Leaf water $\delta^{18}O$ reflects water vapour exchange and uptake by C_3 and CAM epiphytic bromeliads in Panama

Monica Mejia-Chang[A,†], *Casandra Reyes-Garcia*[A,B], *Ulli Seibt*[A,C], *Jessica Royles*[A], *Moritz T. Meyer*[A], *Glyn D. Jones*[A], *Klaus Winter* (iD)[D], *Miquel Arnedo*[E] *and Howard Griffiths* (iD)[A,F]

[A]Physiological Ecology Group, Department of Plant Sciences, University of Cambridge, Cambridge, CB2 3EA, UK.
[B]Unidad de Recursos Naturales, Centro de Investigación Científica de Yucatán, Calle 43 Num. 130 Churburná de Hidalgo, Mérida, 97200, México.
[C]Department of Atmospheric and Oceanic Sciences, UCLA, Los Angeles, CA, USA.
[D]Smithsonian Tropical Research Institute, Apartado 0843-03092, Balboa, Republic of Panama.
[E]Departament de Biologia Evolutiva, Ecologia i Ciències Ambientals, Fac. Biologia, Universitat de Barcelona, Av. Diagonal 645, 08028 Barcelona, Spain.
[F]Corresponding author. Email: hg230@cam.ac.uk

Abstract. The distributions of CAM and C_3 epiphytic bromeliads across an altitudinal gradient in western Panama were identified from carbon isotope ($\delta^{13}C$) signals, and epiphyte water balance was investigated via oxygen isotopes ($\delta^{18}O$) across wet and dry seasons. There were significant seasonal differences in leaf water ($\delta^{18}O_{lw}$), precipitation, stored 'tank' water and water vapour. Values of $\delta^{18}O_{lw}$ were evaporatively enriched at low altitude in the dry season for the C_3 epiphytes, associated with low relative humidity (RH) during the day. Crassulacean acid metabolism (CAM) $\delta^{18}O_{lw}$ values were relatively depleted, consistent with water vapour uptake during gas exchange under high RH at night. At high altitude, cloudforest locations, C_3 $\delta^{18}O_{lw}$ also reflected water vapour uptake by day. A mesocosm experiment with *Tillandsia fasciculata* (CAM) and *Werauhia sanguinolenta* (C_3) was combined with simulations using a non-steady-state oxygen isotope leaf water model. For both C_3 and CAM bromeliads, $\delta^{18}O_{lw}$ became progressively depleted under saturating water vapour by day and night, although evaporative enrichment was restored in the C_3 *W. sanguinolenta* under low humidity by day. Source water in the overlapping leaf base 'tank' was also modified by evaporative $\delta^{18}O$ exchanges. The results demonstrate how stable isotopes in leaf water provide insights for atmospheric water vapour exchanges for both C_3 and CAM systems.

Keywords: C_3, CAM, Crassulacean acid metabolism, *Tillandsia fasciculata*, *Werauhia sanguinolenta*, photosynthetic pathway, gas exchange, epiphyte, oxygen isotopes, altitudinal gradient, mesocosm.

Introduction

Epiphytes provide sensitive climatic indicators, with their distribution reflecting both the microclimate within a particular forest canopy, as well as altitudinal zonation between forest formations (Gómez González *et al.* 2017; Horwath *et al.* 2019). Vascular epiphytes can comprise a significant component of diversity in lower montane forest canopies, and the distribution of C_3 and Crassulacean acid metabolism (CAM) epiphytic bromeliads within neotropical forests has long provided a model system to integrate physiological ecology, life-form and habitat preference (Osmond 1978; Griffiths and Smith 1983; Crayn *et al.* 2015; Males and Griffiths 2018). Here, we analyse

the distribution of C_3 and CAM bromeliads along an altitudinal gradient in western Panama using stable isotopes to evaluate contrasting strategies for water use and exchange of water vapour.

Epiphytic bromeliad species exhibit great diversity in functional forms through an array of morphological and physiological traits. The vegetative body of epiphytic bromeliads is mostly comprised of leaves, whereas roots and stems are highly reduced (Males 2016; Leroy *et al.* 2019). Leaves are displayed in a rosette that often forms a water reservoir or 'tank' between overlapping leaf bases. The water-impounding tank constitutes a stable water source between rain events (North *et al.* 2013; Males 2016) but may be subject to direct evaporation

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(Zotz and Thomas 1999; Males and Griffiths 2018). With extensive leaf trichome cover (Benzing 1976), leaf wetting characteristics (Pierce *et al.* 2001; Leroy *et al.* 2019) also allow bromeliads to inform current studies on direct water uptake by leaves (Dawson and Goldsmith 2018; Berry *et al.* 2019).

Water availability and storage are a major determinant of bromeliad morphology and physiology (North *et al.* 2013; Males 2016; Males and Griffiths 2018). The C_3 pathway tends to predominate in areas where rainfall is more frequent, whereas nocturnal stomatal opening (when transpiration is reduced) allows CAM bromeliads to predominate more in semiarid tropical forests or exposed portions of the canopy (Griffiths and Smith 1983). Bromeliad stomata are particularly sensitive to ambient vapour pressure (Lange and Medina 1979). The stable isotope signals in leaf carbon ($\delta^{13}C$) distinguish those species with CAM (Osmond 1978), whereas oxygen isotopes ($\delta^{18}O$) reflect water use characteristics (Seibt *et al.* 2008; Cernusak *et al.* 2016; Dubbert *et al.* 2017). Precipitation and source water inputs are usually depleted in ^{18}O by around –5 to –10‰ relative to the Vienna standard mean ocean water (VSMOW) mass spectrometric standard but varies on a seasonal basis (Cernusak *et al.* 2016). For C_3 plants, leaf water is normally evaporatively enriched in ^{18}O during transpiration, and then subject to an additional biochemical enrichment of some +27‰ when transferred into organic material (Sternberg *et al.* 2006; Cernusak *et al.* 2016; Lehmann *et al.* 2020).

The $\delta^{18}O$ signal in leaf water and organic material in bromeliads reflects the relative inputs from contrasting water sources such as precipitation, tank water (which may become evaporatively enriched between rain events) and atmospheric water vapour (Farquhar and Cernusak 2005; Seibt *et al.* 2008; Cernusak *et al.* 2016; Lehmann *et al.* 2020; Benettin *et al.* 2021).

The net efflux of water vapour during gas exchange normally leads to evaporative enrichment of ^{18}O in leaf water ($\delta^{18}O_{lw}$) (Harwood *et al.* 1998), whereas water vapour *ingress* under high ambient humidity can lead to more depleted $\delta^{18}O_{lw}$ (Farquhar and Cernusak 2005), allowing niche specialisation to be defined for C_3 and CAM epiphytic bromeliads (Helliker and Griffiths 2007; Reyes-García *et al.* 2008; Helliker 2011; Lehmann *et al.* 2020). Provided that the interplay between water sources, water use and degree of steady-state equilibration for isotopic signals in both C_3 and CAM tissues can be well defined, ^{18}O signals in both leaf water ($\delta^{18}O_{lw}$) and organic material ($\delta^{18}O_{OM}$) of epiphytes can be used to model climatic conditions along altitudinal and latitudinal gradients for the epiphytic bromeliad *Tillandsia usneoides* Sw. (Helliker 2014).

The objectives of this study were, first, to define the distribution of C_3 and CAM epiphytic bromeliads along an altitudinal gradient in western Panama, using $\delta^{13}C$ analyses to identify the metabolic pathway; and second, to use a combination of field sampling and laboratory experimentation to identify the determinants of $\delta^{18}O_{lw}$ and $\delta^{18}O_{OM}$ signals in the two photosynthetic pathways. We expected that CAM species would be more prevalent in the drier lowlands, with a higher abundance of C_3 species in higher elevation forest systems. The contrasting climatic conditions and water source inputs along the altitudinal gradient allowed us to test the hypothesis that net water vapour uptake under high humidity would re-set the $\delta^{18}O_{lw}$ for epiphytes (Farquhar and Cernusak 2005; Helliker and Griffiths 2007; Reyes-García *et al.* 2008; Seibt *et al.* 2008; Lehmann *et al.* 2020). For C_3 bromeliads, we hypothesised that that $\delta^{18}O_{lw}$ would show evaporative enrichment in lowland habitats, but would be more depleted at high altitude cloudforest habitats due to net water vapour inputs. For CAM bromeliads, we predicted that $\delta^{18}O_{lw}$ would also be depleted, due to water vapour inputs associated with stomatal opening at
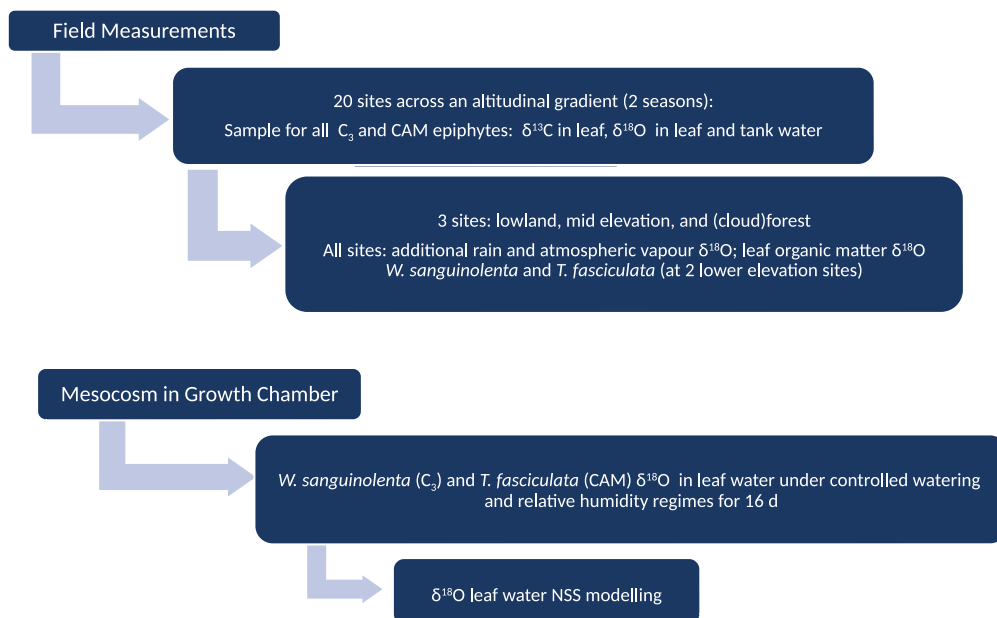

Fig. 1. Summary of the fieldwork and experimental set up showing the scope of sampling and progression from fieldwork to laboratory mesocosm experimentation.

night under high atmospheric humidity (Helliker and Griffiths 2007; Reyes-García *et al*. 2008). Finally, we also set out to test whether water source inputs, from precipitation trapped in the bromeliad tanks, would be affected by growing season and leaf morphology, with higher evaporative ^{18}O enrichment associated with more open leaf rosettes.

To test these hypotheses, and summarised in Fig. 1, we compared $\delta^{18}O_{lw}$, $\delta^{18}O_{OM}$ and source water inputs for epiphytic CAM and C_3 bromeliads along a 1300 m gradient over the Central Cordillera in western Panama, from the Pacific Coast in Chiriquí province, north to the Caribbean coast in Bocas del Toro. Measurements were taken in both rainy and dry seasons to capture the variations in source water $\delta^{18}O$ inputs and likely evaporative demand. A more detailed analysis was undertaken for two sympatric species, *Tillandsia fasciculata* (CAM) and *Werauhia sanguinolenta* (Linden ex Cogniaux & Marchal) J.R. Grant (C_3), that dominate the lower part of the altitudinal transect (between sea level and 600 m; Pierce *et al*. 2002*a*; Zotz *et al*. 2005). These species were also investigated experimentally, using an enclosed mesocosm, to measure the direct contribution from water vapour uptake under well-watered and drought-stressed conditions for C_3 and CAM systems (Helliker and Griffiths 2007). This study confirmed the field work observations, showing that water vapour uptake at high humidities masked evaporative enrichment during transpiration, and was consistent with a mechanistic model of ^{18}O enrichment, explaining depleted $\delta^{18}O_{lw}$ for CAM and upland C_3 bromeliad epiphytes.

Materials and methods

Field measurements

Fieldwork was conducted in 2006 along an altitudinal and precipitation gradient across the Central Cordillera of western Panama, where the dry season extends from January to April (Cavelier *et al*. 1996). The gradient encompassed 20 sites, incorporating lowland coastal sites located within 1 km of the Pacific Coast, up to pristine montane forest sites at 1300 m above sea level and back down to pastureland within 500 m of the Caribbean Coast (see Supplementary material Table S1). Monthly rainfall during the wet season is over 700 mm in the montane forest, but around 400 mm in the lowlands. During the dry season, the lowlands receive minimal rainfall, with montane sites receiving around 150 mm (data provided by Mr Ambrosio Morales, ETESA (Empresa de Transmisión Eléctrica, Panama)).

In order to determine the natural range of spatial and seasonal variation in $\delta^{18}O_{lw}$ and $\delta^{18}O_{OM}$ for C_3 and CAM species, samples of leaf tissue from 36 bromeliad species (Table S2) were collected during the dry (February) and rainy (June) seasons in 2006 along the altitudinal gradient (Fig. 2). Leaf material was taken from the middle portion of fully expanded leaves to reduce isotopic variation associated to different leaf portions (Helliker and Ehleringer 2000; Ogée *et al*. 2007). Leaf samples were stored in sealed glass tubes. To avoid differential effects of transpiration all collections were made between 1100 and 1400 hours. If available, samples of tank water and precipitation were also collected with leaf tissue, for subsequent isotopic analysis.

For three of the sites, representing contrasting environmental conditions, an additional water source, atmospheric vapour, was sampled in order to understand the relationship with $\delta^{18}O_{lw}$. The sites represented lowland (90 m elevation, 8°15′839″N, 81°51′243″W) and mid elevation (650 m elevation, 8°35′134″N, 82°14′125″W) pasture lands with dispersed trees, as well as a cloudforest (978 m elevation, 8°35′125″N, 82°14′151″W). Because $\delta^{18}O$ of atmospheric water vapour can be very variable across the day due to atmospheric conditions, the samples were taken at five times during the day: 0600, 1000, 1400, 1800, and 2200 hours. Values shown here represent the mean of those measurements. Atmospheric vapour samples were collected using a dry ice-ethanol trap (Helliker *et al*. 2002); and were processed at the University of Cambridge, UK.

Humidity chamber measurements

Individuals of *Werauhia sanguinolenta* (Linden ex Cogniaux & Marchal) J.R.Grant (C_3) and *Tillandsia fasciculata* (Sw. var. fasciculata; CAM) adapted to greenhouse conditions in Cambridge, UK were used to investigate the relationship between liquid water and atmospheric water vapour as determinants of C_3 and CAM leaf water isotopic composition. Following Helliker and Griffiths (2007), plants were placed inside 50 L sealed mesocosm within a growth chamber with a 12 h photoperiod (300 $\mu mol\ m^{-2}\ s^{-1}$ at plant height; day/night temperatures within the mesocosm held at 25 and 20°C respectively, monitored with thermocouple thermometer). The epiphytes rested on a mesh above a water-filled tray, with three 5 cm electric fans facing across the reservoir used to generate a high humidity environment; for the low humidity regime, the mesocosm water reservoir was emptied. Two successive 16 day treatments were undertaken with either 'fully hydrated' plants (water in tanks, $n = 14$) or 'water-stressed' treatments (no water in tanks, $n = 15$). Plants were kept at continuously high RH regime (High/High, day: 90%, night: 90%) for 8 days, and for a further 8 days at diurnally variable RH (with water removed from mesocosm reservoir, leading to a Low/High RH regime, day: 50%, night: 90%). The tanks of fully hydrated plants were emptied and refilled each day with local tap water with a $\delta^{18}O$ of −7.0‰; for water stressed plants with empty tanks atmospheric vapour was the only water source. The replicate plants (14 or 15 in the two experiments) had rosettes with many leaves, allowing triplicate leaf samples to be collected at random from different plants on days 0, 3, 7, 8, 9, 10, 11, 15, and 16 for isotopic analyses. Leaf relative water content (RWC) was measured at day 0 and at the end of each 8-day period using the relationship RWC = (fresh weigh − dry weight)/(saturated weight − dry weight) × 100. Over the 16-day experimental period, tissue RWC decreased respectively from 91.6 to 86.6 ± 0.8% (CAM) and 68 to 65.4 ± 2.1% (C_3) for the well-watered treatments (s.e. ranging from 0.8 to 2.1%), with a further decline to 62.3 ± 2.6% (CAM) and 31.3 ± 1.0% (C_3) following the drought treatment. The mean $\delta^{18}O$ of the water used in the reservoir to generate water vapour was −6.2‰ (s.e. ± 0.2‰) ($n = 14$), and the atmospheric water vapour of air passing through the experimental chamber was −19.3‰ (s.e. ± 0.4‰) and −17.8‰ (s.e. ± 0.3‰), for the water-stressed ($n = 15$) and fully hydrated treatments ($n = 14$), respectively.
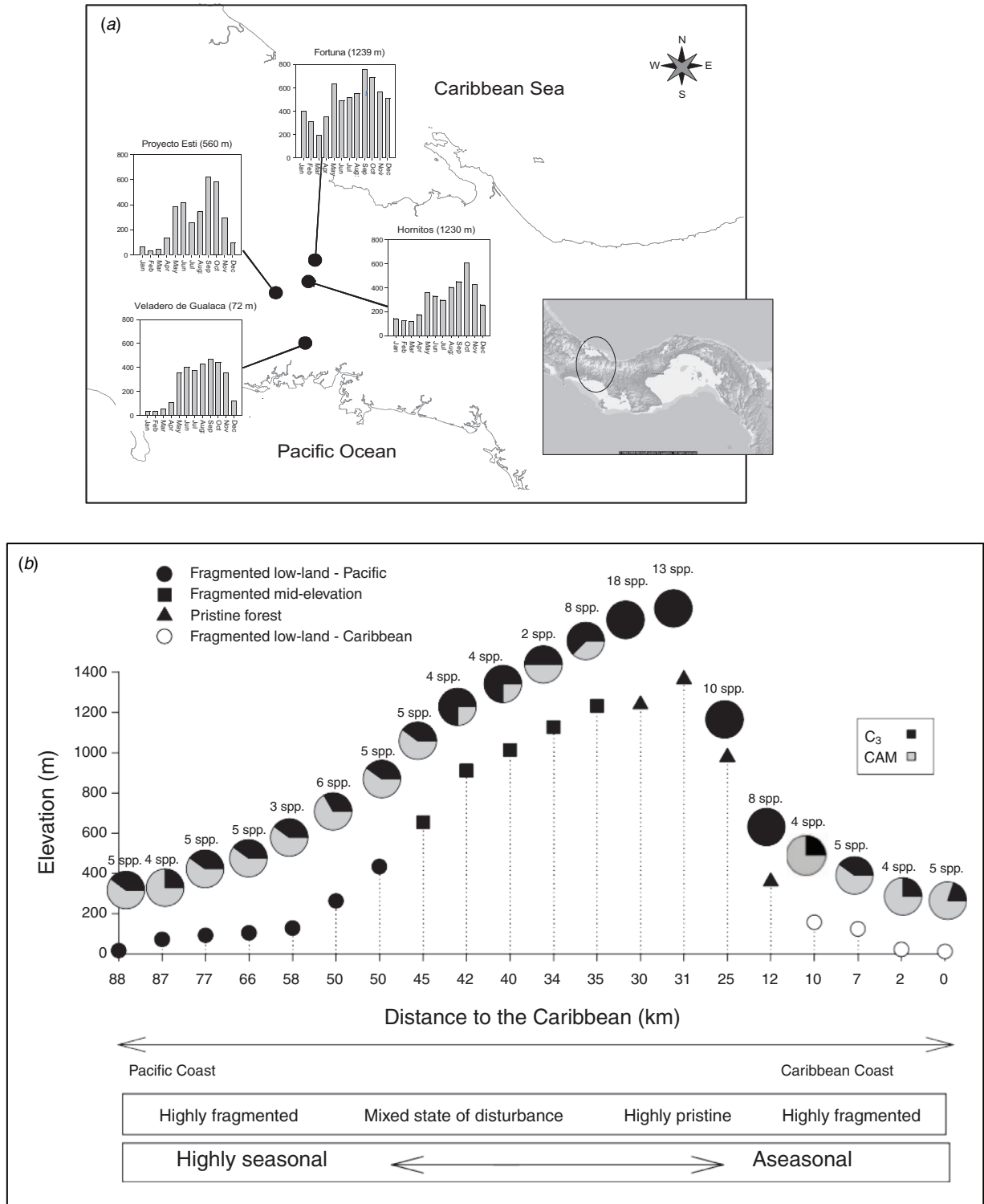
Fig. 2. Map of the study area (*a*) showing monthly precipitation (mm) for selected sites across the Central Cordillera in western Panama. The diagram (*b*) illustrates the relationship between site elevation and distance to the Caribbean (km), the best indicator of rainfall isotopic signature in the region. Each site is classified as Fragmented low-land (Pacific, closed circle), Fragmented mid-elevation (closed square), Pristine forest (closed triangle) and Fragmented low-land (Caribbean, open circle), with the site at 31 km representing the montane cloudforest Fortuna Nature Reserve. Total number of epiphytic bromeliads is shown for each site (pie charts) with C_3 species in black and CAM species in grey, with identification based upon carbon isotope ratio analysis ($\delta^{13}C$) for each specimen. Note that distances between sites are not scaled.

Determination of $\delta^{18}O_{lw}$

Leaf tissues sampled in the field and from the mesocosm experiment were initially stored in 12 mL glass tubes sealed with a screw cap and Viton rubber seal. Bulk leaf water was extracted from leaf tissue using a cryogenic vacuum distillation system and analysed isotopically after equilibration with CO_2 on a mass spectrometer (VG SIRA 10, Modified by ProVac Systems, Crewe, UK), as described by Reyes-García *et al*. (2008). All $\delta^{18}O$ values are reported with respect to VSMOW.

Leaf water $\delta^{18}O$ values were corrected for atmospheric water vapour adsorbed to trichomes using the method of Helliker and Griffiths (2007). The adsorbed water was estimated to be 4 and 11% of the total collected water volume for 50 and 90% relative humidity, increasing $\delta^{18}O_{lw}$ by 0.1 and 0.5‰, respectively.

Organic material isotope composition

A sub-sample of leaves from the most common species collected in the field was oven-dried at 70°C for 3 days and ground to a fine powder and subdivided for isotopic analysis of organic matter. $\delta^{18}O_{OM}$ analysis was completed by Dr C. Keitel, Australian National University, Canberra, following (Farquhar *et al*. 1997). $\delta^{13}C_{OM}$ was determined at the Godwin Laboratory, University of Cambridge, by an elemental analyser (Thermo Finnigan TC/EA) attached to a Thermo Delta V mass spectrometer (for details, see Horwath *et al*. 2019). Organic material was not sampled from the mesocosm experiment as insufficient new growth would have occurred during the short experimental period.

Model simulations

Patterns of $\delta^{18}O_{lw}$ during the chamber experiments were simulated using a non-steady-state (NSS) model of leaf water enrichment (Seibt *et al*. 2008), taking into account the isotopic composition of source water and water vapour, the equilibrium liquid-vapour fractionation (9.8‰ at 20°C), the kinetic fractionation during diffusion of vapour (26.5‰, based on the ratio of stomatal and leaf boundary layer conductances; Farquhar *et al*. 1989; Farquhar and Cernusak 2005; Barbour 2007; Cernusak *et al*. 2016), RH of air, leaf temperature, stomatal conductance, and changes in leaf water content. Calculations started from observed $\delta^{18}O_{lw}$ values. For the well-watered plants, the isotopic composition of tank water was used as the source water term. Stomatal conductance (g_s) was parameterised from gas exchange measurements (using a Li-Cor 6400 system, Li-Cor Biosciences) for *W. sanguinolenta* (C_3) during the day (0.016 mol m^{-2} s^{-1}) and set to a small value at night (0.001 mol m^{-2} s^{-1}). For *T. fasciculata* (CAM), g_s was set to the opposite diurnal pattern (0.016 mol m^{-2} s^{-1} at night, 0.001 mol m^{-2} s^{-1} during the day). The difference between evaporating site and bulk leaf water due to the Péclet effect was also taken into account (eqns 17, 21, and 22 in Farquhar and Cernusak 2005). As the bromeliad leaves had a high leaf water content (22–34 mol m^{-2} for well-watered plants), an effective path length of 30 cm was used to calculate the Péclet number.

For plants under water stressed conditions (no source water input), the model becomes equivalent to that of Helliker and Griffiths (2007). Stomatal conductance was calculated from the observed leaf water loss. Assuming again small g_s at night (C_3) or day (CAM), g_s during the day (C_3) or night (CAM) was adjusted so that the cumulative transpiration matched the observed water loss from the leaves. The resulting g_s values were 0.005 mol m^{-2} s^{-1} and 0.001 mol m^{-2} s^{-1} (C_3), and 0.004 mol m^{-2} s^{-1} and 0.003 mol m^{-2} s^{-1} (CAM), for the two experimental periods (High/High, Low/High RH), respectively. In the absence of advection from source water towards the sites of evaporation, there is no Péclet effect. In addition, the Péclet number would be small (0.02) due to the small transpiration rates, so that the difference between evaporating site and bulk leaf water would only be in the order of 1% (Farquhar and Cernusak 2005).

Statistical analyses

Statistical analysis was carried out in StatView version 5.0 (SAS Institute Inc.) and R (ver. 3.6.3; R Core Team 2018). All analyses were considered significant at $P < 0.05$ and data presented as mean $\pm$ s.e. Depending upon the normality of the data distribution, *t*-test or Wilcoxon analyses were used to compare dry vs rainy season variation in water sources, as well as overall seasonal variation in isotopic composition of leaf water for C_3 and CAM species. Where appropriate, ANOVA's were used and if significant differences were found, Tukey's method for pair-wise comparison was applied to discriminate differences within variables. Paired *t*-test analyses were used to compare the isotopic variation in the humidity chamber experiments. Variation in $\delta^{18}O_{lw}$ in relation to $\delta^{18}O$ tank water along the altitudinal gradient was evaluated with a regression analysis. All analyses were considered significant at $P < 0.05$ and data presented as mean $\pm$ s.e.

Results

Field location and distribution of C_3 and CAM bromeliad epiphytes

In Fig. 2*a*, the seasonality of precipitation is depicted across the continental divide in Panama, running south to north from the Province of Chiriqui (Pacific) to Bocas del Toro (Caribbean). A more distinct seasonal pattern of rainfall is found for the low altitude Pacific Coast, as compared with the high altitude cloudforest site at the Fortuna Nature Reserve (1239 m). Although the focus of this study was primarily on the southern aspect of this altitudinal transect, it is intriguing that the distribution of CAM bromeliads predominates in the lowland habitats on both sides of the continental divide (Fig. 2*b*), consistent with the higher temperatures and evaporative demand likely to be experienced in these habitats. At the higher altitude sites and those facing the prevailing rainfall inputs on the northern slope, the number of bromeliad epiphytes increases and become dominated by those expressing the C_3 pathway (Fig. 2*b*).

Measurements of epiphytic bromeliad isotope components in the field

Water vapour was significantly depleted in $\delta^{18}O$, by around 7‰ relative to rainfall (t-test, $t = -16.47$, $P < 0.001$, Fig. 3*a*,b). Seasonal differences in precipitation, tank water and water vapour were significant (factorial ANOVA, seasonal effect, $P < 0.01$). Dry season water vapour was significantly enriched compared with the wet season (t-test, $t = 2.34$, $P = 0.037$). Water
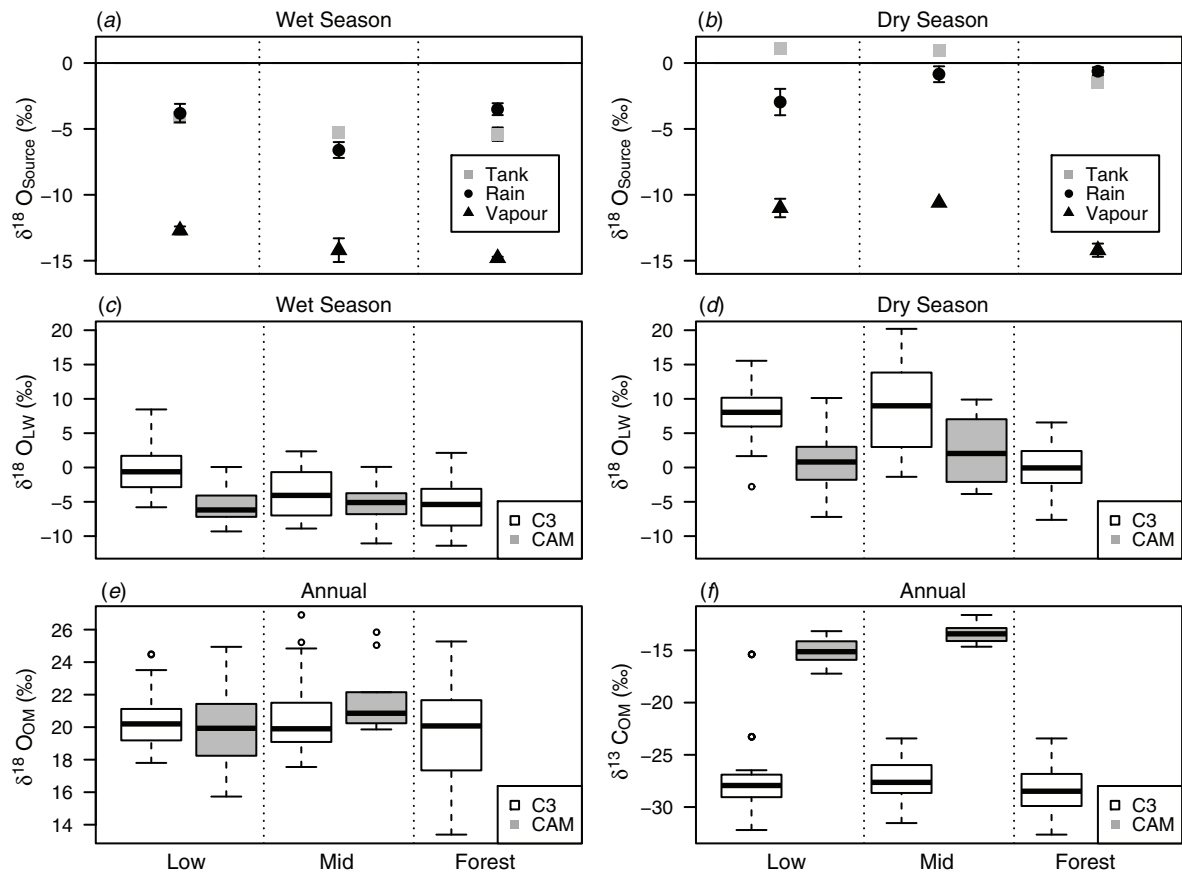
Fig. 3. Mean isotopic components for epiphytic Bromeliads summarised on a seasonal basis along an altitudinal and precipitation gradient in Panama. Data collated as mean values for specimens collected for low-, mid- and high-altitude (cloudforest) sites along a transect from the Pacific Coast to Fortuna; (a) Wet season $\delta^{18}O$ ($\pm$s.e.) in water sources (Legend: rainfall = circles, water vapour = triangles, tank water = grey bars). (b) dry season $\delta^{18}O$ in water sources for C_3 and CAM species (symbols as in Fig. 2a). (c) Boxplot (median and 1.5 × IQR) of $\delta^{18}O$ in leaf water for CAM and C_3 species in the wet season. (d) Boxplot (median and 1.5 × IQR) of $\delta^{18}O$ in leaf water for CAM and C_3 species in the dry season. (e) Boxplot (median and 1.5 × IQR) in annual leaf organic matter for CAM and C_3 species. (f) Boxplot (median and 1.5 × IQR) in annual leaf organic matter for CAM and C_3 species. Data derived from total number of C_3 + CAM samples across sites for Lowland ($n = 35$); Mid altitude ($n = 40$) and (cloud) Forest ($n = 20$), with species representation for each group: lowland (C3 = 2; CAM = 5); Mid altitude (C3 = 6; CAM = 7); (cloud) Forest ($C_3 = 20$).

vapour from the lowland sites was significantly enriched in $\delta^{18}O$, in relation to the cloudforest site (t-test, $t = -4.63$, $P < 0.001$). Rainfall $\delta^{18}O$ was also significantly more enriched during the dry season than during the wet season (W = 1262, $P < 0.0001$). In the wet season there was no significant difference between the tank water and rainfall $\delta^{18}O$ (W = 3310, $P = 0.9$, Fig. 3a). During the dry season, evaporative enrichment increased the tank water $\delta^{18}O$ above that of rainfall at low and mid-altitude sites ($t = -4.47$, $P = 0.0005$), whereas in the cloudforest sites, the two water sources were not significantly different (W = 258.5, $P = 0.06$) so tank water represented precipitation inputs (Fig. 3b).

Within all CAM and C_3 species, $\delta^{18}O_{lw}$ values were significantly isotopically depleted during the wet season (C_3: -2.6 ± 1.2‰, CAM: -7.1 ± 0.7‰; Fig. 3c) than in the dry season (C_3: $+3.7 \pm 0.9$‰, CAM: -0.6 ± 0.8‰), with CAM species having significantly more depleted $\delta^{18}O_{lw}$ than C_3 species (factorial ANOVA seasonal and photosynthetic pathway effects $P < 0.05$; Fig. 3d). The high altitude, montane forest

species had the most isotopically depleted C_3 $\delta^{18}O_{lw}$ values in the wet and dry season (factorial ANOVA, significant season, elevation and photosynthetic pathway effects $P < 0.05$ and Fig. 3c, d). In comparison, for organic material, which integrates wet and dry season precipitation inputs, there was no significant variation in $\delta^{18}O_{OM}$ between elevation for either CAM species (W = 223.5, $P = 0.06$) or C_3 species (ANOVA $P = 0.1318$, $F = 2.095$, D$F = 2$). CAM species growing at mid-elevation sites had significantly less negative values of $\delta^{13}C$ (-13.2‰ $\pm$ 0.2) than plants at the lowlands (-15.0‰ $\pm$ 0.2) (Fig. 3f) (W = 6688, $P < 0.001$), and in contrast, no significant variation was detected for C_3 plants across habitats (ANOVA $P > 0.05$). As would be expected due to the different metabolic pathways, an offset in $\delta^{13}C_{OM}$ of ~10‰ was measured between CAM and C_3 bromeliads.

The overlapping leaf bases 'tanks' provide a reservoir of water to sustain hydraulic supply between rain events, although tank water also tends to evaporate directly during the dry season.
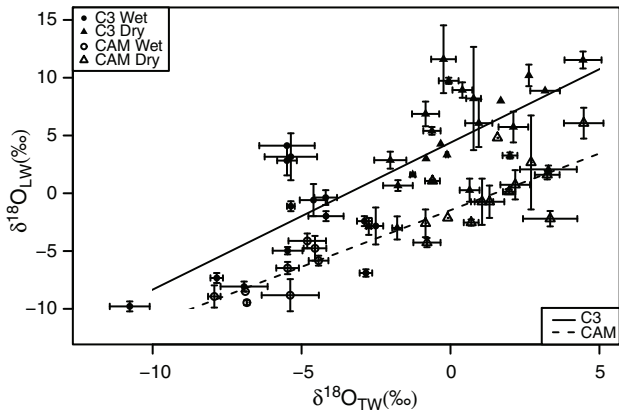
Fig. 4. Variation of $\delta^{18}O$ in bulk leaf water as a function of tank water. Samples from epiphytic bromeliads growing along an altitudinal gradient in western Panama in the wet and dry seasons (data from Fig. 3). Lines represent linear models applied to the C_3 (solid) and CAM (dashed) data. Data include 37 combined paired samples for leaf and tank water, representing C_3 (23 dry; 14 wet season) and 22 CAM (14 dry; 8 wet season).
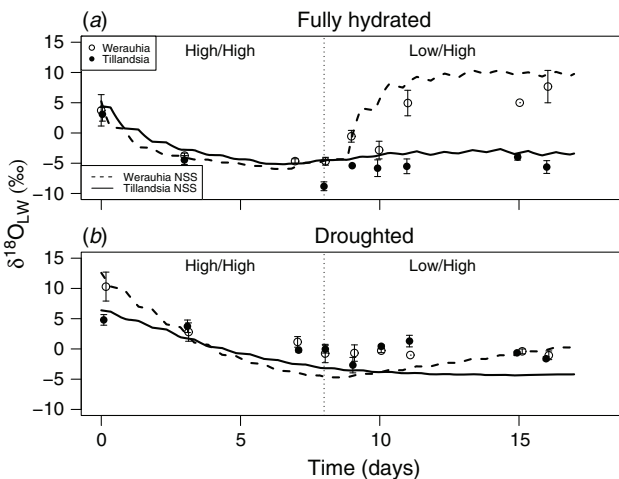

Fig. 5. Fluctuation of leaf water $\delta^{18}O$ for *T. fasciculata* and *W. sanguinolenta* exposed to controlled relative humidity. Plants were subjected to 8 days of high day- and night-time relative humidity (day: 90%, night: 90%), and subsequently for a further 8 days to low daytime only relative humidity (day: 50%, night: 90%). (*a*) Fully hydrated plants; (*b*) water-stressed plants ($n = 3 \pm$ s.e.). Also shown are results from a non-steady-state (NSS) model of leaf water enrichment for each of the species (*Werauhia* NSS, continuous line; *Tillandsia* NSS, dashed line).

Fig. 4 shows the systematic variations in C_3 and CAM $\delta^{18}O_{lw}$ in relation to tank water δ^{18}, for all samples across both wet and dry seasons. For both C_3 and CAM species, when calculated independently of elevation and season, $\delta^{18}O_{lw}$ was positively correlated with the $\delta^{18}O$ of source tank water (Fig. 4, $P < 0.0001$, $r^2 = 0.57$ and $P < 0.0001$, $r^2 = 0.75$ for C_3 and CAM species, respectively). Across the range of tank water $\delta^{18}O_{lw}$ values measured, there is a tendency for C_3 leaf water $\delta^{18}O_{lw}$ to be more enriched than CAM (*t*-test, $t = -3.57$, $P = 0.0007$) with the offset reduced in the more negative values seen for the wet season (Fig. 4). This data is consistent with leaf water values for the two individual C_3 (*W. sanguinolenta*) and CAM (*T. fasciculata*) species shown in Table 1. The $\delta^{18}O_{lw}$ signals of C_3 leaves was enriched in ^{18}O relative to CAM, and were consistent with the overall pattern for all other sympatric C_3 and CAM species (Fig. 3*c, d*). There was also a shift within $\delta^{18}O_{lw}$ for the CAM *T. fasciculata*, which was more pronounced from dry to wet season (depleted by around 9‰ at both altitudes) than in the C_3 *W. sanguinolenta* (Table 1).

Measurements of isotopic exchange by C_3 and CAM tank bromeliads in a humidity chamber

We explored the systematic differences in $\delta^{18}O_{lw}$ between two CAM and C_3 plants under controlled conditions in a mesocosm experiment, and analysed the role of water sources, photosynthetic pathways and evaporative control using a non-steady-state (NSS) model of leaf water enrichment (Farquhar and Cernusak 2005). For plants without source water input (drought stressed), the model is equivalent to that of Helliker and Griffiths (2007).

Although the tank water was replaced on a daily basis in the well-watered treatment (with local tap water with $\delta^{18}O$ –7.0‰), over each 24 h period tank water became enriched for *T. fasciculata* (–4.8‰ $\pm$ 0.1) (n = 32) relative to *W. sanguinolenta* (–5.4‰ $\pm$ 0.1) (n = 40) (*t*-test, $P < 0.0001$), probably reflecting the more open leaf structure and shallower tank in *T. fasciculata*.

For well-watered plants, $\delta^{18}O_{lw}$ decreased over the first 8 days of high RH (12‰ for *T. fasciculata*, 8‰ for *W. sanguinolenta*, $P < 0.003$) (Fig. 5*a*). After the switch to low daytime RH, isotopic enrichment occurred progressively for *W. sanguinolenta*, and after 8 days the $\delta^{18}O_{lw}$ was enriched more in the C_3 (12‰, $P = 0.0007$) than the CAM plant (3‰, $P = 0.04$), relative to the respective values following the high humidity regime (Fig. 5*a*). The model simulations compare well to the observed $\delta^{18}O_{lw}$ values (Fig. 5*a*). The model reproduces the gradual decrease of $\delta^{18}O_{lw}$ in both species during the initial period of constant high RH (High/High), and the subsequent evaporative increase of

Table 1. Comparison of seasonal variations in isotopic composition of mean leaf water and combined tank water for *T. fasciculata* and *W. sanguinolenta*

Data from field sites representing altitudinal extremes of co-occurrence along the altitudinal gradient. Values in parentheses for leaf water ($\delta^{18}O_{lw}$ ‰) are s.e. ($n = 12$–15), with different letters indicating significant differences for a given column

	90 m		600 m	
	Dry	Wet	Dry	Wet
T. fasciculata $\delta^{18}O_{lw}$ (‰) (CAM)	–0.40 (0.33)a	–8.90 (1.04)a	–0.56 (0.51)a	–9.78 (0.92)a
W. sanguinolenta $\delta^{18}O_{lw}$ (‰) (C_3)	3.17 (0.44)b	–1.87 (1.11)b	3.79 (0.81)b	–1.81 (1.17)b

$\delta^{18}O_{lw}$ for the C$_3$ *W. sanguinolenta* but constant with depleted $\delta^{18}O_{lw}$ for the CAM *T. fasciculata* during the final period of low daytime RH (Low/High).

Water-stressed plants also experienced a depletion in $\delta^{18}O_{lw}$ over the first 8 days at high RH (4‰ for *T. fasciculata*, 10‰ for *W. sanguinolenta*, $P < 0.008$) (Fig. 5*b*). No changes in isotopic values were found in either species during the low daytime RH treatment (Fig. 5*b*). For hydrated plants the model simulations reproduce the general patterns in $\delta^{18}O_{lw}$, but under droughted conditions the model under-predicted $\delta^{18}O_{lw}$ for both species towards the end of the high-RH period and for the first few days of the low-RH period (Fig. 5*b*). The model captures reduced level of enrichment seen under droughted conditions in the C$_3$ *W. sanguinolenta*, with minimal gas exchange is occurring under low daytime humidity (Fig. 5*b*) consistent with directly measured stomatal conductance (data not shown).

Discussion

The distribution of CAM and C$_3$ epiphytic bromeliads across the continental divide in western Panama was used as the basis for a comparison of the coupling in water budgets for the two photosynthetic pathways. The study set out to define whether leaf water oxygen stable isotopes ($\delta^{18}O_{lw}$) reflect the seasonal variability in precipitation, changes in $\delta^{18}O$ of tank water reserves, and contrasting daily gas exchange patterns, along an altitudinal transect running north from the Pacific to montane cloud forest at Fortuna (Cavelier *et al*. 1996; Zotz *et al*. 2005; Gómez González *et al*. 2017; Benettin *et al*. 2021). The overall aim was to investigate whether water vapour exchange across leaf surfaces, when atmospheric water vapour concentrations are close to saturation, leads to significant inward fluxes of water which 'reset' the $\delta^{18}O_{lw}$ under natural conditions in the field (Farquhar and Cernusak 2005; Seibt *et al*. 2006; Helliker and Griffiths 2007; Helliker 2011; Lehmann *et al*. 2020) in addition to potential liquid water uptake (Pierce *et al*. 2001; Dawson and Goldsmith 2018; Berry *et al*. 2019).

The distribution of C$_3$ and CAM bromeliad epiphytes did conform to the hypothesis that C$_3$ species would dominate in higher, cloudforest habitats (Fig. 2), and we explore the integration of bromeliad hydraulic properties and $\delta^{18}O$ signals in ecological terms in the final section below. We also set out to test the hypothesis that the gas exchange of C$_3$ and CAM bromeliads would result in contrasting temporal and spatial drivers for $\delta^{18}O_{lw}$, when compared with source water $\delta^{18}O$ inputs measured in terms of precipitation, tank water and water vapour (Dubbert *et al*. 2017; Benettin *et al*. 2021). Depending on seasonal conditions (dry or wet season), we thought that daytime gas exchange associated with C$_3$ species should be subject to evaporative enrichment in $\delta^{18}O_{lw}$ at lower altitudes in the dry season. The impact of net water vapour uptake under high humidities was predicted to result in lower $\delta^{18}O_{lw}$ at high altitude cloudforest sites in the dry season, and for low altitude sites in the wet season, as seen in experimental studies (Helliker and Griffiths 2007; Reyes-García *et al*. 2008; Lehmann *et al*. 2020).

The data shown in Fig. 3 confirm these predictions, where the significant shifts in $\delta^{18}O$ source water inputs as enriched

precipitation, tank water and water vapour in the dry season, relative to the wet season (Fig. 3*a*, *b*). The associated $\delta^{18}O_{lw}$ signals in C$_3$ bromeliads were evaporatively enriched at lower altitudes, and showed a progressive depletion with altitude in the rainy season, consistent with an increasing role for water vapour uptake, particularly in humid cloudforest habitats (Fig. 3*b*, *c*). These shifts are consistent with those seen experimentally with a range of C$_3$ species (Lehmann *et al*. 2020), as well as cloudforest bryophyte communities, where water vapour was also shown to dominate hydrological budgets (Horwath *et al*. 2019).

The night-time stomatal opening associated with the CAM pathway should allow for net water vapour uptake to dominate $\delta^{18}O_{lw}$ signals, although we expected to see contrasting responses between rainy and dry seasons. The data for the range of CAM species found at lower altitude sites were again consistent with these predictions, with significantly lower $\delta^{18}O_{lw}$ values when compared with sympatric C$_3$ species, and the general predictions of water vapour uptake seen previously for CAM plants (Helliker and Griffiths 2007; Reyes-Garcia *et al*. 2008; Helliker 2011, 2014; Lehmann *et al*. 2020).

The $\delta^{18}O_{lw}$ signal was highly correlated with the tank water $\delta^{18}O$ (Fig. 4) and isotopic signatures of tank water reflected evaporative enrichment relative to precipitation under more arid, lowland conditions (Fig. 3*a–d*). At high altitude, the $\delta^{18}O$ of precipitation inputs tended to be similar to regularly replenished tank water ^{18}O in both wet and dry seasons (Fig. 3*a*, *b*). Integration of leaf water signal into organic matter occurs over time (Barbour 2007; Cernusak *et al*. 2016), and thus $\delta^{18}O_{lw}$ represents a more instantaneous marker for coupling of water budgets relative to the organic matter ^{18}O signal (Fig. 3*e*), although the sampling in this study did not show systematic shifts in the $\delta^{18}O_{OM}$ for bromeliad tissues, relative to those seen in other studies (Cernusak *et al*. 2016; Horwath *et al*. 2019).

The study then focussed on two sympatric species, *T. fasciculata* (CAM) and *W. sanguinolenta* (C$_3$), which are widely distributed across the lower altitudes of the transect (from sea level to 600 m) (Pierce *et al*. 2002*a*; Zotz *et al*. 2005). Overall, the variation in $\delta^{18}O_{lw}$ was highly responsive to seasonality, precipitation inputs and ambient humidity (Table 1; Figs 3*a–d*, 4) and, by inference, stomatal sensitivity during gas exchange (Lange and Medina 1979; Griffiths *et al*. 1986). The experimental manipulation of both species in a humidity chamber mesocosm provided additional evidence for the interplay between water vapour inputs, recharge by precipitation, the holding capacity of the bromeliad 'tank', and the regulation of net leaf water use.

Analysing the controls on leaf water enrichment with a NSS model

The experimental component of our study validated the notion that under high humidity, the gross diffusive exchanges of water vapour can dominate leaf water isotopic signatures (Farquhar and Cernusak 2005; Seibt *et al*. 2006; Helliker and Griffiths 2007; Seibt *et al*. 2007; Reyes-García *et al*. 2008; Helliker 2011, 2014; Lehmann *et al*. 2020). Understanding NSS controls on the isotopic signature of plant water and tissue organic material is important during gas exchange by certain species (Farquhar and

Cernusak 2005; Cernusak *et al*. 2008; Lai *et al*. 2008; Cernusak *et al*. 2016; Benettin *et al*. 2021). Our simulations with a NSS leaf water model illustrate how the contrasting diurnal patterns of gas exchange affect $\delta^{18}O_{lw}$ for the two species during the humidity chamber experiments.

For the initial experimental period, despite the distinct stomatal opening patterns of C_3 and CAM, the $\delta^{18}O_{lw}$ became progressively depleted under saturating atmospheric water vapour by day and night. In contrast, for the final period of low RH during the day, which more closely mimics field conditions at lower altitudes, the daytime rates of C_3 gas exchange coincided with low RH, resulting in evaporative leaf water enrichment for the well-watered plants (Fig. 5*a*). As a consequence of the high leaf water content, low stomatal conductance and low leaf hydraulic conductance representative of tank bromeliads (North *et al*. 2013; Males 2016; Males and Griffiths 2018), both species in our study had very low rates of leaf water turnover and thus their $\delta^{18}O_{lw}$ values changed relatively slowly over a time course of days (Helliker and Griffiths 2007; Cernusak *et al*. 2008; Lehmann *et al*. 2020).

Alternatively, under droughted conditions, the sensitivity of bromeliad stomata to humidity (Lange and Medina 1979; Griffiths *et al*. 1986) resulted in reduced water vapour exchange and reduced evaporative enrichment in the C_3 $\delta^{18}O_{lw}$ measured empirically and modelled (Fig. 5*b*). The NSS model also utilised bromeliad tank water as an external water source for leaves similar to soil water in rooted plants. Thus, the gradual evaporative enrichment of tank water over time, seen in both the laboratory mesocosm on a daily basis, and under seasonal conditions in the field (Figs 3, 4) represents an important control on $\delta^{18}O_{lw}$ and organic material in tank bromeliads.

Implications of water vapour for leaf water signatures: from mesocosm to the field

The manipulative component of our study showed that at high RH, leaf water $\delta^{18}O$ signals of both species were dominated by water vapour exchange, even as the water status of the plants, measured as relative water content (RWC), declined (Fig. 4; see 'Materials and methods' for experimental details). Our result is consistent with previous observations demonstrating that $\delta^{18}O_{lw}$ signals in atmospheric CAM bromeliads were dominated by water vapour when exposed to high RH (Helliker and Griffiths 2007; Reyes-García *et al*. 2008; Helliker 2011, 2014; Lehmann *et al*. 2020).

The control of water vapour on $\delta^{18}O_{lw}$ under high humidity conditions did not lead to significant shifts in organic material isotopic signatures in the samples analysed under field conditions. The lower $\delta^{18}O_{lw}$ values for C_3 bromeliads in the high altitude cloudforest sites, relative to lowland sites, were consistent with the more negative $\delta^{13}C$ values found in organic material of those species (Fig. 3*f*), also consistent with higher stomatal conductances (Seibt *et al*. 2008). The comparison of sympatric C_3 and CAM species across the lower altitudinal gradient (Table 1) showed that *T. fasciculata* (CAM) performing gas exchange under high RH at night consistently showed depleted $\delta^{18}O_{lw}$. In contrast, when *W. sanguinolenta* (C_3) was exposed to lower RH during gas exchange and $\delta^{18}O_{lw}$

was evaporatively enriched. The mesocosm manipulations showed that C_3 species are as susceptible to the influx of water vapour as CAM species, which was supported by the field data for bromeliads from the high elevation cloud forest sites, which has been confirmed by the recent manipulations of C_3 and CAM species under saturating water vapour (Lehmann *et al*. 2020). Evidence of water vapour as a main control on $\delta^{18}O_{lw}$ for *Inga* sp., a C_3 understory tree, has been reported for the Amazon rainforest (Lai *et al*. 2008). Furthermore, demonstration of nocturnal transpiration and stomatal conductance across different biomes (Dawson *et al*. 2007; Seibt *et al*. 2007) indicate that the contribution of water vapour might be significant to leaf water at a global scale, particularly when RH is high, in addition to liquid water uptake by wet leaves (Pierce *et al*. 2001; Dawson and Goldsmith 2018; Berry *et al*. 2019; Leroy *et al*. 2019).

Conversely, the relatively small fluctuations observed in $\delta^{18}O_{lw}$ during water stress and low RH in the mesocosm (Fig. 4) reflects the overriding control of stomatal conductance, as stomatal sensitivity to low RH and drought has been consistently shown for bromeliads (Fig. 5, see also Lange and Medina 1979; Griffiths *et al*. 1986; Zotz and Andrade 1998). The temporal separation of gas exchange for C_3 and CAM species is also reflected in the differential signatures for the bromeliads growing along the altitudinal gradient (Fig. 3), where C_3 species consistently showed higher $\delta^{18}O_{lw}$ in comparison with sympatric CAM species. Our findings suggest that $\delta^{18}O_{lw}$ in C_3 bromeliads provide clear markers of local climatic conditions of humidity and water sources.

Ecological implications

The results of this study highlight the sensitivity of epiphytic bromeliads to drought, water stress and changes in local relative humidity, and offers information on the life form and metabolic pathway variation in $\delta^{18}O_{lw}$ for plants exposed to common environmental conditions. The $\delta^{18}O_{lw}$ signal in bromeliad epiphytes can be used as a proxy to understand shifts in the water use of the species which may result from deforestation and its effect on microclimate, as it diminishes rain and fog inputs (Gómez González *et al*. 2017; Horwath *et al*. 2019) and increases canopy openness that in turn increases light incidence and evaporative demand (Williams *et al*. 2020). This type of monitoring is also relevant as temperatures increase and precipitation patterns are affected by global warming (Horwath *et al*. 2019). Shifts in $\delta^{18}O_{lw}$ and $\delta^{18}O_{OM}$ were found to reflect water vapour inputs for the atmospheric CAM *T. usneoides*, as precipitation $\delta^{18}O$ varied along a latitudinal gradient (Helliker 2014).

We explored an altitudinal gradient and did not find a clear trend of higher enrichment organic material at the lower, more open, drier forests, compared with the more moist, upper montane forests. This lack of a correlation may be explained because most of the sites were secondary forests and pastures, with reduced canopy cover below 1000 m (Fig. 2) or differing growing seasons for C_3 and CAM bromeliads. Significant within-site variation was possibly introduced by the different species sampled, which have different tank capacities, plant size, succulence, trichome cover and other ecophysiological traits that

influence water use (Pierce *et al.* 2002*a*, 2002*b*; North *et al.* 2013; Males 2016; Males and Griffiths 2018).

Finally, we also note that the overall distribution of CAM bromeliads across the altitudinal transect is consistent with the predicted capacity to cope with semiarid habitats (Osmond 1978) as seen in lowland habitats on both sides of the continental divide in Panama (Fig. 2) and other tropical coastal locations (Griffiths and Smith 1983; Griffiths *et al.* 1986). Although sadly there were no CAM bromeliads found in the cloud forest habitat at Fortuna (in contrast to that seen in eastern Panama, Pierce *et al.* 2002*b*), the combined use of stable isotopes of oxygen and carbon have helped to de-mistify bromeliads, which continue to set CAM as a curiosity in context (Osmond 1978).

Conflicts of interest

The authors declare no conflicts of interest.

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