{-2}$) | $W/E$ (h) | $W/gw$ (h) |
| **Dry season** | **24 h average** | **Daytime average** | **24 h total** | **Daytime total** |
| Clusia pratensis | 34.4 | 15.1 | 14.6 | 54.7 | 28.3 | 51.3 | 38.9 | 20.5 | 16.1 | 10.6 |
| Clusia rosea | 44.1 | 12.6 | 12.2 | 84.0 | 43.4 | 47.7 | 40.5 | 22.2 | 17.9 | 12.4 |
| Clusia uvitana | 41.7 | 15.9 | 15.1 | 62.9 | 33.1 | 60.2 | 40.4 | 20.7 | 13.0 | 9.9 |
| M. argentea | 9.5 | 62.3 | 60.0 | 3.7 | 1.9 | 147.6 | 131.0 | 1.5 | 0.9 |
| **Wet season** | **24 h average** | **Daytime average** | **24 h total** | **Daytime total** |
| Clusia pratensis | 33.6 | 40.6 | 40.5 | 19.9 | 10.0 | 148.2 | 134.5 | 5.4 | 3.0 |
| Clusia rosea | 46.9 | 15.6 | 15.1 | 72.2 | 37.3 | 87.2 | 70.2 | 12.9 | 8.0 |
| Clusia uvitana | 42.2 | 24.7 | 24.5 | 40.5 | 15.1 | 87.2 | 70.2 | 12.9 | 8.0 |

The net efflux of water vapour from the leaf is given by $E$, whereas the one-way efflux of water vapour is given by $gw$, where $E$ is the total conductance to water vapour of the stomata plus the boundary layer and $gw$ is the intercellular water vapour mole fraction. The $E$ is relevant from a hydration perspective, whereas $gw$ 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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Oxygen isotope composition of Clusia

耕地的有趣结果由Sheshshayee et al. (2005)显示，其中呈现了δ18O与细胞间CO2浓度之间的正相关关系，以及在C3植物中，δ18O的跨膜传输。

δ18O的时常，定义为δ18O的达到稳定状态所需的时长，可能是决定性因素。δ18O的时常为11到18h在干燥季节，与3到8h在湿季节（表2）。δ18O的时常与C3植物偏差不大，为强CAM植物，果实组织被观察到具有C3-type的同位素组成，与之前报道的C3或弱CAM（基于酸度的测量）的植物相符。在植物中，同一类型的CAM活性在干燥植物中更可能在夜间发生（Lüttge 2007）。δ18O的时常也受植物生理学和环境因素的影响。

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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 $\Delta^{18}O_2$ than $C. rosea$, operating in the CAM mode. $C. uvitana$, also operating in the CAM mode, had a daytime $\Delta^{18}O_2$ intermediate between $C. pratensis$ and $C. rosea$. The observed interspecific pattern in $\Delta^{18}O_2$ 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 $\Delta^{18}O_2$ was at an isotopic steady state. As a consequence, the observed relationship between daytime transpiration rate and daytime $\Delta^{18}O_2$ 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 $\Delta^{18}O_2$ from negative to positive for succulent-leaved plants showing varying degrees of midday stomatal closure.

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