



## Tansley review

# Evolutionary trajectories, accessibility and other metaphors: the case of C<sub>4</sub> and CAM photosynthesis

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## Summary

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Are evolutionary outcomes predictable? Adaptations that show repeated evolutionary convergence across the Tree of Life provide a special opportunity to dissect the context surrounding their origins, and identify any commonalities that may predict why certain traits evolved many times in particular clades and yet never evolved in others. The remarkable convergence of C<sub>4</sub> and Crassulacean Acid Metabolism (CAM) photosynthesis in vascular plants makes them exceptional model systems for understanding the repeated evolution of complex phenotypes. This review highlights what we have learned about the recurring assembly of C<sub>4</sub> and CAM, focusing on the increasingly predictable stepwise evolutionary integration of anatomy and biochemistry. With the caveat that we currently understand C<sub>4</sub> evolution better than we do CAM, I propose a general model that explains and unites C<sub>4</sub> and CAM evolutionary trajectories. Available data suggest that anatomical modifications are the 'rate-limiting step' in each trajectory, which in large part determines the evolutionary accessibility of both syndromes. The idea that organismal structure exerts a primary influence on innovation is discussed in the context of other systems. Whether the rate-limiting step occurs early or late in the evolutionary assembly of a new phenotype may have profound implications for its distribution across the Tree of Life.

## I. Introduction

Most characteristics that we identify in organisms have evolved more than once. In plants, nearly every aspect of form – leaf shape, phyllotaxy, floral syndromes, fruit type, growth form, carnivorous and parasitic habits, even basic organs (leaves and roots) – has arisen multiple times (Boyce & Knoll, 2002; Hetherington & Dolan, 2018). Even signature traits that define large extant clades, such as seeds and flowers, show fossil evidence of other lineages having at least approached these purportedly singular adaptations in the past (Scott, 1901). Convergent evolution is a gift to the comparative biologist, as it provides independent instances of a trait of interest. Convergence also illustrates the evolutionary process more generally – both in how natural selection may strongly favor particular organismal configurations – and, in turn, how particular organismal configurations may themselves limit subsequent evolution to only a handful of outcomes. A pattern of repeated convergence can arise from multiple, nonexclusive factors, for example: (1) a given trait may be adaptive under multiple circumstances, thus evolving under a variety of selection pressures (e.g. in plants, leaf trichomes serve multiple functions (Werker, 2000); (2) a single selection pressure favoring a particular trait may be pervasive and experienced by many or all extant lineages (e.g. climate change and the deciduous leaf habit; Edwards *et al.*, 2017); and (3) organismal structure may restrict the range of adaptive solutions that are evolutionary accessible (e.g. it is highly unlikely that a plant will ever evolve flight, but plants have repeatedly evolved convergent fruit types that animals disperse for them; Fleming & Kress, 2011).

$C_4$  photosynthesis and Crassulacean Acid Metabolism (CAM) are two alterations of the plant primary metabolism, and present two of the more striking examples of massive convergence in plants. The precipitous decline in atmospheric  $CO_2$  levels *c.* 30 million years ago (Ma) presented a global selection pressure on terrestrial photosynthesis (Edwards *et al.*, 2010). At low  $CO_2$  and high temperatures, photosynthesis becomes inefficient due to increased levels of photorespiration. Land plants (primarily angiosperms) responded en masse, by evolving – well over 100 times –  $C_4$  and CAM photosynthesis (Fig. 1). Today,  $C_4$  and CAM plants play fundamental ecological roles in the terrestrial biosphere, with  $C_4$  plants contributing up to *c.* 23% of global primary productivity and CAM plants dominating vast areas of the arid landscape (Still *et al.*, 2003; Ogburn & Edwards, 2010). Due to their ecological prominence, physiological uniqueness, and importance in food and biofuel production,  $C_4$  and CAM syndromes have captured the attention of researchers in multiple fields, and many aspects of their ecology, physiology and genetics have been reviewed extensively elsewhere (Sage, 2001, 2004; Keeley & Rundel, 2003; Silvera *et al.*, 2010a; Edwards & Ogburn, 2012; Christin & Osborne, 2014; Winter & Holtum, 2014; Winter *et al.*, 2015; Heyduk *et al.*, 2019), including the very first Tansley review, published in 1985 (Cockburn, 1985).  $C_4$  photosynthesis especially has been well studied, perhaps due to the importance of  $C_4$  grasses in agriculture, and we now understand a considerable amount about the evolutionary trajectory of the  $C_4$  syndrome. The same cannot yet be said of CAM, although this is beginning to change (Silvera *et al.*, 2010b; Horn

*et al.*, 2014; Bone *et al.*, 2015; Heyduk *et al.*, 2016; Goolsby *et al.*, 2018; Males, 2018; Hancock *et al.*, 2019).

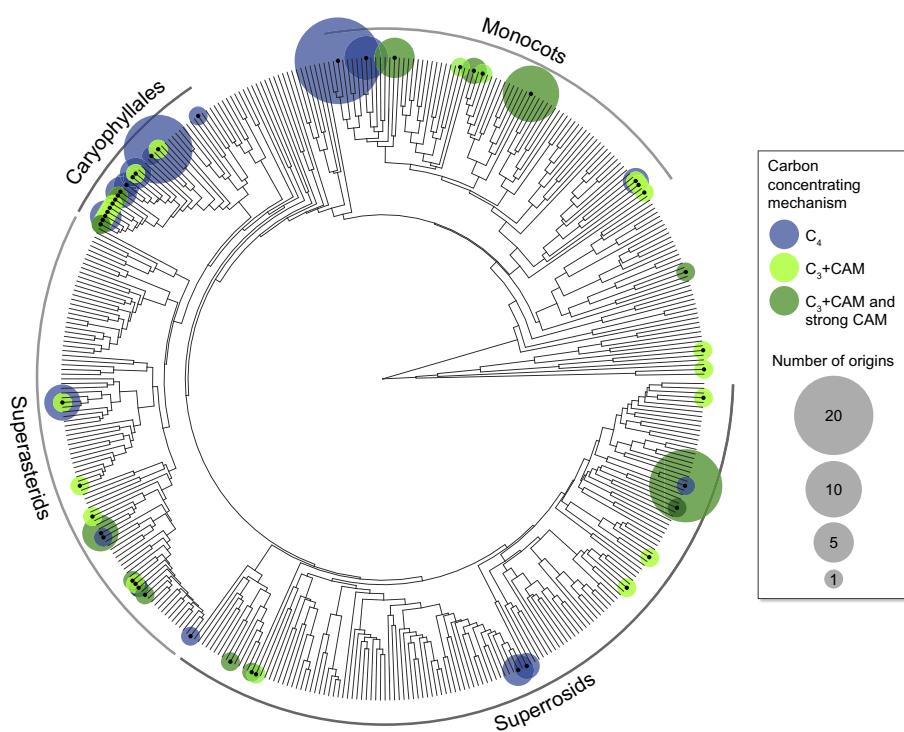
In spite of the great potential these adaptations present as evolutionary model systems, they have yet to fully capture the broader attention of evolutionary biologists. This lack of interest may be due in part to the complexity of the syndromes themselves: in order to understand how they work, one also must understand plant biochemistry and anatomy in some level of detail. But what must also be at least partially to blame is our own focus on the various intricacies of each system, and our lack of connection to more general features of organismal evolution. The relevance of  $C_4$  and CAM evolutionary histories becomes even more significant when they are considered together, as they share many features and yet represent such distinct ecological strategies. They also are remarkable in that they require an integrated, whole-organism consideration of evolutionary change, due to the fact that these syndromes are directly altering the primary metabolism of the organism, which in turn influences every aspect of growth and survival.

In this review I place what we currently understand about both  $C_4$  and CAM evolution into a framework that focuses on the evolutionary integration of anatomy and biochemistry. In this model, which concerns not necessarily the *relative fitness* of intermediate phenotypes, but rather their *evolutionary accessibility*, I propose that the anatomical modifications required for each adaptation are the least accessible elements, and in an analogy to chemical reaction kinetics, their emergence is identified as the ‘rate-limiting step’ in each trajectory. This framework emphasizes the significant influence of organismal structure in shaping alternative evolutionary outcomes.

## II. Evolutionary trajectories, adaptive landscapes and a new evolutionary metaphor: reaction kinetics

I use the term ‘evolutionary trajectory’ to refer to the order of changes that resulted in the evolution of one organismal (phenotypic or genotypic) state from another. Convergent evolution provides a particularly powerful scenario for inferring evolutionary trajectories, in that we may infer this history many times in independent origins with the hopes of finding both commonalities and differences. Evolutionary trajectories are clearly linked to the classical metaphors of fitness landscapes (Wright, 1932), as each evolutionary step along the trajectory should, in most cases, result in a higher fitness than the previous state (but not always; e.g. Woods *et al.*, 2011).

Also related to evolutionary trajectories is the concept of evolutionary accessibility, which may bias trajectories quite independently of relative fitness. Accessibility concerns how ‘far away’ in mutational space one phenotype is from another, and the realization that there is strong bias, at the mutational level, in how evolutionary landscapes are navigated (Stadler *et al.*, 2001; Weinreich *et al.*, 2006). Earlier work on accessibility grew from comparative developmental biology, producing key concepts such as burden (Riedl, 1978) and constraint (Maynard-Smith *et al.*, 1985). More recent experimental work also has demonstrated differential evolutionary accessibility of one phenotype from



**Fig. 1** Phylogenetic distribution of C<sub>4</sub> and Crassulacean Acid Metabolism (CAM) origins. Phylogeny of seed plant families, from Harris & Davies (2016). Blue circles represent C<sub>4</sub> origins, light green circles represent C<sub>3</sub>+CAM origins, and dark green circles represent lineages that contain C<sub>3</sub>+CAM as well as strong CAM. The area of the circle is proportional to the total number of estimated origins in each family, gathered from Sage *et al.* (2011), Horn *et al.* (2014), Goolsby *et al.* (2018), Smith & Winter (1996), Silvera *et al.* (2009) and J.A.C. Smith, K. Winter, J. Holtum, K. Silvera, R. Sage, E. Edwards (unpublished).

another (Weinreich *et al.*, 2006; Finnigan *et al.*, 2012; Meyer *et al.*, 2012), all emphasizing the importance of the ‘starting point’ in influencing evolutionary outcomes in response to a given selection pressure. In the case of phenotypes that are as convergent as CAM and C<sub>4</sub>, we are afforded an elevated statistical power to look for commonalities in the evolutionary ‘starting points,’ and thus potentially identify the organismal attributes that shape their evolutionary accessibility.

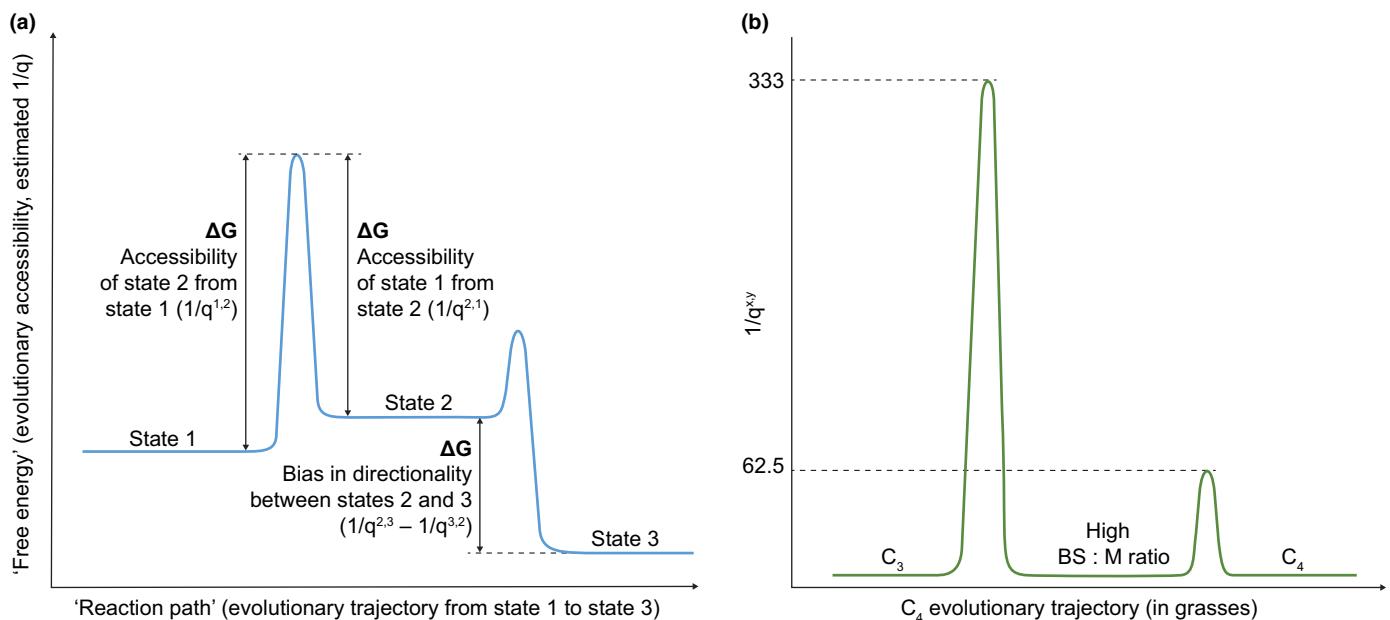
Although we may (and should) aspire to construct an actual mutational map of C<sub>4</sub> and CAM evolutionary accessibility (*sensu* Stadler *et al.*, 2001), macro-evolutionary analyses that infer the frequency of evolutionary transitions between relevant character states currently provide a means for estimating a crude accessibility map. Phylogenies are particularly useful for providing information about the relative frequency and order of different transitions through time and across lineages, and the frequency and order of shifts between certain character states must in some way relate to their relative evolutionary accessibility. Comparative phylogenetic analyses now routinely deal in the currency of estimated character transition rates, and even though we have not explicitly made the connection, thinking about these differential transition rates in the context of chemical reaction kinetics may be a useful analogy for identifying potential ‘rate-limiting steps’ in evolutionary trajectories (Fig. 2). In cases such as C<sub>4</sub> and CAM, where we have identified a set of phenotypes that are likely to be important steps along their respective trajectories, we may consider these as chemical intermediaries – and the activation energy required for certain chemical reactions as analogous to the relative evolutionary accessibility of one phenotype to another. The relative positions of intermediate states along the y-axis represent potential biases

in the direction of character change, and can illustrate the relative reversibility of certain transitions.

The development of this new analogy elevates the role of inferred transition rates in our inference of evolutionary trajectories, but we must be clear about what these rates represent, and be wary of over-interpretation (Edwards *et al.*, 2015; Donoghue and Edwards, 2019). Inferred transition rates are simply a way of summarizing past events in evolution, and are only something that can be estimated from a phylogeny after the evolutionary transitions have already occurred. Organisms do not possess transition rates, only attributes; these attributes exist within a complex evolutionary arena over long periods of time, and it is the interaction of these attributes and other external factors that produced a set of transitions from which we can estimate an average rate of change for that lineage. Despite these complexities, I’d argue that estimated transition rates are still informative in helping to distinguish relative evolutionary accessibility between particular phenotypes. At the same time, parameters estimated from macroevolutionary analyses appear especially susceptible to reification, and it is important to clarify what terms like ‘transition rates’ and ‘rate-limiting steps’ actually signify, and specifically how they relate (or, more precisely, how they do *not* relate) to underlying evolutionary processes.

### III. Background on CAM and C<sub>4</sub> syndromes

Most terrestrial plants perform C<sub>3</sub> photosynthesis, where the enzyme Rubisco catalyzes the reaction between atmospheric CO<sub>2</sub> and ribulose 1,5-biphosphate (RuBP) to form a 3-carbon (3-C) molecule. This is the first step of the Calvin cycle that eventually produces glyceraldehyde-3-phosphate, the building block for all carbohydrates. Under high temperatures and low internal CO<sub>2</sub>



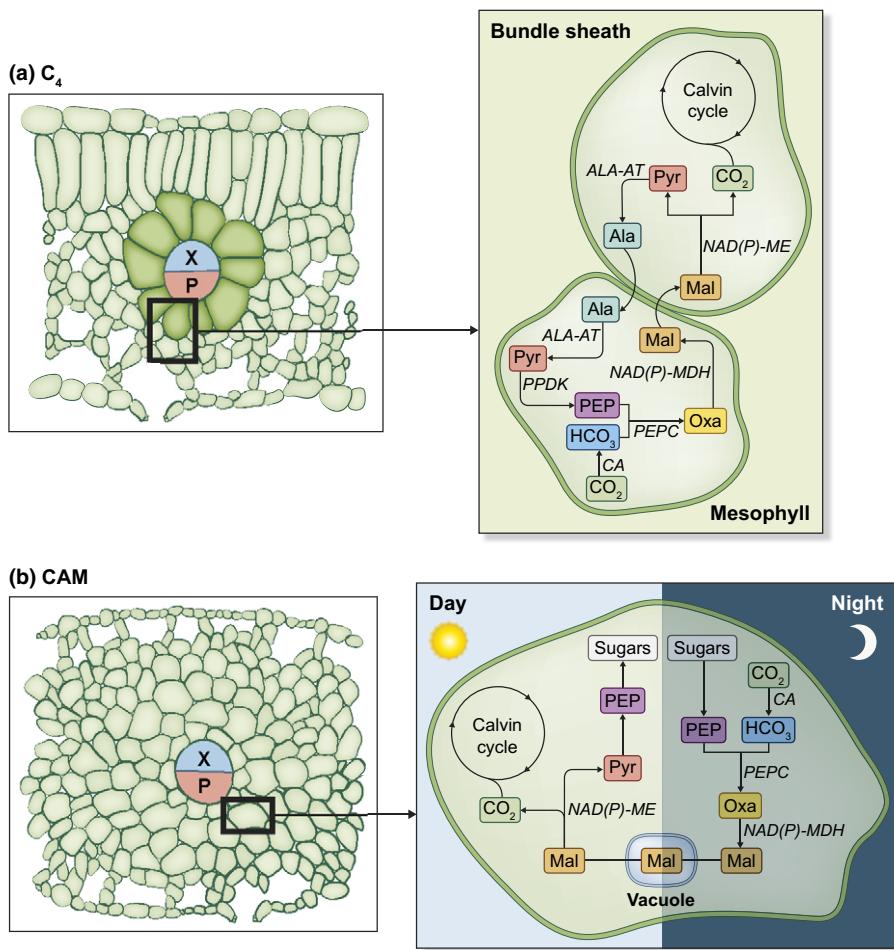
**Fig. 2** Rate limiting steps in evolutionary trajectories. (a) A hypothetical ‘reaction path’ borrowed from chemical reaction kinetics, where ‘state 1’, ‘state 2’ and ‘state 3’ are phenotypic states occupied during the evolutionary transition from state 1 to state 3 (state 2 is akin to a chemical intermediate). The height of the curves separating the states (the ‘free energy released’) are analogous to the evolutionary accessibility of one state from another, as estimated by evolutionary transition rate  $q_{x,y}$ . Nonsymmetrical transition rates (e.g.  $q_{1,2} < q_{2,1}$ ) result in shifts of phenotypic states along the y-axis, and indicate the reversibility of particular transitions. Complex trajectories with multiple transitions will include a rate-limiting step, which is the step with the highest  $\Delta G$ . (b) Reaction kinetics of the  $C_4$  trajectory in grasses, using transition rate estimates from Christin *et al.* (2013). The evolution of high bundle sheath to mesophyll ratio (BS : M) in leaves is the rate-limiting step; afterward, the  $C_4$  pathway evolves repeatedly.

concentrations, Rubisco becomes inefficient, interacting with  $O_2$  and initiating an energetically costly process known as photorespiration. All plants have a functional photorespiratory cycle, which may be beneficial in reducing photooxidation in high-light environments (Kozaki & Takeba, 1996). However, under low atmospheric  $CO_2$  concentrations, drought stress, and/or hot temperatures, levels of photorespiration can become debilitating, and plants have responded by repeatedly evolving internal  $CO_2$  concentrating mechanisms ( $C_4$  and CAM) to promote Rubisco carboxylation (Sage *et al.*, 2012). The general approach of both  $C_4$  and CAM is to first fix atmospheric  $CO_2$  into a 4-C molecule, typically malate, catalyzed by the enzyme phosphoenolpyruvate carboxylase (PEPC). This metabolite is later de-carboxylated in the proximity of Rubisco and the Calvin cycle. The release of  $CO_2$  during this step results in a localized, elevated  $CO_2$  concentration, which suppresses photorespiration and allows the Calvin cycle to proceed efficiently. All enzymes involved are already found in all plants and were co-opted for this new purpose; in fact, PEPC plays an important role in supplying malate to the TCA cycle, and as such is already being expressed in photosynthetic tissue (Aubry *et al.*, 2011).

A major difference between  $C_4$  and CAM syndromes lies in how they have isolated Rubisco and PEPC activity. In  $C_4$  plants (Fig. 3a), both PEPC and Rubisco operate during the day, when stomata are open: PEPC is active in the mesophyll tissue, and 4-C acids must be transported intercellularly to Rubisco, which is restricted to an inner compartment, typically the bundle sheath cells that surround veins. Major exceptions to this anatomical arrangement have appeared in the Amaranthaceae (including

Chenopodiaceae), a phylogenetic hot-bed of  $C_4$  evolution that also presents a diverse array of spatial configurations of Rubisco isolation within the leaf (Kadereit *et al.*, 2003, 2012), including multiple origins of single-cell  $C_4$  photosynthesis, where PEPC operates in one region of the cell and Rubisco in another (Edwards *et al.*, 2004). Regardless of the exact spatial arrangement, however, the reduction of photorespiration and the temporal coordination with photosynthetic light reactions create a ‘fuel-injected’ photosynthetic engine, and  $C_4$  plants typically achieve very high photosynthetic capacity (Sage & Zhu, 2011). They include several important crops (maize, sugarcane), are often found as fast growing weeds in disturbed environments (e.g. purslane, crabgrass, spurge), and are thought to be especially well adapted to monsoon climates with a hot growing season (Sage, 2004; Sage *et al.*, 2011).  $C_4$  grasses especially are productive elements of tropical grasslands and savannahs – combined with croplands, it is estimated that  $C_4$  grasses contribute up to 23% of annual terrestrial GPP (Still *et al.*, 2003).

Unlike  $C_4$  plants, the CAM biochemical pathway (Fig. 3b) separates PEPC and Rubisco activity temporally, rather than spatially. PEPC is active at night, and 4-C acids accumulate in the vacuole. In the morning, acids move out of the vacuole and are decarboxylated to release  $CO_2$ , which is then fixed by Rubisco. Thus, PEPC and Rubisco operate in the same cells, but during different periods of a diurnal cycle. In plants that fix most of their carbon with CAM, this diurnal pattern is accompanied by an inverted stomatal behavior: stomata open at night when PEPC is active, and are closed for a large portion of the day, when Rubisco is active. Having stomata closed during the day increases water use



**Fig. 3** The basics of C<sub>4</sub> and Crassulacean Acid Metabolism (CAM) biochemistry and anatomy. In C<sub>4</sub> plants, PEP Carboxylase fixes CO<sub>2</sub> in mesophyll cells, and malate is transferred to the bundle sheath for decarboxylation and subsequent fixation by Rubisco and the Calvin cycle. C<sub>4</sub> plants need a high bundle sheath to mesophyll ratio (BS : M) for efficient photosynthesis. In CAM plants, PEP Carboxylase operates at night, and malate is stored overnight in vacuoles. During the day it is decarboxylated and fixed by Rubisco in the same cells. A strong CAM cycle is facilitated by large cells and low mesophyll conductance, which aid in malate storage and limiting CO<sub>2</sub> loss during the day. P, phloem; X, xylem.

efficiency (WUE), as lowered night-time temperature and higher relative humidity will reduce transpiration. Predictably, CAM plants dominate the landscapes of many arid and semiarid ecosystems (Nobel, 1988; Arakaki *et al.*, 2011). CAM also is common in tropical forest epiphytes, which occupy water-limited microhabitats (Zotz & Ziegler, 1997), and in aquatic plants, where low CO<sub>2</sub> diffusion in water places strong C limitations on photosynthesis (Keeley, 1998). CAM is an inherently more flexible photosynthetic system than C<sub>4</sub>, because mesophyll cells still possess a functional C<sub>3</sub> cycle, and so additional CO<sub>2</sub> may be taken up from the atmosphere directly via C<sub>3</sub> photosynthesis, even in strong CAM plants. In fact, a flexible 'C<sub>3</sub>+CAM' phenotype, where a plant is typically C<sub>3</sub> but also can engage a CAM cycle, may actually be more common than the emblematic strong CAM plants such as cacti and agaves, but identifying the C<sub>3</sub>+CAM phenotype currently requires controlled experiments on living plants (Winter & Holtum, 2002, 2014).

At last count, C<sub>4</sub> has evolved an estimated minimum of 67 times (Sage *et al.*, 2011; Sage, 2016), and only in angiosperms, though the actual number may well turn out to be much greater. The evolutionary history of CAM is less well known, in part because it is prevalent in highly speciose plant lineages that pose challenges to comprehensive taxon sampling, such as orchids (Silveira *et al.*, 2009), euphorbias (Horn *et al.*, 2014) and bromeliads (Crayn *et al.*,

2004), and in part because many species only perform small amounts of CAM, which can be difficult to detect. Based on its broad taxonomic distribution (Smith & Winter, 1996), including *Isoetes*, ferns, *Welwitschia* and many distinct clades of angiosperms, it seems likely that some type of CAM has evolved even more frequently than C<sub>4</sub>.

In summary, C<sub>4</sub> and CAM utilize similar biochemistry, but the spatial and temporal configurations of enzymatic reactions are very different. Importantly, each pathway requires an unique suite of anatomical characters in order to work efficiently, which will be discussed in detail below. Conventional wisdom holds that these characters are antagonistic, such that anatomy which facilitates efficient C<sub>4</sub> will simultaneously disadvantage a CAM metabolism (Sage, 2002). Likewise, C<sub>4</sub> and CAM have typically been thought to solve distinct problems: because CAM requires succulence and also increases WUE, it is assumed to be an adaptation to drought (Raven & Spicer, 1996; Keeley & Rundel, 2003; Edwards & Ogburn, 2012); because C<sub>4</sub> results in a higher maximum photosynthetic capacity, it is assumed to be an adaptation to high light and hot temperatures (Ehleringer *et al.*, 1991, 1997; Sage, 2004). Thus, although low atmospheric CO<sub>2</sub> and climate change may have provided the global backdrop to both of these adaptations, it is thought that their evolutionary trajectories are largely independent, and that C<sub>4</sub> and CAM

syndromes are more or less incompatible from both functional and ecological perspectives.

This view has been challenged elsewhere (Edwards & Ogburn, 2012), and the environmental selection pressures potentially associated with each syndrome is purposefully not addressed in this review. It is quite common to find C<sub>4</sub> and CAM plants co-occurring across many landscapes, albeit often presenting different life histories and growth strategies. From a functional point of view, it also is important to briefly mention *Portulaca*, a facultative CAM lineage that has likely evolved C<sub>4</sub> photosynthesis three times in parallel while maintaining a functional CAM cycle (Koch & Kennedy, 1980; Kraybill & Martin, 1996; Mazen, 2001; Guralnick *et al.*, 2002; Lara, 2004; Christin *et al.*, 2014; Holtum *et al.*, 2017a; Winter *et al.*, 2019). Although C<sub>4</sub> and CAM evolutionary trajectories are largely presented here as distinct, *Portulaca* demonstrates that they also can overlap, and furthermore, that the two syndromes are even compatible at the organ level. As most phenotypes in plants appear to have evolved more than once, it seems likely that if we look, we will discover other C<sub>4</sub> lineages that also operate a facultative CAM cycle.

#### IV. The C<sub>4</sub> evolutionary trajectory: anatomy first, biochemistry second

We know a fair amount about how C<sub>4</sub> plants have evolved from their C<sub>3</sub> ancestors, precisely because of the development of multiple model clades with diverse photosynthetic phenotypes and resolved phylogenies (e.g. (McKown *et al.*, 2005; Christin *et al.*, 2011, 2013; Edwards, 2014). This trajectory has been reviewed extensively elsewhere (Sage, 2001, 2004; Gowik & Westhoff, 2011), and is based largely on shared macroevolutionary patterns discovered in grasses and a great diversity of eudicots, including *Flaveria*, *Heliotropium*, *Molluginaceae*, *Portulaca*, *Anticharis* and *Blepharis* (Ku *et al.*, 1983; Vogan *et al.*, 2007; Christin *et al.*, 2011; Khoshravesh *et al.*, 2012; Ocampo *et al.*, 2013; Williams *et al.*, 2013; Fisher *et al.*, 2015). The vast majority of C<sub>4</sub> origins have incorporated the bundle sheath cells (BS) that surround veins as the location for the Calvin cycle, and a key anatomical configuration for an efficient C<sub>4</sub> cycle is a high bundle sheath to mesophyll ratio (BS : M) in photosynthetic tissue. Without a high BS : M, the proportion of leaf volume with an operational Calvin cycle will be too low, limiting sugar production. Likewise, it is advantageous to maintain a short diffusive pathway for malate transfer from where it is formed (mesophyll) to where it will be decarboxylated (BS). A high BS : M which can be achieved by either high venation density, or very wide BS cells, or both. In all lineages investigated, a high BS : M has been found in C<sub>3</sub> plants that are close relatives of C<sub>4</sub> plants, suggesting that a high BS : M trait is ancestral to the clade in question, and thus evolves before any implementation of a C<sub>4</sub> biochemical cycle. This does seem to be the most parsimonious explanation, although any sort of formal ancestral state reconstruction analysis is rarely performed (Hancock & Edwards, 2014).

A notable exception is Christin *et al.* (2013), who constructed a large anatomical dataset of species from across the grass phylogeny and modeled the evolution of various anatomical characters. They identified the branch on the phylogeny where a C<sub>4</sub>-like BS : M ratio

first appeared and, remarkably, it was at the base of the PACMAD clade, a large grass lineage (*c.* 5000 species) that includes all known C<sub>4</sub> grass origins (*c.* 24; Grass Phylogeny Working Group II, 2012). As the similarly speciose BEP grass lineage never achieved the same BS : M ratios, and also never evolved C<sub>4</sub> photosynthesis, the authors interpreted high BS : M as an evolutionary ‘enabler’: a phenotype that affords a higher evolutionary accessibility to the C<sub>4</sub> syndrome. Although this study is the most rigorous analysis of these patterns (Christin *et al.*, 2013), the qualitative conclusions made by other studies are consistent with this order of events, as is a meta-analysis of various C<sub>4</sub>-evolving lineages by Williams *et al.* (2013). In other words, although more work should be done, there is already ample evidence that the most essential element of C<sub>4</sub> anatomy evolves before the development of even a rudimentary C<sub>4</sub> biochemical cycle, and no evidence as yet to suggest otherwise. Concomitant with high BS : M in certain groups is an increase in the number of chloroplasts and mitochondria in the BS cells. The function of high BS : M in C<sub>3</sub> plants is not known, although several hypotheses have been suggested (Sage, 2001, 2004; Griffiths *et al.*, 2013). One particularly compelling idea is that large BS cells may act as high capacitance cells, situated directly between the leaf vasculature and the transpirational demand driven by open stomata. These cells may act to protect the vein xylem from cavitation during pulses of extremely high vapor pressure deficit, which would occur in hot, sunny, semi-arid environments, precisely the environments where we assume C<sub>4</sub> is adaptive.

With a high BS : M in place, the next purported step toward C<sub>4</sub> is the development of a less-efficient C-concentrating mechanism in bundle sheath cells, called ‘C<sub>2</sub>’ photosynthesis (Monson & Rawsthorne, 2000; Sage *et al.*, 2012). In C<sub>2</sub> plants, the photorespiratory cycle has been partially arrested in mesophyll cells, before the conversion of glycine to serine and CO<sub>2</sub> by glycine decarboxylase (GDC). Glycine is instead shuttled to the bundle sheath where it re-enters the photorespiratory cycle, eventually producing a CO<sub>2</sub> molecule and thus elevating CO<sub>2</sub> concentrations in the BS. This gradient in CO<sub>2</sub> concentration within the leaf promotes higher carbon fixation rates in the BS cells, which may reshape the fitness landscape toward building BS cells as the primary location for the Calvin cycle. The fitness landscape between C<sub>2</sub> and C<sub>4</sub> photosynthesis has been modeled as a ‘Mt Fuji’ slope, with clear and strong directional selection toward a full C<sub>4</sub> cycle once a C<sub>2</sub> phenotype has evolved (Heckmann *et al.*, 2013). Furthermore, in a remarkable modeling experiment, Mallmann *et al.* (2014) proposed a direct mechanism for the assembly of the C<sub>4</sub> biochemical cycle. In C<sub>2</sub> plants, the processing of photorespiratory CO<sub>2</sub> in the BS creates a nitrogen (N) imbalance between BS and M cells that requires a redistribution of metabolites out of the BS and back to M. They found that the C<sub>4</sub> biochemical cycle was the most likely and efficient means of rectifying the N imbalance. The Mallmann model suggests that C<sub>2</sub> plants should also operate a low-level C<sub>4</sub> cycle, not for primary C fixation, but rather as a means for recycling N-rich metabolites between cells. It is quite straightforward, then, to simply upregulate PEPC and further downregulate Rubisco in mesophyll cells, and the entire C<sub>4</sub> phenotype has been assembled. To support their metabolic model, they presented gene expression profiles of C<sub>2</sub>, C<sub>4</sub>-like and full C<sub>4</sub> species of *Flaveria*, which indeed

show the predicted gradient of weak to strong expression of C<sub>4</sub> genes. If the Mallman model is accurate, then C<sub>4</sub> evolution is a textbook example of ‘exaptation’ (Gould & Vrba, 1982), where a trait initially evolved for one function is successfully recruited into another.

A simple pattern emerges from the C<sub>4</sub> evolutionary trajectory. Before any major biochemical components are assembled, a certain anatomical configuration of photosynthetic tissue must be established. We can consider this state to be the relatively ‘rare’ phenotype that, once evolved, opens up new possibilities for biochemical experimentation. The subsequent establishment of a rudimentary CCM in the bundle sheath cells creates a new axis of selection for increased Rubisco activity there, and very quickly the remaining elements of the C<sub>4</sub> biochemical cycle can be assembled into their new function.

This is an appealing and testable model, and predicts many patterns that have already been observed. For instance, jointly considering the Heckmann and Mallman models would suggest that the C<sub>2</sub> phenotype would be an evolutionarily short-lived state, as there is both a strong selection gradient toward C<sub>4</sub>, and a highly biased set of metabolic scenarios that would consistently facilitate the emergence of the C<sub>4</sub> biochemical cycle. Indeed, it seems that C<sub>2</sub> species are relatively rare, and most are evolutionarily young. There are some exceptions: in the Molluginaceae, for example, the *Mollugo verticillata* group may have evolved C<sub>2</sub> photosynthesis upwards of 15 Ma – and a full C<sub>4</sub> pathway was never assembled (Christin *et al.*, 2011). If C<sub>2</sub> necessarily creates an N imbalance that must be solved, did *Mollugo* recruit an alternative set of enzymes to do so, thereby redirecting its own evolutionary trajectory?

The C<sub>2</sub> model is compelling in large part because it is so logical, and also is supported by theory, modeling, and the phylogenetic proximity of C<sub>2</sub> and C<sub>4</sub> species in multiple lineages. But could C<sub>4</sub> also have evolved *without* passing through the C<sub>2</sub> state? The C<sub>2</sub> model was developed in large part by the intense study of *Flaveria*, which remains undoubtedly the most influential lineage in our models of C<sub>4</sub> evolution. In a recent review, (Sage *et al.*, 2018) provided a helpful list of C<sub>4</sub> lineages for which closely related C<sub>2</sub> species have been identified, which emphasized that the majority of C<sub>4</sub> lineages have no known close C<sub>2</sub> relatives. This could be due to several reasons: they exist but simply have not been discovered; the C<sub>2</sub> phase was short lived and the C<sub>4</sub> cycle emerged before a subsequent speciation event, leaving no record of this intermediate state in living taxa; C<sub>2</sub> species have since gone extinct; or, C<sub>4</sub> evolved without passing through a C<sub>2</sub> stage. With over 70 origins of the C<sub>4</sub> pathway currently identified, and very few of them carefully studied, it seems likely that all of these options are important to consider. Ideally, we would develop multiple model clades, each to the level of detail that we have for *Flaveria*, which would help clarify whether we are simply missing living C<sub>2</sub> taxa because we have not yet investigated the right species. The other possibilities are more difficult to differentiate. With increasingly sophisticated genomic tools available for analysis of any organism, might we be able to identify the ‘ghost of C<sub>2</sub> past’ in the genomes of C<sub>4</sub> species that have no living C<sub>2</sub> relatives?

We also need to develop alternative hypothetical C<sub>4</sub> trajectories that do not involve a C<sub>2</sub> state. New ideas are materializing from continued study of *Alloteropsis*, a newly emerging model clade for

C<sub>4</sub> evolution (Dunning *et al.*, 2017). *Alloteropsis* may be the finest model clade yet for identifying the very early stages of C<sub>4</sub> emergence, as there is a full C<sub>3</sub> to C<sub>4</sub> physiological spectrum found within a single species, *Alloteropsis semialata* (Lundgren *et al.*, 2016). One potentially new realization is that the C<sub>4</sub> optimization stage may be longer and more elaborate than we have thought, and that many of the differences between distantly related C<sub>3</sub> and C<sub>4</sub> species evolved later in C<sub>4</sub> lineages, long after the first emergence of the pathway – and thus are not *necessary* components, but rather are *optimizations* of the pathway (Heyduk *et al.*, 2019). In *Alloteropsis*, for example, several populations received several fully optimized C<sub>4</sub> genes via lateral gene transfer from co-occurring C<sub>4</sub> grass species (Christin *et al.*, 2012), which they preferentially utilize, and their vertically inherited genes show very low levels of expression.

## V. The CAM evolutionary trajectory (part 1): defining ‘C<sub>3</sub>+CAM’ and ‘strong CAM’ phenotypes

In spite of the significant amount of research into the basics of CAM physiology, ecology and molecular biology, I would argue that we currently know far less about how CAM has evolved than we do about C<sub>4</sub>, and in fact do not have even the beginnings of a proposed model such as the ‘anatomy-first’ and C<sub>2</sub> models discussed above. This is due to several reasons. The most critical may be that the flexibility of CAM physiology has made it difficult to definitively circumscribe what a CAM plant is. All plants with a CAM cycle also have a fully functional C<sub>3</sub> cycle in their mesophyll cells, which means that the degree of expression of C<sub>3</sub> vs CAM is flexible, and can change depending on the age and physiological status of an individual plant. A C<sub>4</sub> leaf does not sometimes become a C<sub>3</sub> leaf; even a C<sub>2</sub> plant doesn’t sometimes run its CCM and sometimes not. Thus, there are more clearly identifiable phenotypes along the C<sub>3</sub>–C<sub>4</sub> trajectory that do not, as yet, have clear analogs in CAM biology.

There have been many attempts to categorize different kinds of CAM behavior, especially in plants that primarily use C<sub>3</sub> metabolism yet also express small amounts of CAM (Box 1). For the purposes of this review, I will lump these categories into a single ‘C<sub>3</sub>+CAM’ phenotype (similar to the ‘C<sub>3</sub>-CAM’ category of Winter *et al.*, 2015). The C<sub>3</sub>+CAM category can be further delineated further by distinguishing whether the CAM cycle is constitutively (albeit at a low level) or facultatively expressed (Winter & Holtum, 2014). This is surely a functionally significant distinction, but it is still unclear how fixed these behaviors are within a given species. Because most species labeled as low-level constitutive are *also* facultative, showing increased CAM expression under stress (e.g. Hancock *et al.*, 2019), the only trait distinguishing these phenotypes is whether there is statistically significant nocturnal malate accumulation under well-watered conditions – and this also could presumably vary with plant age, other environmental conditions, and even the precision of our methods to quantify malate accumulation. For these reasons, and because there are very few studies documenting the phylogenetic distribution of constitutive low-level vs facultative CAM (but see Hancock *et al.*, 2019), for now I am considering both as simply C<sub>3</sub>+CAM. C<sub>3</sub>+CAM cannot be identified with stable C isotope surveys, as these species

will carry the signature of a C<sub>3</sub> plant. Nor is C<sub>3</sub>+CAM currently recognizable by any particular morphological or anatomical feature (e.g. Silvera *et al.*, 2005; Males, 2018); unfortunately, the only way to identify this phenotype is through drought experiments on living plants. For these reasons, we still do not know much about the real phylogenetic distribution and possible abundance of C<sub>3</sub>+CAM plants. New species are continuously being identified (Winter & Holtum, 2014; Heyduk *et al.*, 2018; Holtum *et al.*, 2017b; Holtum *et al.*, 2018), and from an astonishing diversity of lineages (e.g. fungus-induced CAM activity in *Camellia* (Theaceae); Yuan *et al.*, 2012). It seems reasonable to think that C<sub>3</sub>+CAM physiology is far more common than is generally appreciated.

And so, what is a ‘strong CAM’ phenotype? Here I define strong CAM as plants who have committed to utilizing the CAM cycle daily as their primary metabolism. Like C<sub>4</sub> plants they are easily detected from the <sup>13</sup>C/<sup>12</sup>C isotope ratio of their tissues, as PEPC and Rubisco show differential discrimination against the heavier <sup>13</sup>C isotopes (Farquhar *et al.*, 1982; O’Leary, 1988). Also like C<sub>4</sub>, strong CAM species tend to present a recognizable set of anatomical specializations. Their photosynthetic tissues are notably succulent – in many cases, strong CAM plants have developed a succulent stem cortical tissue as their primary photosynthetic organ (e.g. cacti, *Euphorbia*), whereas in other lineages, leaves have become noticeably succulent (e.g. agaves, aloes and orchids). Succulence and strong CAM are clearly associated across the tree of life (Kluge & Ting, 1978; Ogburn & Edwards, 2010; Nyffeler *et al.* 2008) – but is this because they are two distinct adaptations to water-limited environments, and so become associated simply because they are co-selected? This argument was favored by de Santo *et al.* (1983), who found no relationship between mesophyll succulence and the strength of the CAM cycle in several species of *Cissus* and *Peperomia*. This view is in stark contrast to the majority of studies that have documented significant differences in various metrics of succulence between CAM and non-CAM species, as outlined below. Furthermore, there are sound theoretical arguments for a mechanistic link between photosynthetic succulence and CAM function.

## VI. The CAM evolutionary trajectory (part 2): identifying the relevant anatomical parameters

There are multiple anatomical attributes of succulent plants that have direct and significant influence on plant carbon fixation and the relative efficiencies of C<sub>3</sub> vs CAM photosynthesis. Perhaps most directly, succulent plants typically have large cells with thin cell walls and large vacuoles, which allows for increased cellular water storage and high tissue capacitance. In strong CAM plants, the vacuole has been estimated to comprise upwards of 97% of the cell volume (Steudle *et al.*, 1980). It is thought that the size of the vacuole may place a physical limitation on the amount of malic acid stored at night, which in turn limits the amount of carbohydrate produced the following day, and there are reported clear differences between cell size (and by proxy vacuole size) between strong CAM and other species (Nelson *et al.*, 2005; Nelson & Sage, 2008; Heyduk *et al.*, 2016; Males, 2018).

### Box 1 Variations of CAM photosynthesis referred to here as ‘C<sub>3</sub>+CAM’.

**CAM-cycling:** CAM biochemistry is used to re-fix respiratory CO<sub>2</sub>, but does not fix atmospheric CO<sub>2</sub>.

**CAM-idling:** CAM-cycling, but plant keeps stomata closed during both day and night.

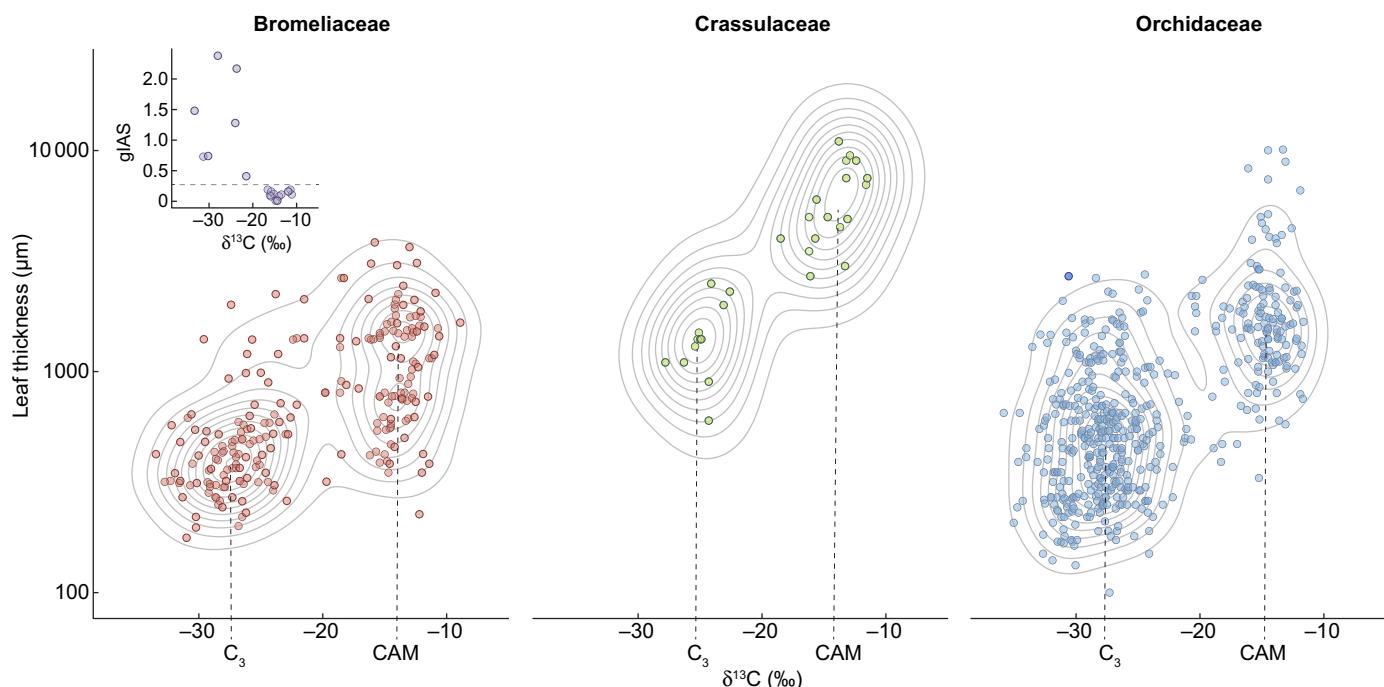
**Low-level CAM or weak CAM:** CAM cycle fixes small amount of CO<sub>2</sub> at night; could be respiratory and/or atmospheric CO<sub>2</sub>.

**Facultative CAM:** CAM cycle is upregulated as a stress response (typically drought); response can be reversed when stress is alleviated.

**Developmental CAM:** young tissue performs C<sub>3</sub> photosynthesis and CAM cycle is predictably induced as the tissue ages.

The total thickness of photosynthetic tissue and volumetric percentage of intercellular airspace (%IAS) are two additional traits that are both tightly associated with succulence and also carry physiological consequences for efficient carbon fixation (Ogburn & Edwards, 2013; Borland *et al.*, 2018). Thickness and low %IAS both reduce CO<sub>2</sub> diffusion through the mesophyll, which can lower C<sub>i</sub> and thus limit photosynthesis in C<sub>3</sub> plants (Evans, 1996). By contrast, low mesophyll conductance can actually boost CAM efficiency, as the high internal CO<sub>2</sub> concentrations that are reached during daytime decarboxylation will present less risk of loss of CO<sub>2</sub> back to the atmosphere if the conductance of the pathway is low (Maxwell *et al.*, 1997; Griffiths *et al.*, 2007; Nelson & Sage, 2008; Barrera Zambrano *et al.*, 2014; Borland *et al.*, 2018). These key traits (cell size, %IAS, tissue thickness) together present a potential anatomical antagonism within a plant between the efficiencies of its C<sub>3</sub> and CAM pathways, which must play a significant role in evolutionary transitions between C<sub>3</sub>+CAM and strong CAM states. Is the trade-off between C<sub>3</sub> and CAM optimality continuous, such that somewhere within a multi-dimensional morphospace, there is an area where both pathways are equally efficient? Or is this better modeled as a C<sub>3</sub>/CAM threshold, where certain anatomical configurations present steep changes in relative photosynthetic efficiencies between the two pathways?

We are still severely lacking detailed comparative data on this problem, and CAM photosynthesis models do not typically include anatomical parameters (e.g. Shameer *et al.*, 2018). In an unique study of a set of *Clusia* species that include C<sub>3</sub>, C<sub>3</sub>+CAM and strong CAM phenotypes, Barrera Zambrano *et al.* (2014) discovered a positive linear relationship between the size of photosynthetic palisade cells and the percentage of CO<sub>2</sub> uptake that occurred at night. The authors suggest that *Clusia* is distinct from other CAM-evolving groups in maintaining relatively high %IAS, which allows them to maintain C<sub>3</sub> function. They propose that *Clusia* solves the C<sub>3</sub>–CAM antagonism by maintaining high %IAS in the spongy mesophyll, to facilitate CO<sub>2</sub> diffusion in C<sub>3</sub> mode, while evolving large palisade cells to allow for sufficient malate storage in CAM mode. This particular leaf structure could possibly allow for efficient C<sub>3</sub> and CAM cycles and might explain the extreme photosynthetic flexibility of many *Clusia* species (Lüttge, 2006). Indeed, even *Clusia* species that have been categorized as ‘obligate



**Fig. 4** Contour density plots of leaf thickness and  $^{13}\text{C}$  values. The bimodal distribution of  $^{13}\text{C}$  values in each lineage is evident from the two distinct peaks in each contour plot. In some cases (Crassulaceae and, to a lesser degree, orchids) this is coupled with bimodality in leaf thickness, suggesting an anatomical threshold. This is not evident in bromeliads. However, Earles *et al.* (2018) recently presented more precise methods for estimating relevant anatomical parameters. The insert is their modeled intercellular airspace conductance (glAS,  $\text{mol m}^{-2} \text{s}^{-1} \text{bar}^{-1}$ ) in bromeliads; strong Crassulacean Acid Metabolism (CAM) bromeliads occupy a unique and limited range of glAS relative to bromeliads with  $\text{C}_3$ -like  $^{13}\text{C}$  values, suggestive of a strong anatomical threshold. Data from Males (2018), Silvera *et al.* (2005), Teeri *et al.* (1981), Earles *et al.* (2018).

CAM' fix a large proportion of carbon during the day (e.g. *Clusia rosea* > 50%; Barrera Zambrano *et al.*, 2014), and broader isotope surveys show that the majority of field-grown *Clusia* species primarily use  $\text{C}_3$  photosynthesis (Holtum *et al.*, 2004).

There are other lineage-based surveys that rely on cruder (but still informative) measures of both succulence and CAM activity, and they provide varying levels of support for a threshold model of  $\text{C}_3$ –CAM antagonism. Tissue thickness and  $^{13}\text{C}$  isotope values are the easiest traits to measure, and unsurprisingly are the best sampled (Fig. 4). In certain CAM-evolving clades, such as the Crassulaceae, a threshold model appears likely (Teeri *et al.*, 1981), whereas in others (Bromeliaceae), strong CAM plants are indistinguishable in leaf thickness from plants with a  $\text{C}_3$ -like  $^{13}\text{C}$  isotope value. In groundbreaking new work, Earles *et al.* (2018) present an anatomical study of a handful of  $\text{C}_3$  (or more likely,  $\text{C}_3$ +CAM) and strong CAM bromeliads using micro-CT scanning, providing the first 3D anatomical reconstructions of a succulent leaf. The authors developed several potentially significant new parameters for estimating  $\text{CO}_2$  diffusion constraints, including the tortuosity of the  $\text{CO}_2$  diffusion pathway and the total connectivity of the intercellular airspace. Remarkably, their strong CAM species occupied a very small and extremely low range of values for conductance of the intercellular airspace (glAS), which did not overlap with the very wide range of their  $\text{C}_3$  (or, more likely,  $\text{C}_3$ +CAM) species (Fig. 4 insert). This contrasts sharply with the bromeliad leaf thickness data, and provides strong support for a threshold evolutionary model, where the  $\text{C}_3$  pathway is favored

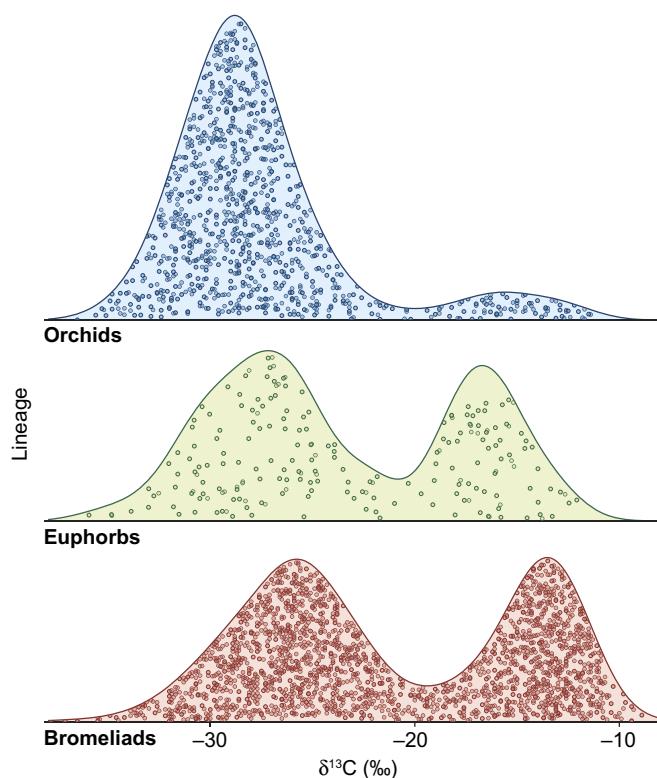
across a broad anatomical context, and a primarily CAM metabolism is favored only when  $\text{CO}_2$  diffusion becomes extremely limiting.

## VII. The CAM evolutionary trajectory (part 3): biochemistry first, anatomy second

As emphasized throughout, there is still not much concrete evidence for a clear CAM evolutionary trajectory; yet it is also possible, based on what we know about the biology and distribution of the  $\text{C}_3$ +CAM and strong CAM phenotypes, to generate some testable hypotheses. Available evidence suggests that  $\text{C}_3$ +CAM is an evolutionarily accessible phenotype and, further, that it may often act as a precursor to strong CAM (Edwards & Donoghue, 2006; Heyduk *et al.*, 2018). In a recent essay, Bräutigam *et al.* (2017) went so far as to suggest that *all* plants are essentially  $\text{C}_3$ +CAM, because several  $\text{C}_3$  species have been shown to accumulate malic and citric acids at night, which are then incorporated into amino acid synthesis during the day. The premise that the organization of a rudimentary CAM cycle is relatively simple has been proposed before (e.g. Winter *et al.*, 2015); however, Bräutigam *et al.* (2017) further imply that evolving strong CAM from a  $\text{C}_3$ +CAM state is just as simple, and proposed a continuous and smooth upregulation of CAM metabolism to a strong CAM phenotype. Silvera *et al.* (2010a) also present the 'CAM evolutionary continuum', suggesting continuous variation between  $\text{C}_3$  and strong CAM states. And yet we know from

extensive isotopic surveys that, in reality, there is a striking bimodal pattern to photosynthetic metabolism – in multiple CAM-evolving lineages most species use either mostly C<sub>3</sub>, or mostly CAM (Fig. 5). This suggests a more complicated evolutionary landscape.

Unlike the C<sub>4</sub> trajectory, which infers that the early phenotypes evolve rarely, creating an early rate-limiting step in C<sub>4</sub> evolution, I would argue that during the evolution of strong CAM, the early C<sub>3</sub>+CAM phenotypes are common and accessible, and the later anatomical changes that optimize a CAM metabolism happen more rarely. Thus the evolution of CAM is not a ‘continuum’ or simple ‘upregulation’ – like C<sub>4</sub>, it contains rate-limiting steps, and like C<sub>4</sub>, the rate-limiting step lies squarely in organismal structure (Fig. 6). In this model, the C<sub>3</sub>+CAM phenotype is both common and also evolutionarily stable. There are many advantages to being C<sub>3</sub>+CAM (Winter & Holtum, 2014), and most C<sub>3</sub>+CAM plants will likely never evolve into strong CAM species. This may be especially true for annual C<sub>3</sub>+CAM species, which often employ their CAM cycle at the end of the growing season, perhaps as a way to extend their reproductive output. From the broad phenotypic space that C<sub>3</sub>+CAM plants occupy, there may only be a subset of this space from which strong CAM has subsequently evolved. This space is likely only occupied by perennial species, with long-lived photosynthetic tissue, as there are no known strong CAM annual species. In this subset of perennial C<sub>3</sub>+CAM lineages, further



**Fig. 5** Isotopic surveys in Crassulacean Acid Metabolism (CAM)-evolving lineages. Bromeliads, euphorbias and orchids all contain a mix of C<sub>3</sub>, C<sub>3</sub>+CAM and strong CAM species, and are the best sampled lineages for <sup>13</sup>C isotopic values. All show a strong bimodal distribution of values, with most species showing either strong C<sub>3</sub>-like or strong CAM values, providing support for a threshold-like model of strong CAM evolution. Data from Silveira *et al.* (2010b), Crayn *et al.* (2015), Horn *et al.* (2014).

increases in succulence are required, and it is not until the C<sub>3</sub> pathway becomes limited by CO<sub>2</sub> diffusion that the evolution of a strong CAM phenotype is realized.

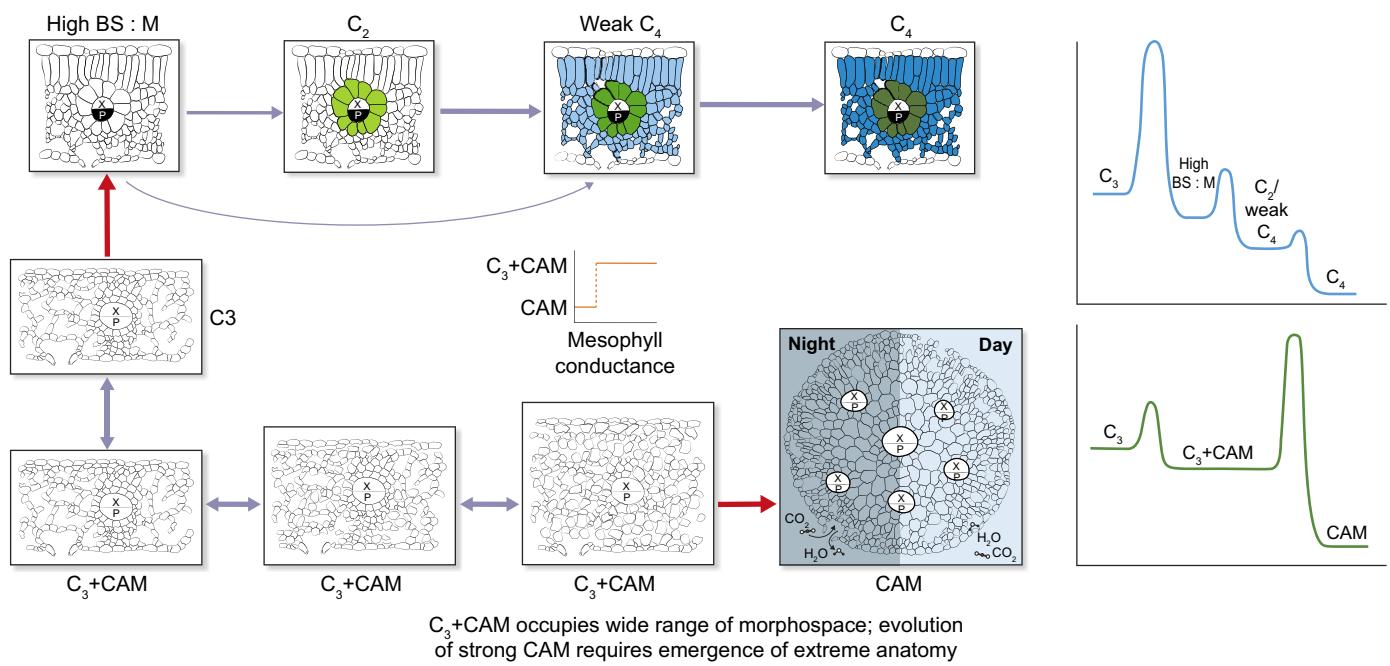
In this model, the tight functional link between extreme succulence and strong CAM makes it difficult to unpack which traits are selected for and which are playing the supporting role. Does increased succulence evolve under selection for increased water storage, and strong CAM subsequently evolves to overcome CO<sub>2</sub> limitation? Does strong CAM evolve to increase photosynthetic WUE, which requires increased succulence to improve the efficiency of CAM? Or are they co-selected, as increased WUE and increased water storage function perfectly together as elements of a drought-avoidance water use strategy (Ogburn & Edwards, 2010)? The hope in presenting these open questions is that they will motivate the multiple integrative phylogenetic studies that are so desperately needed.

### VIII. Conclusions: C<sub>4</sub> and CAM trajectories as mirrored images of evolutionary accessibility

The purported C<sub>4</sub> and CAM evolutionary trajectories I am favoring here have striking similarities and differences (Fig. 6). In both cases, I am arguing that the anatomical components of these adaptations are what limits their evolution, and the biochemical cycles are not particularly difficult to assemble. And yet their trajectories are nearly mirror images of one another. In the case of C<sub>4</sub>, the structural properties must be established first, in order to implement the spatial separation of PEPC and Rubisco; in the case of CAM, a low-level CAM cycle can occur without much anatomical specialization – rather, an extreme anatomy is required for the evolution of strong CAM. Whether the rate-limiting step occurs early or late in an evolutionary trajectory could profoundly influence phylogenetic patterns in the distribution of any complex adaptation. The long-recognized clustered origins of C<sub>4</sub> photosynthesis, for example (Sage *et al.*, 2011) are fully consistent with an early rate-limiting step, that once realized, results in many parallel origins. Although this has still not been explicitly tested, CAM evolution has long been assumed to be more phylogenetically diffuse (Smith & Winter, 1996). A greater phylogenetic scattering of strong CAM origins would be consistent with a late rate-limiting step, as the elevated accessibility afforded by a C<sub>3</sub>+CAM state is more readily realized by a greater diversity of lineages. This framework for thinking about evolutionary dynamics might help to explain how other convergent adaptations are more or less clustered across the tree of life (e.g. symbiotic N fixation (Soltis *et al.*, 1995), parasitism (Conn *et al.*, 2015), floral symmetry (Citerne *et al.*, 2010)).

### IX. Conclusions: organismal structure, anatomical pleiotropy and evolutionary innovation

How often do changes to the physical structure of an organism act as the rate-limiting step in evolutionary innovation? Pollination syndromes provide a useful analogy to C<sub>4</sub> and CAM: they consist of both structural (floral morphology) and biochemical (scent, pigmentation, nectar production) elements, and particular



**Fig. 6** Proposed  $C_4$  and Crassulacean Acid Metabolism (CAM) evolutionary trajectories. Rate-limiting steps indicated by a red arrow. In  $C_4$ , the rate-limiting step is early in the trajectory; once a certain anatomy is in place, a  $C_4$  metabolism is more evolutionarily accessible. A key intermediary is  $C_2$  photosynthesis, which creates an elevated  $CO_2$  concentration inside bundle sheath (BS) cells, facilitating more efficient Rubisco activity there. A weak  $C_4$  cycle quickly emerges, perhaps to correct a nitrogen imbalance between BS and mesophyll (M) cells, and from there a strong  $C_4$  cycle is all but inevitable. The arrow connecting high BS:M and weak  $C_4$  states indicates that the  $C_2$  stage, whereas perhaps the most common evolutionary path to  $C_4$ , is unlikely to be the only path. By contrast, during the evolution of strong CAM, a full CAM biochemical cycle evolves early and often and does not require much anatomical specialization, but plays a minor role in total carbon fixation. The evolution of a strong CAM plant, with CAM acting as the primary photosynthetic pathway, is dependent on further anatomical modifications associated with extreme photosynthetic succulence. This transition may be modeled as a threshold, where increases in succulence only result in 'tipping' toward CAM at the lowest limits of M conductance (e.g. Earles *et al.*, 2018). P, phloem; X, xylem.

syndromes have evolved multiple times independently. In a study of floral evolution in *Iochroma*, Smith *et al.* (2008) determined that floral tube length was less evolutionarily labile than either pigmentation or nectar production; remarkably, in this system also, morphological changes appear to act as the rate-limiting step in the repeated assembly of a complex phenotype.

These examples bear more generally on the role of development and resulting organismal structure in biasing evolutionary outcomes (Maynard-Smith *et al.*, 1985). At certain levels, this bias is obvious and uncontroversial. The remarkable diversity in floral form, for example, has evolved within the confines of an ordered set of whorled organs; only the Triuridaceae has managed to break this structure (possibly twice; Silva *et al.*, 2015). The evolution of a unifacial vs a bifacial cambium carried serious consequences for the types of arborescent life forms that different land plant lineages have managed to occupy (Donoghue, 2005). And the  $C_4$  and CAM evolutionary models presented here suggest that structural biases may be more pervasive than the handful of well-known examples, and operating at much finer scales. Why might organismal structure act as a primary influence on evolutionary trajectories? As Hutchinson (1965) likened ecology to a theater and evolution to a play, the physical structure of an organism may be thought of as another type of theater, one that hosts a biochemical play of immense complexity and an enormous cast. It is logical that the possibilities of what the characters might do is limited by the set

of the stage; it also is logical that any changes to the set will affect all character interactions, even unintended ones. This might be considered analogous to pleiotropic effects of individual genes and the role of pleiotropy in constraining or enabling evolutionary change. Returning to bundle sheath cells for a moment, one can immediately recognize the concept of 'anatomical pleiotropy': these cells are the nexus of interaction between the leaf mesophyll and the vascular bundle, and as such have pivotal roles in leaf development, protection and repair of hydraulic integrity, carbohydrate transport into and out of phloem, ion storage and N metabolism (Leegood, 2008; Griffiths *et al.*, 2013). It stands to reason that, considering all of the roles that these cells already play in all plants, any modification that would enable a new function to evolve might also significantly disrupt many other ongoing and essential functions. A strong anatomical 'pleiotropy' is one possible explanation of why structural changes may commonly emerge as rate-limiting steps in evolutionary trajectories.

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