Intrinsic water-use efficiency and heterotrophic investment in tropical leaf growth of two Neotropical pioneer tree species as estimated from $\delta^{13}C$ values

V. J. Terwilliger,[1] K. Kitajima,[2] D. J. Le Roux-Swarthout,[1,4] S. Mulkey[2] and S. J. Wright[3]

[1]Department of Geography, University of Kansas, Lawrence, KS 66045, USA; [2]Department of Botany, University of Florida, Gainesville, FL 32611, USA; [3]Smithsonian Tropical Research Institute, PO Box 2072, Balboa, Panamá; [4]Present address: Biology Department, Hope College, Holland, MI 49423, USA

Author for correspondence:
V. J. Terwilliger
Tel: +1 785 864 5547
Fax: +1 785 864 5378
Email: Terwilli@ukans.edu

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Summary

- ^{13}C enrichment in emerging leaves and its effect on carbon isotopic composition ($\delta^{13}C$) of mature leaves were investigated in the neotropical pioneer tree species, *Cecropia longipes* and *Urera caracasana*, in Panama.
- Leaves of all ages were analysed for $\delta^{13}C$, gas exchange, nitrogen, and leaf mass per area. Low intercellular to atmospheric carbon dioxide (CO_2) partial pressure ($p_i : p_a$); and high $\delta^{13}C$ of CO_2 in air, intrinsic water-use efficiency, internal resistance, and carboxylation capacity were discounted as causes of ^{13}C enrichment.
- ^{13}C enrichment might occur when leaf growth is from imported organic carbon but might not reflect $\delta^{13}C$ values at the leaf producing the carbon. Results support hypotheses that: *de novo* sucrose synthesis causes ^{13}C enrichment of mobile sugars after export from source leaves; and high ratios of PEP carboxylase (PEPc) : PEPc + Rubisco cause emerging leaves to be ^{13}C enriched relative to their growth substrate. Carbon contributions of ^{13}C-enriched early growth could yield inaccurate $p_i : p_a$ estimates from $\delta^{13}C$ in mature leaves.
- A model estimated investment of imported organic carbon to leaf growth and improved estimates of $p_i : p_a$ from mature leaves. With such adjustment, $\delta^{13}C$ analyses provide valuable information about age-related source–sink relations in leaves.

Key words: *Cecropia longipes*, *Urera caracasana*, carbon isotopes, leaf development.

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Introduction

The carbon use properties of trees are considered to be among the most fundamental factors linked to the habitats they can successfully occupy and to their role in the carbon cycle (Givnish, 1999; Malhi & Grace, 2000). Characteristics of carbon use may vary with seasonal water availability and leaf age among individuals and species of tropical trees (Lortie & Aarssen, 1996; Dalling *et al.*, 1999, Valladares, 2000). Knowledge of not only the average carbon use tendencies but also the variability in those characteristics is thus critical to understanding where tree species can be distributed and forest roles in the carbon cycle.

Sophisticated ecophysiological measurements can estimate carbon exchanges from leaf to whole forest scales (Field & Ehleringer, 1993; Malhi *et al.*, 1998, 1999). Most are impractical for assessing variability, *per se*, of carbon uses among trees in all but the most accessible tropical forests. Stable carbon isotopic analyses of leaves, however, are a potential source of information about carbon use properties that could be compared among many trees and within remote forests. One common application of stable carbon analyses is to interpret effects of environment on integrated carbon use vs water loss tradeoffs of plants (Mulkey, 1986; Mooney *et al.*, 1989; Martinelli *et al.*, 1991; Kapos *et al.*, 1993; Huc *et al.*, 1994; Buchmann *et al.*, 1997; Sobrado & Ehleringer, 1997; Guehl *et al.*, 1998; Bonal *et al.*, 2000).

A specific use of leaf carbon isotopic compositions ($\delta^{13}C$) is to estimate intercellular to atmospheric partial pressures of CO_2 ($p_i : p_a$) integrated over the lives of leaves. $P_i : p_a$ is

inversely related to intrinsic water use efficiency, a measure of carbon use/water loss tradeoffs that is insensitive to variations in vapor pressure deficits (Von Caemmerer & Farquhar, 1981). Most commonly, $p_i : p_a$ is inferred according to the model and constants (Farquhar *et al.*, 1989):

$$\delta^{13}C_{LEAF} = \delta^{13}C_{CO_{2A}} - a - (b - a)p_i : p_a + d \qquad \text{Eqn 1}$$

(CO_{2A}, CO_2 in air; a, changes in isotopic composition favouring $^{12}CO_2$ during diffusion (4.4‰, Craig, 1953); b, changes in isotopic composition during carboxylation (27‰, see below); and d, 'other' isotopic changes (see below)).

In C_3 plants, 'b' is predominantly caused by Rubisco, whose superior ability to fix $^{12}CO_2$ over $^{13}CO_2$ can lead to isotopic changes (b_3) of 29‰ (Roeske & O'Leary, 1984). Some proportion (β) of the CO_2 entering the leaf will be fixed by PEPcarboxylase (PEPc) causing isotopic changes (b_4) of –5.7‰ (O'Leary, 1993). In C_3 plants, β is assumed to be about 0.057 (Holbrook *et al.*, 1984) so that (Farquhar *et al.*, 1989):

$$b = (1 - \beta)(b3) + \beta(b4) = 27‰ \qquad \text{Eqn 1a}$$

The term 'd' is for other internal factors that affect $\delta^{13}C_{LEAF}$ values, and may be small enough to be ignored (O'Leary, 1993). In sum, a and b are assumed to be constant, and d is ignored when interpreting $p_i : p_a$ from eqn 1.

Nonetheless, $\delta^{13}C_{LEAF}$ values do not always correspond to trends in $p_i : p_a$. An important example in trees is that $\delta^{13}C_{LEAF}$ values are often least negative (highest) as leaves first emerge (Lowden & Dyck, 1974) even though $p_i : p_a$ can also be highest then (Terwilliger, 1997). It is likely that the high $\delta^{13}C_{LEAF}$ values occur when large inputs of translocated organic carbon are fueling most of the growth of newly emerging leaves.

The causes of ^{13}C enrichment during growth from imported organic carbon are not fully known. The simplest explanation is that the ^{13}C enrichment exactly reflects the $\delta^{13}C$ value of the organic substrate for growth. The substrate might then have the $\delta^{13}C$ value of the leaf in which it was produced. If the substrate is formed in an environment conducive to high $p_i : p_a$, then new leaf growth from it might reflect the $p_i : p_a$ of that earlier time. Findings that leaves emerging at the onset of rainy season had higher $\delta^{13}C$ values than those of mature leaves in Venezuelan dry forest (Sobrado & Ehleringer, 1997) and that newly emerging leaves of deciduous species were more ^{13}C enriched than those of evergreens in Mediterranean spring (Damesin *et al.*, 1998) support this possibility. Nonetheless, ^{13}C enrichment also occurred in new leaves whose imported substrate for growth was produced in the rainy season, thereby suggesting that prior environment is not the sole cause of the high $\delta^{13}C$ values (Terwilliger, 1997).

Another possibility is that the imported substrate for early leaf growth becomes ^{13}C enriched at an enzymatic branchpoint during metabolism in its leaf of origin (O'Leary, 1981; O'Leary *et al.*, 1992). However, findings that completely heterotrophic tobacco (*Nicotiana tabacum* L. cv. Wisconsin 38) was enriched in ^{13}C over the sucrose medium upon which it was cultured suggest that the substrate is, at best, not the sole source of ^{13}C enrichment in early leaf growth (Terwilliger & Huang, 1996). In sum, factors in addition to imported organic carbon or $p_i : p_a$ apparently contribute to ^{13}C enrichment of new leaves.

Two hypotheses have been forwarded to explain cases where $\delta^{13}C_{LEAF}$ values corresponded poorly with $p_i : p_a$ in more mature foliage. First, carboxylation capacities may adjust to environment (Körner *et al.*, 1988). Second, internal resistance to diffusion may cause $p_i : p_a$ at the site of carboxylation to be lower than what could be measured in thick leaves (Vitousek *et al.*, 1990). These hypotheses have arisen from analyses of $\delta^{13}C$, instantaneous $p_i : p_a$, nitrogen relations, and leaf mass per area (Vitousek *et al.*, 1990; Cordell *et al.*, 1999).

The $\delta^{13}C$ signal that heterotrophic growth is occurring is valuable for quantifying source-sink relations in leaves. Furthermore, better knowledge of the mechanisms producing this signal may prove carbon isotopic analyses to be valuable sources of information about metabolite partitioning and source-sink relations in field situations. Finally, understanding the causes of disparities between $\delta^{13}C_{LEAF}$ values and $p_i : p_a$ may suggest limits to the resolution of integrated $p_i : p_a$ and intrinsic water use efficiency inferences possible from isotopic analyses alone.

This paper examines developmental influences on $\delta^{13}C_{LEAF}$ values and their significance for interpreting $p_i : p_a$ of two tree species, *Cecropia longipes* and *Urera caracasana* in Panamá. We address four questions. First, can the input of imported organic carbon contribute to the ^{13}C enrichment of new leaves? Second, to what extent can leaf mass per area and nitrogen relations influence the $\delta^{13}C$ values of leaves during development? Third, can the input of ^{13}C enriched tissue at leaf initiation be sufficient to lower the resolution of an integrated $p_i : p_a$ estimate when the leaf is fully expanded? Fourth, can advantage be taken of the ^{13}C enrichment effect on newly emerging leaves to obtain information about heterotrophic and autotrophic inputs to leaf growth?

Materials and Methods

Study sites and design

The study was conducted in the upper canopy of a moderately dry seasonal forest in Parque Natural Metropolitáno (8°59′ N, 79°31′ W) bordering Panama City, Panamá. The forest is 75–150 yr secondary growth and receives an average rainfall of 1798 mm yr^{-1}, 92% of which usually falls during the May–December portion of its April–December rainy season (S. Paton, pers. comm.). We worked in its upper canopy from a gondola attached to a 42-m tall construction crane (Parker *et al.*, 1992). Fieldwork was conducted between July and

Table 1 Growth properties of study species

Species*	Family	Mature height (m)	Guild	New leaves produced	Time to full leaf expansion (d)	**Leaf lifespan (d)
Cecropia longipes Pitt.	Cecropiaceae	10–15	Pioneer	Apr–Dec	21	95 ± 2.5
Urera caracasana (Jacq.) Griseb	Urticaceae	5–10	Pioneer	Apr–Nov	28	121 ± 2.7

*Nomenclature follows Correa *et al.* (1997), mature heights and guilds are based on Croat (1978). **Leaf lifespan is the mean number of days (± sd) from leaf emergence until leaf loss for leaves emerging in May and June 1996. Leaf phenologies are from Kitajima *et al.* (1997).

August 1996, a year of somewhat higher than average rainfall (2016 mm, S. Paton, unpublished).

Species and field design

We examined changes in carbon isotopic composition, gas exchange and leaf nitrogen that take place as leaves of *Cecropia longipes* and *Urera caracasana* develop. These pioneer character species have large leaves with similar photosynthetic characteristics (Kitajima *et al.*, 1997). Height and leaf phenology characteristics are summarized in Table 1.

Changes in leaf development with age were examined according to two protocols. For the first, leaves of different known ages were collected one morning (5 August 1996) from single branches of three *Cecropia longipes* and two *Urera caracasana* trees (referred to hereafter as same-day samples). The second sampling protocol consisted of tagging several newly emerging leaves on each of three *Cecropia longipes* trees, sampling some at emergence, and sampling the remainder 4, 12 or 19 d thereafter (referred to hereafter as cohort over time samples).

Leaf ages of same-day sampled *U. caracasana* and *C. longipes* were determined by weekly visits to the study trees during another study. Thus their ages were known to a minimum accuracy of 6 d when they were collected. The longevity of the leaf cohorts marked for this study was estimated with the Kaplan–Meier method of survival analysis accounting for right-censored samples with JMP statistical software (SAS Institute, Cary, NC, USA) (Table 1). The longevities were within the error range of values estimated in previous years (Kitajima *et al.*, 1997; S. J. Wright, unpublished).

Separation of organic fractions

To examine possible effects of imported organic carbon inputs to leaf isotopic composition; starch, soluble sugars (mostly sucrose, other important sugars include raffinose, glucose, and fructose) and cellulose were extracted from individual leaves of each species. Specifically, soluble sugars were extracted to track the isotopic composition of the mobile substrata that are most available for growth or export. Starch is the most common storage form of organic carbon. Cellulose, because of its long turnover time, provides a particularly time integrated fraction for isotopic analysis.

Leaves were frozen in liquid nitrogen immediately upon collection to halt all further enzymatic action. The methods of Bonicel *et al.* (1987), were used to extract soluble sugars. Specifically, soluble sugars were extracted by boiling 100 mg of dried, ground leaf tissue in 80% (v/v) ethanol, centrifuging at 12000 ***g*** for 10 min, separating the supernatant, and repeating the procedure for the residual. The supernatants were then combined and filtered through activated charcoal. Some (3 ml) of the extract was used to determine soluble sugar concentrations by the dinitrosalicylic method of Miller (1959). This aliquot was added to a 3-ml reagent consisting of 1% dinitrosalicylic acid, 0.2% phenol, 0.05% sodium sulfite, 1% sodium hydroxide, heated, and 6% Rochelle salt added. The colour intensity of the resulting liquid was measured at 575 nm and compared with a glucose standard curve. A separate portion of the extract was isotopically analysed after evaporating it to dryness, first at 40°C on a hotplate and then in a lyophilizer.

Starch was extracted according to the methods described in Brugnoli *et al.* (1988) and Ehleringer (1991) by adding 10 ml of 20% HCl (v/v) to 100 mg of dried, ground tissue, agitating for 30 min, and centrifuging at 12 000 ***g***. The supernatant was collected and the procedure was repeated on the residual. The combined supernatants were mixed in 80% ethanol for 24 h. Further centrifugation converted the starch to a pellet, which was then lyophilized. Starch concentrations were determined from an additional sample of the same leaf using methods described in Luethy-Krause and Landolt (1990). The starch sediment from the extraction procedure was dissolved by heating it in 1 ml of 1 M $HClO_4$ at 60°C for 1.5 h. The mixture was then neutralized with 1 ml of 1 M NaOH and 4 ml of 0.5 M acetate and centrifuged for 15 min at 2500 ***g***. A sample of 3 ml starch was mixed with 50 µl of a 0.2% iodine, 2% potassium iodide solution. Absorbance of this mixture at 536 nm was linear to the starch concentration.

Alpha-cellulose, the most time-integrating fraction, was extracted from delipified leaves with sodium chlorite, glacial acetic acid, and 17% sodium hydroxide as described by Sternberg (1989). The resulting cellulose was rinsed thoroughly via centrifugation and lyophilized. Cellulose yield was determined

as the dry mass of cellulose recovered divided by the dry mass of leaf used for the extraction.

Air samples

Air samples were extracted for isotopic analyses of CO_2 from 2 l, pre-evacuated flasks. Each flask had two arms with stopcocks to control air entry and egress. Air was pumped through each flask using a hand pump attached with viton tubing. Four air samples were taken at mid-morning to early afternoon (*c.* 10 : 00–13 : 00) from upper canopy areas at about weekly intervals.

Carbon isotopic analyses

Dried, ground whole leaf tissue, soluble sugars, starch and cellulose samples of *c.* 2 mg were automatically combusted on a continuous flow elemental analyser (Heraeus CHN-O Rapid, Hanqu, Germany) coupled to a stable isotope ratio mass spectrometer (Finnigan Delta-S, Bremen, Germany). Carbon dioxide was extracted cryogenically on a gas extraction vacuum line, isotopically analysed on the dual inlet system of the mass spectrometer, and corrected for possible N_2O as described in Sternberg *et al.* (1989). All preparations for isotopic analyses were performed in the Stable Isotope Facility, Geography Department, University of Kansas, Lawrence, KS, USA and all mass spectrometry work was at the Department of Biochemistry, University of Nebraska, Lincoln, NB, USA.

Carbon isotope ratios are expressed in ‰ as δ – values where:

$$\delta^{13}C = [(R_{c\ sample}/R_{c\ standard}) - 1]1000 \qquad \text{Eqn 2}$$

and

$$R_c = [^{13}C]/[^{12}C] \qquad \text{Eqn 2a}$$

An acetanilide reference was used to obtain $\delta^{13}C$ values from the automated combustion system. The reference gas for $\delta^{13}C$ values obtained using the dual inlet was from Oztech (Dallas, TX, USA). References were calibrated against the international Pee Dee Belemnite standard. The precision of continuous flow measurements was 0.2‰ and from 0.1 to 0.2‰ using the dual inlet.

Laboratory physiological and anatomical measurements

Leaf mass per area (LMA in g m^{-2}) was determined from dried 3.43 cm^2 leaf disks taken from leaves collected for isotopic analyses in the present study or estimated from trends established on leaves of the same trees as part of a longer study. Total d. wt (g $leaf^{-1}$) of entire leaves were also obtained. Nitrogen contents were determined for the same leaf disks used for LMA determination with a Perkin-Elmer CHNO/S Model II elemental analyser (Perkin-Elmer, Norwalk, CT, USA). Additional LMA data were collected from whole leaf area and dry mass in 2000 for expanding leaves (1–21 d from emergence). Ash contents were determined from pooled samples of disks of the same age after heating in a muffle furnace at 500°C for 6–12 h.

Gas exchange

We measured light saturated (PPFD = 1500 µmol m^{-2} s^{-1}) net photosynthesis rates (A_{max}), conductance (g) and computed internal partial pressures of CO_2 (p_i) on leaves of both species with a portable infra-red gas analyser (IRGA) (LI 6400, Li-Cor, Lincoln, NE, USA). The IRGA was equipped with a red LED light source (LI6400-02) and a CO_2 mixer control unit. All gas exchange data were collected in the mornings between 8 : 00 and 12 : 00. The CO_2 concentration of the reference air entering the leaf chamber was adjusted so that the 'sample' air exiting the chamber was 350 ppm of CO_2. This resulted in CO_2 concentration of the reference air to be in the range of 360–388 ppm and most typically at close to 370 ppm. The chamber temperature was controlled by maintaining the Peltier block temperature at 28°C. The relative humidity of the reference air was kept as close to ambient (usually 70–85%) as possible and was manually adjusted where necessary to be below 90%. The air flow rate was 500 ml min^{-1}.

Some gas exchange values were measured immediately before the whole-leaf sampling, while additional values were obtained from leaves of similar age on the same trees. Although a large number of measures were repeated on leaves of contrasting ages after their full expansion between June and August 1996 as part of another study, the sample sizes for expanding leaves were limited. Therefore, supplementary gas exchange data were collected in June 2000 for expanding leaves. The only and slight differences in the gas exchange method in 2000 were the use of blue-red LED (LI6200–02B) and of constant reference CO_2 of 380 ppm (mean sample air CO_2 = 365 ppm when net A_{max} was positive). Dark respiration rates were obtained to provide qualitative inferences about CO_2 partial pressures at the site of Rubisco and to estimate gross photosynthesis. Dark respiration rates were measured as steady state rates after turning the lamp off for recently expanded leaves (both species) and expanding leaves (of *C. longipes* only).

Statistical analyses

Comparisons of $\delta^{13}C$ values between very young and older leaves of species were made with Mann Whitney *u*-tests. Comparisons of $\delta^{13}C$ values of whole leaves and their extracted fractions were made with paired *t*-tests. $\delta^{13}C$ values were compared between species in specific age classes using Student *t*-tests. Trends in leaf mass and nitrogen relations with leaf age were examined using simple linear regression.

Smoothing spline curves (Lambda = 10 000) were fit to gas exchange measurements and how well they fit was evaluated with r^2 values. Comparative statistical tests and linear regressions were analysed using Minitab. Smoothing spline curves were fit and evaluated using JMP Statistical Software (SAS Institute, Cary, NC, USA).

Estimating $p_i : p_a$ at different intervals of leaf development from $\delta^{13}C$ and from gas exchange

Values of $p_i : p_a$ estimated from $\delta^{13}C$ values and from gas exchange measurements for subintervals of leaf lives were compared. We were specifically interested in whether $\delta^{13}C$ values of leaf growth that accrued after early emergence gave a better estimate of $p_i : p_a$ than $\delta^{13}C$ values of whole leaves. The following protocol was used to achieve this objective (Terwilliger *et al.*, 2001).

The $\delta^{13}C$ value of a whole leaf (TOTAL LEAF) of a given age is almost equal to the sum of the proportional contributions (ρ) of leaf carbon mass with a given $\delta^{13}C$ value in each successive time interval of leaf development (t) or:

$$\delta^{13}C_{\text{TOTAL LEAF}} = \sum_{i=1}^{N} \rho t\, \delta^{13}C_t \qquad \text{Eqn 3}$$

An error of < 0.02 occurs in eqn 3 because a δ-value is not a fractional abundance (Hayes, 1982). We made two assumptions that also introduce error. First, we assumed that ash-free leaf masses (M) were proportional to the mass of carbon in a leaf. Second, the ash-free leaf mass of a younger leaf on a branch was assumed to equal the ash-free leaf mass of an older leaf when it was the same age. Using these assumptions, eqn 3 was rearranged as follows to estimate the $\delta^{13}C$ value of carbon that accumulated in a leaf after early emergence:

$$\delta^{13}C_{(d-h)} = (M_d\delta^{13}C_d - M_h\delta^{13}C_h)/(M_d - M_h) \qquad \text{Eqn 4}$$

(d, the age of the leaf; and h, the age of the leaf at the end of early emergence (14 d in this study, see Results and Discussion)).

Integrated $p_i : p_a$ for the time interval following emergence was then calculated using eqn 1 using the average $\delta^{13}C_{CO_2\ Air}$ obtained during the study (–8.1‰). Corresponding estimates of integrated $p_i : p_a$ were made from gas exchange measurements by first converting instaneous $p_i : p_a$ values to weighted means, and then subtracting the weighted influence of early leaf $p_i : p_a$.

Results

Carbon isotopic analyses: same day sampling

The $\delta^{13}C$ values were higher in newly emerging leaves than in older leaves. Specifically, whole leaves of *Cecropia longipes* measured on 5 Aug 1996 were significantly more enriched in ^{13}C in ≤ 28 d old leaves than in > 28 d old leaves ($P < 0.004$, no exceptions) (Fig. 1). Differences in $\delta^{13}C$ values between newly emerging and older leaves ranged from 2 to 3‰ on a single tree. Whole leaves of *Urera caracasana* collected on 5 Aug were significantly more enriched in ^{13}C in ≤ 14 d than in > 14 d old leaves ($P < 0.04$, no exceptions on a tree by tree basis) (data in Fig. 2). Differences in $\delta^{13}C$ values of ≤ 14 d and > 14 d old leaves ranged from 0.7 to 3‰ on an individual tree. There are not enough data to statistically evaluate whether ^{13}C enrichment truly persists for longer in *C. longipes* than in *U. caracasana*. There was little variation in $\delta^{13}C$ values (< 1‰) among older leaves of each species and no trend in these values over time.

The $\delta^{13}C$ values of cellulose and soluble sugars were also higher in the younger (≤ 28 d) leaves than in the older leaves of *C. longipes* (for cellulose, $P < 0.02$; for soluble sugars, $P < 0.03$) (Fig. 1). Differences in $\delta^{13}C$ values of cellulose

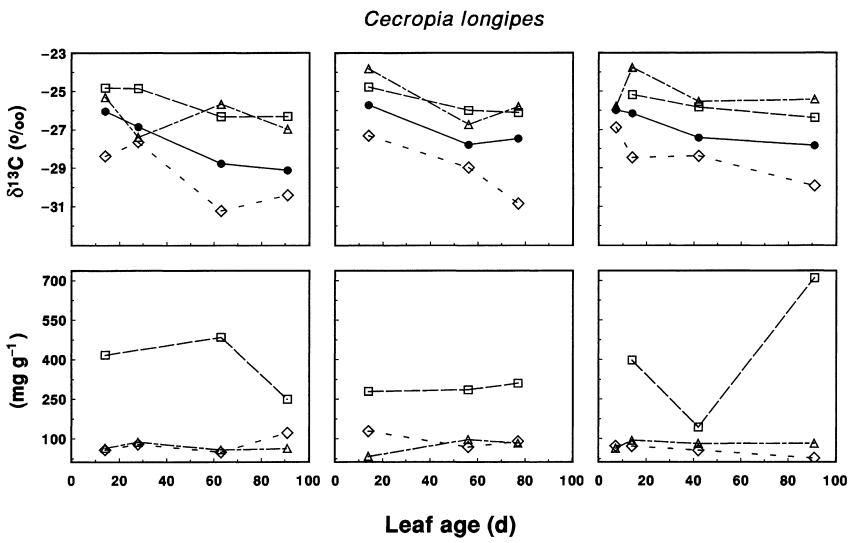

Fig. 1 $\delta^{13}C$ values of leaves and $\delta^{13}C$ values and yields (mg g^{-1} d. wt leaf) of leaf cellulose, starch, and soluble sugars of *Cecropia longipes*. Leaves were all collected during mid-morning of one day (5 August 1996) from single branches of each of three trees. Each column of figures represents a single branch of an individual tree. Missing cellulose $\delta^{13}C$ values are where insufficient tissue existed for all analyses. Tissue loss may also have caused higher error in cellulose yield values than in yield values for other fractions. Missing yields for cellulose fractions are where excessive tissue loss occurred in rinsing during the extraction procedure. Closed circles represent whole leaf data, open squares represent cellulose, open triangles represent starch, and open diamonds represent the soluble sugars. (n = 1 leaf per age, i.e. metabolic fractions and whole tissue are all from the same leaf of a given age.)

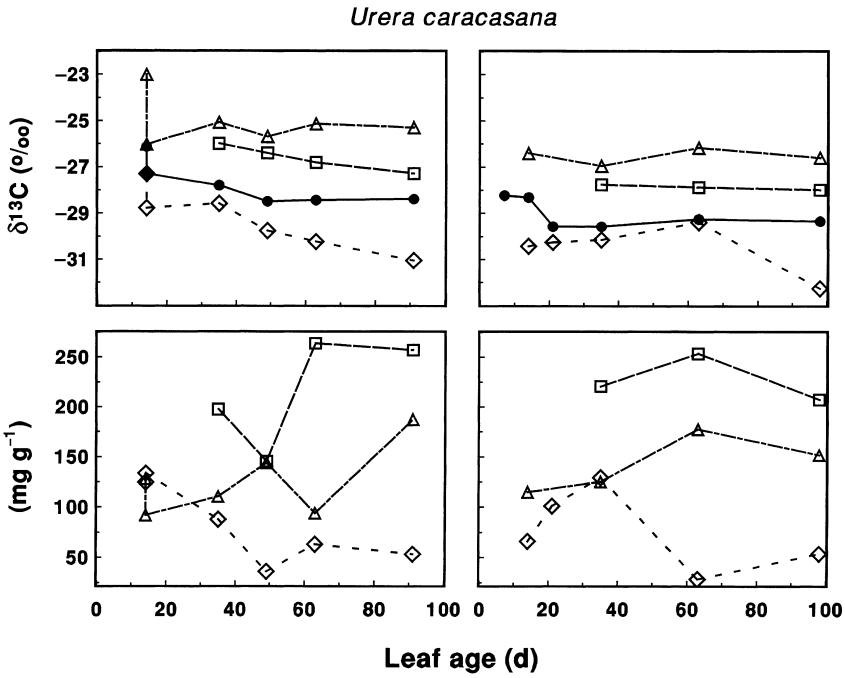

Fig. 2 $\delta^{13}C$ values of leaves and $\delta^{13}C$ values and yields (mg g^{-1} d. wt leaf) of leaf cellulose, starch and soluble sugars of *Urera caracasana*. Leaves were all collected during mid-morning of one day (5 August 1996) from single branches of each of two trees. Each column of figures represents a single branch of an individual tree. Missing $\delta^{13}C$ values and yields of metabolites are where insufficient tissue existed for all analyses. Closed circles represent whole leaf data, open squares represent cellulose, open triangles represent starch and open diamonds represent the soluble sugars. (n = 1 leaf per age, i.e. metabolic fractions and whole tissue are all from the same leaf of a given age. Exception = day 14 on the first tree where two leaves emerged during the same week.)

Table 2 Summary of Paired-*t*-test comparisons of $\delta^{13}C$ values among metabolites in leaves of various ages on *Cecropia longipes* and *Urera caracasana* sampled on a single day

	Cecropia longipes			*Urera caracasana*		
	Cellulose	Starch	Soluble sugars	Cellulose	Starch	Soluble sugars
Whole leaf	***	**	***	***	***	**
Cellulose	–	ns	***	–	**	***
Starch	–	–	***	–	–	**

Significant differences: *P < 0.05; **P < 0.001; ***P < 0.0001.

between younger and older leaves of a tree were smaller (maximum = 1.2‰) than between younger and older whole leaves, however. The range in differences of $\delta^{13}C$ values of soluble sugars between younger and older leaves (from < 1‰ to 3.8‰) was greater than for whole leaves. The $\delta^{13}C$ values of starch did not differ significantly between younger and older leaves of either species (Figs 1 and 2). There was no difference in $\delta^{13}C$ between younger and older leaves of *U. caracasana*, although younger leaves were always more ^{13}C enriched than the oldest (> 90 d) leaves. The youngest leaves of *U. caracasana* that yielded adequate cellulose for analysis had higher $\delta^{13}C$ values than did those of the oldest leaves, although cellulose could not be recovered from = 28 d old leaves.

The $\delta^{13}C$ values of all metabolic fractions differed significantly from the $\delta^{13}C$ values of whole leaves (Table 2). The magnitude of differences in $\delta^{13}C$ between specific metabolites and whole leaves varied among samples, however (Figs 1 and 2). Cellulose and starch $\delta^{13}C$ values were significantly higher than were those of whole leaves. The soluble sugars had significantly lower $\delta^{13}C$ values than did whole leaves. Cellulose $\delta^{13}C$ values did not differ significantly from those of starch in *C. longipes* but were significantly higher than those of starch in *U. caracasana*.

The $\delta^{13}C$ values did not differ between *C. longipes* and *U. caracasana* of older (> 21 d) tissues (P < 0.12) but were higher in *C. longipes* than in *U. caracasana* if all values were compared (P < 0.018, data in Figs 1 and 2). The cellulose of older (> 28 d) *C. longipes* had significantly higher $\delta^{13}C$ values than those of *U. caracasana* (P < 0.0009). No differences in $\delta^{13}C$ values of starch or soluble sugars occurred, on average, between species.

Carbon isotopic analyses: sampling over time

The $\delta^{13}C$ values of cohort over time sampled leaves of *Cecropia longipes* collected 19 d after being tagged were lower than $\delta^{13}C$ values of young emerging members of their cohort 4 and 9 d after tagging (Mann Whitney u-tests, P < 0.05, no individual exceptions) (Fig. 3). The similarity of this trend to that of the age trend for leaves of this species collected on a single day suggests that $\delta^{13}C$ values decreased after first emergence for developmental rather than environmental reasons. No differences in $\delta^{13}C$ values were found between leaves collected from the tops and bottoms of the crowns of each *C. longipes* tree sampled. The $\delta^{13}C$ values of cellulose did not vary significantly in cohort over time sampled *C. longipes*.

$\delta^{13}C_{AIR}$ values did not correspond to changes in $\delta^{13}C_{LEAF}$ over time. The four $\delta^{13}C_{AIR}$ values obtained from mid-July to mid-August averaged –8.1‰ and ranged from –8.3 to –8.0‰.

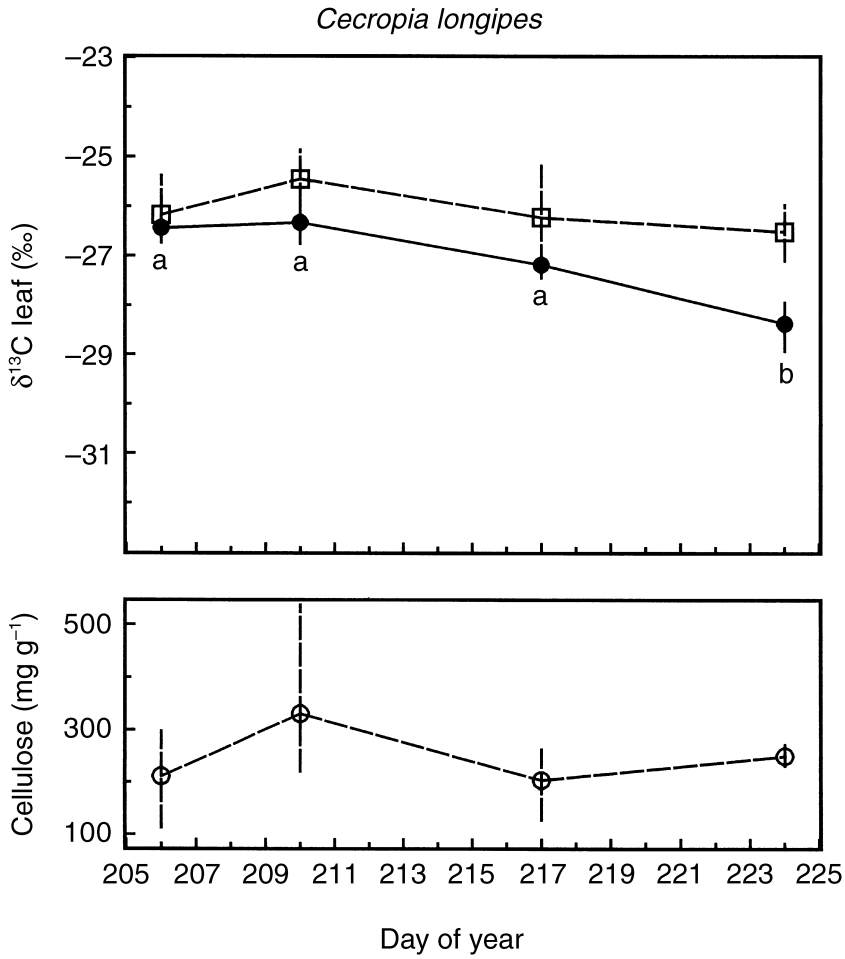

Fig. 3 $\delta^{13}C$ values leaves (closed circles) and cellulose (open circles), and cellulose concentrations of *Cecropia longipes* where values track leaf chronology at weekly intervals beginning with newly emerging leaves. Bars represent highest and lowest values of a data set. Data points of *Cecropia longipes* represent means of two leaves on each of three trees or a total of six values per mean. Leaves were collected from the highest and lowest elevation of the crown. There was no positional effect on $\delta^{13}C$ values of *C. longipes*. Different letters indicate significantly different $\delta^{13}C$ values of whole leaves (none occur for $\delta^{13}C$ values of cellulose) ($P < 0.05$).

Gas exchange properties

For a given vapor pressure deficit, water use efficiency (WUE) is inversely related to $p_i : p_a$ (von Caemmerer & Farquhar, 1981). The comparatively high $\delta^{13}C$ values of newly emerging leaves were not the result of lower $p_i : p_a$ and higher WUE at that time, however. Both vapor pressure deficit sensitive instantaneous WUE (net photosynthesis at light saturation : transpiration rate or A_{max} : E) (data not shown) and vapor pressure insensitive intrinsic WUE (A_{max} : conductance or g) were lower and $p_i : p_a$ was higher during early leaf expansion of *C. longipes* than later (Fig. 4). Gas exchange trends with leaf age were identical in 1996 and 2000 for both species. In both species, A_{max} increased until about 2 wk after leaves completed expansion (*c.* 40 d after emergence), then declined gradually afterwards with leaf age. Although these patterns were similar in the two species, *U. caracasana* had positive A_{max} at leaf emergence, whereas A_{max} of *C. longipes* was negative for the first 5–10 d (Fig. 4). The negative A_{max} of *C. longipes* reflects its high dark respiration during the early phase of leaf expansion (–9, –6.1, and –2.9 μmol m^{-2} s^{-1} for 1, 7 and 31-d-old leaves) indicating that gross A_{max} was positive during leaf expansion (3.7, 5.7 and 16.4 μmol m^{-2} s^{-1}). Conductance (g) rates averaged 0.50 and 0.52 mol m^{-2} s^{-1} for *C. longipes* and *U. caracasana*, respectively, and were positively correlated with A_{max}, that is lower at first emergence and senescence than at intermediate stages ($P < 0.001$ in both species). Like A_{max}, instantaneous and intrinsic WUE increased for about 40 d (Fig. 4, intrinsic WUE only). Instantaneous $p_i : p_a$ was highest at leaf emergence, declined rapidly until full expansion, and increased slightly towards leaf senescence (Fig. 4).

Leaf mass and nitrogen changes with age

The consistent finding of highest $\delta^{13}C$ values when leaves first emerge did not correspond to any systematic trends with age in leaf mass per area (LMA), nitrogen concentration on a dry leaf weight basis [N], or nitrogen content per unit leaf area (N : area). In both species, LMA decreased during early leaf expansion (< 21 d from emergence), then increased linearly with leaf age after leaves completed expansion (Fig. 5). LMA increase after leaves had fully expanded was much greater in *C. longipes* than *U. caracasana*.

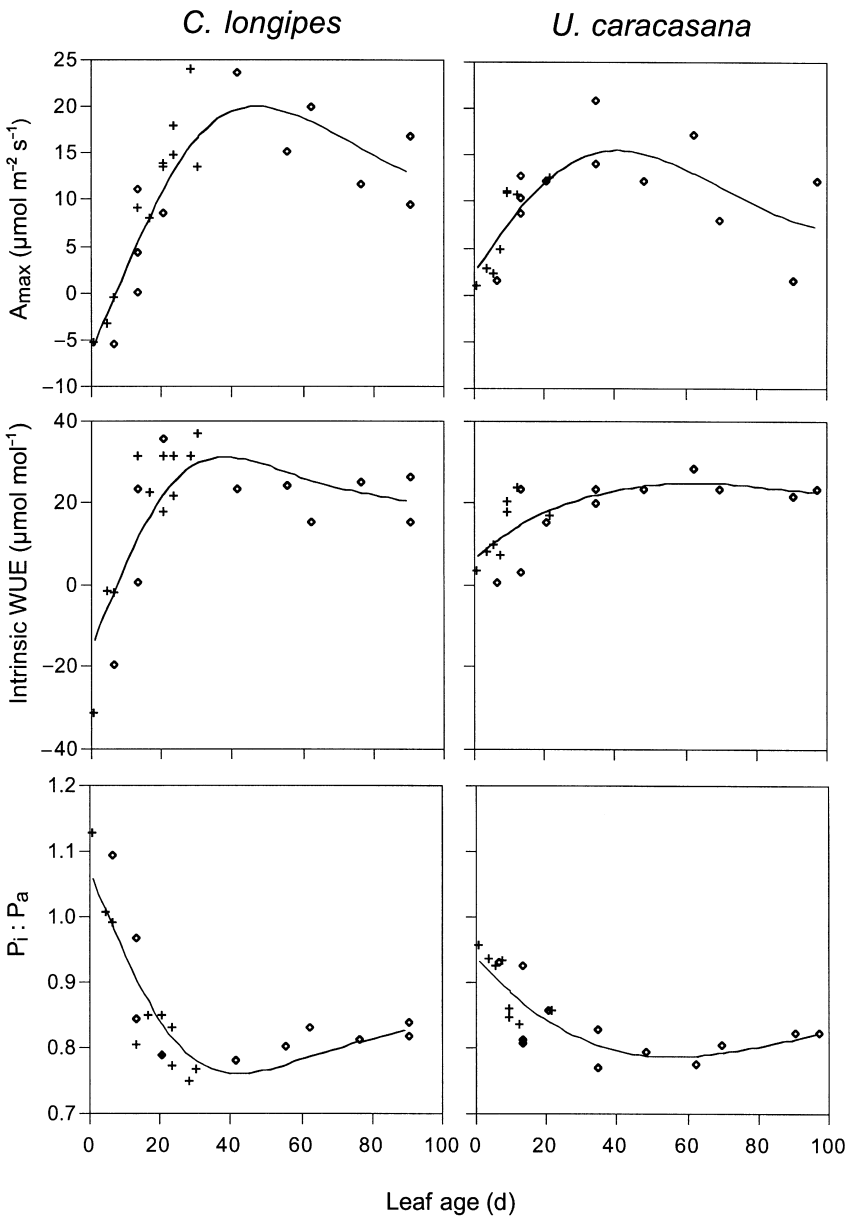

Fig. 4 Change of maximum photosynthesis rates (A_{max}), intrinsic water use efficiency (WUE_{INTR}) and intercellular to atmospheric CO_2 partial pressure ($p_i : p_a$) at light saturation with leaf age in *Cecropia longipes* and *Urera caracasana*. The circles indicate measurements June–August 1996, while crosses indicate supplemental measurements collected in June 2000. Leaves were fully expanded at 21 and 28 d for *C. longipes* and *U. caracasana*, respectively, while physiological maturity (= peak A_{max}) was not achieved for an additional 2 wk in both species.

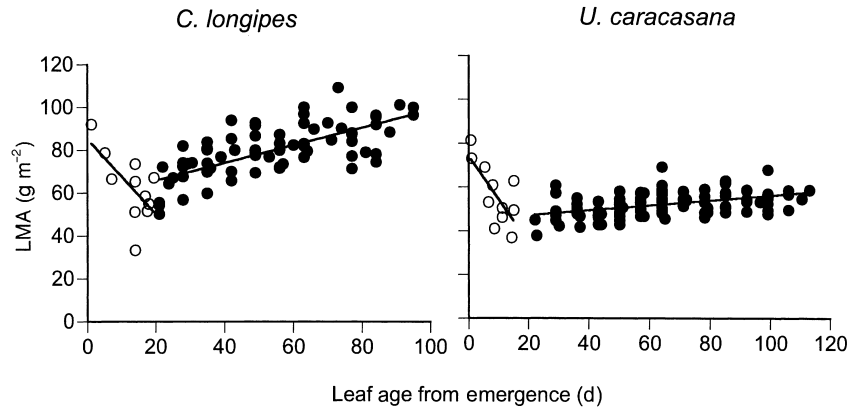

Fig. 5 Relationship between leaf mass per area (LMA) and leaf age from emergence for *Cecropia longipes* (N = 85) and *Urera caracasana* (N = 116) (in 1996 and 2000). The decrease of LMA during early leaf expansion (< 21 d, open circles) was significant ($P < 0.04$) in *C. longipes* and close to significant ($P < 0.08$) in *U. caracasana*. The increase of LMA with leaf ageing in older leaves (> 21 d, closed circles) was very strong in both species ($P < 0.001$), although the degree of increase was much greater in *C. longipes* (> 40% increase between age 0 and 90 d) than *U. caracasana* (< 20% increase between age 0 and 100 d).

Table 3 Leaf mass and nitrogen in *Cecropia longipes* (*Cecl*) and *Urera caracasana* (*Urec*) leaves of different ages that were measured on a single day

Species or stat	Tree	Age (d)	Tot leaf mass (g leaf^{-1})	Ash-free leaf mass (g leaf^{-1})	Maximum ash-free leaf mass (%)	[N] (mg N g d. wt^{-1})	N : area (gN m^{-2})
Cecl	3	7	0.76***	0.72	3.1	31.57***	ns
	1	14	8.18	7.71	31.3	33.70^{+}	2.20
	2	14	5.48	5.16	22.4	32.30^{+}	2.37
	3	14	7.92	7.41	32.1	31.20^{+}	1.56
	1	28	15.26	14.16	57.5	35.40	1.94
	3	42	18.36	16.64	72.0	26.95	1.89
	2	56	16.54	14.73	63.9	22.66	2.06
	1	63	19.71	17.56	71.3	27.40	2.10
	2	77	26.53	23.04	100.0	19.45	1.94
	1	91	28.83	24.61	100.0	24.20	1.91
	3	91	27.06	23.10	100.0	21.40	1.67
r^2			0.88			0.78	ns
Urec	2	7	0.17***	0.14	10.0	34.79***	1.82
	1	14	1.64	1.38	29.8	42.00^{+}	3.06*
	1	14	2.08	1.72	32.4	34.00	2.06
	2	14	0.78	0.65	12.2	33.61^{+}	1.76
	2	21	2.01	1.65	30.9	36.00	2.10
	1	35	3.13	2.50	67.7	31.26	1.79
	2	35	2.90	2.32	43.4	31.10	1.39
	1	49	5.94	4.63	100.0	26.55	1.52
	1	63	6.00	4.55	93.7	24.20	1.40
	2	63	4.77	3.62	100.0	26.40	1.62
	1	91	6.41	4.61	100.0	20.67	1.21
	2	98	7.53	5.34	100.0	23.80^{+}	1.27
r^2			0.88			0.78	0.48

Nitrogen concentrations ([N]) per unit dry leaf mass (d. wt) and per area (N : area) were measured on the same leaf used for isotopic analyses where plus superscripts appear in the [N] column. All total (tot) leaf masses correspond to leaves used for isotopic analyses. Ash-free leaf mass was estimated from the trends of ash accumulation with leaf age. The ash-free leaf mass of a given age divided by the maximum ash-free leaf mass measured for a given tree was used to compute percentage of maximum leaf mass. Results of regression analyses of changes in variables with leaf age are noted by the first value of the variable for each species (*$P < 0.05$; ***$P < 0.001$; ns, not significant and no superscript means regression not performed for that variable). r^2 values are given where a regression was performed and the variable significantly changed with age.

[N] decreased significantly with leaf age in both species ($P < 0.0005$ for both species). N : area decreased significantly with leaf age in *U. caracasana* ($P < 0.013$) but not in *C. longipes* (Table 3).

Masses of whole leaves increased linearly with age in both species ($P < 0.0005$). Roughly 15% and 57% of increase in LMA was from the linear increase in mineral ash with leaf age (% ash = 0.106 $\sqrt{}$ leaf age + 5.11 for *C. longipes*; % ash = 0.142 × leaf age + 1.56 for *U.* caracasana, $P < 0.0001$; K. Kitajima, unpublished). The nonash portion of whole leaf biomass was estimated using these relationships (Table 3). To estimate contributions of carbon at each age to the maximum carbon attained by a leaf, ash-free mass of a leaf of a particular age was divided by the highest ash-free leaf mass obtained for a tree and converted to percentage. Using this estimate, the percentage of maximum carbon gained by the first 2 wk of growth when $\delta^{13}C$ values were highest ranged from 12 to 32% (Table 3).

Discussion

Our results support the hypothesis that measurable isotope effects occur as leaves first emerge that are not accounted for with the constants usually applied to eqns 1 and 1a. Greater ^{13}C enrichment occurred during early leaf development than later (Figs 1–3). The changes in $\delta^{13}C$ values of leaves with age did not correspond to changes in $p_i : p_a$ or associated gas exchange properties. Instantaneous measures of $p_i : p_a$ decreased and WUE increased from earliest emergence to full leaf expansion. Unless $\delta^{13}C$ values of atmospheric CO_2 changed with leaf age (Buchmann *et al.*, 1997), the observed trends in $\delta^{13}C_{LEAF}$ values should have corresponded to an increase in $p_i : p_a$ and decrease in intrinsic WUE during leaf development.

Two lines of evidence suggest that the $\delta^{13}C$ trends with leaf development were not caused by changes in $\delta^{13}C_{AIR}$, however. First, our limited measurements of $\delta^{13}C_{AIR}$ taken during

the study varied by < 0.3‰. Second, $\delta^{13}C$ trends with leaf age were similar regardless of sampling protocol. The same-day sampled leaves of *C. longipes* corresponded to the final week's air sample. The cohort over time sampled leaves of *C. longipes* corresponded to the entire period during which air was sampled. If undetected changes in $\delta^{13}C_{AIR}$ had caused the observed differences in $\delta^{13}C_{LEAF}$ values, the direction of change of $\delta^{13}C_{LEAF}$ values in same-day sampled *C. longipes* leaves would have been opposite those of cohort over time samples. We now consider possible causes of ^{13}C enrichment of newly emerging leaves and evaluate the importance of this effect for ecological interpretations from $\delta^{13}C$ analyses.

Can the input of an organic reserve account for ^{13}C enrichment of newly emerging leaves?

Tracer studies using $^{13}CO_2$ divide leaf development into three stages (Deleens *et al.*, 1994; Maillard *et al.*, 1994a; Maillard *et al.*, 1994b; Ameziane *et al.*, 1997). The first stage is predominantly heterotrophic, that is fuelled by organic carbon imported from elsewhere in the plant. The second stage is predominantly autotrophic, that is growth occurs from the products of the leaf's own photosynthesis. In the third stage, organic carbon is exported.

Enrichment in ^{13}C is most evident during the heterotrophic growth stage. A logical possibility is that the ^{13}C enrichment comes from the heterotrophic source of carbon for growth. The results of this study, however, suggest that the carbon that can be exported from an older leaf will be depleted in ^{13}C compared with the carbon that can be used at the site of new leaf growth.

A source of carbon for early leaf growth is the soluble sugar from adjacent, older leaves. Soluble sugars of the oldest leaves of *C. longipes* and *U. caracasana* had $\delta^{13}C$ values at least 2‰ lower than those in new leaves and at least 3.5‰ lower than $\delta^{13}C$ values of whole leaves under 2 wk old. Thus, some ^{13}C enrichment seems to occur to soluble sugars after they exit their site of production. This is not an isotopic imprint from the conversion of sucrose to starch since starch had 4–7‰ higher $\delta^{13}C$ values than did the corresponding soluble sugars of leaves.

Two particularly plausible explanations exist for why $\delta^{13}C$ values are higher in newly emerging leaves than in older source leaves. First, the relative abundance of individual compounds with discrete $\delta^{13}C$ values could be different in the soluble sugars of new leaves than in old leaves. Sucrose is usually the predominant compound in this fraction. Results of work on poplars suggest that although relative amounts of sucrose in the souble sugars do change during leaf development, they are similar (*c.* 60%) in new and old leaves (Bonicel *et al.*, 1987; Bonicel & Verçosa de Médeiros Raposo, 1990). The possibilities that these results are not universal for all species under all conditions or that differences in concentrations for other compounds are causing the $\delta^{13}C$ increases in soluble sugars from old to new leaves merit further study, however.

A second explanation could result even if the relative isotopic contributions of all compounds to the soluble sugars were identical. Work by Gleixner *et al.* (1993, 1998) suggests that differences in relative amounts of *de novo* synthesized sucrose contribute to higher $\delta^{13}C$ values of sucrose in heterotrophic than photosynthetic tissue. As occurred in the predominantly sucrose containing soluble sugars of *C. longipes* and *U. caracasana*, they found $\delta^{13}C$ values of sucrose to be at least 3‰ lower than those of starch in fully expanded leaves of both beet and potato. They also found $\delta^{13}C$ values of sucrose to be lower in potato leaves than in the completely heterotrophic tubers. If this disparity is because the tuber is heterotrophic and the leaf autotrophic, then the mechanisms producing their results may have also caused the $\delta^{13}C$ values of soluble sugars to be lower in older than in newly emerging leaves in our study.

In Fig. 6, we modified the explanations of Gleixner *et al.* (1993, 1998) to illustrate a logical cause of differences in $\delta^{13}C$ values of the soluble sugars between old and newly emerging leaves. The explanation specifically applies to the sucrose portion of the soluble sugars and so only sucrose is referred to in Fig. 6. The vertical arrow at the left is a qualitative relative scale for $\delta^{13}C$ values. The ^{13}C enrichment of starch is shown in the chloroplast of the older leaf. Under equilibrium conditions, the reaction of triose phosphate with aldolase can yield ^{13}C enriched starch. In addition, when carboxylation rates by Rubisco are high, residual triose P may become high in the chloroplast as lighter triose P is preferentially transported to the cytosol. Transitory starch would then acquire the ^{13}C enrichment of its triose P precursor.

As indicated in the old leaf of Fig. 6, sucrose that is first formed after photosynthesis has a similar carbon isotopic composition to its triose P precursor. A potentially large partitioning of carbon isotopes occurs thereafter during oxidation of pyruvate. The principal kinetic isotope effect for the differentiation is driven by pyruvate dehydrogenase (PDH) which yields ^{13}C enriched *de novo* synthesized sucrose as well as other carbohydrates, and ^{13}C depleted Acetyl CoA (DeNiro & Epstein, 1977).

The total sucrose in the older leaf of Fig. 6 has a higher $\delta^{13}C$ than first-formed sucrose because of the additions of *de novo* synthesized sucrose. Some of the total sucrose of the older leaf is then exported to fuel the emergence of the new leaf. We conservatively made the $\delta^{13}C$ of sucrose entering the new leaf the same as the total sucrose of the older leaf that produced it but caution that some ^{13}C enrichment could have occurred en route during interactions in the stem. The new leaf is entirely heterotrophic to clearly illustrate that a higher proportion of the total sucrose is ^{13}C enriched *de novo* sucrose in the new leaf than in the older, more autotrophic leaf.

Figure 6 may explain why $\delta^{13}C$ values of the soluble sugars are higher in new leaves than old but not why whole new leaves

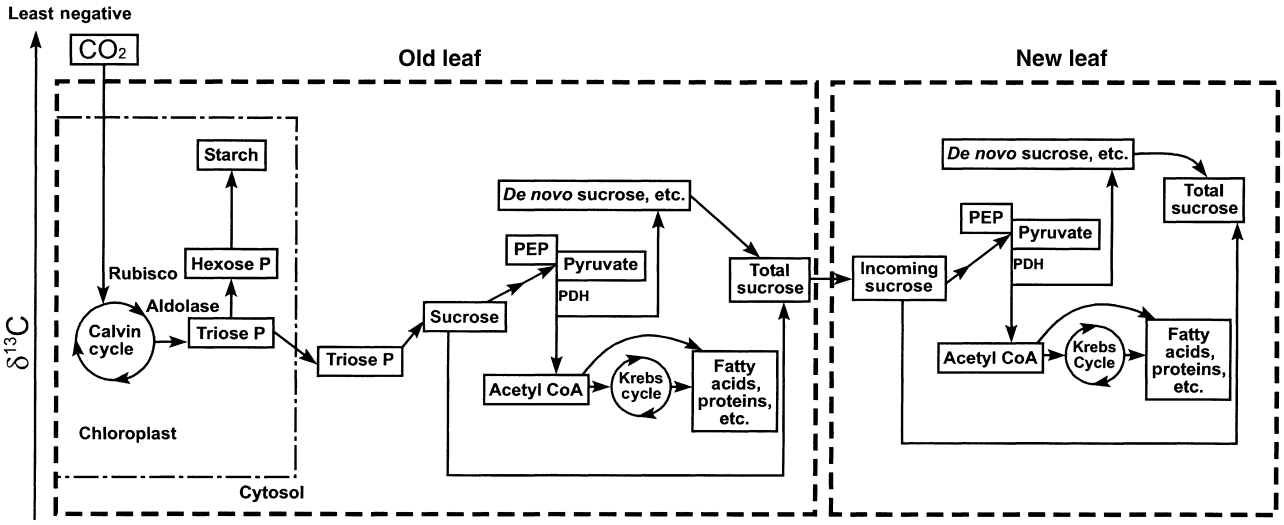

Fig. 6 Possible mechanisms causing differences in $\delta^{13}C$ values between soluble sugars of older leaves and new leaves (inspired by DeNiro & Epstein, 1977; Gleixner *et al.*, 1998).

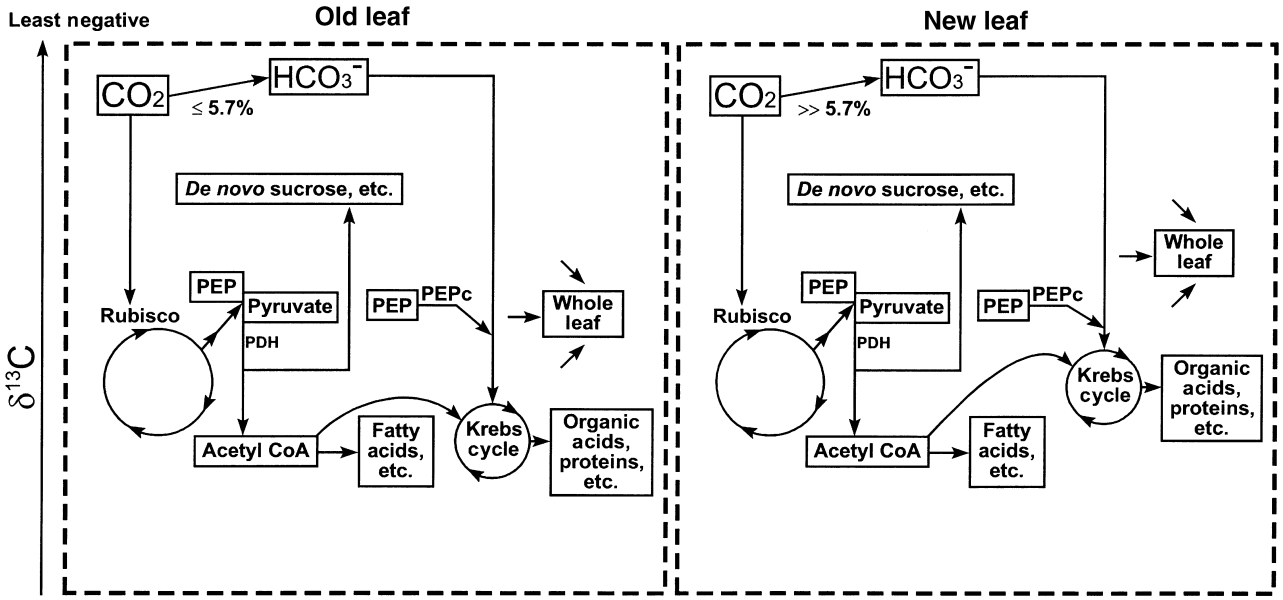

Fig. 7 Possible mechanisms causing $\delta^{13}C$ values to be higher in new leaves than in older leaves.

had higher $\delta^{13}C$ values than the soluble sugars of the oldest (stage 3) leaves. Enrichment in ^{13}C of soluble sugars during transport could have produced this result. The findings of Le Roux-Swarthout *et al.* (2000) that the $\delta^{13}C$ values of completely heterotrophic leaves covary with $\delta^{13}C$ values of atmospheric CO_2 suggest two more mechanisms for the ^{13}C enrichment of newly emerging leaves, however. First, ^{12}C may preferentially exit during respiration, causing ^{13}C enrichment in the leaf. In the only recent evidence for measurable isotope effects during respiration, the work of Duranceau *et al.*, (1999) under drought conditions showed the opposite situation to occur. A more facile loss of ^{13}C during respiration than acquisition during photosynthesis led to ^{13}C depletion in the leaf. The second possibility has no counterevidence. In a new leaf, PEPc fixed carbon could constitute far more than the 5.7% usually assumed in eqn 1a.

Figure 7 illustrates how PEPc fixed carbon could cause newly emerging leaves to be ^{13}C enriched relative to older leaves. Isotope effects during sucrose translocation are not repeated. In the older leaf, all but *c.* 5.7% of carboxylation is by Rubisco, as per the standard interpretation of eqn 1. In the new leaf, Rubisco causes little or none of the carboxylation so PEPc

fixed carbon necessarily becomes much greater than 5.7% of the total carboxylation. Only a portion of PEP is shown to be carboxylated (Salisbury & Ross, 1992) to ultimately cause ^{13}C enrichment to the products of the Krebs cycle. These products of anaplerotic replenishment would be a greater proportion of the organic carbon and hence lead to higher $\delta^{13}C$ values in a new leaf than an old leaf. A study of differences in $\delta^{13}C$ of products of the Krebs cycle, such as fatty acids, would be an appropriate next test of this hypothesis.

Our and other evidences strongly suggest that ^{13}C enrichment in newly emerging leaves is not a *de facto* imprint of the original heterotrophic substrate for growth. Nevertheless, these evidences neither support nor negate that environmental effects on the isotopic composition of imported organic carbon during its production contribute to $\delta^{13}C$ value of a newly emerging leaf.

Do LMA and nitrogen concentrations influence the $\delta^{13}C$ values of leaves during development?

Nitrogen relations and differences in internal resistance to CO_2 have been proposed to explain another counterintuitive case of ^{13}C enrichment: increasing $\delta^{13}C_{LEAF}$ values with the increase in cold and moisture on an elevation gradient (Vitousek *et al.*, 1990; Cordell *et al.*, 1999). One cause of increasing $\delta^{13}C_{LEAF}$ values with elevation may be increased carboxylation capacity by nitrogen rich Rubisco. This hypothesis has been supported for some species by findings that N : area and LMA increase and photosynthesis rates do not vary with elevation (Evans, 1989; Field & Mooney, 1986; Cordell *et al.*, 1999).

Increases in $\delta^{13}C_{LEAF}$ may also be caused by increased internal resistance to diffusion as suggested by higher LMA and no decline in $p_i : p_a$ with elevation in *Metrosideros polymorpha* (Vitousek *et al.*, 1990). Lower p_i at the sites of carboxylation by Rubisco than at the substomatal cavities may result from high internal resistance to diffusion. In this case, $\delta^{13}C_{LEAF}$ values would be reflecting $p_i : p_a$ at the site of Rubisco as per eqn 1 but a calculation from gas exchange measurements would best reflect $p_i : p_a$ in the substomatal cavities (Evans *et al.*, 1986).

Our results do not convincingly support either hypothesis. Low A_{max}, high dark respiration rates, high instantaneous $p_i : p_a$, and pale colour as leaves of both species first emerge are evidences against high carboxylation, even though new leaves had the highest [N] and $\delta^{13}C$ values. These results conform to other findings that young leaves have high [N] but low carboxylation capacities (Kursar & Coley, 1992; Miyazawa *et al.*, 1998). We cannot discard the possibility that high LMA fostered high internal resistances, causing ^{13}C enrichment in early leaf growth. Nonetheless, the combination of high substomatal $p_i : p_a$ calculated from gas exchanges, with low net photosynthesis, and high dark respiration rates of leaves during early emergence conform to findings that recycled carbons from high dark respiration rates of developing leaves raise CO_2 partial pressure in the developing chloroplast (Miyazawa *et al.*, 1998).

Can the input of ^{13}C enriched tissue at leaf initiation be sufficient to lower the resolution of an integrated $p_i : p_a$ estimate of a fully expanded leaf?

Our results suggest that ^{13}C enrichment in newly emerging leaves is more than just a powerful signal of leaf processes associated with more heterotrophic stages of growth. These early effects may significantly and variably influence the $\delta^{13}C$ values of whole leaves in later stages of growth, thereby lowering the accuracy of $p_i : p_a$ estimates from standard use of eqn 1. Support for this hypothesis is derived from the evidence of ^{13}C enrichment in leaves of *Urera caracasana* and *Cecropia longipes* through their first 14 d of growth. Growth after 14 d appears to mark the end of the early heterotrophic growth stage as the $\delta^{13}C$ values of both species descended thereafter (Figs 1 and 2) and A_{max} became substantially greater than zero (Fig. 4). Growth during the early (< 14 d) stage could constitute between 12 and 32% of the maximum ash-free mass and isotopic signature attained by a leaf according to our simple estimate (Table 3). Leaves in later stages of development could be identical in $p_i : p_a$ to one another yet still have different $\delta^{13}C$ values due to differences in the percentage of the mass they accrued during their more heterotrophic stage of development. We demonstrate this more quantitatively in the next section when we explore means of isolating early effects on the $\delta^{13}C$ values of leaves from the second, most autotrophic stage of growth.

Can advantage be taken of the ^{13}C enrichment effect on newly emerging leaves to obtain information about heterotrophic and autotrophic inputs to leaf growth?

Crude estimators of heterotrophic investment in early leaf growth are the mass of carbon of a leaf at the end of its ^{13}C enriched phase and the percentage contribution of this input to the maximum mass of carbon a leaf will attain. Using the ash-free masses of leaves at 14 d as indicators of the absolute heterotrophic investment, more carbon was invested in early leaf growth in *C. longipes* than in *U. caracasana*. The percent investment in maximum leaf mass by early heterotrophic growth did not differ between the two species, however (Table 3, values for 14 d). These simple estimators would be practical for studying variation in investment of imported carbon among individuals and between populations in forests.

Large disparities existed between integrated $p_i : p_a$ estimated from $\delta^{13}C$ values of whole leaves at all ages (eqn 1) and integrated $p_i : p_a$ estimated from gas exchange measurements (see Material and Methods). As indicated by the upper frames of Fig. 8, data points for leaves < 28 d of age (circles) are farthest from the 1 : 1 slope line they would fall along if $\delta^{13}C$

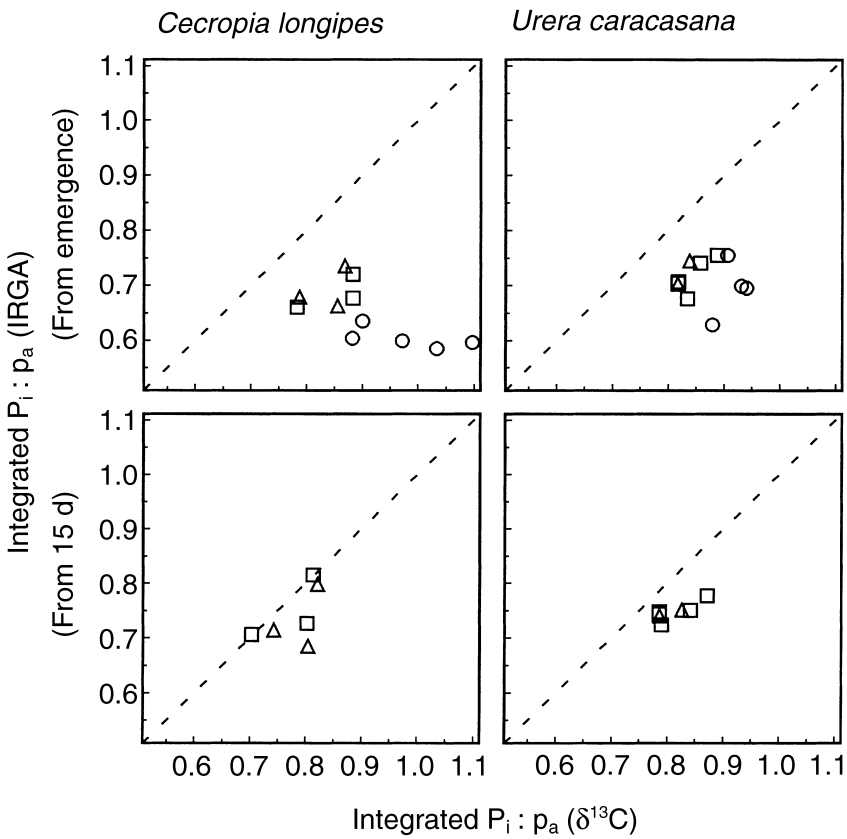

Fig. 8 Comparison of integrated $p_i : p_a$ as calculated from $\delta^{13}C$ values (x-axes) and gas exchange (IRGA) (y-axes) as a function of leaf age in *Cecropia longipes* and *Urera caracasana*. Upper frames compare $p_i : p_a$ calculated from whole leaf $\delta^{13}C$ values with corresponding weighted average $p_i : p_a$ calculated from gas exchange measurements and leaf masses. Lower frames compare $\delta^{13}C$ derived and gas exchange derived estimates of integrated $p_i : p_a$ that excludes the most heterotrophic phase of growth. Circles, < 29 d old leaves; squares, are 29–75 d old leaves; triangles, > 75 d old leaves.

values and gas exchange measurements yielded the same $p_i : p_a$ estimate. Correspondence between $p_i : p_a$ estimates was consistently < 1 : 1 in leaves of all ages, however, suggesting that $p_i : p_a$ was always underestimated from $\delta^{13}C$ values. One explanation for the underestimate is that the error introduced by ^{13}C enriched carbon during early heterotrophic growth is never fully erased by subsequent growth.

Fig. 8 supports the hypothesis that the accuracy of $p_i : p_a$ estimates from $\delta^{13}C$ values of older, more autotrophic leaves can be greatly improved by subtracting the isotopic influence of carbon required during the early, heterotrophic stage of leaf growth. The lower frames of Fig. 8 estimate $p_i : p_a$ for leaf growth intervals after the early heterotrophic stage (leaf age minus 14 d, leaves < 28-d-old not shown; see eqn 4). Subtraction of the estimated influence of early carbon yielded a close to 1 : 1 correspondences in $p_i : p_a$ between $\delta^{13}C$ and gas exchange calculations. Correspondence between the two estimates did not significantly differ between leaves of intermediate age (> 28 d to = 75 d) and the oldest (> 75 d) leaves. We thus have no evidence of isotopic changes during the carbon export phase that would, in addition to heterotrophic inputs, lower the accuracy of $p_i : p_a$ estimates from eqn 1.

Additional study may further improve estimates of imported carbon inputs to early leaf growth and $p_i : p_a$ of later growth from $\delta^{13}C$ values. Specifically, losses of carbon will occur as mobile sugars are converted to more durable compounds. Better knowledge of the isotopic consequences of turnover may further improve inferences about carbon use from $\delta^{13}C$ analyses.

The integrated $p_i : p_a$ of tropical tree species is more accessible to study using stable carbon isotopic methods than to other means of analysis. Our findings show, however, that the standard interpretation of integrated $p_i : p_a$ from $\delta^{13}C$ values of tree leaves can be significantly inaccurate. Our results also strongly suggest that ^{13}C enrichment after organic carbon is imported for leaf growth causes some of the inaccuracy. Disparities between integrated $p_i : p_a$ calculated from $\delta^{13}C$ values and true $p_i : p_a$ are greatest as leaves first emerge, leading to a new use of $\delta^{13}C$ values for estimating heterotrophic investments in leaf growth. Inferences from $\delta^{13}C$ requiring estimates of integrated $p_i : p_a$ can be improved by subtracting the approximate isotopic contributions of early leaf growth when these heterotrophic inputs are high.

Empirically, the period of leaf growth with ^{13}C enrichment corresponds roughly to the time of leaf area expansion before leaves achieve physiological maturity. How the pattern may hold for nonpioneer species with slower leaf growth is unknown. Many tropical trees with delayed greening and nitrogen allocation to leaves (Kursar & Coley, 1991, 1992), may have an even longer period of heterotrophic leaf development. Further understanding of the processes leading to ^{13}C enrichment will likely increase the power of stable carbon isotopic

analyses to understand tree resource use characteristics and ecosystem biogeochemistry in tropical forests.

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