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Limited photosynthetic plasticity in the leaf-succulent CAM plant *Agave angustifolia* grown at different temperatures

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Abstract. In *Agave angustifolia* Haw., a leaf-succulent constitutive crassulacean acid metabolism (CAM) plant of tropical Panama, we tested whether nocturnal CO_2 uptake and growth were reduced at night temperatures above 20°C. Unlike some CAM model species from habitats with pronounced day-night temperature variations, in *A. angustifolia* temperature affected little the relative contributions of CAM and C_3 photosynthesis to growth. In plants grown under 12 h light/dark regimes of 25/17, 30/22 and 35/27°C, biomass increased with temperature. Maintaining day temperature at 35°C and reducing night temperature from 27 to 17°C markedly lowered growth, a reduction partially reversed when roots were heated to 27°C. Across all treatments, whole-shoot $\delta^{13}C$ values ranged between –14.6 and –13.2 ‰, indicating a stable proportion of CO_2 was fixed at night, between 75 and 83%. Nocturnal acidification reflected growth, varying between 339 and 393 μmol H^+ g^{-1} fresh mass and 63–87 μmol H^+ cm^{-2}. In outdoor open-top chambers, warming the air 3°C above ambient at night did not reduce biomass accumulation. The persistence of a high capacity for nocturnal CO_2 fixation at the expense of a limited capacity for switching between C_3 and CAM probably makes this *Agave*, and others like it, potential species for biomass production in seasonally-dry landscapes.

Additional keywords: biofuel, C_3 photosynthesis, climate change, crassulacean acid metabolism, open-top chamber.

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Introduction

Crassulacean acid metabolism (CAM) in terrestrial plants is a water-use efficient photosynthetic adaptation that assists plants to occupy habitats subject to periodic water stress (Osmond 1978; Winter 1985). CAM is water-use efficient because CO_2 is assimilated at night when temperatures and rates of evaporation are at daily minima (Winter *et al*. 2005).

In addition to fixing CO_2 during the night, many species with CAM can also incorporate atmospheric CO_2 during the light via C_3 photosynthesis, a pathway that is less water-use efficient than CAM. It is not uncommon for CAM species with a capacity for light fixation to adjust the proportions of carbon fixed at night and during the day in response to the availability of water (Winter 1985; Borland *et al*. 2011; Dodd *et al*. 2002) such that CO_2 fixation in the light increases when water is available and decreases when water is scarce. The ability to regulate CO_2 uptake during the dark and the light thus enables many CAM species to maintain some daily carbon gain as they reduce water-loss, whereas C_3 and C_4 plants may not be able to maintain a positive carbon balance under similar stressful conditions.

Evaporative water loss increases with the difference in vapour pressure between the leaf and the atmosphere, and thus responds to increasing temperature. It is therefore not surprising that in CAM species the proportion of carbon fixed at night and during the day responds to temperature (De Vries 1884; Kluge and Ting 1978). In CAM plants, the temperature responses of CO_2 fixation during the night and the day are not the same (Nobel 1988; Yamori *et al*. 2014), as might be expected when two biochemically distinct processes are involved. CO_2 uptake in the dark is enhanced when daytime temperature maxima are typically above 30°C, and night maxima are below 20°C. As a result, night temperatures significantly above 20°C are not only widely considered suboptimal for nocturnal CO_2 uptake (Neales 1973*a*, 1973*b*; Kluge and Ting 1978; Neales *et al*. 1980; Medina and Osmond 1981) but, by extrapolation, suboptimal for growth. The night-time temperature responses are at odds with the expression of pronounced CAM in the many species (both epiphytes and terrestrial plants) that inhabit the tropics (Wong and Hew 1976; Holtum and Winter 1999; Crayn *et al*. 2004; Silvera *et al*. 2005) where night temperatures are commonly around 20−25°C.

Early studies proposed that the mechanistic basis for reduced nocturnal CO_2 uptake at higher night temperatures is a product of differing thermal optima of malate synthesising and consuming reactions and of increased respiration (Brandon 1967; Kaplan *et al*. 1976) but these ideas have not been further pursued experimentally. Similarly, there is little experimental evidence that the negative effects of high night temperatures on CO_2 uptake in the dark that have been observed by several authors ultimately adversely affect growth, even amongst the extensive

Agave and cactus research published by Nobel and his colleagues (see Nobel 1988 for a review).

Here we explored the relationship between growth, $\delta^{13}C$ (as indicator of the relative contributions of light and dark fixation to carbon gain), and day and night temperatures in *Agave angustifolia* Haw., a constitutive strong-CAM species from the tropics and subtropics of Central America and Mexico. *A. angustifolia* is a putative wild ancestor of the domesticated agaves (Gentry 1982; Colunga-García Marín *et al*. 1999), *Agave fourcroydes* Lem. (henequen) which is used for fibre production, and *Agave tequilana* F.A.C.Weber, the source of tequila. Both *A. fourcroydes* and *A. tequilana* have been proposed as biofuel feedstock crops for seasonally dry landscapes in tropical northern Australia and elsewhere (Chambers and Holtum 2010; Holtum *et al*. 2011; Owen and Griffiths 2013). In a warming world containing roughly 400 million ha of abandoned agricultural land much of which is in the semiarid tropics and sub-tropics (Campbell *et al*. 2008), it could be expected that the area suitable for growing appropriate *Agave* species for biofuels might be expanding. However, if the expression of CAM in *Agave* is progressively reduced with increasing night temperature, and the expression of daytime CO_2 uptake is reduced as well, then growing CAM *Agave* for biomass may become a less attractive proposition.

Here we demonstrate that *A. angustifolia* accumulates biomass more rapidly at a relatively high day/night temperature regime of 37/27°C than it does at 25/17°C. At a high daytime temperature of 35°C, growth increased when night temperature was increased from 17 to 27°C. In addition, the proportional contribution of light and dark CO_2 uptake to net carbon gain, measured using $\delta^{13}C$ values as a proxy (Winter and Holtum 2002), responded little to temperatures to which *A. angustifolia* might be exposed in its natural habitat. We suggest that this lack of photosynthetic plasticity may be a desirable trait when growing constitutive strong CAM plants for biomass production in seasonally-dry environments in the tropics and subtropics.

Materials and methods

Plant material

Agave angustifolia Haw. was collected from Playa Majagual, Panama (8°43′N, 79°45′E) and maintained outdoors in forest topsoil at the Smithsonian Tropical Research Institute, Santa Cruz Experimental Research Facility, Gamboa, Republic of Panama (9°07′N, 79°42′W).

Experiment using growth chambers

Bulbils of 0.68 ± 0.09 (s.e.) g DM with leaf areas of 33.04 ± 7.13 cm^2 were planted in 6 L pots (16 cm internal diameter) containing 80% forest topsoil, 20% sand and 2 g Osmocote Plus fertiliser (Scotts-Sierra Horticultural Products, OH, USA). The plants ($n = 6$ per treatment) were grown from 1/7/2012 until 24/9/2012 inside five controlled-environment chambers (Model GC15, Environmental Growth Chambers, OH, USA) operating under 12 h light/12 h dark cycles of 25/17°C, 30/22°C, 35/27°C, 35/17°C, and 35/17°C and maintaining soil temperature at ≥27°C (Table 1). Photon flux density was 500 µmol m^{-2} s^{-1} at the top of the plants.

Air temperatures inside growth chambers, measured using copper-constantan fine wire thermocouples, were essentially identical to chamber set-temperatures and assumed their new value within 10 min of light/dark and dark/light transitions.

Soil temperatures within all pots reflected the day/night air temperatures except for pronounced lags as they gradually heated or cooled following switchovers between temperature regimes (Fig. 1). For the treatment in which roots were heated, pots containing plants were placed in a water-bath equipped with a heating thermostat (Model Alpha, Lauda, NJ, USA) set to 27°C. Soil temperature was measured in each pot using two copper-constantan thermocouples embedded in silicone sealant.

Open-top chamber experiment

Agave angustifolia was grown outdoors in six open-top chambers between 1 November 2012 and 25 June 2013 at the Santa Cruz Experimental Research Facility. Each chamber

Table 1. Growth chamber environments

Leaf temperatures of *Agave angustifolia* were measured in the middle of the light period and in the dark (1 h before onset of the light) using an infrared thermometer (MT6; Raytek, CA, USA), the readings of which were verified using fine wire copper-constantan thermocouples. Values are means of six measurements ± s.e.

Air temperature (°C)		Relative humidity (%)		Leaf temperature (°C)	
Light	Dark	Light	Dark	Light	Dark
25	17	56	76	27.4 ± 0.4	15.1 ± 0.1
30	22	40	57	32.8 ± 0.3	20.5 ± 0.0
35	27	29	41	38.5 ± 0.2	26.1 ± 0.1
35	17	29	77	37.5 ± 0.3	15.6 ± 0.0
35[A]	17[A]	29	77	38.2 ± 0.4	17.7 ± 0.2

[A]In this treatment, the roots were heated to achieve 27°C during the dark. Heating was maintained during the light.

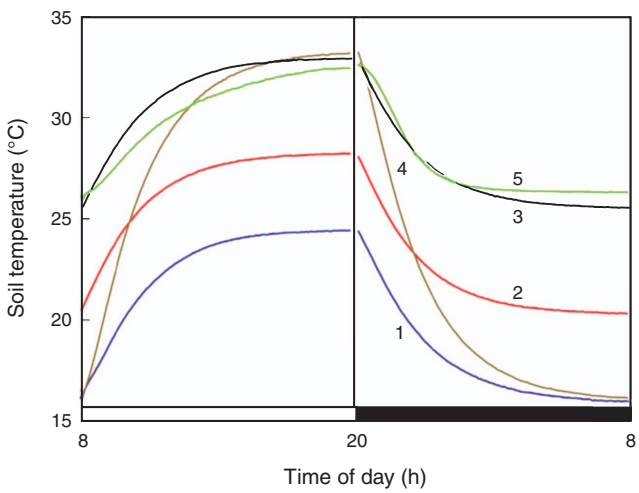

Fig. 1. A 24 h day-night cycle of soil temperatures in pots containing *Agave angustifolia* plants exposed in growth cabinets to 12 h day/night temperature regimes of 25/17°C (1), 30/22°C (2), 35/27°C (3), 35/17°C (4) and 35/17°C plus roots warmed to achieve 27°C (5). Open bar denotes light period, closed bar denotes dark period. Measurements were at 5 min intervals.

contained six 19 L containers each with one bulbil planted in forest soil. At the onset of the experiment, the average weight of bulbils was 0.99 ± 0.10 g DM and the average area of bulbil leaves was 41.38 ± 3.20 cm^2. The chambers and environmental control within the chambers are described by Cheesman and Winter (2013). In three chambers night temperature was maintained at 3°C above the ambient night temperature. Pots received ambient rainfall with additional daily watering as required to maintain soils at close to field capacity.

At harvest, soil was washed by hand from the roots. Leaf area was measured using a leaf area meter (Model LI-3100, Li-Cor, Lincoln, NE, USA). Roots, leaves and stems were oven-dried at 70°C.

Titratable acidity

From mature leaves of each plant, two discs, each of 0.9 cm diameter, were excised using a cork borer at the end of the light and dark periods and were frozen in liquid nitrogen. Organic acids were extracted by sequentially boiling samples in 50% methanol and in water for 5 min. Extracts were cooled to room temperature and titrated with 10 mM KOH to pH 6.5 (Holtum *et al*. 2004).

Stable isotope analysis

The $\delta^{13}C$ values of finely-ground homogeneous powder from the pooled dried leaves of whole plants were measured using an isotope ratio mass spectrometer (Delta V; Thermo Fisher Scientific, Ottawa, ON, Canada) in the Stable Isotope Laboratory of the Smithsonian Tropical Research Institute (Cernusak *et al*. 2011; Winter *et al*. 2014).

Results

Whole-plant mean dry biomass recorded at the two higher day/night temperature treatments, 30/22 and 35/27°C, was significantly greater than that of plants grown at 25/17°C (Fig. 2*a*), with plants at 35/27°C, the warmest regime, having a mean biomass that was 27% greater than for plants at 25/17°C, the coolest regime.

Compared with 35/27°C, maintaining the day temperature at 35°C and reducing the night temperature to 17°C lowered mean biomass from 8.8 to 5.9 g $plant^{-1}$, a significant drop of 33%. In contrast, the mean biomass of plants at 25/17°C and 35/17°C was similar.

The response pattern of leaf area to temperature was qualitatively similar to the biomass pattern but overall amplified (Fig. 2*b*). Compared with the mean leaf area of plants at 25/17°C, leaf area was 25% greater at 30/22°C and 49% greater at 35/27°C. As with biomass, the mean leaf area of plants grown at 35/17°C was 32% less than for plants grown at 35/27°C. Leaf area ratio (LAR) increased with temperature (Fig. 2*c*) whereas leaf mass/area (LMA), a measure of leaf density, remained constant or decreased slightly (Fig. 2*d*).

Maintaining roots at 27°C during a 35/17°C air temperature regime increased leaf area and LAR significantly (Fig. 2*b*, *c*), whereas LMA decreased slightly (Fig. 2*d*). The heating of roots was accompanied by an associated 2.1°C increase in leaf temperature at night (Table 1), presumably the product of

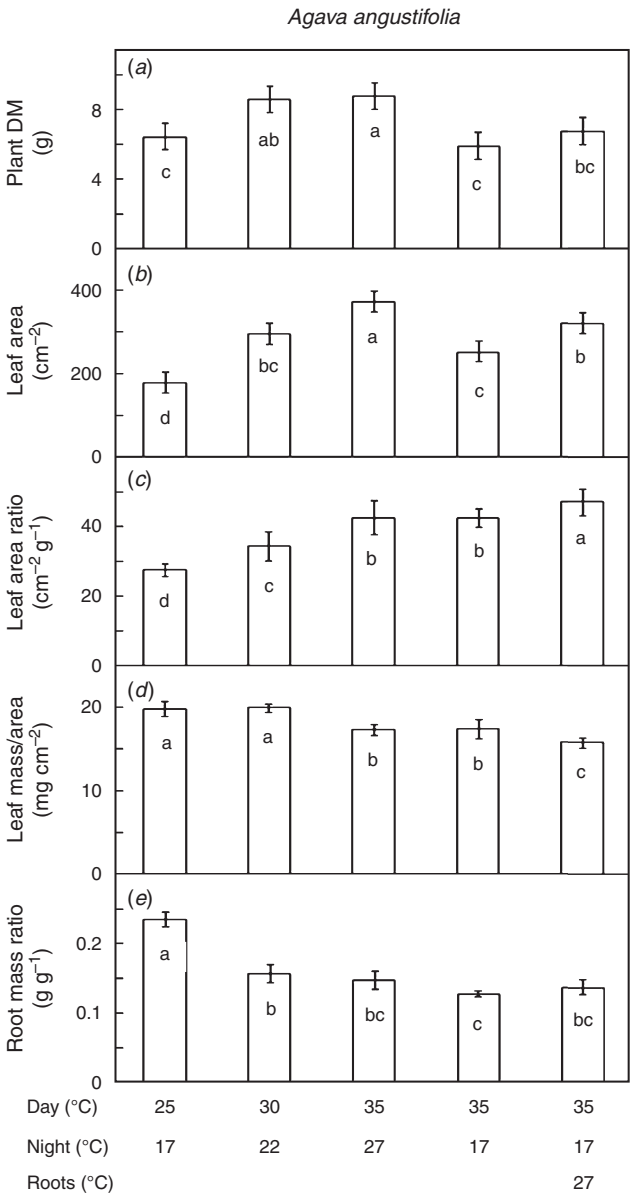

Fig. 2. Biomass (*a*), leaf area (*b*), leaf area ratio (leaf area per whole-plant dry mass) (*c*), leaf mass/area (leaf dry mass per unit leaf area) (*d*) and root mass ratio (root dry mass per whole-plant dry mass) (*e*) of *Agave angustifolia* plants exposed to 12 h day/night temperature regimes of 25/17°C, 30/22°C, 35/27°C, 35/17°C, and 35/17°C plus roots warmed to 27°C. Bars indicate 95% confidence levels of the means ($n = 6$ plants). Letters within columns indicate statistical significance, categories not sharing a letter differ significantly at $P < 0.05$ (Tukey's HSD).

thermal dissipation from H_2O in the circulating bath used to heat the roots.

Nocturnal acid accumulation (ΔH^+) ranged between 339 and 393 µmol H^+ g^{-1} FM and 63–87 µmol H^+ cm^{-2} across treatments (Table 2). Acid levels were similar in plants grown at 25/17, 30/22 and 35/27°C, with the largest mean acid accumulation measured for plants at 30/22°C. In plants grown at 35°C during the light, mean acidification values were not significantly affected when the night temperature

Table 2. Nocturnal increases in leaf tissue acidity of *Agave angustifolia* grown under five 12 h light/12 h dark temperature regimes

Samples were taken from mature leaves at the end of the light and end of the dark periods. Values are means ± s.e. ($n = 6$ plants). Means followed by the same letter do not differ (Tukey's HSD test, $P < 0.05$)

Titratable acidity	Light/dark temperature (°C)				
	25/17	30/22	35/27	35/17	35/17 + root warming[A]
			μmol g^{-1} fresh mass		
Light	34 ± 4	13 ± 1	0 ± 0	0 ± 0	0 ± 0
Dark	399 ± 7	406 ± 10	371 ± 5	343 ± 8	340 ± 12
Δ	364 ± 10ab	393 ± 10a	371 ± 5ab	343 ± 8b	339 ± 12b
			μmol cm^{-2}		
Light	8 ± 4	3 ± 3	0 ± 2	0 ± 3	0 ± 2
Dark	88 ± 4	90 ± 3	75 ± 2	67 ± 3	63 ± 2
Δ	80 ± 4ab	87 ± 3a	75 ± 2abc	67 ± 3bc	63 ± 2c

[A]In this treatment, the roots were heated to achieve 27°C during the dark. Heating was maintained during the light.

Table 3. $\delta^{13}C$ values of pooled leaves of *Agave angustifolia* plants grown under five 12 h light/12 h dark temperature regimes

Values are means ± s.e. ($n = 6$ plants). Means followed by the same letter do not differ (Tukey's HSD test, $P < 0.05$)

	Light/dark temperature (°C)				
	25/17	30/22	35/27	35/17	35/17 + root warming[A]
$\delta^{13}C$ (‰)	–14.6 ± 0.1a	–14.5 ± 0.0a	–14.4 ± 0.1a	–13.2 ± 0.1b	–13.2 ± 0.1b

[A]In this treatment, the roots were heated to achieve 27°C during the dark. Heating was maintained during the light.

was reduced from 27 to 17°C or when roots were warmed to 27°C during 17°C nights.

As an indicator of the proportional contribution of CO_2 uptake in the dark and the light to shoot carbon gain, $\delta^{13}C$ values differed by only 1.4 ‰ across all treatments (Table 3). Plants cultured under day/night regimes of 25/17, 30/22 and 35/27°C had similar $\delta^{13}C$ values of between –14.4 and –14.6 ‰. The least negative $\delta^{13}C$ value, –13.2 ‰, were observed for plants at 35/17°C irrespective of whether the roots were heated.

For well-watered plants grown in open-top chambers under ambient light, elevating night temperature by 3°C did not affect plant dry mass or leaf area (Table 4).

Table 4. Dry biomass and leaf area of whole plants of *Agave angustifolia* grown in six open-top chambers under ambient conditions or ambient days with nights elevated by 3°C at the Smithsonian Tropical Research Institute, Santa Cruz Experimental Research Facility, Gamboa, Republic of Panama (9°07′N, 79°42′W)

Significance of difference of the means ($n = 3$) was tested using a Students t-test (2-tailed, equal variance). n.s. = not significant, $P \geq 0.05$

Parameter	Temperature treatment		Test of significance
	Ambient	Night temperature elevated by 3°C	
Dry mass (g)	36.2 ± 0.4	42.9 ± 5.7	n.s. ($P = 0.321$)
Leaf area (cm^2)	713 ± 12	886 ± 153	n.s. ($P = 0.323$)

Discussion

The growth response of the constitutive strong CAM plant *A. angustifolia* to a range of day-night temperature regimes contrasted with an often-cited generalisation that high day temperatures combined with low night temperatures favour CAM and enhance growth (Kluge and Ting 1978; Owen and Griffiths 2013). In *A. angustifolia*, biomass was greater for plants grown at day temperatures above 25°C and night temperatures above 17°C. Nocturnal accumulation of titratable acidity was similar for plants grown at night temperatures of 17 and 27°C.

Biomass accumulation, leaf area and LAR increased as day and night temperatures were increased from 25/17 to 35/27°C, whereas unchanged $\delta^{13}C$ values indicated that the relative contribution of nocturnal CO_2 uptake to carbon gain remained around 75% (using the calibration of Winter and Holtum 2002). In general agreement with the CAM-type $\delta^{13}C$ values, nocturnal acidification was substantial under the three treatments. As growth is driven by overall assimilation rate and LAR, and nocturnal CO_2 assimilation remained relatively constant, the principal contributor to increased growth between 25/17, 30/22 and 35/27°C appears to have been increased LAR.

Compared with the highest temperature regime, 35/27°C, maintaining the day temperature at 35°C and reducing the night temperature to 17°C markedly reduced biomass accumulation. As LAR was similar for both treatments it follows that the overall rates of CO_2 uptake were lower in plants under 17°C nights, a conclusion supported by marginally lower nocturnal acidification at 17°C. $\delta^{13}C$ values suggest that over the life of the plants under 27 and 17°C nights, 76 and 83% of the carbon, that is, similar proportions, were obtained in the dark respectively.

Biomass accumulation was similar between plants subjected to 17°C nights and days of 25 or 35°C but LAR was greater at

the higher day temperature. As was deduced following the comparison of plant growth at 35/27 and 35/17°C, it appears that the overall rate of CO_2 uptake per unit area was lower in the 35/17°C plants than in the 25/17°C plants, a deduction consistent with the lower nocturnal acidification in plants at 35/17°C.

In the 35/17°C treatment, warming the soil to 27°C increased total leaf area and LAR but biomass, acidification and $\delta^{13}C$ values remained constant. Interpretation of the effects on growth of soil heating were complicated by an associated increase of nocturnal leaf temperature by 2.1°C (Table 1), as explained in 'Materials and Methods'. Stimulation of growth following root heating has previously been reported for the C_3 species, cotton, possibly the consequence of temperature shifting source–sink relationships (Königer and Winter 1993).

Warming of *A. angustifolia* grown outdoors by 3°C during the night did not adversely affect growth. In fact, higher means for whole-plant dry mass (19% increase) and leaf area (24% increase) of heated plants, although not statistically different, suggest that a positive statistically significant correlation between night temperature and growth might well be observed in a more extensively replicated experiment. Significantly increased growth rates have been reported for the C_3 pioneer tree species, *Ficus insipida* and *Ochroma pyramidale*, maintained at 3°C above ambient at night (Cheesman and Winter 2013).

The perception that CAM activity in general is stimulated by pronounced differences in day and night temperature stems in the main from studies of species of *Kalanchoë*, *Agave* and *Opuntia* from habitats where cool nights and hot days predominate (Queiroz 1966; Neales 1973*a*; Szarek and Ting 1974; Kluge and Ting 1978; Nobel and McDaniel 1988), although broadly similar observations have been reported for *Ananas comosus*, a tropical species that has been modified by breeding for desirable agronomic characters (Neales 1973*b*; Neales *et al*. 1980; Zhu *et al*. 1999). One might expect any requirement for substantial day/night temperature differences to be less evident in species like *A. angustifolia* that are native to the humid tropics where nights are typically warm and day/night temperature fluctuations are less pronounced (Milburn *et al*. 1968; Wong and Hew 1976; Griffiths and Smith 1983; Winter 1985; Holtum and Winter 1999; Crayn *et al*. 2004; Silvera *et al*. 2005). Nobel and Hartsock (1978, 1981) demonstrated that CO_2 exchange by *Agave* species can rapidly acclimate to new temperature regimes but the implications of these short-term responses for growth are not clear.

On the basis that nocturnal CO_2 uptake is reflected in growth, minimum night-time temperatures have been used to form the temperature index components of environmental productivity indexes (EPI) designed to predict the productivities of various *Agave* and *Opuntia* in the field (Nobel 1984, 1985, 1988, 1989, 1991; Nobel and Meyer 1985; Nobel and Quero 1986; Nobel and Valenzuela 1987; Pimienta-Barrios *et al*. 2001). Similarly, a model that integrates EPI estimates with soil water retention characters and GIS methodology to predict potential productivity of *Agave* species in Australia, apportioned to minimum night-time temperatures 95% of the effects of temperature on productivity in *A. fourcroydes* (Owen and Griffiths 2013). The observations on the growth of *A. angustifolia* reported here suggest that EPI modelling based on minimum temperatures may be a too simplistic approach, particularly for tropical CAM species.

The responses of growth in *A. angustifolia* to day and night temperatures will not only reflect the effects of temperature on the CAM cycle but also the effects of temperature on other cell processes. Diel patterns of leaf and cladode expansion have been observed in CAM *M. crystallinum*, and in CAM perennials such as *Opuntia engelmanii*, *O. oricola* and *Kalanchoë beharensis* (Gouws *et al*. 2005) but the interaction between the demands of CAM cells for carbon skeletons and the demands by the rest of the plant for carbon skeletons and energy are not well understood, even for long-studied models such as the annual *Mesembryanthemum crystallinum* and perennial *Kalanchoë* spp. (Borland and Dodd 2002; Antony and Borland 2009). It has been proposed that the substantial requirements for carbon skeletons in CAM cells limits the export of carbohydrates for growth during acidification at night, and during deacidification in the light (Gouws *et al*. 2005; Haider *et al*. 2012). Growth could be particularly sensitive to temperature when carbon export for growth is expected to be most evident – during the first and last phases of the light, and perhaps late at night when the accumulation of malic acid is close to completion and turgor pressures are high.

Photosynthetic plasticity, which is the ability to switch between C_3 and CAM photosynthesis, contributes to the ecological success of some CAM species, particularly annuals, trees and hemi-epipiphytes in seasonally-dry habitats (Zotz and Winter 1994*a*, 1994*b*; Winter and Holtum 2014). These photosynthetically highly flexible species often exhibit reduced rates of growth when CAM is the predominate photosynthetic pathway. In contrast, many open-field CAM perennials such as *A. angustifolia* and its close relatives, *A. fourcroydes* and *A. tequilana*, combine strong CAM with substantial growth rates. In these species, the persistence of CAM throughout large parts of the dry season can enable appreciable rates of growth in concert with high water-use efficiency (Nobel 1985, 1988, 1991; Nobel and Valenzuela 1987). It is this ability to exhibit year-round water-use efficient growth in seasonally-dry conditions that enables agaves to accumulate biomass at sizeable annualised rates and has resulted in them being considered as biofuel feedstock crops in regions where growth of traditional food and bioenergy crops is suboptimal (Nobel 1988; Borland *et al*. 2009; Davis *et al*. 2011; Holtum *et al*. 2011).

In conclusion, consistent with its tropical occurrence, *A. angustifolia* successfully grew at high day/night temperatures, and low night temperatures combined with high day temperatures adversely affected growth. In all situations, CAM was the principal contributor to carbon gain, suggesting limited photosynthetic plasticity in this leaf-succulent constitutive CAM species.

There is substantial literature on the effects of temperature on daily CO_2 exchange patterns of CAM plants. What is clearly needed is research that extends these studies to temperature effects on growth, particularly of species native to habitats with contrasting temperature regimes.

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