



## SYMPOSIUM

# The Evolution of CAM Photosynthesis in Australian *Calandrinia* Reveals Lability in C<sub>3</sub>+CAM Phenotypes and a Possible Constraint to the Evolution of Strong CAM

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**Synopsis** Australian *Calandrinia* has radiated across the Australian continent during the last 30 Ma, and today inhabits most Australian ecosystems. Given its biogeographic range and reports of facultative Crassulacean acid metabolism (CAM) photosynthesis in multiple species, we hypothesized (1) that CAM would be widespread across Australian *Calandrinia* and that species, especially those that live in arid regions, would engage in strong CAM, and (2) that Australian *Calandrinia* would be an important lineage for informing on the CAM evolutionary trajectory. We cultivated 22 Australian *Calandrinia* species for a drought experiment. Using physiological measurements and  $\delta^{13}\text{C}$  values we characterized photosynthetic mode across these species, mapped the resulting character states onto a phylogeny, and characterized the climatic envelopes of species in their native ranges. Most species primarily utilize C<sub>3</sub> photosynthesis, with CAM operating secondarily, often upregulated following drought. Several phylogenetically nested species are C<sub>3</sub>, indicating evolutionary losses of CAM. No strong CAM was detected in any of the species. Results highlight the limitations of  $\delta^{13}\text{C}$  surveys in detecting C<sub>3</sub>+CAM phenotypes, and the evolutionary lability of C<sub>3</sub>+CAM phenotypes. We propose a model of CAM evolution that allows for lability and reversibility among C<sub>3</sub>+CAM phenotypes and C<sub>3</sub> and suggest that an annual life-cycle may preclude the evolution of strong CAM.

## Introduction

Crassulacean acid metabolism (CAM) is a water-use efficient form of photosynthesis that is characterized by a capacity to fix CO<sub>2</sub> in the dark and to store the carbon overnight in malic acid. In contrast to most plants with C<sub>3</sub> and C<sub>4</sub> photosynthesis, when CAM is expressed in a plant the pathway is rarely the sole contributor to carbon gain. It almost always co-exists with C<sub>3</sub> photosynthesis (or, very rarely, with C<sub>4</sub>-photosynthesis in *Portulaca* [Koch and Kennedy 1982; Ocampo et al. 2013; Winter et al. 2019; Christin et al. 2015; Holtum et al. 2017a; Holtum and Winter 2017; Winter 2019]).

In comparison to CO<sub>2</sub> uptake during the light by C<sub>3</sub> or C<sub>4</sub> photosynthesis, the proportion of carbon

obtained during the night in species with CAM may contribute anywhere between close to 0% and 100% of 24-h carbon uptake (Winter and Holtum 2002, 2014). Plants with CAM therefore exhibit a spectrum of phenotypes that range between what have been designated as C<sub>3</sub>+CAM, in which daytime C<sub>3</sub> photosynthesis contributes the majority of carbon, and strong CAM (perhaps thought of as CAM+C<sub>3</sub>), in which nocturnal CAM-type CO<sub>2</sub> uptake contributes the majority of carbon (Winter et al. 2015, Edwards 2019). The C<sub>3</sub>+CAM phenotype includes species which, when unstressed, obtain carbon exclusively or predominantly via C<sub>3</sub> photosynthesis but in which CAM is induced or up-regulated following exposure to a stress, generally water limitation (see

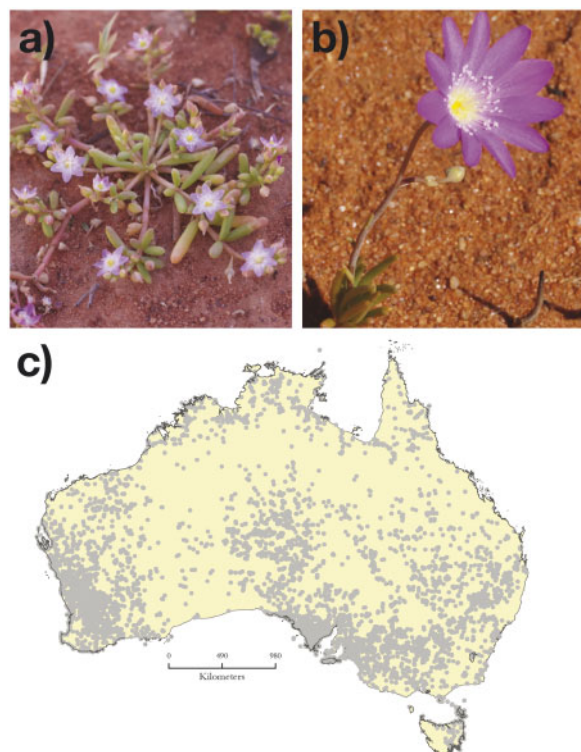
Winter 2019, for a review). If the stress-related increase in nocturnal acidification is lost when the stress is removed, the induced CAM-component is said to be facultative. An important ecological facet of  $C_3$ +CAM is thus that the relative contribution of CAM to 24-h carbon gain often varies in response to environmental stress (Griffiths 1989; Cushman 2001; Winter et al. 2015; Holtum et al. 2017b), with the proportion of  $CO_2$  obtained via CAM being greatest when a plant is exposed to stress.

It has been hypothesized that the facultative and constitutive  $C_3$ +CAM phenotypes may represent early steps in the evolution of more pronounced CAM (Guralnick and Jackson 2001; Silvera et al. 2010; Edwards and Ogburn 2012; Hancock and Edwards 2014; Bräutigam et al. 2017; Edwards 2019; Heyduk et al. 2019). This hypothesis is reasonably built on a set of observations: (1) the CAM phenotype is too complex to appear via a single mutation, so it must be assembled incrementally, (2)  $C_3$ +CAM phenotypes are found in close phylogenetic association with strong-CAM species (Guralnick et al. 2007; Silvera et al. 2009; Crayn et al. 2015; Goolsby et al. 2018), and (3) in the evolution of  $C_4$  photosynthesis, a related photosynthetic pathway, there are well described  $C_3$ – $C_4$  intermediate phenotypes that are thought to represent transitional states along the  $C_4$  evolutionary trajectory (Christin et al. 2011; Sage et al. 2012; Edwards 2019). The characterization of  $C_3$ – $C_4$  intermediates, combined with densely sampled and well-resolved phylogenies, has provided significant insight into the evolutionary assembly of the  $C_4$  syndrome in the Molluginaceae (Christin et al. 2011), Neurachninae (Christin et al. 2012), Portulacaceae (Ocampo et al. 2013), Chenopodiaceae (Kadereit et al. 2003, 2010), Poaceae (Christin et al. 2013), and *Flaveria* (McKown et al. 2005). In contrast to  $C_4$ , the evolutionary dynamics of CAM are not well understood, in large part because we still have not fully developed a model lineage that possesses all relevant phenotypes and is of a moderate size that will allow complete physiological and phylogenetic sampling. Many lineages that possess both  $C_3$ +CAM and strong CAM species are extremely species-rich clades (e.g., orchids and bromeliads), and a lack of phylogenetic resolution has prevented clear identification of key evolutionary transitions. Unambiguous transitions from  $C_3$  to  $C_3$ +CAM to strong CAM in a well sampled clade have yet to be demonstrated for any lineage (Hancock and Edwards 2014; Winter et al. 2015).

Australian *Calandrinia* (Montiaceae) is a lineage of about 70 species of small leaf-succulent herbs

endemic to Australia (Obbens 2006; Tahir and Carolin 2011; West and Chinnock 2013; Hancock et al. 2018). The few species that have been examined experimentally are  $C_3$ +CAM species (Winter and Holtum 2011, 2014; Holtum et al. 2017b). During the past ~30 million years, while the Australian landmass has been subject to dramatic climate change and aridification, the Australian *Calandrinia* have persisted and diversified into six clades that have radiated across the continent, colonizing the majority of Australian ecosystems (Hancock et al. 2018). Most species are annual but roughly 15% are perennial. Although most speciose along the coastal fringes of western/southwestern Australia and the extensive semi-arid and arid regions of central Australia, Australian *Calandrinia* also inhabit savannas and savanna-woodlands in the summer-rainfall tropics of northern Australia; the winter-rainfall Mediterranean regions of southern Australia; and several species extend into southern temperate areas including the Bass Strait islands and Tasmania. Australian *Calandrinia* are only absent from the high alpine and rainforest systems of Australia, neither of which are particularly extensive.

Given the length of time that Australian *Calandrinia* has been present in Australia, the extensive changes in climate and aridification through which Australian *Calandrinia* have persisted, the biogeographic range of Australian *Calandrinia*, its phylogenetic placement within the CAM-rich Portulacineae (Caryophyllales) (Goolsby et al. 2018; Moore et al. 2018), and recent reports of  $C_3$ +CAM within at least five species, we hypothesized that the presence and the extent of expression of CAM in Australian *Calandrinia* would reflect the habitats in which various species are found (e.g., CAM might be more pronounced in a perennial lineage that inhabits the hottest/driest regions of Australia). In order to test this hypothesis, and to test whether *Calandrinia* might be a useful CAM model lineage, we quantified the capacity for nocturnal acidification over the course of a drought experiment in 22 species of Australian *Calandrinia* that represent both the biogeographic and phylogenetic breadth of the lineage. We also conducted a more exhaustive survey of  $\delta^{13}C$  isotope values across the lineage, which should identify species with more pronounced CAM. Using a recently resolved, comprehensive phylogeny for Australian *Calandrinia* (Hancock et al. 2018), we investigated the relationships between the photosynthetic phenotype and the environment in which these plants live, and tested hypotheses that  $C_3$ +CAM phenotypes represent early stages in the evolution of more pronounced CAM from  $C_3$  ancestors.



**Fig. 1** Representative Australian *Calandrinia* species, (a) *C. stagnensis* and (b) *C. pentivalvis*, and (c) a map showing all databased Australian *Calandrinia* collections (gray dots) from the Australian Virtual Herbarium.

## Materials and methods

### Study species, growth conditions and experimental parameters

Species included in the experiment represent the phylogenetic and biogeographic diversity of the Australian *Calandrinia* lineage (Figs. 1, 2). Plant material and seeds were obtained from wild plants, plants in cultivation (garden), from herbarium specimens, and herbarium seed collections (Table 1). Seeds were germinated in 24-cell seedling trays, with Osmocote seed-raising mix during the winter of 2015 (July–August). Roughly 3 weeks after germination, seedlings were transplanted to 0.8 L plastic pots, with Osmocote potting mixture. All plants were grown under semi-natural conditions in a closed-sided shade house at James Cook University (Queensland, Australia). Daily temperature within the shade house ranged from 36°C to 23°C and Photosynthetically Active Radiation (PAR) was close to 50% ambient. All plants were watered daily until the experiment commenced.

The discovery and characterization of CAM requires measurements of day–night changes in titratable acidity and/or 24-h gas-exchange over the course of drought experiment. The drying-down,

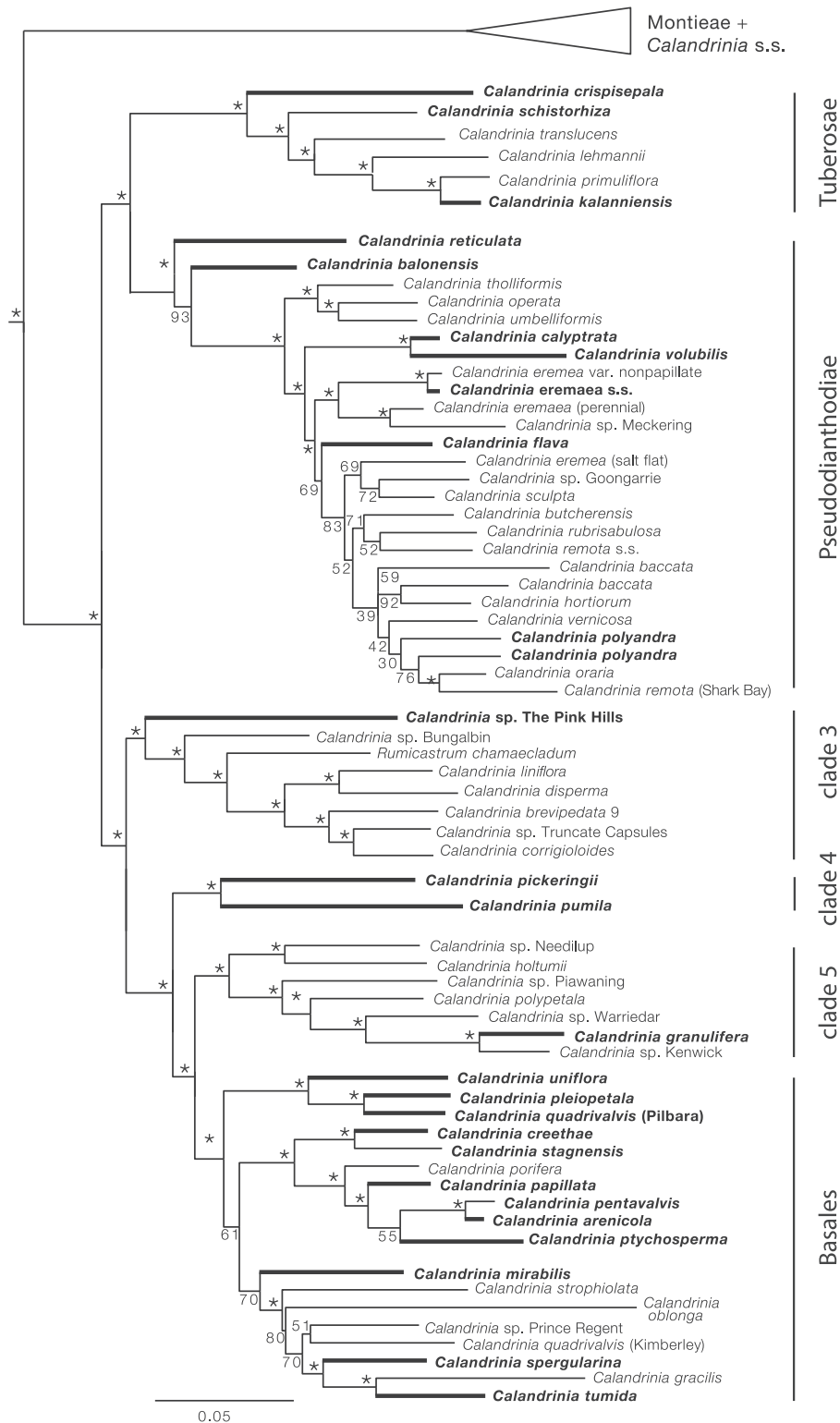
re-watering experiment was staggered over the months of September and October 2015. Staggering the experiment was necessary (1) as species germinated and matured at different rates, (2) to ensure that all plants were in the same location within the shade house over the duration of the experiment, and (3) to facilitate rapid sampling of time-sensitive biochemistry (i.e., malic acid degradation with sunrise). Because of variation in germination success, we sampled a range of 3–10 individuals per species over the course of the experiment. Leaves were harvested from plants at dusk on Day 1 and dawn on Day 2 (when plants were well-watered), after which watering ceased. Droughted tissue was harvested at dusk on Day 11 and dawn on Day 12, following which the plants were re-watered and sampled further at dusk on Day 20 and dawn on Day 21 (re-watered).

### Titratable acidity

Harvested leaves were immediately weighed and frozen in liquid nitrogen. Organic acids were extracted by boiling leaf tissue in ~80 mL of 50% ethanol in distilled water. Once half of the total volume boiled off, an additional ~40 mL of distilled water was added to each beaker. Beakers were removed from the hotplates when the final volume reached 40 mL. The H<sup>+</sup> pools were determined by measuring the volume of 5 mM NaOH required to neutralize each sample to pH 6.5. To calculate nocturnal increase in tissue acidity ( $\Delta\text{H}^+$ ), we subtracted the titratable acidity at the end of the light period from the titratable acidity at the end of the dark period.  $\Delta\text{H}^+$  was then averaged for each species at each time point (i.e., watered, droughted, and re-watered). A student's *t*-test was performed to determine if  $\Delta\text{H}^+$  values were significantly different and greater than zero, as well as to check for significant differences when plants were watered, droughted, and re-watered.

### Net CO<sub>2</sub> exchange

The 24-h gas-exchange measurements capture patterns of net CO<sub>2</sub> exchange and stomatal conductance. To measure nocturnal CO<sub>2</sub> uptake, gas-exchange by four Australian *Calandrinia* species, *C. balonensis*, *C. mirabilis*, *C. pickeringii*, and *C. ptychosperma*, was monitored using a LiCOR-6400 portable gas analyzer (LiCOR, Lincoln, NE, USA). Mature leaf tissue was placed into a standard clear bottom chamber (model 6400-08) and the leaf was exposed to 12 h light (500  $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ , 28°C)/12 h dark (22°C) cycles. Gas-exchange values were



**Fig. 2** The Australian *Calandrinia* phylogeny from Hancock et al. (2018) generated from 297 loci (matrix length) using a targeted gene enrichment approach. Topology was generated with Astral and branch lengths from concatenated maximum likelihood analysis (RAxML). Bootstrap support values from 1000 bootstrap replicates with greater than 95% support are indicated with a star. The 22 species included in the experiment are highlighted with bold lettering and thicker branches. Species not included in the experiment, but which have been phenotyped using either gas-exchange or titratable acidity elsewhere (Winter and Holtum 2011, 2014; Holtum et al. 2017b), are highlighted with only bold lettering.

**Table 1** Voucher specimen table and collection localities for Australian *Calandrinia* species included in the experiment.

| Species                               | Voucher ID | Collector | Origin  | Latitude (S) | Longitude (E) |
|---------------------------------------|------------|-----------|---|--------------|---------------|
| <i>Calandrinia arenicola</i>          | JAH 137    | LB        | ~5 km NW of Laura, QLD  | −15.501      | 144.484       |
| <i>Calandrinia balonensis</i>         | LPH 85     | LPH       | ~7 km south of Alice Springs  | −23.813      | 133.779       |
| <i>Calandrinia calyptrata</i>         | JAH191     | LPH       | Long Forest Reserve, Victoria   | −37.644      | 144.497       |
| <i>Calandrinia creethae</i>           | JAH 16     | AK        | Willuna, WA   | −26.591      | 120.152       |
| <i>Calandrinia crispispala</i>        | LPH 196    | LPH       | South of Yalgoo, WA   | −28.999      | 117.125       |
| <i>Calandrinia eremaea</i>            | JAH 5      | AK        | You Yang Ranges, VIC  | −37.953      | 144.423       |
| <i>Calandrinia flava</i>              | JAH 6      | AK        | Mt. Magnet, WA  | −28.065      | 117.848       |
| <i>Calandrinia granulifera</i>        | JAH 7      | AK        | from AK, east of Northam WA   | −31.655      | 116.671       |
| <i>Calandrinia kalanensis</i>         | LPH 198    | LPH       | 41 km N along Yalgoo Road from junction w/Geraldton, Mt. Magnet Rd                                    | −28.013      | 116.721       |
| <i>Calandrinia mirabilis</i>          | JAH 21     | AK        | from AK, RJC10295   |              |               |
| <i>Calandrinia</i> sp. The Pink Hills | LPH 178    | LPH       | ~5.3 km N of Cobra Daily Creek Homestead  | −25.258      | 115.890       |
| <i>Calandrinia papillata</i>          | LPH 191    | LPH       | 3.2 km S of Meka Homestead  | −27.423      | 116.827       |
| <i>Calandrinia pickeringii</i>        | JAH 40     | JAH       | Near peak of Mt Bald, northern NSW  | −28.852      | 152.045       |
| <i>Calandrinia pleiopetala</i>        | JAH 3      | JAH       | QH568091, QH699641 or QH726961  |              |               |
| <i>Calandrinia ptychosperma</i>       | JAH 36     | JAH       | From seeds in BNE (AQ0736128 - 202.7 km by road W of St George on road to Cunnamulla)                 | −28.004      | 146.538       |
| <i>Calandrinia pumila</i>             | JAH 10     | JAH       | from AK, Sth Wongan Hills WA  | −31.034      | 116.930       |
| <i>Calandrinia quadrivalvis</i>       | JAH 66     | JAH       | Durack River crossing about 100 m west side of xing—side of road on southern side—in sand deposition. | −15.942      | 127. 219      |
| <i>Calandrinia reticulata</i>         | JAH 12     | AK        | from AK, Meekatharra, WA  | −26.025      | 118.698       |
| <i>Calandrinia spurgularina</i>       | JAH 204    | LPH 130   | Along Savannah Way, east of Burketown, NT   | −10.043      | 136.399       |
| <i>Calandrinia tumida</i>             | JAH 138    | Leesa     | ~5 km NW of Laura, QLD  | −15.501      | 144.484       |
| <i>Calandrinia uniflora</i>           | JAH 56     | JAH       | Stuart Hwy roadside—ca 1.5 m from road—43 km past Larramie  | −15.207      | 133.105       |
| <i>Calandrinia volubilis</i>          | JAH 194    | LB        | Bob Chinnock, Adelaide, SA  | −34.938      | 138.841       |

recorded every 10 min over the course of the experiment, with each value being the mean  $\pm$  standard deviation (SD) of 600 measurements taken at 1-s intervals.

### Phenotyping species

A significant  $\Delta H^+$  value indicated CAM activity. A species was characterized as C<sub>3</sub> if their  $\Delta H^+$  values were not significant when watered or droughted. A species was denoted as facultative CAM if their  $\Delta H^+$  values were not significant when watered but significant when droughted. Species that had significant but low  $\Delta H^+$  values, regardless of experimental conditions, were designated as low-level constitutive CAM. A strong CAM species—of which none were observed—would be indicated by significant and high  $\Delta H^+$  values, regardless of experimental conditions. In strong CAM species, the majority of 24-h carbon gain occurs at night, and this carbon

gain is reflected in the  $\Delta H^+$  values and stable isotope analysis (see below).

### Stable carbon isotope analysis

Stable carbon isotope ratio ( $\delta^{13}C$ ) is often used to distinguish between C<sub>3</sub> and strong CAM plants as  $\delta^{13}C$  value is correlated linearly with the percentage of plant carbon obtained in the light and in the dark (Winter and Holtum 2002). For plants grown under controlled conditions, those that obtain 100% of their carbon during the light via C<sub>3</sub> photosynthesis have a  $\delta^{13}C$  value of about  $-27\text{‰}$ , and plants that fix 100% of their carbon at night have a  $\delta^{13}C$  value of about  $-9\text{‰}$  (Winter and Holtum 2002). For plants growing in the field, the contribution of nocturnal CO<sub>2</sub> fixation to long-term carbon gain can range from close to 0% to 100%, depending upon the species, its developmental age, and the growth environment (Winter et al. 2015). Surveys of  $\delta^{13}C$

values of field-grown plants often show a pronounced bimodal frequency distribution with peaks around  $-26$  and  $-13\text{‰}$ , and a minimum at around  $-20\text{‰}$ . The latter value approximates a tissue carbon pool for which 50% was obtained at night.

Given these distributions, we initially classified anything with a  $\delta^{13}\text{C}$  value less negative than  $-20\text{‰}$  as strong CAM,  $-21\text{‰}$  to  $-24\text{‰}$  as potentially  $\text{C}_3+\text{CAM}$ , and tissues with  $\delta^{13}\text{C}$  values more negative than  $-24\text{‰}$  as  $\text{C}_3$ . The  $\delta^{13}\text{C}$  values of dried, finely-ground leaves collected from whole plants were measured using a Finnigan Delta plusXL mass spectrometer (Boston University, Providence, RI, USA). For the majority of species,  $\delta^{13}\text{C}$  values were measured for two to five individuals and the average ( $\pm\text{SD}$ ) value was calculated (Table 2). Comparison of the classifications between  $\delta^{13}\text{C}$  and drought experiment approaches allowed us to evaluate the accuracy of  $\delta^{13}\text{C}$  in identifying  $\text{C}_3+\text{CAM}$  phenotypes.

### Ancestral character state reconstruction

For analyses of character evolution, we used the Australian *Calandrinia* phylogeny from Hancock et al. (2018) (Fig. 2). To reconstruct ancestral character states at all internal nodes for our qualitative character states, inferred from both the carbon isotope ratio and titratable acidity measurements, we used Maximum Likelihood reconstruction in the APE package as implemented in the R Project for Statistical Computing (Paradis et al. 2004). For both character state reconstructions, we compared an evolutionary model with equal transition rates between states (ER) with models that allowed the transition rates to vary (ARD and SYM). We chose the model with the lowest AIC value when reconstructing ancestral character states.

Since the  $\text{C}_3+\text{CAM}$  phenotypic spectrum can also be interpreted as a continuous trait, with  $\Delta\text{H}^+$  as the measure of CAM activity, we plotted  $\Delta\text{H}^+$  when plants were watered and droughted across the tips of the phylogeny. We used Phytools (Revell 2012) as implemented in R to reconstruct ancestral character states under each condition.

### Locality and climate data

A total of 13,590 georeferenced records for Australian *Calandrinia* were downloaded from the Australian Virtual Herbarium (<http://avh.chah.org.au/>) on February 10, 2016. We followed a number of steps to clean these records, removing records with identical coordinates for a given taxon, records with latitude and longitude coordinates of 0, 0, records with low precision, and records that matched

the latitude/longitude of herbaria and botanical gardens (Edwards et al. 2015); [https://github.com/ejwards/reanalysis\\_zanne2014](https://github.com/ejwards/reanalysis_zanne2014)). After cleaning, we were left with 11,087 unique records. The remaining localities were imported into ArcGIS 4.0 and overlain on a map of Australia, and any records that were in the ocean were removed.

Climate layers (ASCII format) for mean annual/semi-annual temperature, mean annual/semi-annual precipitation, precipitation variability, evapotranspiration, maximum annual temperature, minimum annual temperature, average sun hours, number of rain days above 10 mm, number of rain days above 5 mm, and relative humidity were downloaded from the Australian Government Bureau of Meteorology (<http://Calandrinia.bom.gov.au/>). Layers were converted into Rasters using ArcMAP 4.0.1 and clipped to match the Australian continent, which included a 35 km buffer in efforts to capture surrounding islands. Finally, an attributes table was constructed containing all georeferenced localities, with corresponding species name and the climate data.

Species means for each climate variable were calculated for further comparative analysis. Principal components analysis (Supplementary Fig. S1) showed that temperature and more so precipitation explained most of the variation in the data, and since all Australian *Calandrinia* (even perennials) are seasonal in their growth (Obbens 2012), we focused our analyses on mean growing season temperature (MGST) and mean growing season precipitation (MGSP). The growing season was determined for each species based on species descriptions, distribution maps from the literature (Obbens 2006, 2011, 2012; Tahir and Carolin 2011; West and Chinnock 2013), and localities from the Australian Virtual Herbarium ([www.avh.org](http://www.avh.org)). To visualize relationships between photosynthetic mode and climate, we plotted mean values ( $\pm\text{SD}$ ) in an X–Y coordinate space with MGST on the X-axis and MGSP on the Y-axis. MANOVA and Tukey tests were run to determine if phenotypes occupy significantly distinct temperature and precipitation climatic space.

To test for these trait correlations within a phylogenetic context, we used the *brunch* function in the R package Caper (Orme 2013). The *brunch* function performs phylogenetically independent contrast (PIC) correlations between discrete and continuous traits by assessing contrasts of continuous variables on nodes of the tree where sister taxa have contrasting values for the discrete trait. To estimate the evolutionary correlation between continuous characters  $\Delta\text{H}^+$  and MGST and MGSP using PIC, we used the *pic* function in the R package Phytools (Revell 2012).

**Table 2** Mean ( $\pm$ SD) carbon isotope ratios and the photosynthetic phenotype these values confer for 67 species of Australian Calandrinia. For the species in which photosynthetic metabolism has been measured using physiological tools (i.e., titratable acidity or gas-exchange) the phenotype is denoted (references given for phenotypic data generated outside the scope of this study)

| Species                                     | SD $\delta^{13}\text{C}$ | SD $\delta^{13}\text{C}$ | $\delta^{13}\text{C}$ phenotype | Physiology phenotype                                  |
|---|--------------------------|--------------------------|---------------------------------|---|
| <i>Calandrinia arenicola</i>                | -27.9                    | 0.7                      | C3                              | C3  |
| <i>Calandrinia baccata</i>                  | -27.1                    | 1.9                      | C3                              |   |
| <i>Calandrinia balonensis</i>               | -27.7                    | 0.8                      | C3                              |   |
| <i>Calandrinia brevipedata</i>              | -29.7                    | 0.9                      | C3                              |   |
| <i>Calandrinia calyptata</i>                | -27.9                    | 1.4                      | C3                              | C3+CAM  |
| <i>Calandrinia corrigioloides</i> (coastal) | -28.6                    | NA                       | C3                              |   |
| <i>Calandrinia corrigioloides</i> (inland)  | -28.4                    | NA                       | C3                              |   |
| <i>Calandrinia creethae</i>                 | -28.4                    | 1.6                      | C3                              | C3+CAM (Holtum et al. 2017b)                          |
| <i>Calandrinia crispisepala</i>             | -24.8                    | 1.3                      | C3                              | C3+CAM  |
| <i>Calandrinia disperma</i>                 | -28.4                    | 2.2                      | C3                              |   |
| <i>Calandrinia eremaea</i> (salt flat)      | -25.7                    | 1.4                      | C3                              |   |
| <i>Calandrinia eremaea</i> s.s.             | -25.7                    | 2.3                      | C3                              |   |
| <i>Calandrinia eremaea</i> (perennial)      | -25.5                    | 2.9                      | C3                              |   |
| <i>Calandrinia flava</i>                    | -22.1                    | 2.0                      | C3+CAM                          | C3+CAM  |
| <i>Calandrinia gracilis</i>                 | -30.5                    | 1.8                      | C3                              |   |
| <i>Calandrinia granulifera</i>              | -29.5                    | 0.5                      | C3                              | C3  |
| <i>Calandrinia holtumii</i>                 | -22.7                    | 2.7                      | C3+CAM                          | C3+CAM (L. Hancock et al., manuscript in preparation) |
| <i>Calandrinia hortiorum</i>                | -26.7                    | NA                       | C3                              |   |
| <i>Calandrinia kalanniensis</i>             | -27.6                    | 0.8                      | C3                              | C3+CAM  |
| <i>Calandrinia lehmannii</i>                | -27.2                    | 1.4                      | C3                              |   |
| <i>Calandrinia liniiflora</i>               | -28.9                    | 0.3                      | C3                              |   |

(continued)

**Table 2** Continued

| Species                                   | SD $\delta^{13}\text{C}$ | SD $\delta^{13}\text{C}$ | $\delta^{13}\text{C}$ phenotype | Physiology phenotype                                  |
|---|--------------------------|--------------------------|---------------------------------|---|
| <i>Calandrinia mirabilis</i>              | -25.7                    | 0.7                      | C3                              | C3+CAM  |
| <i>Calandrinia oblonga</i>                | -29.3                    | NA                       | C3                              |   |
| <i>Calandrinia operta</i>                 | -24.6                    | 1.6                      | C3                              |   |
| <i>Calandrinia oraria</i>                 | -28.2                    | 0.5                      | C3                              |   |
| <i>Calandrinia papillata</i>              | -24.9                    | NA                       | C3                              | C3+CAM  |
| <i>Calandrinia pentavalvis</i>            | -26.5                    | NA                       | C3                              | C3+CAM  |
| <i>Calandrinia pickeringii</i>            | -28.2                    | NA                       | C3                              | C3+CAM  |
| <i>Calandrinia pleiopetala</i>            | -27.2                    | 1.9                      | C3                              | C3+CAM  |
| <i>Calandrinia polyandra</i>              | -26.5                    | 0.6                      | C3                              | C3+CAM (Winter and Holtum 2011)                       |
| <i>Calandrinia polypetala</i>             | -27.4                    | NA                       | C3                              |   |
| <i>Calandrinia porifera</i>               | -26.7                    | 1.1                      | C3                              |   |
| <i>Calandrinia primuliflora</i>           | -28.6                    | NA                       | C3                              |   |
| <i>Calandrinia ptychosperma</i>           | -27.5                    | 1.0                      | C3                              | C3+CAM  |
| <i>Calandrinia pumila</i>                 | -28.6                    | 1.1                      | C3                              | C3+CAM  |
| <i>Calandrinia quadrivalvis</i> (Pilbara) | -28.4                    | 0.7                      | C3                              | C3+CAM  |
| <i>Calandrinia remota</i> s.s.            | -28.3                    | NA                       | C3                              | C3+CAM  |
| <i>Calandrinia remota</i> (Shark Bay)     | -21.1                    | NA                       | C3+CAM                          |   |
| <i>Calandrinia reticulata</i>             | -26.5                    | 2.5                      | C3                              | C3+CAM  |
| <i>Calandrinia rubrisabulosa</i>          | -24.4                    | 1.6                      | C3+CAM                          |   |
| <i>Calandrinia schistorhiza</i>           | -24.1                    | NA                       | C3+CAM                          | C3+CAM (L. Hancock et al., manuscript in preparation) |
| <i>Calandrinia sculpta</i>                | -26.2                    | 0.8                      | C3                              |   |
| <i>Calandrinia</i> sp. Bungalbin          | -26.9                    | NA                       | C3                              |   |
| <i>Calandrinia</i> sp. Goongarrie         | -23.2                    | NA                       | C3+CAM                          |   |
| <i>Calandrinia</i> sp. Kenwick            | -28.8                    | 1.9                      | C3                              |   |
| <i>Calandrinia</i> sp. Meckering          | -24.1                    | 0.9                      | C3+CAM                          |   |

(continued)

Table 2 Continued

| Species                                    | SD<br>$\delta^{13}\text{C}$ | SD<br>$\delta^{13}\text{C}$ | $\delta^{13}\text{C}$<br>phenotype | Physiology<br>phenotype               |
|--|-----------------------------|-----------------------------|------------------------------------|---------------------------------------|
| <i>Calandrinia</i><br>Needilup             | sp. -26.6                   | 0.6                         | C3                                 |                                       |
| <i>Calandrinia</i><br>Ongerup              | sp. -28.9                   | NA                          | C3                                 |                                       |
| <i>Calandrinia</i><br>Piawaning            | sp. -30.5                   | 0.1                         | C3                                 |                                       |
| <i>Calandrinia</i><br>Prince Regent        | sp. -30.5                   | NA                          | C3                                 |                                       |
| <i>Calandrinia</i><br>Southern<br>Granites | sp. -28.2                   | NA                          | C3                                 |                                       |
| <i>Calandrinia</i><br>The Pink Hills       | sp. -25.8                   | 1.2                         | C3                                 | C3+CAM                                |
| <i>Calandrinia</i><br>Truncate<br>capsules | sp. -27.4                   | 2.6                         | C3                                 |                                       |
| <i>Calandrinia</i><br>Warrieder            | sp. -26.1                   | NA                          | C3                                 |                                       |
| <i>Calandrinia</i><br>Widgiemooltha        | sp. -22.1                   | 0.1                         | C3+CAM                             |                                       |
| <i>Calandrinia</i><br>Yinberrie Hills      | sp. -29.3                   | NA                          | C3                                 |                                       |
| <i>Calandrinia</i><br><i>spergularina</i>  | -28.7                       | 1.3                         | C3                                 | C3+CAM                                |
| <i>Calandrinia</i><br><i>stagnensis</i>    | -28.4                       | 0.5                         | C3                                 | C3+CAM<br>(Holtum<br>et al. 2017b)    |
| <i>Calandrinia</i><br><i>strophiolata</i>  | -26.9                       | 0.8                         | C3                                 |                                       |
| <i>Calandrinia</i><br><i>tholiformis</i>   | -28.3                       | 0.3                         | C3                                 |                                       |
| <i>Calandrinia</i><br><i>translucens</i>   | -27.0                       | 0.5                         | C3                                 |                                       |
| <i>Calandrinia tumida</i>                  | -31.2                       | NA                          | C3                                 | C3                                    |
| <i>Calandrinia</i><br><i>umbelliformis</i> | -21.5                       | 1.8                         | C3+CAM                             |                                       |
| <i>Calandrinia uniflora</i>                | -28.8                       | 1.4                         | C3                                 | C3+CAM                                |
| <i>Calandrinia</i><br><i>vernicosa</i>     | -25.5                       | 2.2                         | C3                                 |                                       |
| <i>Calandrinia volubilis</i>               | -23.5                       | 3.0                         | C3+CAM                             | C3+CAM<br>(Winter and<br>Holtum 2014) |
| <i>Rumicrastrum</i><br><i>chamaecladum</i> | -30.1                       | NA                          | C3                                 |                                       |

## Results

### Titrateable acidity measurements

#### Watered plants

Measurements of nocturnal acid accumulation ( $\Delta\text{H}^+$ ) were used as an indicator of the presence

or absence of CAM activity in leaves (Fig. 3a, b; Supplementary Table S1). The  $\Delta\text{H}^+$  values ranged from  $-5.8$  to  $13.7 \mu\text{mol H}^+ \text{g}^{-1}$  fresh leaf mass when plants were well watered. Ten of the 22 *Calandrinia* species exhibited significant ( $P < 0.05$ ) differences between evening and morning titrateable acidities (Fig. 3; Supplementary Table S1). The remaining 12 species did not have significant  $\Delta\text{H}^+$  values.

#### Droughted plants

After  $\sim 12$  days of drought there was an increase (Fig. 3a) in titrateable acidity across the majority of species (17 of 22), and nocturnal acid accumulation ranged between 0 and  $82.2 \mu\text{mol H}^+ \text{g}^{-1}$  fresh leaf mass (Fig. 3; Supplementary Table S1). Of the 10 species that showed significant  $\Delta\text{H}^+$  when watered, six of them showed a significant increase in titrateable acidities when droughted: *C. crispisepala*, *C. eremaea*, *C. flava*, *C. kalannensis*, *C. pleiopetala*, and *C. ptychosperma*. The other four species, *C. balonensis*, *C. pickeringii*, *C. pumila*, and *C. reticulata*, showed no significant increase in  $\Delta\text{H}^+$  when water-limited compared with well-watered.

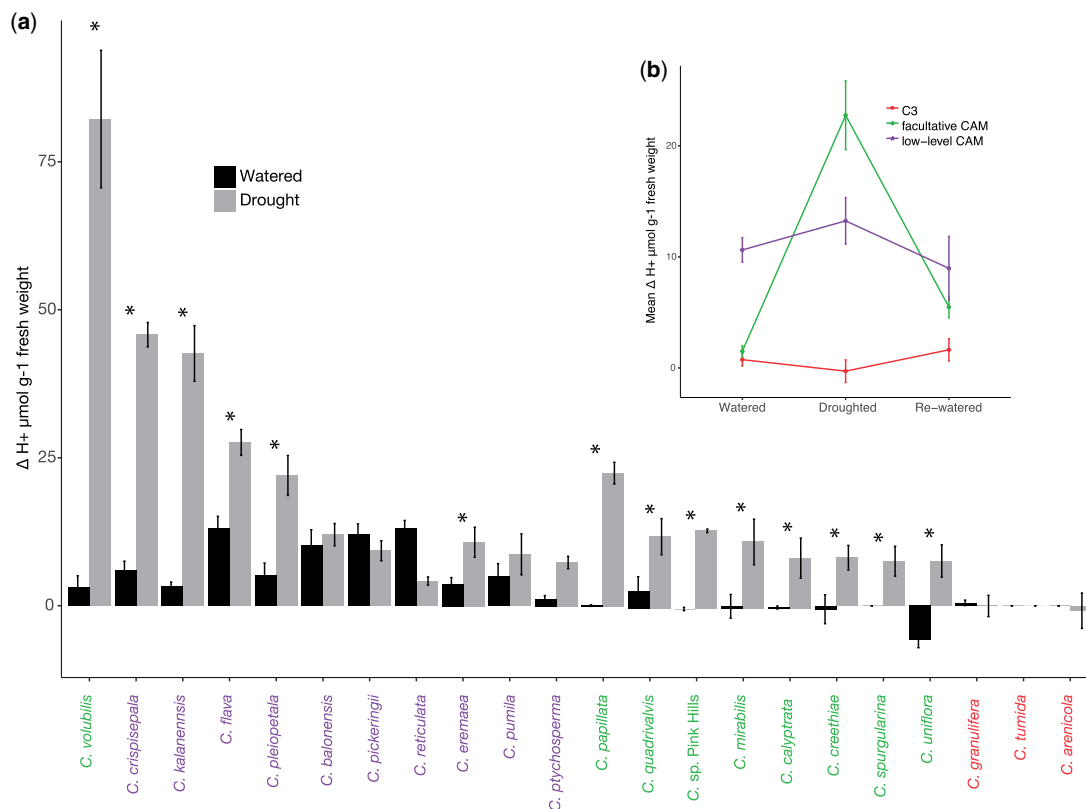
Nine of the 12 species that had non-significant  $\Delta\text{H}^+$  when watered, had significant  $\Delta\text{H}^+$  when water-limited (i.e., exhibited facultative CAM). These species include *C. calyptrata*, *C. creethae*, *C. mirabilis*, *C. sp.* The Pink Hills, *C. papillata*, *C. uniflora*, *C. quadrivalvis*, *C. spergularina*, and *C. volubilis*.  $\Delta\text{H}^+$  ranged between  $7.6$  and  $82.2 \mu\text{mol g}^{-1}$  fresh leaf mass. Significant nocturnal acid accumulation was not detected in three species of *Calandrinia* (*C. arenicola*, *C. granulifera*, and *C. tumida*) either when the plants were watered or when water-limited (Fig. 2a, b).

#### Re-watered plants

After re-watering, nocturnal acid accumulation generally declined (Fig. 3b; Supplementary Table S1). Of the 10 species with significant  $\Delta\text{H}^+$  values when watered, all returned to low-level CAM except for *C. balonensis*, *C. crispisepala*, and *C. pumila*. These three species no longer significantly accumulated malic acid overnight after re-watering. In the case of *C. balonensis* and *C. pumila*, it is likely that these droughted, re-watered individuals were no longer healthy, as the leaves had lost their turgidity and appeared to be senescing.

Of the nine species that showed a significant increase in titrateable acidity (facultative CAM) with drought stress but no CAM activity when watered, six of them showed no appreciable ( $P > 0.05$ ) nocturnal acid accumulation when re-watered (Fig. 3b;





**Fig. 3** (a) Mean ( $\pm$ SE) nocturnal CO<sub>2</sub> fixation ( $\Delta H^+$   $\mu\text{mol g}^{-1}$  fwt) by well-watered plants (gray bars) and droughted plants (black bars). The 22-species names are colored according to phenotypes in Fig. 5. (b) Mean ( $\pm$ SE) nocturnal CO<sub>2</sub> fixation ( $\Delta H^+$   $\mu\text{mol g}^{-1}$  fwt) when species were watered, droughted, and re-watered. Species phenotyped as C<sub>3</sub> (red), constitutive low-level CAM (purple), and facultative CAM (green) based on  $\Delta H^+$  values over the course of the experiment.

Supplementary Table S1). The other three facultative CAM species, *C. creethae*, *C. sp. The Pink Hills*, and *C. volubilis*, continued accumulating H<sup>+</sup> at night when re-watered (Supplementary Table S1); it is possible that our sampling protocol—titratable acidity measurements made on Day 20—simply did not capture the reversal of CAM in these three species. Another possibility is that the leaves were still experiencing some stress (e.g., high light intensity and too much water) and so still engaging in some CAM.

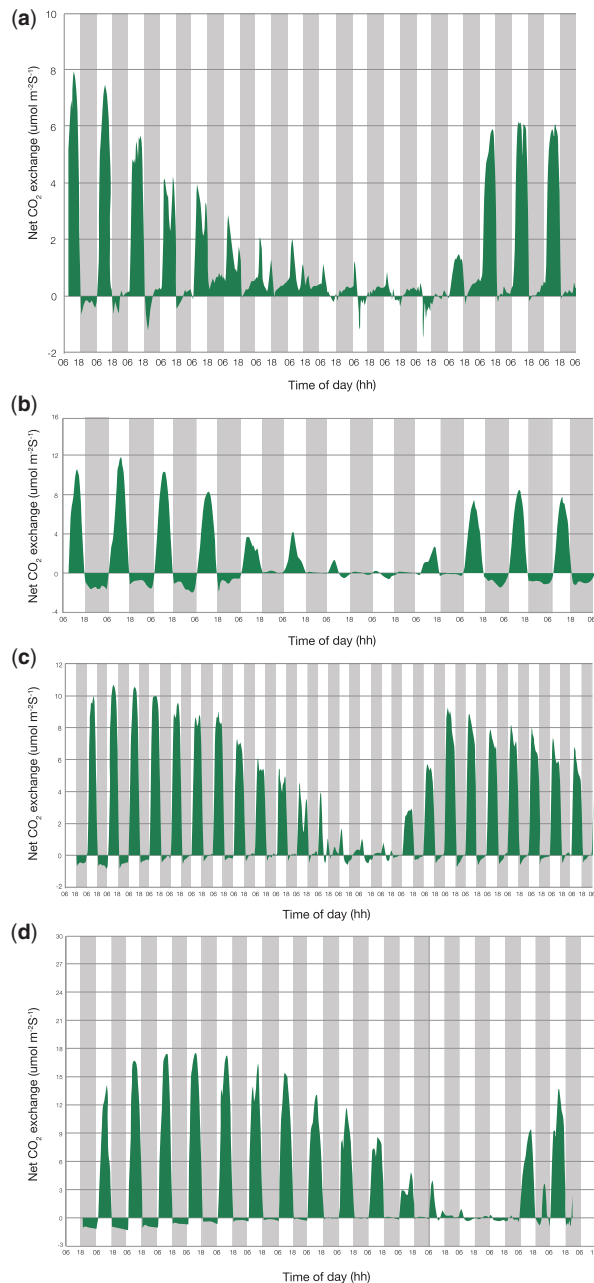
#### Gas-exchange

Continuous whole-leaf gas-exchange measurements on four species of Australian *Calandrinia* over the experiment demonstrated net CO<sub>2</sub> gas-exchange patterns indicative of C<sub>3</sub> and CAM photosynthesis (Fig. 4a–d). All four species engaged in daytime CO<sub>2</sub> assimilation (C<sub>3</sub> photosynthesis) when watered. With the imposition of drought, there was a precipitous decline in daytime net CO<sub>2</sub> fixation and a general decrease in the rate of CO<sub>2</sub> loss at night. After ~5 days of water-limitation all of the species showed net nocturnal CO<sub>2</sub> uptake, although the rates were

low and varied between species (Fig. 4a–d). With re-watering, all four species returned to primarily daytime CO<sub>2</sub> uptake (C<sub>3</sub> photosynthesis).

#### Photosynthetic types and ancestral character state reconstruction

Species were assigned to discrete photosynthetic categories based on both physiological measurements (titratable acidity and gas-exchange, when available) and stable carbon isotope content ( $\delta^{13}\text{C}$ ). On the basis of physiological measurements, species were initially characterized as either C<sub>3</sub>, C<sub>3</sub>+CAM, or strong CAM. We further divided the C<sub>3</sub>+CAM phenotype into either low-level CAM or facultative CAM (Fig. 5). Species phenotyped as C<sub>3</sub> did not have significant  $\Delta H^+$  values when watered or water-limited and included three species, *C. arenicola*, *C. granulifera*, and *C. tumida*. Ten species were scored as constitutive low-level CAM: *C. balonensis*, *C. crispisepala*, *C. eremaea*, *C. flava*, *C. kalanensis*, *C. pickeringii*, *C. pleiopetala*, *C. ptychosperma*, *C. pumila*, and *C. reticulata*. Species characterized as facultative CAM showed an induction of CAM activity when water-limited and, in most cases,



**Fig. 4** (a) Sixteen days of net CO<sub>2</sub> exchange by a leaf of a ~2-month-old *C. balonensis*. Watering was withheld from Day 2 onwards. Shaded areas indicate the dark periods. Re-watering commenced on Day 13. (b) Twelve days of net CO<sub>2</sub> exchange by a leaf of a ~2-month-old *C. mirabilis*. Watering was withheld from Day 2 onwards. Shaded areas indicate the dark periods. Re-watering commenced on Day 9. (c) Twenty-four days of net CO<sub>2</sub> exchange by a leaf of a ~2-month-old *C. pickeringii*. Watering was withheld from Day 2 onwards. Shaded areas indicate the dark periods. Re-watering commenced on Day 17. (d) Seventeen days of net CO<sub>2</sub> exchange by a leaf of a ~2-month-old *C. Ptychosperma* plant. Watering was withheld from Day 3 onwards. Shaded areas indicate the dark periods. Re-watering commenced on all plants on Day 15.

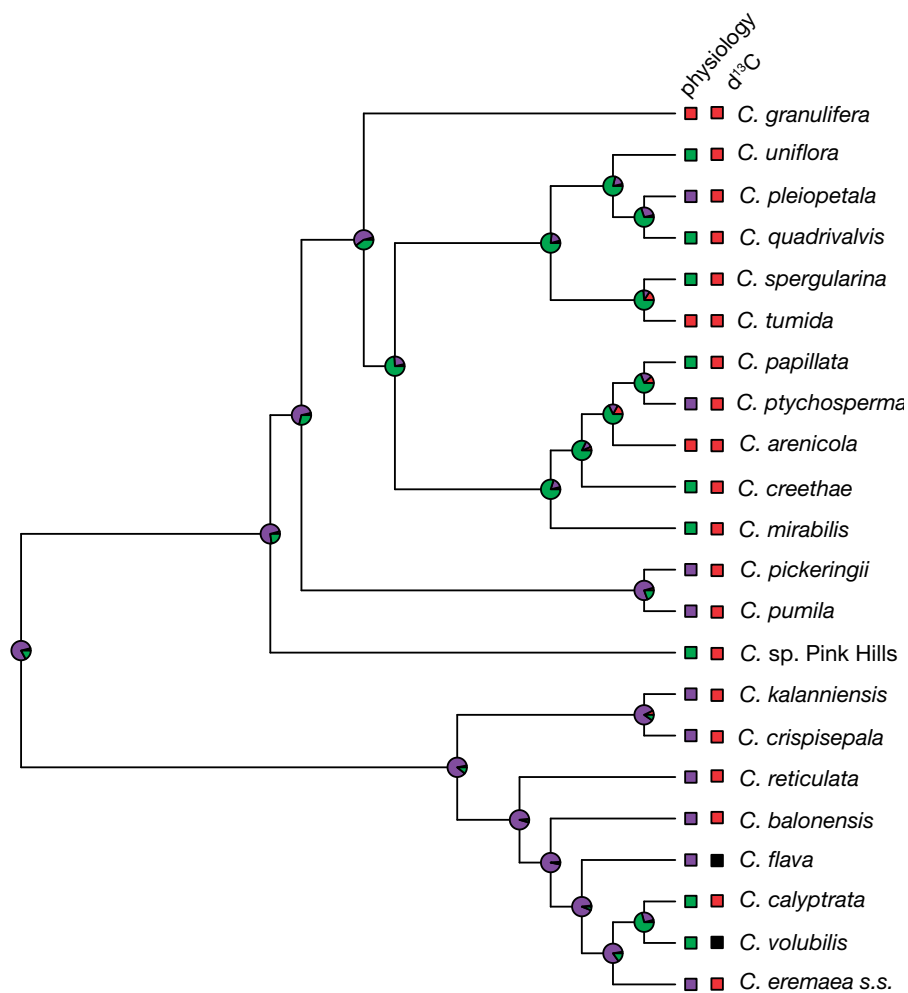
significant reduction of CAM when re-watering resumed. Based on these criteria, 9 of the 22 species were scored as facultative CAM: *C. calyptata*, *C. crethae*, *C. mirabilis*, *C. sp. The Pink Hills*, *C. papillata*, *C. uniflora*, *C. quadrivalvis*, *C. spergularina*, and *C. volubilis*. No species was phenotyped as strong CAM. In addition, we used  $\delta^{13}\text{C}$  values to score species as either C<sub>3</sub> (more negative than  $-24\text{‰}$ ), C<sub>3</sub>+CAM ( $-21\text{‰}$  to  $-24\text{‰}$ ), or strong-CAM (less negative than  $-20\text{‰}$ ). Twenty of the 22 species included in the drought experiment presented C<sub>3</sub>-like  $\delta^{13}\text{C}$  values. *Calandrinia flava* and *C. volubilis*, both of which have  $\delta^{13}\text{C}$  values within the  $24\text{‰}$  to  $-20\text{‰}$  window, were phenotyped as C<sub>3</sub>+CAM. Of the remaining ~45 Australian *Calandrinia* species measured, all but six species presented C<sub>3</sub>-like  $\delta^{13}\text{C}$  values. *Calandrinia holtumii*, *C. umbelliformis*, *C. schistorhiza*, *C. remota* (Shark Bay), *C. sp. Meckering*, and *C. sp. Widgiemooltha* had  $\delta^{13}\text{C}$  values within the  $24\text{‰}$  to  $-20\text{‰}$  window, indicating the presence of C<sub>3</sub>+CAM photosynthesis (Table 2).

Not surprisingly, ancestral character state reconstructions of photosynthetic mode differed significantly depending whether they were based upon  $\delta^{13}\text{C}$  values or physiological measurements (Fig. 4). Based on  $\delta^{13}\text{C}$  values, the ancestral character state of Australian *Calandrinia* (under the slightly favored ARD transition model) is C<sub>3</sub> and there have been two transitions to C<sub>3</sub>+CAM (*C. flava* and *C. volubilis*).

When using physiological methods, the ancestral state under an equal rates model (AIC > 2) is reconstructed as low-level constitutive CAM (scaled likelihood = 0.71), with multiple shifts to facultative CAM. There are also shifts back from facultative CAM to low-level constitutive CAM, as seen in *C. pleiopetala* and *C. Ptychosperma* (Fig. 4). Analyses also indicate multiple evolutionary reversals from C<sub>3</sub>+CAM back to C<sub>3</sub> photosynthesis (i.e., *C. tumida*, *C. arenicola*, and *C. granulifera*). The ancestral character state reconstructions of CAM activity as a continuous trait ( $\Delta\text{H}^+$ ) tells a similar evolutionary story (Fig. 6): the degree of CAM expression, as measured by the magnitude of  $\Delta\text{H}^+$ , is evolutionarily labile, though the highest levels of  $\Delta\text{H}^+$  mostly cluster in one subclade (lineages Tuberosae + Pseudodiantodiace).

#### CAM types and environment

Australian *Calandrinia* species included in the study span the entire temperature/precipitation climatic envelope of the lineage, which occupies considerable breadth in climatic space, from localities that



**Fig. 5** Australian *Calandrinia* pruned to the 22 species included in the drought experiment. The colored boxes (red = C<sub>3</sub>, black = C<sub>3</sub>+CAM, purple = constitutive low-level CAM, green = facultative CAM) mapped onto the tips indicate the discrete photosynthetic phenotype for each species derived from titratable acidity measurements (first column) compared with  $\delta^{13}\text{C}$  values (second column). Ancestral character states were reconstructed in APE under an equal rates model using the photosynthetic phenotype derived from titratable acidity measurements.

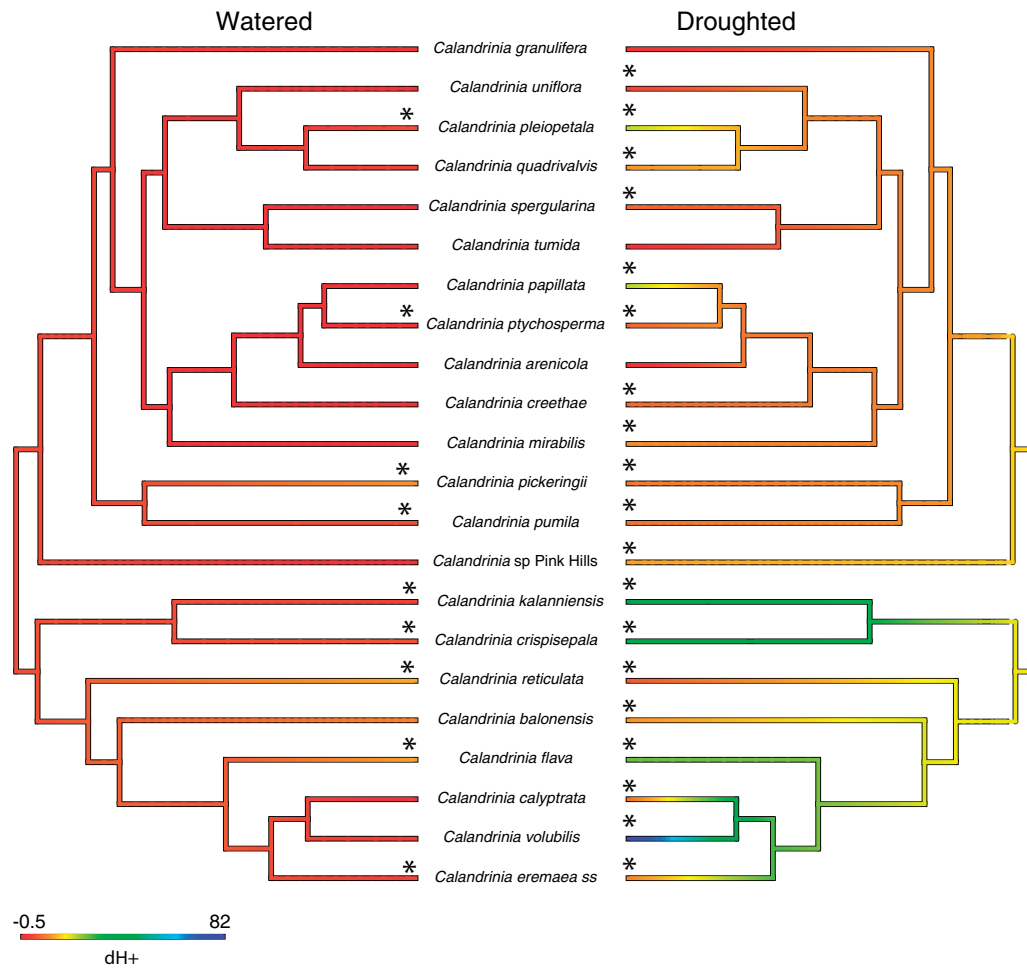
experience over 1500 mm of rain to places with long-term averages of only 20 mm of rain per year (Fig. 7a, b). Only three species (*C. tumida*, *C. spergularina*, and *C. arenicola*) inhabit the monsoonal tropics of the northern Australia, whereas most species grow in the hot, dry regions of Australia (lower right quadrant of Fig. 7a, b). Results from the MANOVA and Tukey tests show that precipitation ( $P < 0.001$ ), but not temperature ( $P = 0.7$ ), significantly varies between photosynthetic types, with both low-level CAM ( $P = 0.005$ ) and facultative CAM ( $P = 0.023$ ) occupying significantly drier regions than C<sub>3</sub> species. There was no difference in precipitation or temperature between low-level and facultative CAM phenotypes ( $P = 0.63$  and  $0.41$ , respectively), however, facultative CAM species occupy a significantly broader climate range (Euclidean distance norm = 9.405) than low-level

CAM species (Euclidean distance norm = 6.307); low-level constitutive CAM occupies only a portion of the facultative CAM climate envelope (Supplementary Fig. S1). When we examined C<sub>3</sub> and C<sub>3</sub>+CAM phenotypes within a phylogenetic framework there was no significant difference in the precipitation and temperature space occupied by C<sub>3</sub> species compared with C<sub>3</sub>+CAM species. PICs between  $\Delta\text{H}^+$  and MSGT and  $\Delta\text{H}^+$  and MSGP were not significant, although there was a stronger relationship between  $\Delta\text{H}^+$  and MSGP ( $P = 0.11$ ) than  $\Delta\text{H}^+$  and MSGT ( $P = 0.75$ ).

## Discussion

### Phenotypic variation and a model for CAM evolution

Characterizing species into discrete photosynthetic phenotypes is, unsurprisingly, quite sensitive to the

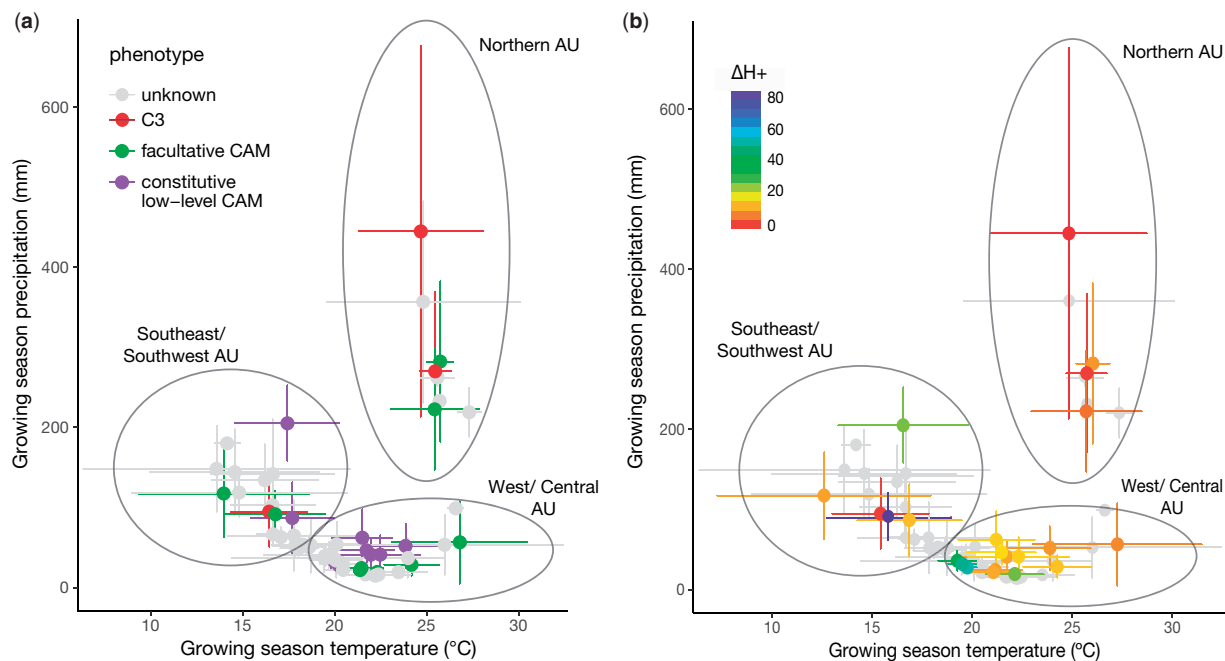


**Fig. 6**  $\Delta H^+$  mapped as a continuous character across the 22-tip Australian *Calandrinia* phylogeny when plants were well-watered (left) or droughted (right). Asterisks above branches indicate significant  $\Delta H^+$  values.

method used to detect CAM. On the basis of  $\delta^{13}\text{C}$  values we inferred  $\text{C}_3+\text{CAM}$  in only two of the 22 species included in the greenhouse experiment, while titratable acidity measurements revealed  $\text{C}_3+\text{CAM}$  in 19 species (Fig. 5). Extrapolating this disparity to our broader isotopic survey (Table 2), we suspect that most Australian *Calandrinia* species are likely to have a  $\text{C}_3+\text{CAM}$  metabolism, in spite of their “ $\text{C}_3$ -like” isotopic values. These results once again illustrate the shortcomings of the carbon isotope ratio method to detect  $\text{C}_3+\text{CAM}$  phenotypes and reinforce the requirement of physiological measurements to truly understand the phylogenetic distribution and prevalence of  $\text{C}_3+\text{CAM}$  plants. With that said, there is certainly value to carbon isotope surveys, as they uncover strong CAM species, and thus guide us to fruitful phylogenetic neighborhoods in which to investigate CAM evolution (e.g., Silveira et al. 2005).

Reconstructing the ancestral character state with physiological measurements (i.e., titratable acidity and 24 h gas-exchange) reveals a different story

about the expression and evolution of CAM in Australian *Calandrinia* (Fig. 5). We show that the ancestral state of Australian *Calandrinia* is likely to be low-level CAM, with multiple transitions to a purely facultative state (and several reversions back to low-level CAM). Remarkably, we have documented several losses of detectable CAM activity, with three putative  $\text{C}_3$ -only species (*C. tumida*, *C. arenicola*, and *C. granulifera*) nested within  $\text{C}_3+\text{CAM}$  clades. Reversion to  $\text{C}_3$  physiology from CAM, particularly strong CAM, is likely to be rare. Although it has been reported at least once in the Agavoideae (Heyduk et al. 2016; although see a re-analysis by Heyduk et al. 2018) and Bromeliaceae (Crayn et al. 2004) and multiple times in the Orchidaceae (Silveira et al. 2009) and *Clusia* (Vaasen et al. 2002; Gehrig et al. 2003), phylogenetic sampling and phenotyping (all of which were performed using  $\delta^{13}\text{C}$  values) within these studies were insufficient to confidently confirm a complete loss of CAM.



**Fig. 7** Comparison of ecological niche space of Australian *Calandrinia* species across Australia as a function of mean seasonal temperature and mean seasonal precipitation. Each dot represents a species mean ( $\pm$ SD). (a) Species are colored according to photosynthetic phenotype (C<sub>3</sub> = red, low-level CAM = purple, facultative CAM = green, unknown = gray). (b) Species means are colored according to  $\Delta H^+$  values with higher  $\Delta H^+$  represented in cooler colors and lower  $\Delta H^+$  represented in warmer colors.

Here, we have discovered C<sub>3</sub> species nested well within C<sub>3</sub>+CAM clades, using a well-resolved phylogeny and experimental physiological data. The multiple reversals to C<sub>3</sub> and the considerable lability detected between C<sub>3</sub>, low-level, and facultative CAM suggests that transitions between these character states are not uncommon and are evolutionarily accessible (*sensu* Edwards and Donoghue 2013; Edwards 2019). Continuous character state reconstructions of  $\Delta H^+$  support this deduction and indicate that species from the Tuberosae (*C. crispisepala* and *C. kalanniensis*) and Pseudodanthodiae (*C. reticulata*, *C. balonensis*, *C. flava*, *C. calyptrata*, *C. volubilis*, and *C. eremaea*) clades generally have higher  $\Delta H^+$  values regardless of conditions than the rest of Australian *Calandrinia* studied (Figs. 5, 6). Both Pseudodanthodiae and the perennial Tuberosae live in particularly arid regions of western and central Australia (Obbens 2006, 2011; Obbens et al. 2017) (bottom right corner of Fig. 7a, b) where more CAM activity, whether it is low-level or facultative, would likely be advantageous.

It could be argued that the species in which we observed solely C<sub>3</sub> photosynthesis might be able to exhibit CAM when grown under different environmental conditions, or that CAM may be present at levels below our level of detection, which is about 1–2  $\mu\text{mol H}^+ \text{g}^{-1}$  fresh mass. Both arguments are

unlikely to substantially affect our conclusions. If CAM is exhibited under some environmental conditions in *C. arenicola*, *C. granulifera*, or *C. tumida* the expression is likely to be minimal as *in situ*  $\delta^{13}\text{C}$  values for the species are  $-28$ ,  $-29$ , and  $-31$ ‰, respectively (Table 2). Acid titration is arguably our most sensitive method for detecting CAM-type acid fluctuations. It is more sensitive than gas-exchange and isotopic analyses and although it does not distinguish between acids, as do some forms of chromatography or spectroscopy, the method measures the accumulation of acids, not their anions. Transcriptome profiling of these species may reveal low-level expression of key elements of the CAM cycle (e.g., Heyduk et al. 2018); though, if the induction of a CAM cycle does not result in any measurable malic acid accumulation, its benefit is currently unclear to us.

Since we did not detect strong CAM in Australian *Calandrinia*, we cannot corroborate or refute hypotheses that these C<sub>3</sub>+CAM phenotypes represent states along a C<sub>3</sub> to CAM evolutionary trajectory. We do, however, suggest a model of C<sub>3</sub>-to-CAM evolution which allows for lability and reversibility among C<sub>3</sub>+CAM phenotypes and C<sub>3</sub> photosynthesis. Continued phylogenetic and physiological investigation into both Australian *Calandrinia* and other Portulacineae may help to further resolve the evolutionary significance of C<sub>3</sub>+CAM variation.

### Climatic space of C<sub>3</sub>-CAM phenotypes

Non-phylogenetic climate space analyses of the 22 species included in the study indicate that both low-level CAM and facultative CAM species occupy significantly drier regions than C<sub>3</sub> species, and facultative CAM species occupy a broader climate envelope than low-level CAM species (Supplementary Fig. S1). In spite of these general patterns, independent contrasts showed no relationship between photosynthetic type, when coded as either a qualitative or quantitative trait, and climate. These findings are not entirely surprising given (1) the lack of statistical power that results from having only three contrasts (three C<sub>3</sub> species) in the data and (2) our experiment included only a sub-sample of Australian *Calandrinia* (i.e., only 22 of ~70 species), so inferred sister species relationships in the analysis are not necessarily true sister species. Perhaps most importantly, the lack of correlation between photosynthesis and climate also reflects the occupation of C<sub>3</sub> species in two distinct climate zones. Two of the C<sub>3</sub> species, *C. arenicola* and *C. tumida*, inhabit the hot, wet tropics of northern Queensland, whereas the third C<sub>3</sub> species, *C. granulifera*, inhabits cool, drier climates of southern Australia, including northern Tasmania and islands in Bass Strait (Fig. 7a, b). All three species germinate toward the end of the wet season, when temperatures are cooler and water is readily available. C<sub>3</sub> plants are well adapted to moist and cool climates and so it is not surprising, from an ecological perspective, that these species do not engage in CAM photosynthesis at a detectable level.

Facultative CAM plants tend to inhabit arid environments where precipitation is variable or distinctly seasonal, though in some cases (i.e., *Clusia*), facultative CAM plants are epiphytic or hemi-epiphytic in wet forests (Zotz and Winter 1993, 1994; Winter and Holtum 2014). Under such environmental conditions, plants use C<sub>3</sub> photosynthesis when water is available, allowing for higher rates of CO<sub>2</sub> fixation and vegetative growth than when CAM. When water becomes limiting, plants engage a CAM cycle, which provides prolonged net carbon gain at a lower water cost, presumably allowing for higher reproductive output. This increase in fecundity with CAM has been well demonstrated in the facultative CAM annual *Mesembryanthemum crystallinum*. In one experiment, *M. crystallinum* exposed to air without CO<sub>2</sub> during the night—and therefore unable to engage fully in CAM—exhibited a 90% decline in seed production compared to those plants that were provided CO<sub>2</sub> at night (Winter and Ziegler 1992). Furthermore, it has been shown that mutants

without facultative CAM had lower fecundity than wild-type individuals (Cushman et al. 2008). Beyond prolonged carbon gain and increases in fecundity, switching to CAM may help plants survive periods of water-stress until they can start using C<sub>3</sub> photosynthesis again. The adaptive advantage of constitutive low-level CAM, however, is not fully understood. One hypothesis is that by engaging in low-level CAM, plants can reduce daytime transpiration rates and thereby increase water use efficiency and overall plant fitness (Harris and Martin 1991; Herrera 2009). Another hypothesis is that by always engaging in low-level CAM, these plants can more quickly upregulate CAM expression in the face of water deficit.

Despite the possible advantages of low-level CAM, there are also likely to be trade-offs associated with maintaining this phenotype, and it has presumably been lost multiple times in Australian *Calandrinia* (Fig. 4). One trade-off to consider is that CAM is energetically more expensive than C<sub>3</sub>, requiring an additional 2.5 or 3.5 more ATP per CO<sub>2</sub> than required by the C<sub>3</sub> pathway (assuming zero photorespiration in C<sub>3</sub>) (Nobel 1991; Winter and Smith 1996). Another possible compromise may involve anatomical traits that enhance the degree or efficiency of CAM but limit the efficiency of C<sub>3</sub> photosynthesis (Edwards 2019). Reduction in intercellular airspace is known to be correlated to CAM function, as this physical trait limits CO<sub>2</sub> flux and thereby increases the likelihood of CO<sub>2</sub> recapture from both respiration and malate decarboxylation (Maxwell et al. 1997; Nelson et al. 2005; Nelson and Sage 2007; Earles et al. 2018; Males and Griffiths 2018). However, the reduction in mesophyll conductance associated with reduced intercellular air space limits the uptake of atmospheric CO<sub>2</sub>, the concentration of CO<sub>2</sub> around the active sites of Rubisco, and thus the photosynthetic efficiency of the C<sub>3</sub> pathway (Evans and von Caemmerer 1996; Maxwell et al. 1997).

It might just be that maintaining or engaging in low-level CAM, regardless of environmental conditions, may not be of benefit for species growing in regions that do not experience intermittent drought but instead have short life-cycles that occur during a period of gradual drying. For example, annuals *C. uniflora* and *C. spergularina* germinate following the monsoonal summer rains that characterize the tropics of northern Australia. These species typically complete their life-cycle in 6–8 weeks, during which time the soil slowly dries out. Only toward the end of their lives do these species contend with severe water limitation. It is conceivable that these plants

(which use facultative CAM) only engage CAM toward the end of life thereby extending carbon gain for reproductive output.

### Why no strong CAM?

Australian *Calandrinia* is a lineage of plants capable of employing C<sub>3</sub> and C<sub>3</sub>+CAM photosynthesis. The strong CAM phenotype was neither detected in any of the species included in the drought experiment (Fig. 4a–d; Supplementary Table S1), nor was it detected in our broader isotopic survey. Although additional physiological investigation of a few candidate species is warranted (i.e., species with more positive  $\delta^{13}\text{C}$  values such as *C. umbelliformis* and *C. holtumii*) we do not believe, based on current evidence, that strong CAM has evolved in Australian *Calandrinia*. This hypothesis is based on both the present findings and previous work in which only C<sub>3</sub>+CAM was detected in five additional Australian *Calandrinia* species (Winter and Holtum 2011, 2014; Holtum et al. 2017b); thus, of the ~70 Australian *Calandrinia* species, three are currently experimentally phenotyped as C<sub>3</sub>, 26 as C<sub>3</sub>+CAM, and zero as strong CAM. It should be noted that new species of *Calandrinia* continue to be described (e.g., Obbens 2018).

Although the lack of strong CAM in Australian *Calandrinia* is puzzling, it is not entirely surprising. To begin with, there appear to be no known strong CAM species that are also annuals. Although there are perennial Australian *Calandrinia* species (i.e., section Tuberosae and species in Pseudodiantodiaceae), these perennials are geophytes with aboveground parts that last only a single growing season (i.e., their photosynthetic organs are functionally annual). We hypothesize that strong CAM may not have evolved in this lineage because strong CAM does not appear to be selected for in species with this life history strategy. Annual plants must grow rapidly and transfer carbon to reproductive structures in order to complete their life cycle in one season. C<sub>3</sub> photosynthesis allows for more rapid growth than strong CAM, particularly early on when water is typically not a limiting factor. In a plant that must complete its life in a single, short growing-season in a low-nutrient environment, the development of strong CAM and the anatomical traits often associated with it (e.g., leaf and stem succulence, increased vacuole and cell size, defense mechanisms such as spines and secondary compounds) may divert the allocation of energy, carbon, and water away from reproductive structures.

Australia is the most arid vegetated continent, and thus a landscape in which one might expect terrestrial CAM species to be well-represented, but CAM is an uncommon mode of photosynthesis, apparently expressed by fewer than 1% of its terrestrial vascular plant species (Holtum et al. 2016). This study triples the number of documented C<sub>3</sub>+CAM plants native to Australia, and highlights how annual C<sub>3</sub>+CAM phenotypes, but not perennial strong-CAM phenotypes, dominate the known terrestrial Australian CAM flora. The previous estimate that Australia supports perhaps 80 terrestrial species of CAM plants assumed that perhaps a third of *Calandrinia* were CAM (Holtum et al. 2016). A revised estimate of between 150 and 170 terrestrial Australian CAM species would be more consistent with the proportion of *Calandrinia* shown to exhibit CAM in this study, the rate of discovery of new *Calandrinia* species (Obbens 2014, 2018; Obbens et al. 2017), the demonstration of facultative CAM in Australian *Portulaca* (Holtum and Winter 2017; Holtum et al. 2017a), and the demonstration of CAM in other Australian genera (J.A.M. Holtum and L. Hancock unpublished).

Why there are so few terrestrial strong CAM plants, either large or small in stature, and considerably more C<sub>3</sub>+CAM plants in Australia remains unclear. Ellenberg (1981) suggested that precipitation in arid Australia is too variable to support the large perennial succulent life-form, most of which exhibit strong CAM. By comparing the climate space of regions that harbor large succulents around the world (i.e., the Americas and Africa), Ellenberg identified a rainfall predictability envelope that he hypothesized was required for large succulent life-forms such as the *Agave*, cacti and euphorbs. In Australia, only a small region in South Australia and Victoria fits within this climatic envelope. However, non-native large succulent CAM plants can grow well in Australia, flourishing in environments outside Ellenberg's rainfall predictability envelope (Holtum et al. 2016). Introduced cacti have become invasive weeds (Mann 1970; Chinnock 2015) and strong CAM has been demonstrated in all four of the Australian taxa of perennial pencil-stemmed succulents that attain a height of 0.5 m or greater, *Cynanchum brevipedicellatum* (P.I.Forst.) Liede and Meve, two subspecies of *C. viminalis* (Apocynaceae) and *Euphorbia sarcostemmoides* (Euphorbiaceae) (Holtum et al. 2016; J.A.M. Holtum and L. Hancock unpublished).

An alternative argument is that perhaps some Australian climates are suited to strong CAM, but terrestrial lineages with the propensity to evolve strong CAM are simply not present. This does not

necessarily appear to be the case either. Although there are no native *Agave*, *Aloe*, Bromeliaceae, Cactaceae, or Didiereaceae in Australia, there are genera in Australia for which strong CAM stem- or leaf-succulents inhabit other continents, for example, *Anacampseros* (Guralnick et al. 2007), *Crassula* (Pilon-Smits et al. 1996), and *Euphorbia* (Horn et al. 2014). Perhaps filters associated with transoceanic movement, the timing of arrival in Australia, the presence of fire, or competition effects affected selection for CAM strategies. In spite of the history of research into CAM biology (Ranson and Thomas 1960) the evolutionary biology of strong CAM is not well understood—how often it has evolved, whether it is reversible, and the anatomical requirements that facilitate its assembly. We predict that the transition to a strong CAM phenotype may be rare relative to transitions between  $C_3$  and  $C_3$ +CAM, and perhaps requires both a long-lived photosynthetic tissue and significant anatomical evolution (e.g., Edwards and Donoghue 2006; Heyduk et al. 2016; Edwards 2019).

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## Supplementary data

Supplementary data available at *ICB* online.

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