



Tansley review

Evolutionary trajectories, accessibility and other metaphors: the case of C₄ and CAM photosynthesis

Author for correspondence:
Erika J. Edwards
Tel: +1 203 432 3869
Email: Erika.edwards@yale.edu

Erika J. Edwards

Department of Ecology and Evolutionary Biology, Yale University, 165 Prospect St, New Haven, CT 06520-8105, USA

Received: 3 December 2018

Accepted: 18 March 2019

Contents

Summary	1742	VII. The CAM evolutionary trajectory (part 3): biochemistry first, anatomy second	1750
I. Introduction	1743	VIII. Conclusions: C ₄ and CAM trajectories as mirrored images of evolutionary accessibility	1751
II. Evolutionary trajectories, adaptive landscapes and a new evolutionary metaphor: reaction kinetics	1743	IX. Conclusions: organismal structure, anatomical pleiotropy and evolutionary innovation	1751
III. Background on CAM and C ₄ syndromes	1744	Acknowledgements	1752
IV. The C ₄ evolutionary trajectory: anatomy first, biochemistry second	1747	References	1753
V. The CAM evolutionary trajectory (part 1): defining 'C ₃ +CAM' and 'strong CAM' phenotypes	1748		
VI. The CAM evolutionary trajectory (part 2): identifying the relevant anatomical parameters	1749		

Summary

New Phytologist (2019) **223**: 1742–1755
doi: 10.1111/nph.15851

Key words: C₄ photosynthesis, convergence, Crassulacean Acid Metabolism (CAM) photosynthesis, evolution, leaf anatomy, succulence.

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 C₄ 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 C₄ and CAM, focusing on the increasingly predictable stepwise evolutionary integration of anatomy and biochemistry. With the caveat that we currently understand C₄ evolution better than we do CAM, I propose a general model that explains and unites C₄ 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).

C₄ 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 CO₂ levels *c.* 30 million years ago (Ma) presented a global selection pressure on terrestrial photosynthesis (Edwards *et al.*, 2010). At low CO₂ 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 – C₄ and CAM photosynthesis (Fig. 1). Today, C₄ and CAM plants play fundamental ecological roles in the terrestrial biosphere, with C₄ 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, C₄ 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; Silveira *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). C₄ photosynthesis especially has been well studied, perhaps due to the importance of C₄ grasses in agriculture, and we now understand a considerable amount about the evolutionary trajectory of the C₄ syndrome. The same cannot yet be said of CAM, although this is beginning to change (Silveira *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 C₄ 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 C₄ 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

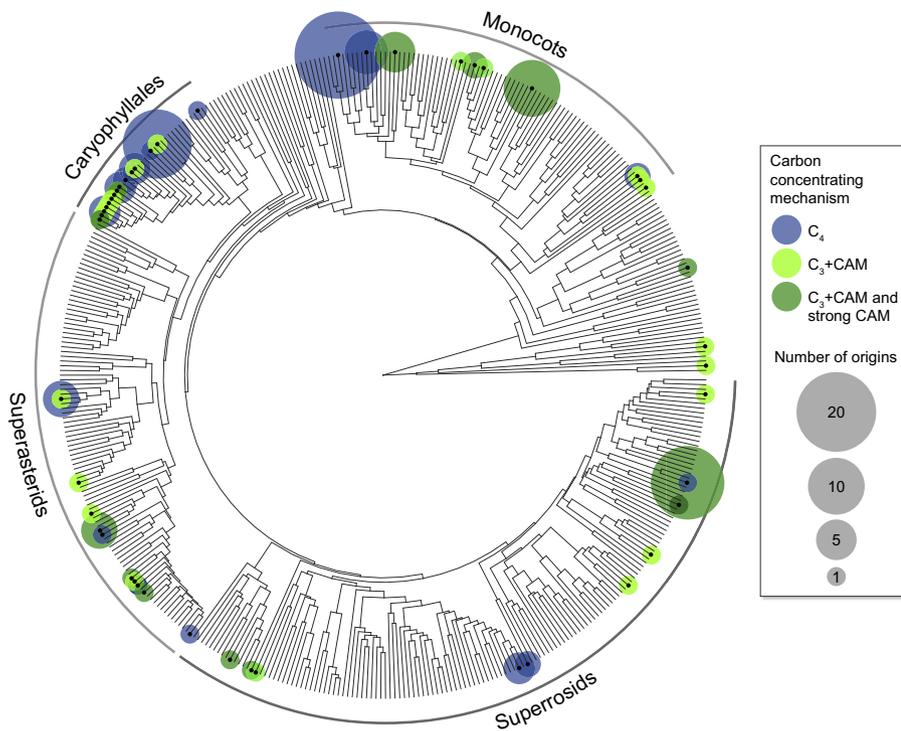


Fig. 1 Phylogenetic distribution of C_4 and Crassulacean Acid Metabolism (CAM) origins. Phylogeny of seed plant families, from Harris & Davies (2016). Blue circles represent C_4 origins, light green circles represent C_3 +CAM origins, and dark green circles represent lineages that contain C_3 +CAM as 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_4 , 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_4 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_4 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_4 syndromes

Most terrestrial plants perform C_3 photosynthesis, where the enzyme Rubisco catalyzes the reaction between atmospheric CO_2 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_2

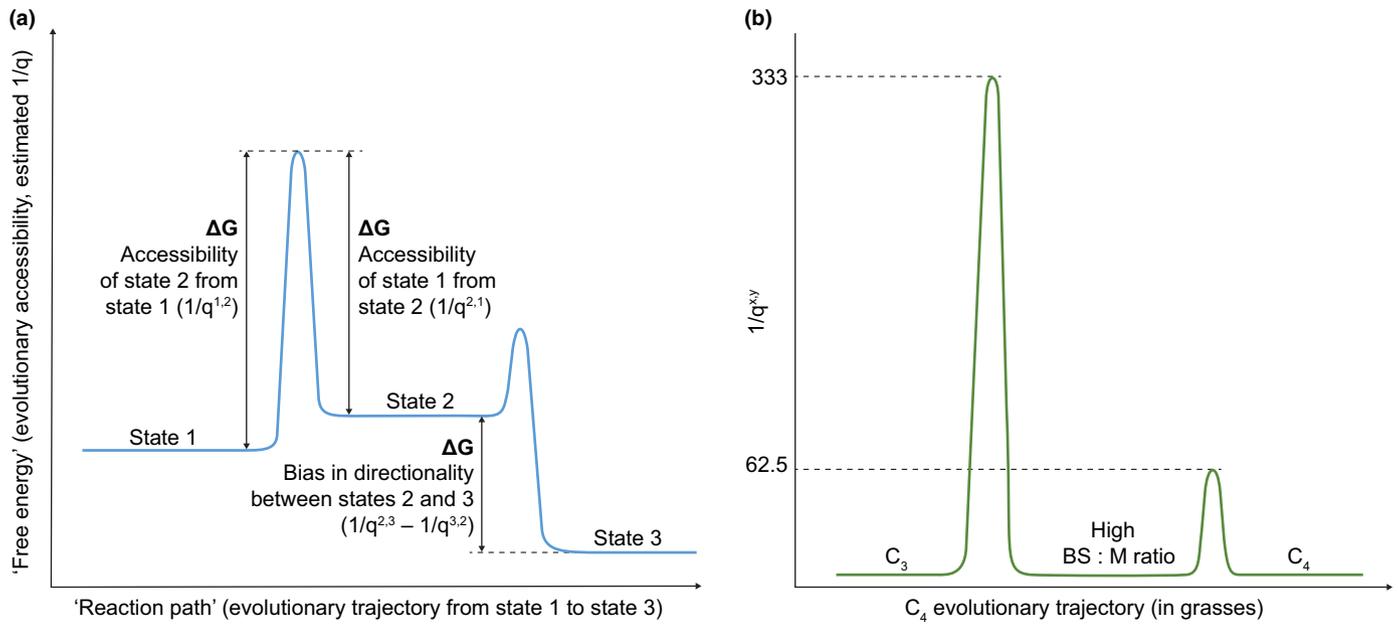


Fig. 2 Rate limiting steps in evolutionary trajectories. (a) A hypothetical 'reaction path' borrowed from chemical reaction kinetics, where 'state 1', 'state 2' and 'state 3' are phenotypic states occupied during the evolutionary transition from state 1 to state 3 (state 2 is akin to a chemical intermediate). 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 ΔG . (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

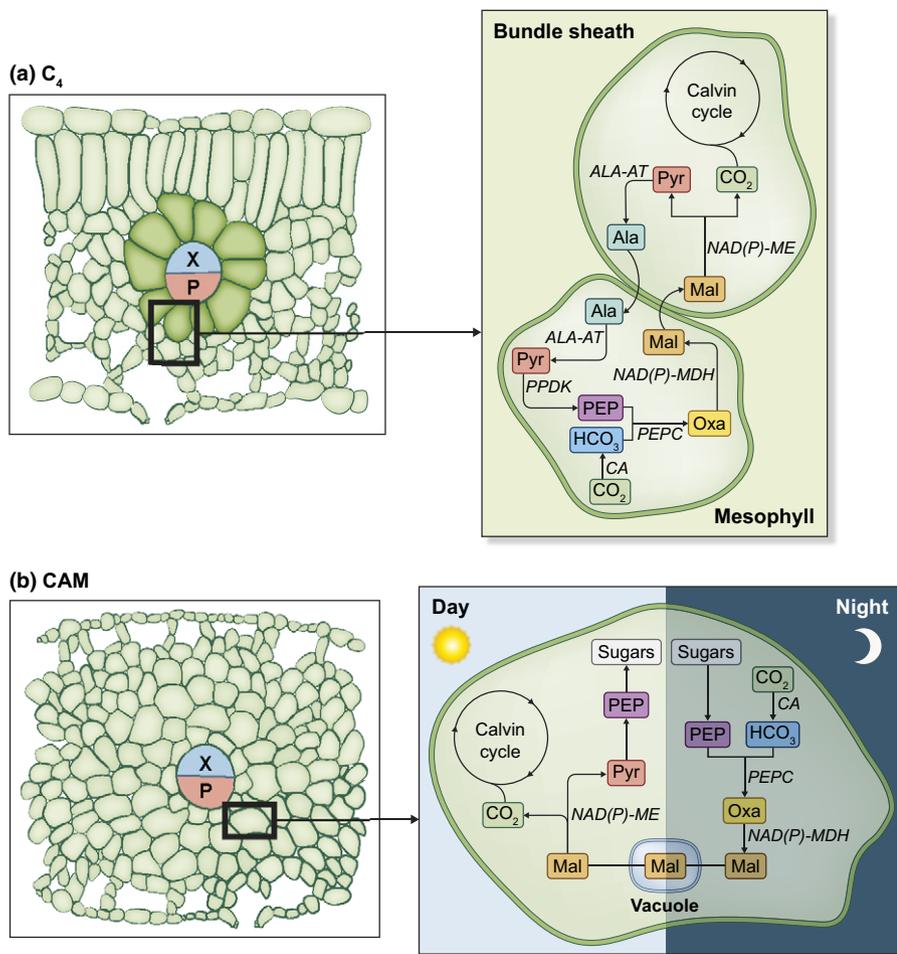


Fig. 3 The basics of C_4 and Crassulacean Acid Metabolism (CAM) biochemistry and anatomy. In C_4 plants, PEP Carboxylase fixes CO_2 in mesophyll cells, and malate is transferred to the bundle sheath for decarboxylation and subsequent fixation by Rubisco and the Calvin cycle. C_4 plants need a high bundle 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_2 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_2 diffusion in water places strong C limitations on photosynthesis (Keeley, 1998). CAM is an inherently more flexible photosynthetic system than C_4 , because mesophyll cells still possess a functional C_3 cycle, and so additional CO_2 may be taken up from the atmosphere directly via C_3 photosynthesis, even in strong CAM plants. In fact, a flexible ' C_3 +CAM' phenotype, where a plant is typically C_3 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_3 +CAM phenotype currently requires controlled experiments on living plants (Winter & Holtum, 2002, 2014).

At last count, C_4 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 (Silvera *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_4 .

In summary, C_4 and CAM utilize similar biochemistry, but the spatial and temporal configurations of enzymatic reactions are very different. Importantly, each pathway requires a 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_4 will simultaneously disadvantage a CAM metabolism (Sage, 2002). Likewise, C_4 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_4 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_2 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_4 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_4 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_4 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_4 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_4 lineages that also operate a facultative CAM cycle.

IV. The C_4 evolutionary trajectory: anatomy first, biochemistry second

We know a fair amount about how C_4 plants have evolved from their C_3 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_4 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_4 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_3 plants that are close relatives of C_4 plants, suggesting that a high BS : M trait is ancestral to the clade in question, and thus evolves before any implementation of a C_4 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_4 -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_4 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_4 photosynthesis, the authors interpreted high BS : M as an evolutionary 'enabler': a phenotype that affords a higher evolutionary accessibility to the C_4 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_4 -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_4 anatomy evolves before the development of even a rudimentary C_4 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_3 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_4 is adaptive.

With a high BS : M in place, the next purported step toward C_4 is the development of a less-efficient C-concentrating mechanism in bundle sheath cells, called ' C_2 ' photosynthesis (Monson & Rawsthorne, 2000; Sage *et al.*, 2012). In C_2 plants, the photorespiratory cycle has been partially arrested in mesophyll cells, before the conversion of glycine to serine and CO_2 by glycine decarboxylase (GDC). Glycine is instead shuttled to the bundle sheath where it re-enters the photorespiratory cycle, eventually producing a CO_2 molecule and thus elevating CO_2 concentrations in the BS. This gradient in CO_2 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_2 and C_4 photosynthesis has been modeled as a 'Mt Fuji' slope, with clear and strong directional selection toward a full C_4 cycle once a C_2 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_4 biochemical cycle. In C_2 plants, the processing of photorespiratory CO_2 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_4 biochemical cycle was the most likely and efficient means of rectifying the N imbalance. The Mallmann model suggests that C_2 plants should also operate a low-level C_4 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_4 phenotype has been assembled. To support their metabolic model, they presented gene expression profiles of C_2 , C_4 -like and full C_4 species of *Flaveria*, which indeed

show the predicted gradient of weak to strong expression of C_4 genes. If the Mallman model is accurate, then C_4 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_4 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_4 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_2 phenotype would be an evolutionarily short-lived state, as there is both a strong selection gradient toward C_4 , and a highly biased set of metabolic scenarios that would consistently facilitate the emergence of the C_4 biochemical cycle. Indeed, it seems that C_2 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_2 photosynthesis upwards of 15 Ma – and a full C_4 pathway was never assembled (Christin *et al.*, 2011). If C_2 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_2 model is compelling in large part because it is so logical, and also is supported by theory, modeling, and the phylogenetic proximity of C_2 and C_4 species in multiple lineages. But could C_4 also have evolved *without* passing through the C_2 state? The C_2 model was developed in large part by the intense study of *Flaveria*, which remains undoubtedly the most influential lineage in our models of C_4 evolution. In a recent review, (Sage *et al.*, 2018) provided a helpful list of C_4 lineages for which closely related C_2 species have been identified, which emphasized that the majority of C_4 lineages have no known close C_2 relatives. This could be due to several reasons: they exist but simply have not been discovered; the C_2 phase was short lived and the C_4 cycle emerged before a subsequent speciation event, leaving no record of this intermediate state in living taxa; C_2 species have since gone extinct; or, C_4 evolved without passing through a C_2 stage. With over 70 origins of the C_4 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_2 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_2 past' in the genomes of C_4 species that have no living C_2 relatives?

We also need to develop alternative hypothetical C_4 trajectories that do not involve a C_2 state. New ideas are materializing from continued study of *Alloteropsis*, a newly emerging model clade for

C_4 evolution (Dunning *et al.*, 2017). *Alloteropsis* may be the finest model clade yet for identifying the very early stages of C_4 emergence, as there is a full C_3 to C_4 physiological spectrum found within a single species, *Alloteropsis semialata* (Lundgren *et al.*, 2016). One potentially new realization is that the C_4 optimization stage may be longer and more elaborate than we have thought, and that many of the differences between distantly related C_3 and C_4 species evolved later in C_4 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_4 genes via lateral gene transfer from co-occurring C_4 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 'C₃+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_4 , and in fact do not have even the beginnings of a proposed model such as the 'anatomy-first' and C_2 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_3 cycle in their mesophyll cells, which means that the degree of expression of C_3 vs CAM is flexible, and can change depending on the age and physiological status of an individual plant. A C_4 leaf does not sometimes become a C_3 leaf; even a C_2 plant doesn't sometimes run its CCM and sometimes not. Thus, there are more clearly identifiable phenotypes along the C_3 – C_4 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_3 metabolism yet also express small amounts of CAM (Box 1). For the purposes of this review, I will lump these categories into a single 'C₃+CAM' phenotype (similar to the 'C₃-CAM' category of Winter *et al.*, 2015). The C₃+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 C₃+CAM. C₃+CAM cannot be identified with stable C isotope surveys, as these species

will carry the signature of a C_3 plant. Nor is C_3 +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_3 +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_3 +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_4 plants they are easily detected from the $^{13}C/^{12}C$ isotope ratio of their tissues, as PEPC and Rubisco show differential discrimination against the heavier ^{13}C isotopes (Farquhar *et al.*, 1982; O'Leary, 1988). Also like C_4 , 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_3 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_3 +CAM'.

- CAM-cycling*: CAM biochemistry is used to re-fix respiratory CO_2 , but does not fix atmospheric CO_2 .
- 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_2 at night; could be respiratory and/or atmospheric CO_2 .
- 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_3 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_2 diffusion through the mesophyll, which can lower C_i and thus limit photosynthesis in C_3 plants (Evans, 1996). By contrast, low mesophyll conductance can actually boost CAM efficiency, as the high internal CO_2 concentrations that are reached during daytime decarboxylation will present less risk of loss of CO_2 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_3 and CAM pathways, which must play a significant role in evolutionary transitions between C_3 +CAM and strong CAM states. Is the trade-off between C_3 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_3 /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_3 , C_3 +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_2 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_3 function. They propose that *Clusia* solves the C_3 -CAM antagonism by maintaining high %IAS in the spongy mesophyll, to facilitate CO_2 diffusion in C_3 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_3 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

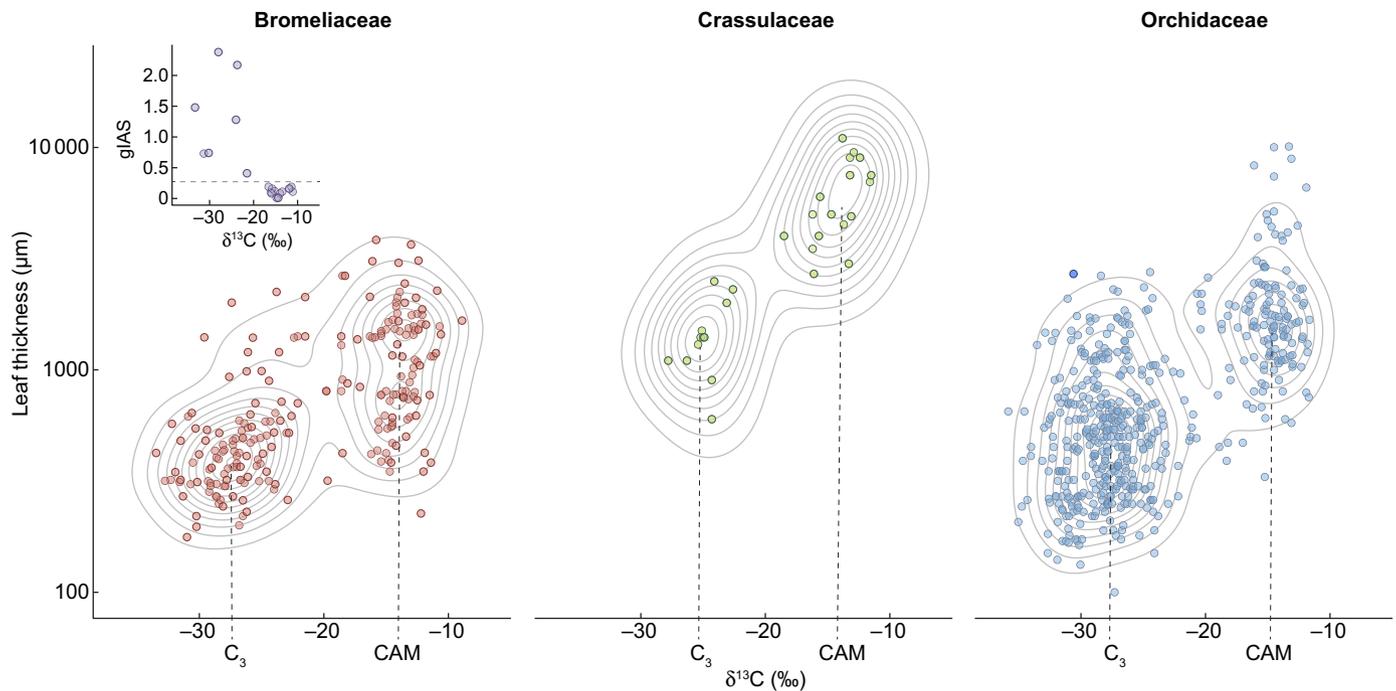


Fig. 4 Contour density plots of leaf thickness and ^{13}C values. The bimodal distribution of ^{13}C values in each lineage is evident from the two distinct peaks in each contour plot. In some cases (Crassulaceae and, to a lesser degree, orchids) this is coupled with bimodality in leaf thickness, suggesting 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 (g_{IAS} , $\text{mol m}^{-2} \text{s}^{-1} \text{bar}^{-1}$) in bromeliads; strong Crassulacean Acid Metabolism (CAM) bromeliads occupy a unique and limited range of g_{IAS} relative to bromeliads with C_3 -like ^{13}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 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 C_3 –CAM antagonism. Tissue thickness and ^{13}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 C_3 -like ^{13}C isotope value. In groundbreaking new work, Earles *et al.* (2018) present an anatomical study of a handful of C_3 (or more likely, 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 CO_2 diffusion constraints, including the tortuosity of the 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 (g_{IAS}), which did not overlap with the very wide range of their C_3 (or, more likely, 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 C_3 pathway is favored

across a broad anatomical context, and a primarily CAM metabolism is favored only when 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 C_3 +CAM and strong CAM phenotypes, to generate some testable hypotheses. Available evidence suggests that 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 C_3 +CAM, because several 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 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 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_3 , or mostly CAM (Fig. 5). This suggests a more complicated evolutionary landscape.

Unlike the C_4 trajectory, which infers that the early phenotypes evolve rarely, creating an early rate-limiting step in C_4 evolution, I would argue that during the evolution of strong CAM, the early C_3 +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_4 , it contains rate-limiting steps, and like C_4 , the rate-limiting step lies squarely in organismal structure (Fig. 6). In this model, the C_3 +CAM phenotype is both common and also evolutionarily stable. There are many advantages to being C_3 +CAM (Winter & Holtum, 2014), and most C_3 +CAM plants will likely never evolve into strong CAM species. This may be especially true for annual C_3 +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_3 +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_3 +CAM lineages, further

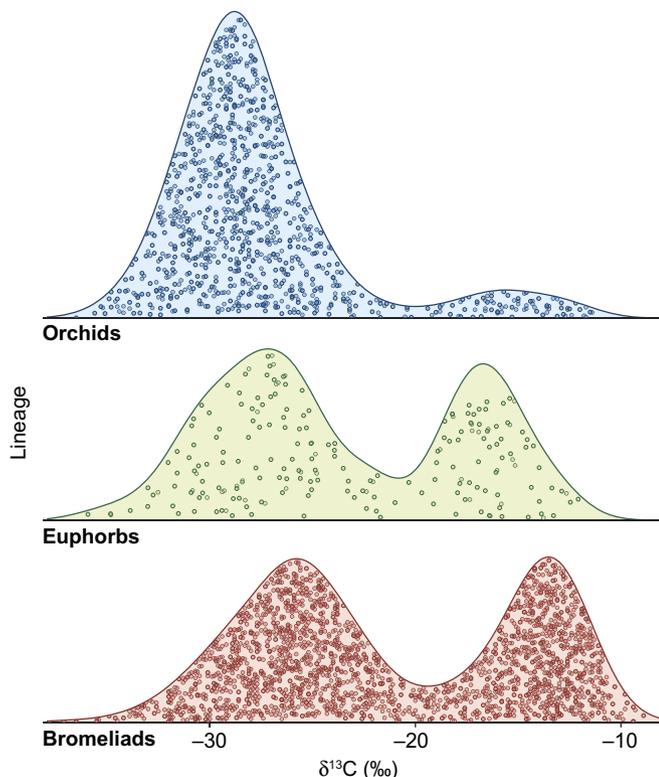


Fig. 5 Isotopic surveys in Crassulacean Acid Metabolism (CAM)-evolving lineages. Bromeliads, euphorbias and orchids all contain a mix of C_3 , C_3 +CAM and strong CAM species, and are the best sampled lineages for ^{13}C isotopic values. All show a strong bimodal distribution of values, with most species showing either strong C_3 -like or strong CAM values, providing support for a threshold-like model of strong CAM evolution. Data from Silvera *et al.* (2010b), Crayn *et al.* (2015), Horn *et al.* (2014).

increases in succulence are required, and it is not until the C_3 pathway becomes limited by CO_2 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_2 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_4 and CAM trajectories as mirrored images of evolutionary accessibility

The purported C_4 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_4 , 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_4 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_3 +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_4 and CAM: they consist of both structural (floral morphology) and biochemical (scent, pigmentation, nectar production) elements, and particular

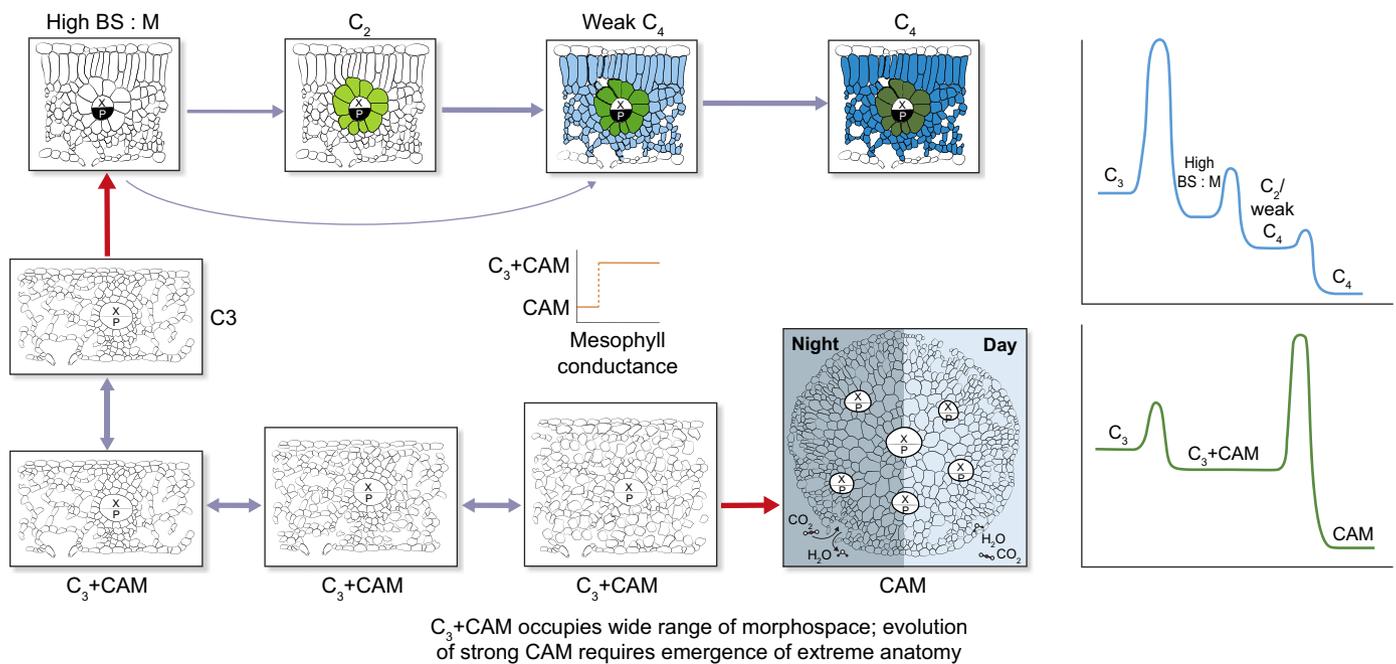


Fig. 6 Proposed C₄ and Crassulacean Acid Metabolism (CAM) evolutionary trajectories. Rate-limiting steps indicated by a red arrow. In C₄, the rate-limiting step is early in the trajectory; once a certain anatomy is in place, a C₄ metabolism is more evolutionarily accessible. A key intermediary is C₂ photosynthesis, which creates an elevated CO₂ concentration inside bundle sheath (BS) cells, facilitating more efficient Rubisco activity there. A weak C₄ cycle quickly emerges, perhaps to correct a nitrogen imbalance between BS and mesophyll (M) cells, and from there a strong C₄ cycle is all but inevitable. The arrow connecting high BS : M and weak C₄ states indicates that the C₂ stage, whereas perhaps the most common evolutionary path to C₄, 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 C₄ 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.

Acknowledgements

I would first like to thank *New Phytologist* for the invitation to write a Tansley review, and for having published such an inspiring set of thought-provoking review articles in this venue over the years. The ideas presented here have developed over time and only because of the great luck I have had in working with so many talented people

on these problems, including Monica Arakaki, Pascal-Antoine Christin, Ian Gilman, Lillian Hancock, Karolina Heyduk, Joseph Holtum, Jose Moreno-Villena, Matt Ogburn, Colin Osborne, Rowan Sage, Caroline Stromberg, Klaus Winter and all members of the Edwards lab, past and present. I would like to especially thank Miriam Diaz and Christopher Still for first introducing me to the worlds of CAM and C_4 , respectively. The broader C_4 and CAM research communities are such dynamic, exciting, and welcoming groups of scientists, and I'm so grateful to be a part of them. A special thank you to Michael Donoghue for his unwavering support over the last 20 years, and his endless enthusiasm for sitting around and talking through an idea. Finally, I thank Joel Abraham and Elissa Martin for their great help with figures, and Editor Mark Rausher and 2 anonymous reviewers, whose suggestions significantly improved an earlier draft. This work was supported in part by the National Science Foundation (DEB-1026611, DEB-1252921 and IOS-1754662).

References

- Arakaki M, Christin PA, Nyffeler R, Lendel A, Egli U, Ogburn RM, Spriggs E, Moore MJ, Edwards EJ. 2011. Contemporaneous and recent radiations of the world's major succulent plant lineages. *Proceedings of the National Academy of Sciences, USA* 108: 8379–8384.
- Aubry S, Brown NJ, Hibberd JM. 2011. The role of proteins in C_3 plants prior to their recruitment into the C_4 pathway. *Journal of Experimental Botany* 62: 3049–3059.
- Barrera Zambrano VA, Lawson T, Olmos E, Fernández-García N, Borland AM. 2014. Leaf anatomical traits which accommodate the facultative engagement of crassulacean acid metabolism in tropical trees of the genus *Clusia*. *Journal of Experimental Botany* 65: 3513–3523.
- Bone RE, Smith JAC, Arrigo N, Buerki S. 2015. A macro-ecological perspective on crassulacean acid metabolism (CAM) photosynthesis evolution in Afro-Madagascan drylands: Eulophiinae orchids as a case study. *Journal of Integrative and Comparative Biology* 208: 469–481.
- Borland AM, Leverett A, Hurtado-Castano N, Hu R, Yang X. 2018. Functional anatomical traits of the photosynthetic organs of plants with crassulacean acid metabolism. Including bioenergy and related processes. In: Adams WW, Terashima I, eds. *The leaf: a platform for performing photosynthesis*. Cham, Switzerland: Springer Nature, 281–305.
- Boyce CK, Knoll AH. 2002. Evolution of developmental potential and the multiple independent origins of leaves in Paleozoic vascular plants. *Paleobiology* 28: 70–100.
- Bräutigam A, Schlüter U, Eisenhut M, Gowik U. 2017. On the Evolutionary Origin of CAM Photosynthesis. *Plant Physiology* 174: 473–477.
- Christin P, Sage T, Edwards E, Ogburn R, Khoshravesh R, Sage R. 2011. Complex evolutionary transitions and the significance of C_3 - C_4 intermediate forms of photosynthesis in Molluginaceae. *Evolution; International Journal of Organic Evolution* 65: 643–660.
- Christin PA, Arakaki M, Osborne CP, Braeutigam A, Sage RF, Hibberd JM, Kelly S, Covshoff S, Wong GK-S, Hancock L *et al.* 2014. Shared origins of a key enzyme during the evolution of C_4 and CAM metabolism. *Journal of Experimental Botany* 65: 3609–3621.
- Christin PA, Edwards EJ, Besnard G, Boxall SF, Gregory R, Kellogg EA, Hartwell J, Osborne CP. 2012. Adaptive evolution of C_4 photosynthesis through recurrent lateral gene transfer. *Current Biology* 22: 445–449.
- Christin PA, Osborne CP. 2014. The evolutionary ecology of C_4 plants. *Journal of Integrative and Comparative Biology* 204: 765–781.
- Christin PA, Osborne CP, Chatelet DS, Columbus JT, Besnard G, Hodkinson TR, Garrison LM, Vorontsova MS, Edwards EJ. 2013. Anatomical enablers and the evolution of C_4 photosynthesis in grasses. *Proceedings of the National Academy of Sciences, USA* 110: 1381–1386.
- Citerne H, Jabbour F, Nadot S, Damerval C. 2010. The Evolution of Floral Symmetry. *Advances in Botanical Research* 54: 85–137.
- Cockburn W. 1985. Tansley Review No 1. Variation in photosynthetic acid metabolism in vascular plants: CAM and related phenomena. *New Phytologist* 101: 3–24.
- Conn CE, Bythell-Douglas R, Neumann D, Yoshida S, Whittington B, Westwood JH, Shirasu K, Bond CS, Dyer KA, Nelson DC. 2015. Convergent evolution of strigolactone perception enabled host detection in parasitic plants. *Science* 349: 540–543.
- Crayn DM, Winter K, Schulte K, Smith JAC. 2015. Photosynthetic pathways in Bromeliaceae; phylogenetic and ecological significance of CAM and C_3 based on carbon isotope ratios for 1893 species. *Botanical Journal of the Linnean Society* 178: 169–221.
- Crayn DM, Winter K, Smith JAC. 2004. Multiple origins of crassulacean acid metabolism and the epiphytic habit in the Neotropical family Bromeliaceae. *Proceedings of the National Academy of Sciences, USA* 101: 3703–3708.
- Donoghue MJ. 2005. Key innovations, convergence, and success: macroevolutionary lessons from plant phylogeny. *Paleobiology* 31: 77–93.
- Donoghue MJ, Edwards EJ. 2019. Model clades are vital for comparative biology, and ascertainment bias is not a problem in practice: a response to Beaulieu and O'Meara (2018). *American Journal of Botany* 106: 327–330.
- Dunning LT, Lundgren MR, Moreno Villena JJ, Namaganda M, Edwards EJ, Nosil P, Osborne CP, Christin PA. 2017. Introgression and repeated co-option facilitated the recurrent emergence of C_4 photosynthesis among close relatives. *Evolution; International Journal of Organic Evolution* 71: 1541–1555.
- Earles JM, Theroux-Rancourt G, Roddy AB, Gilbert ME, McElrone AJ, Brodersen CR. 2018. Beyond porosity: 3D leaf intercellular airspace traits that impact mesophyll conductance. *Plant Physiology* 178: 148–162.
- Edwards EJ. 2014. The inevitability of C_4 photosynthesis. *eLife* 3: e03702.
- Edwards EJ, Chatelet DS, Chen B-C, Ong JY, Tagane S, Kanemitsu H, Tagawa K, Teramoto K, Park B, Chung K-F *et al.* 2017. Convergence, consilience, and the evolution of temperate deciduous forests. *The American Naturalist* 190: S87–S104.
- Edwards EJ, Donoghue MJ. 2006. *Pereskia* and the origin of the cactus life-form. *American Naturalist* 167: 777–793.
- Edwards GE, Franceschi VR, Voznesenskaya EV. 2004. Single-cell C_4 photosynthesis versus the dual-cell (Kranz) paradigm. *Annual Review of Plant Biology* 55: 173–196.
- Edwards EJ, Ogburn RM. 2012. Angiosperm responses to a low- CO_2 world: CAM and C_4 photosynthesis as parallel evolutionary trajectories. *International Journal of Plant Sciences* 173: 724–733.
- Edwards E, Osborne C, Stromberg C, Smith S, C_4 Grasses Consortium. 2010. The origins of C_4 grasslands: integrating evolutionary and ecosystem science. *Science (New York, N.Y.)* 328: 587–591.
- Edwards EJ, de Vos JM, Donoghue MJ. 2015. Doubtful pathways to cold tolerance in plants. *Nature* 521: E5–E6.
- Ehleringer JR, Cerling TE, Helliker BR. 1997. C_4 photosynthesis, atmospheric CO_2 and climate. *Oecologia* 112: 285–299.
- Ehleringer JR, Sage RF, Flanagan LB, Pearcy RW. 1991. Climate change and the evolution of C_4 photosynthesis. *Trends in Ecology & Evolution (Personal Edition)* 6: 95–99.
- Evans JR. 1996. Carbon dioxide diffusion inside leaves. *Plant Physiology* 110: 339.
- Farquhar GD, O'Leary MH, Berry JA. 1982. On the relationship between carbon isotope discrimination and the intercellular carbon dioxide concentration in leaves. *Australian Journal of Plant Physiology* 11: 539–552.
- Finnigan GC, Hanson-Smith V, Stevens TH, Thornton JW. 2012. Evolution of increased complexity in a molecular machine. *Nature* 481: 360–364.
- Fisher AE, McDade LA, Kiel CA, Khoshravesh R, Johnson MA, Stata M, Sage TL, Sage RF. 2015. Evolutionary history of *Blepharis* (Acanthaceae) and the origin of C_4 photosynthesis in section *Acanthodium*. *International Journal of Plant Sciences* 176: 770–790.
- Fleming TH, Kress WJ. 2011. A brief history of fruits and frugivores. *Acta Oecologica* 37: 521–530.
- Goolsby EW, Moore AJ, Hancock LP, de Vos JM, Edwards EJ. 2018. Molecular evolution of key metabolic genes during transitions to C_4 and CAM photosynthesis. *American Journal of Botany* 105: 602–613.
- Gould SJ, Vrba ES. 1982. Exaptation—a missing term in the science of form. *Paleobiology* 8: 4–15.

- Gowik U, Westhoff P. 2011. The path from C₃ to C₄ photosynthesis. *Plant Physiology* 155: 56–63.
- Grass Phylogeny Working Group II. 2012. New grass phylogeny resolves deep evolutionary relationships and discovers C₄ origins. *New Phytologist* 193: 304–312.
- Griffiths H, Robe WE, Girnus J, Maxwell K. 2007. Leaf succulence determines the interplay between carboxylase systems and light use during Crassulacean acid metabolism in *Kalanchoe* species. *Journal of Experimental Botany* 59: 1851–1861.
- Griffiths H, Weller G, Toy LFM, Dennis RJ. 2013. You're so vein: bundle sheath physiology, phylogeny and evolution in C₃ and C₄ plants. *Plant, Cell & Environment* 36: 249–261.
- Guralnick LJ, Edwards G, Ku MSB, Hockema B, Franceschi V. 2002. Photosynthetic and anatomical characteristics in the C₄–crassulacean acid metabolism–cycling plant *Portulaca grandiflora*. *Functional Plant Biology* 29: 763–773.
- Hancock L, Edwards EJ. 2014. Phylogeny and the inference of evolutionary trajectories. *Journal of Experimental Botany* 65: 3491–3498.
- Hancock LP, Holtum JAM, Edwards EJ. 2019. The evolution of CAM photosynthesis in Australian Calandrinia reveals lability in C₃+CAM phenotypes and a possible constraint to the evolution of strong CAM. *Integrative and Comparative Biology* icz089.
- Harris LW, Davies TJ. 2016. A complete fossil-calibrated phylogeny of seed plant families as a tool for comparative analyses: testing the 'time for speciation' hypothesis. *PLoS ONE* 11: e0162907.
- Heckmann D, Schulze S, Denton A, Gowik U, Westhoff P, Weber APM, Lercher MJ. 2013. Predicting C₄ photosynthesis evolution: modular, individually adaptive steps on a Mount Fuji fitness landscape. *Cell* 153: 1579–1588.
- Hetherington AJ, Dolan L. 2018. Stepwise and independent origins of roots among land plants. *Nature* 561: 235.
- Heyduk K, McKain MR, Lalani F, Leebens-Mack J. 2016. Evolution of a CAM anatomy predates the origins of Crassulacean acid metabolism in the Agavoideae (Asparagaceae). *Molecular Phylogenetics and Evolution* 105: 102–113.
- Heyduk K, Moreno-Villena JJ, Gilman I, Christin PA, Edwards EJ. 2019. The genetics of convergent evolution: insights from plant photosynthesis. *Nature Reviews Genetics*. doi: 10.1038/s41576-019-0107-5.
- Heyduk K, Ray JN, Ayyampalayam S, Mack JL. 2018. Shifts in gene expression profiles are associated with weak and strong Crassulacean acid metabolism. *American Journal of Botany* 105: 587–601.
- Holtum JAM, Aranda J, Virgo A, Gehrig HH, Winter K. 2004. $\delta^{13}\text{C}$ values and crassulacean acid metabolism in *Clusia* species from Panama. *Trees—Structure and Function* 18: 658–668.
- Holtum JAM, Hancock LP, Edwards EJ, Winter K. 2017a. Optional use of CAM photosynthesis in two C₄ species, *Portulaca cyclophylla* and *Portulaca digyna*. *Journal of Plant Physiology* 214: 91–96.
- Holtum JAM, Hancock LP, Edwards EJ, Winter K. 2017b. Facultative CAM photosynthesis (crassulacean acid metabolism) in four species of *Calandrinia*, ephemeral succulents of arid Australia. *Photosynthesis Research* 134: 17–25.
- Holtum JAM, Hancock LP, Edwards EJ, Winter K. 2018. Crassulacean acid metabolism in the Basellaceae (Caryophyllales) (R Leegood, Ed.). *Plant Biology* 20: 409–414.
- Horn JW, Xi Z, Riina R, Peirson JA, Yang Y, Dorsey BL, Berry PE, Davis CC, Wurdack KJ. 2014. Evolutionary bursts in *Euphorbia* (Euphorbiaceae) are linked with photosynthetic pathway. *Evolution* 68: 3485–3504.
- Hutchinson GE. 1965. *The ecological theater and the evolutionary play*. New Haven, CT, USA: Yale University Press.
- Kadereit G, Ackerly D, Pirie MD. 2012. A broader model for C₄ photosynthesis evolution in plants inferred from the goosefoot family (Chenopodiaceae s.s.). *Proceedings of the Royal Society of London. Series B: Biological Sciences* 279: 3304–3311.
- Kadereit G, Borsch T, Weising K, Freitag H. 2003. Phylogeny of Amaranthaceae and Chenopodiaceae and the evolution of C₄ photosynthesis. *International Journal of Plant Sciences* 164: 959–986.
- Keeley J. 1998. CAM photosynthesis in submerged aquatic plants. *The Botanical Review* 64: 121–175.
- Keeley J, Rundel P. 2003. Evolution of CAM and C₄ carbon-concentrating mechanisms. *International Journal of Plant Sciences* 164: S55–S77.
- Khoshravesh R, Akhani H, Sage TL, Nordenstam B, Sage RF. 2012. Phylogeny and photosynthetic pathway distribution in *Anticharis* Endl. (Scrophulariaceae). *Journal of Experimental Botany* 63: 5645–5658.
- Kluge M, Ting IP. 1978. Morphology, anatomy, and ultrastructure of CAM plants. In: Kluge M, Ting IP, eds. *Crassulacean acid metabolism: analysis of an ecological adaptation*. Berlin, Germany: Springer, 29–44.
- Koch K, Kennedy RA. 1980. Characteristics of Crassulacean Acid Metabolism in the succulent C₄ Dicot, *Portulaca oleracea* L. *Plant Physiology* 65: 193–197.
- Kozaki A, Takeba G. 1996. Photorespiration protects C₃ plants from photooxidation. *Nature* 384: 557–560.
- Kraybill AA, Martin CE. 1996. Crassulacean acid metabolism in three species of the C₄ genus *Portulaca*. *International Journal of Plant Sciences* 157: 103–109.
- Ku MSB, Monson RK, Littlejohn RO Jr, Nakamoto H, Fisher DB, Edwards GE. 1983. Photosynthetic characteristics of C₃–C₄ intermediate *Flaveria* species: I. Leaf anatomy, photosynthetic responses to O₂ and CO₂ and activities of key enzymes in the C₃ and C₄ pathways. *Plant Physiology* 71: 944–948.
- Lara MV. 2004. Induction of a Crassulacean Acid-like Metabolism in the C₄ succulent plant, *Portulaca oleracea* L.: study of enzymes involved in carbon fixation and carbohydrate metabolism. *Plant and Cell Physiology* 45: 618–626.
- Leegood RC. 2008. Roles of the bundle sheath in leaves of C₃ plants. *Journal of Experimental Botany* 59: 1663–1673.
- Lundgren MR, Christin PA, Escobar EG, Ripley BS, Besnard G, Long CM, Hattersley PW, Ellis RP, Leegood RC, Osborne CP. 2016. Evolutionary implications of C₃–C₄ intermediates in the grass *Alloterospis semialata*. *Plant, Cell & Environment* 39: 1874–1885.
- Lüttge U. 2006. Photosynthetic flexibility and ecophysiological plasticity: questions and lessons from *Clusia*, the only CAM tree, in the neotropics. *New Phytologist* 171: 7–25.
- Males J. 2018. Concerted anatomical change associated with crassulacean acid metabolism in the Bromeliaceae. *Functional Plant Biology* 45: 681–695.
- Mallmann J, Heckmann D, Bräutigam A, Lercher MJ, Weber AP, Westhoff P, Gowik U, Weigel D. 2014. The role of photorespiration during the evolution of C₄ photosynthesis in the genus *Flaveria*. *eLife* 3: e02478.
- Maxwell K, von Caemmerer S, Evans JR. 1997. Is a low internal conductance to CO₂ diffusion a consequence of succulence in plants with Crassulacean Acid Metabolism? *Functional Plant Biology* 24: 777–786.
- Maynard-Smith JM, Burian R, Kauffman S, Alberch P, Campbell J, Goodwin B, Lande R, Raup D, Wolpert L. 1985. Developmental constraints and evolution: a perspective from the Mountain Lake Conference on Development and Evolution. *The Quarterly Review of Biology* 60: 265–287.
- Mazen AMA. 2001. Changes in properties of phosphoenolpyruvate carboxylase with induction of Crassulacean Acid Metabolism (CAM) in the C₄ plant *Portulaca oleracea*. *Photosynthetica* 38: 385–391.
- McKown AD, Moncalvo J-M, Dengler NG. 2005. Phylogeny of *Flaveria* (Asteraceae) and inference of C₄ photosynthesis evolution. *American Journal of Botany* 92: 1911–1928.
- Meyer JR, Dobias DT, Weitz JS, Barrick JE, Quick RT, Lenski RE. 2012. Repeatability and contingency in the evolution of a key innovation in phage lambda. *Science (New York, N.Y.)* 335: 428–432.
- Monson R, Rawsthorne S. 2000. CO₂ assimilation in C₃–C₄ intermediate plants. In: Leegood R, Sharkey T, von Caemmerer S, eds. *Photosynthesis: physiology and metabolism*. Dordrecht, the Netherlands: Kluwer Academic, 533–550.
- Nelson EA, Sage RF. 2008. Functional constraints of CAM leaf anatomy: tight cell packing is associated with increased CAM function across a gradient of CAM expression. *Journal of Experimental Botany* 59: 1841–1850.
- Nelson EA, Sage TL, Sage RF. 2005. Functional leaf anatomy of plants with crassulacean acid metabolism. *Functional Plant Biology* 32: 409–419.
- Nobel PS. 1988. *Environmental biology of agaves and cacti*. New York, NY, USA: Cambridge University Press.
- Nyffeler R, Eggli U, Ogburn RM, Edwards EJ. 2008. Variations on a theme: repeated evolution of succulent life forms in the Portulacaceae. *Haseltonia* 14: 26–36.
- Ocampo G, Koteyeva NK, Voznesenskaya EV, Edwards GE, Sage TL, Sage RF, Columbus JT. 2013. Evolution of leaf anatomy and photosynthetic pathways in Portulacaceae. *American Journal of Botany* 100: 2388–2402.
- Ogburn RM, Edwards EJ. 2010. The ecological water-use strategies of succulent plants. *Advances in Botanical Research* 55: 179–225.

- Ogburn RM, Edwards EJ. 2013. Repeated origin of three-dimensional leaf venation releases constraints on the evolution of succulence in plants. *Current Biology* 23: 722–726.
- O'Leary MH. 1988. Carbon isotopes in photosynthesis. *BioScience* 38: 328–336.
- Raven JA, Spicer RA. 1996. The evolution of crassulacean acid metabolism. *Ecological Studies: Analysis and Synthesis* 114: 360–385.
- Riedl R. 1978. *Order in living organisms*. New York, NY: John Wiley & Sons.
- Sage RF. 2001. Environmental and evolutionary preconditions for the origin and diversification of the C₄ photosynthetic syndrome. *Plant Biology* 3: 202–213.
- Sage RF. 2002. Are crassulacean acid metabolism and C-4 photosynthesis incompatible? *Functional Plant Biology* 29: 775–785.
- Sage R. 2004. The evolution of C₄ photosynthesis. *New Phytologist* 161: 341–370.
- Sage RF. 2016. A portrait of the C₄ photosynthetic family on the 50th anniversary of its discovery: species number, evolutionary lineages, and Hall of Fame. *Journal of Experimental Botany* 67: 4039–4056.
- Sage RF, Christin PA, Edwards EJ. 2011. The C₄ plant lineages of planet Earth. *Journal of Experimental Botany* 62: 3155–3169.
- Sage RF, Monson RK, Ehleringer JR, Adachi S, Pearcy RW. 2018. Some like it hot: the physiological ecology of C₄ plant evolution. *Oecologia* 187: 941–966.
- Sage RF, Sage TL, Kocacinar F. 2012. Photorespiration and the evolution of C₄ photosynthesis. *Annual Review of Plant Biology* 63: 19–47.
- Sage RF, Zhu X-G. 2011. Exploiting the engine of C₄ photosynthesis. *Journal of Experimental Botany* 62: 2989–3000.
- de Santo AV, Alfani A, Russo G, Fiochetto A. 1983. Relationship between CAM and succulence in some species of Vitaceae and Piperaceae. *Botanical Gazette* 144: 342–346.
- Scott DH. 1901. On the structure and affinities of fossil plants from the Palaeozoic rocks. IV. The seed-like fructification of Lepidocarpon, a genus of Lycopodiaceae cones from the Carboniferous Formation. *Philosophical Transactions of the Royal Society of London. Series B: Biological Sciences* 194: 291–333.
- Shameer S, Baghalian K, Cheung CYM, Ratcliffe RG, Sweetlove LJ. 2018. Computational analysis of the productivity potential of CAM. *Nature Plants* 4: 165–171.
- Silva FV, Matías SE, Ambrose BA, Santana SV, Mena AM, Guzmán JM, Martínez E, Meyerowitz EM, Buylia ERA. 2015. Inside-out flowers characteristic of *Lacandonia schismatica* evolved at least before its divergence from a closely related taxon, *Triuris brevistylis*. *International Journal of Plant Sciences* 164: 345–357.
- Silvera K, Neubig KM, Whitten WM, Williams NH, Winter K, Cushman JC. 2010a. Evolution along the crassulacean acid metabolism continuum. *Functional Plant Biology* 37: 995–1010.
- Silvera K, Santiago LS, Cushman JC, Winter K. 2009. Crassulacean Acid Metabolism and epiphytism linked to adaptive radiations in the Orchidaceae. *Plant Physiology* 149: 1838–1847.
- Silvera K, Santiago LS, Cushman J, Winter K. 2010b. The incidence of crassulacean acid metabolism in Orchidaceae derived from carbon isotope ratios: a checklist of the flora of Panama and Costa Rica. *Botanical Journal of the Linnean Society* 163: 194–222.
- Silvera K, Santiago LS, Winter K. 2005. Distribution of crassulacean acid metabolism in orchids of Panama: evidence of selection for weak and strong modes. *Functional Plant Biology* 32: 397–407.
- Smith JAC, Winter K. 1996. Taxonomic distribution of crassulacean acid metabolism. In: Smith JAC, Winter K, eds. *Crassulacean acid metabolism: biochemistry, ecophysiology and evolution*. Berlin, Germany: Springer, 427–436.
- Smith SD, Ané C, Baum DA. 2008. The role of pollinator shifts in the floral diversification of *Ipomoea* (Solanaceae). *Evolution; International Journal of Organic Evolution* 62: 793–806.
- Soltis DE, Soltis PS, Morgan DR, Swensen SM, Mullin BC, Dowd JM, Martin PG. 1995. Chloroplast gene sequence data suggest a single origin of the predisposition for symbiotic nitrogen fixation in angiosperms. *Proceedings of the National Academy of Sciences, USA* 92: 2647–2651.
- Stadler BM, Stadler PF, Wagner GP, Fontana W. 2001. The topology of the possible: formal spaces underlying patterns of evolutionary change. *Journal of Theoretical Biology* 213: 241–274.
- Stedle E, Smith JAC, Lüttge U. 1980. Water-relation parameters of individual mesophyll cells of the crassulacean acid metabolism plant *Kalanchoë daigremontiana*. *Plant Physiology* 66: 1155–1163.
- Still CJ, Berry JA, Collatz GJ, Defries RS. 2003. Global distribution of C₃ and C₄ vegetation: carbon cycle implications. *Global Biogeochemical Cycles* 17: e1006.
- Teeri JA, Tonsor SJ, Turner M. 1981. Leaf thickness and carbon isotope composition in the Crassulaceae. *Oecologia* 50: 367–369.
- Vogan PJ, Frohlich MW, Sage RF. 2007. The functional significance of C₃ C₄ intermediate traits in *Heliotropium* L. (Boraginaceae): gas exchange perspectives. *Plant, Cell & Environment* 30: 1337–1345.
- Weinreich DM, Delaney NF, DePristo MA, Hartl DL. 2006. Darwinian evolution can follow only very few mutational paths to fitter proteins. *Science* 312: 111–114.
- Werker E. 2000. Trichome diversity and development. *Advances in Botanical Research* 31: 1–35.
- Williams BP, Johnston IG, Covshoff S, Hibberd JM. 2013. Phenotypic landscape inference reveals multiple evolutionary paths to C₄ photosynthesis. *eLife* 2: e00961.
- Winter K, Holtum JAM. 2002. How closely do the δ¹³C values of crassulacean acid metabolism plants reflect the proportion of CO₂ fixed during day and night? *Plant Physiology* 129: 1843–1851.
- Winter K, Holtum JAM. 2014. Facultative crassulacean acid metabolism (CAM) plants: powerful tools for unravelling the functional elements of CAM photosynthesis. *Journal of Experimental Botany* 65: 3425–3441.
- Winter K, Holtum JAM, Smith JAC. 2015. Crassulacean acid metabolism: a continuous or discrete trait? *Journal of Integrative and Comparative Biology* 208: 73–78.
- Winter K, Sage RF, Edwards EJ, Virgo A, Holtum JAM. 2019. Facultative CAM in a C₃-C₄ intermediate. *Journal of Experimental Botany*. doi: 10.1093/jxb/erz085.
- Woods RJ, Barrick JE, Cooper TF, Shrestha U, Kauth MR, Lenski RE. 2011. Second-order selection for evolvability in a large *Escherichia coli* population. *Science (New York, N.Y.)* 331: 1433–1436.
- Wright S. 1932. The roles of mutation, inbreeding, crossbreeding and selection in evolution. In: *Proceedings of the sixth international congress of genetics*. Brooklyn NY, USA: Brooklyn Botanical Gardens, 356–366.
- Yuan M, Xu F, Wang S-D, Zhang D-W, Zhang Z-W, Cao Y, Xu X-C, Luo M-H, Yuan S. 2012. A single leaf of *Camellia oleifera* has two types of carbon assimilation pathway, C₃ and crassulacean acid metabolism. *Tree Physiology* 32: 188–199.
- Zotz G, Ziegler H. 1997. The occurrence of crassulacean acid metabolism among vascular epiphytes from Central Panama. *Journal of Integrative and Comparative Biology* 137: 223–229.