Photosynthetic and growth responses of C_3 and C_4 grasses to short-duration sunflecks and resultant consequences for their performance in understory environments.

Thesis submitted in fulfilment of the requirements for the degree of

MASTER OF SCIENCE of RHODES UNIVERSITY

By

CLAIRE ELIZABETH ADAMS

2016

Abstract

Low C_4 grass species abundance in understory environments is thought to be as a result of their high-light requirements, lack of photosynthetic advantage relative to C_3 species in cooler environments, and an inability to adequately utilise sunflecks. This study sets out to investigate this theory, hypothesizing that C_3 grass species outperform C_4 grass species under the canopy, not as a result of quantum efficiency temperature effects, but as a result of C_4 species inability to utilize short-duration sunflecks. Short sunflecks could result in a breakdown in assimilate movement between the mesophyll (MSC) and bundle sheath (BSC) cells. The role of BSC leakiness, stomata and PSII efficiency on the ability of C_3 and C_4 *Alloteropsis semialata* to utilize short-duration sunflecks was investigated using gaseous exchange and chlorophyll fluorescence techniques, while the growth of both subspecies under a simulated flecking-light environment tested whether these measured responses translated into effects on growth. As C_3 grasses are known to possess higher levels of stomatal conductance in relation to C_4 species, results showed that C_3 *A. semialata* was able to utilize short-duration sunflecks as a result of increased stomatal conductance and an ability to induce photosynthesis under various light flecking conditions. In contrast, C_4 *A. semialata* was unable to utilize sunflecks possibly as a result of energetic limitations of the carboxylation mechanism (PSII) and not because of increased bundle sheath leakiness. These photosynthetic responses translated into growth differences when both types were grown in an artificially flecking light environment. The photosynthetic differences noted for C_3 and C_4 *A.semialata* were also evident in shade adapted understory grasses; *Erharta erecta* (C_3), *Dactylotenium australe* (C_4) and *Brachiaria chusqueoides* (C_4). As photosynthetic induction was marginally more rapid in all species relative to *A. semialata* it suggests some degree of adaptation in shade grasses, however the inability of the C_4 species to utilise short sunflecks remained. As a result, it was hypothesized that C_3 shade

adapted *E. erecta* and C_4 *B. chusqueoides*, co-inhabiting the same forest understory, are able to do so because of differences in light micro-environments associated with each species. However, canopy openness and light profiles determined for theses micro-climates showed no differences and that both species have persisted within a relatively stable understory environment. The co-existence was possible as the understory was supplied with a significant proportion of its daily light in flecks sufficiently long as to not compromise C_4 productivity. A survey of 10 species of grasses under various canopy densities and in the open showed a strong negative linear relationship between canopy openness and the rate at which photosynthesis was induced by flecking light, which has not been shown before. This did not result from the phylogenetic relationship between species and could be shown for a single species (*E. erecta)* growing in a range of light environments. This demonstrates that C_4 grasses, despite adaptation, would be limited from sunfleck environments if a significant proportion of the daily light available consists of flecks of short-duration. The generation of such environments may occur as a result of woody thickening and could help explain the observed decline in C_4 grasses under these conditions.

Table of Contents

List of Tables

List of Figures

Acknowledgements

- Firstly, I would like to say thank you to my incredible family! Mom and Dad, I could never have accomplished this without all of your support (both financial and emotional). Thank you for all your words of encouragement and investing so much of your energies into me, giving me belief in myself when I never thought I would be able to complete a Masters. Rossy, my sibling, thank you for always being there for me when I needed help, an ear to listen to me and for your endless amounts of wisdom.
- Brad, thank you so much for agreeing to take me on as a Masters student! For your guidance, advice and copious amounts of support and patience. Thank you for making me believe in myself again and restoring my love for science.
- To my best friends, Lara and Holly. Thank you for your endless support and time... I would never have been able to get through this without you. Sean, thank you for putting up with me during this time, helping me laugh and always being there for me (especially at home and driving me around). To all my other friends, Connor and Dustin Laming, Lance Ho, Adam Kramer, Morgan Hunter, Greg Woolf, Tessa Gallagher, Sam Beckley, Amy Richardson, Gaby Ayres, Lavinia Perumal and Cara Thorne, thank you for all your support (especially helping motivate me at the Rat when my morale was low!).
- Thank you to all the staff at the Botany Department for all of your help and positive attitudes.
- Thank you to all the people who helped contribute and analyze parts of my data. Kimberley Simpson for your endless help with stats or every time I had any question. Pascal-Antoine for all your help with the phylogenetic work, Michael Cramer for writing copious amounts of R code for my photo diode analysis and together with Simon C. Power access to one of your data sets.

Declaration

This dissertation, submitted for the degree of Master of Science in the Department of Botany, Rhodes University, represents original work by the author and has not been submitted in any form to any other institution. Where mention has been made of the work of others, it has been duly acknowledged in the text.

.....

CLAIRE ELIZABETH ADAMS

2016

I certify that the above statement is correct.

.....

PROFESSOR BRAD. S RIPLEY

Supervisor

CHAPTER ONE: LOW C_4 ABUNDANCE IN SHADE ENVIRONMENTS AND POTENTIAL UNDERLYING MECHANISMS.

1.1. C_4 grasses are excluded from shade environments

Although the majority of terrestrial plants utilize the C_3 photosynthetic pathway, the pathway is limited by low concentrations of CO_2 and high temperatures which exacerbate photorespiration. The issue of photorespiration was solved by the evolution of the C_4 pathway, whereby morphological and biochemical adaptations allowed for the concentration of CO_2 around the O_2 sensitive enzyme, ribulose-1, 5-bisphosphate carboxylase/oxygenase (Rubisco) in the bundle sheath allowing for increased rates of photosynthesis at lower mesophyll CO_2 concentrations, decreased stomatal conductance and warmer leaf temperatures(Chaffey, 1999; Sage and Kubien, 2007). This evolution of C_4 photosynthesis in response to increasing temperatures and decreasing levels of carbon dioxide (CO_2) experienced by plants in the early Oligocene (32-25 Myr) has been viewed by many scientists as an adaptation to open and arid environments (Osborne and Freckleton, 2009). As a result of being a 'sun specialist', C_4 grasses are not commonly found in shaded environments below the canopy and, under these cooler, lower light conditions, are unable to compete against other C_3 grass species (Ehleringer and Björkman, 1977; Pearcy and Calkin, 1983; Chazdon and Pearcy, 1986*a*; Ehleringer *et al.*, 1997).

The distribution of C_4 grasses has been shown to be influenced by various climatic variables and temperature effects have been a focal point explaining these patterns from North

America to Australia (Chazdon and Fetcher, 1984*a*; Klink and Joly, 1989). These studies place C_4 species in hot, dry areas where they have increased carboxylation efficiency (CE) when compared to C_3 species and show the converse relationship in cooler environments where C_3 species are no longer limited by the cost of photorespiration. However other studies have shown that temperature alone cannot explain the lack of C_4 species in the cooler, shaded environments and have begun to look at the role that light has on species distributions (Hofstra *et al.*, 1972; Ehleringer and Björkman, 1977; Chazdon and Pearcy, 1986*a*; Klink and Joly, 1989; Horton and Neufeld, 1998; Edwards and Smith, 2010).

In order to look at the effect of light under different thermal conditions, Ehleringer, (1978) constructed a model to calculate primary production of C_3 and C_4 grasses based on a) the light and temperature intensities experienced by each leaf, b) the light absorption and attenuation within the canopy, c) the temperature experienced throughout the day and d) the daily path of light at different latitudes and declinations (Ehleringer, 1978). With these calculations he was able to simulate both the effect of canopy size (LAI) and different environmental regimes on distribution in order to predict C_4 abundance in different habitats. When simulating shaded environments (light that was 10% of light experienced at the top of the canopy) C_4 grasses were not found in cold, shaded areas but were found in the shade when temperatures exceeded 32^{o}C. This therefore highlights that the decrease in quantum yield limits C_4 distribution in cooler climates but cannot explain the exclusion of C_4 grasses based on quantum efficiency from shaded canopies in warmer, tropical climates (Ehleringer, 1978).

The exclusion of C_4 grasses based on light was further investigated by Klink & Joly (1989) in open and closed forest patches in Brazil. During this investigation, 55 C_4 grass species were identified, of which 48 species were found to be full sun species and the remaining 7 shade species were found in less dense areas of the closed canopy. In contrast, 23 C_3 species were identified, where the 3 sun species identified were found in open areas with

water saturated soils (Klink and Joly, 1989). This exclusion of C_4 species from shaded environments was further illustrated by Hofstra (1972), identifying only 1 of the 77 C_4 grass species on the tropical island, Java (Indonesia), in the shade (Hofstra *et al.*, 1972).

1.2. Why are C_4 species excluded from the shade? - Two major hypotheses

The majority of studies have hypothesized that C_4 species would be excluded from shaded environments based on quantum yield, while fewer have proposed a different mechanism, based on limitations imposed on the carbon concentrating mechanism employed by C_4 species.

1.2.1. Quantum efficiency

Previous literature has focused on the C_3 pathway as conferring a competitive advantage in low light environments as C_3 plants are able to function at higher quantum efficiency in low light and cooler environments, when compared to C_4 plants (Ehleringer and Björkman, 1977; Sage, 2004; Rabinowitch, 1951; Singsaas *et al.*, 2001). Quantum efficiency depends on the carboxylase and oxygenase activities of Rubisco, the former producing carbohydrates and the latter wasting energy via photorespiration. High temperature favours the oxygenation reaction, initiating photorespiration and making C_3 plants less efficient under these conditions. In contrast, the C_4 photosynthetic system greatly reduces photorespiration by concentrating CO_2 around Rubisco in the bundle sheath, almost eliminating the oxygenation reaction of Rubisco. However, the C_4 concentrating mechanism carries additional costs and makes quantum efficiency lower than that of C_3 plants in cooler conditions (Ehleringer and Björkman, 1977; Ehleringer *et al.*, 1997; Chaffey, 1999).

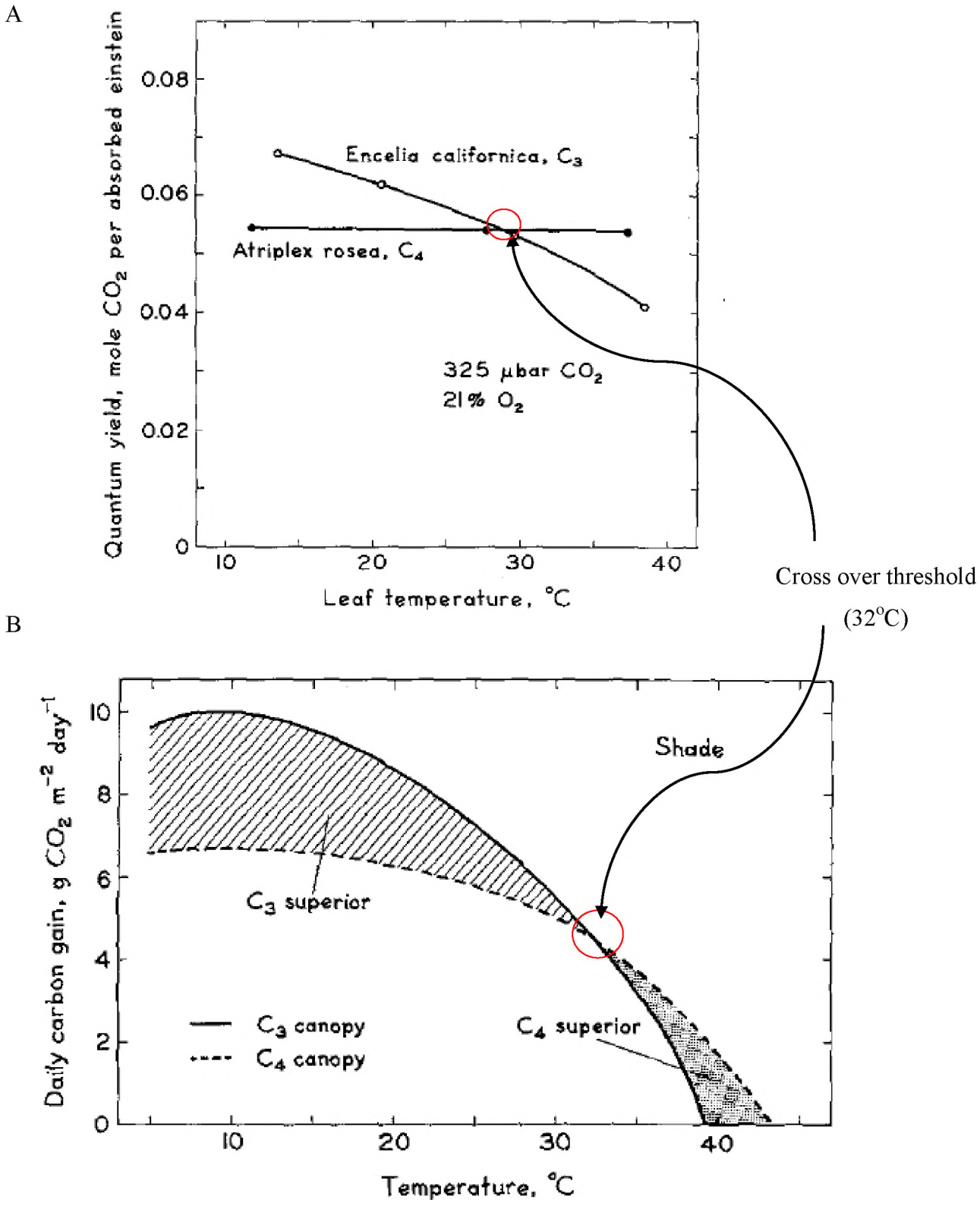

Figure 1-1. Simulated responses of selected C_3 and C_4 grass species as a function of leaf temperature. This figure was adapted from data presented by Ehleringer, 1977 (Ehleringer

and Björkman, 1977). (A) Differences in Quantum yield of CO_2 between C_3 and C_4 species as a function of temperature. (B) Total daily carbon gain for identical C_3 and C_4 grass canopies in a shaded habitat (10% full sun) as a function of temperature. The red circle highlights the cross over threshold temperature modelled by Ehleringer, 1977; where C_3 species lose their competitive ability against C_4 species based on temperature (32°C).

Hence, authors have argued that because shaded environments are cooler, the energetics of C_3, over C_4 photosynthesis, would be favoured (Ehleringer and Björkman, 1977; Sage, 2004). This competitive advantage experienced by C_3 species in the shade will only hold in areas with a temperature below 32°C. At higher temperatures, C_4 energetics would be more efficient (Ehleringer and Björkman, 1977).

1.2.2. Biochemical limitation of the CCM

Another hypothesis is that the complex biochemistry of the C_4 system, which has a high energetic cost, would lead to a slower utilization of sunflecks, direct flashes of saturating light which penetrate the canopy to reach the ground below. The inability to utilize these sunflecks would explain why C_4 plants are not generally found in shaded environments (Pearcy *et al.*, 1985). Slower utilization of sunflecks may result from the inhibition of the coordination between the mesophyll and bundle sheath metabolism which is spatially separated within the leaves (Pearcy, 1983; Sage, 2014; Sage and Stata, 2015). This spatial separation of the dual-cell photosynthetic mechanism utilised by C_4 species, requires that large fluxes of metabolites are moved between the mesophyll (MSC) and bundle sheath (BSC) cells, where C_4 acids are decarboxylated in the MSC, providing high concentrations of CO_2 to the C_3 cycle (Fig. 1-2).

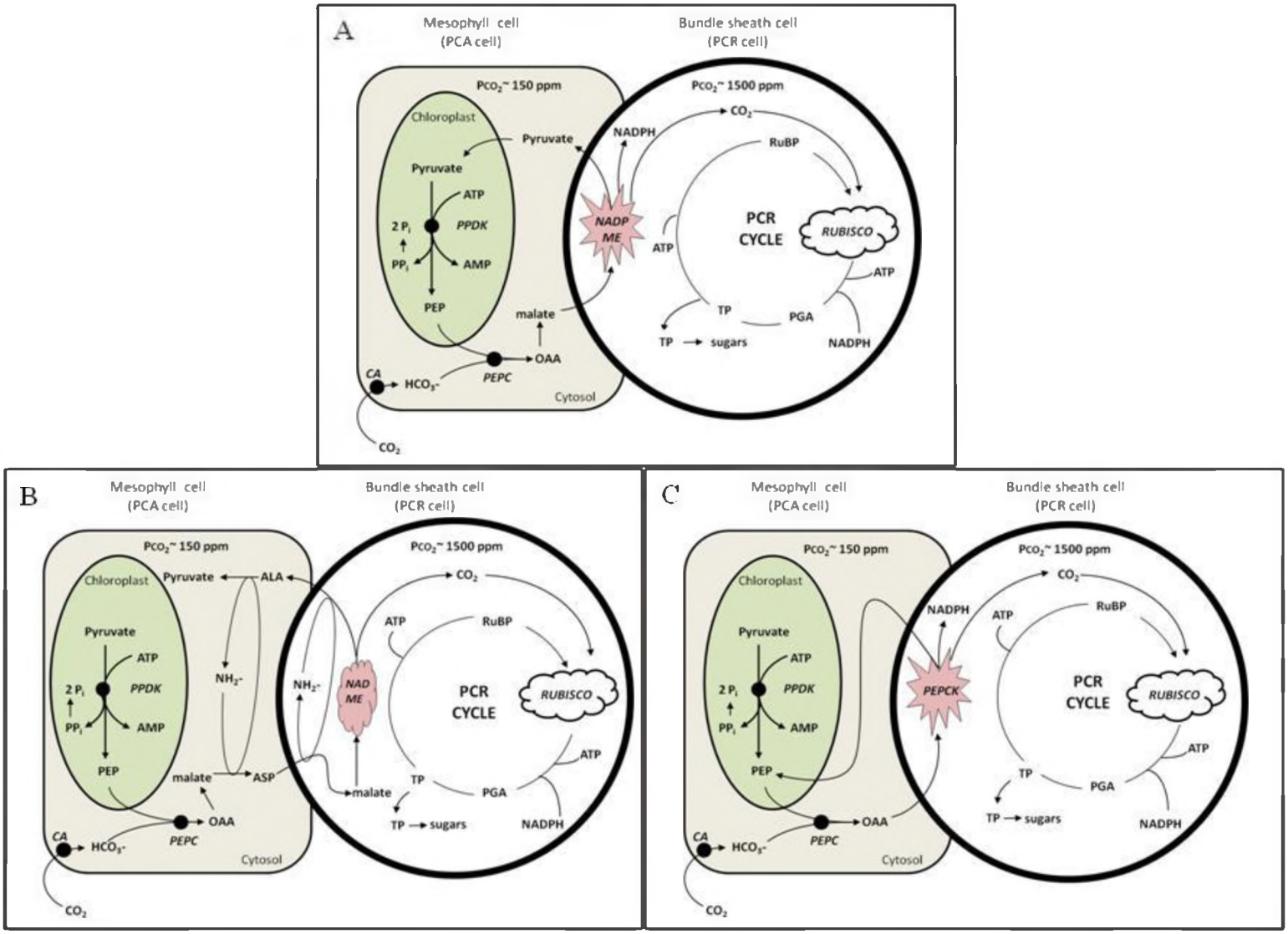

Figure 1-2. Diagrammatic representation of the various C_4 photosynthetic pathways based on their biochemical subtype. a. NADP-malic enzyme (NADP-ME) subtype; b. NAD-malic enzyme subtype (NAD-ME) and c. PEP carboxykinase (PEPCK) subtype. ASP: aspartate; ALA: alanine; AMP: adenosine monophosphate; ATP: adenosine triphosphate; CA: carbonic anhydrase; NADPH: nicotinamide adenine dinucleotide phosphate; OAA: oxaloacetic acid; PEPC: PEP carboxylase; PGA: 3-phosphoglyceric acid; PPDK: pyruvate phosphate dikinase; PPi: pyrophosphate; RuBP: ribulose-1, 5-bisphosphate; TP: triose phosphate. NH2- and circular arrows indicate the presence of transamination cycles in both PCA and PCR cell types. Green ovals indicate chloroplasts while red stars indicate the enzyme by which each subtype is classified. Italicised words indicate enzymes. The cycle begins in the same manner in all C_4 pathways. Carbonic anhydrase (CA) converts CO_2 into bicarbonate, which is subsequently turned into PEP via PEPCase, producing a four-carbon

organic acid, oxaloacetic acid (OAA) in the cytosol of the MSC. From this point, three biochemical subtypes are recognized based on the principle enzyme that decarboxylates OAA in the BSC. (Sage, 2004; Gowik and Westhoff, 2011; Sage et al., 2012).These include the NADP-malic enzyme (NADP-ME) subtype, the NAD-malic enzyme subtype (NAD-ME) and the PEP carboxykinase (PEPCK) subtype. The C_4 subtype using NADP-ME possesses BS cells that are altered to possess lower numbers of mitochondria and chloroplasts. In this pathway OAA is converted into malate which then diffuses to the site of Rubisco. During this decarboxylation, the pyruvate formed is shuttled back to the MSC where it is converted back into PEP (A). In contrast, the BS cells of plants utilizing the NAD-ME subtype contain numerous numbers of mitochondria and chloroplasts (Sage, 2004; Gowik and Westhoff, 2011; Sage et al., 2012). In this subtype, OAA is converted into aspartate which then diffuses to the site of Rubisco. The pyruvate formed during the carboxylation step is first transaminated to form alanine before being shuttled back to the MSC and converted back into PEP (B). The last subtype PEPCK differs from the others, where PEP is formed during the decarboxylation step and can therefore move straight back into the outer compartment to be carboxylated by PEPCase (C) (Sage, 2004; Gowik and Westhoff, 2011; Sage et al., 2012). Ultimately, 18 ATP and 12 NADPH molecules are required to fix 6 molecules of CO2 for the synthesis of one molecule of hexose sugar in the PCR cycle (Sage, 2004; Gowik and Westhoff, 2011; Sage et al., 2012).

The open nature of this system allows for leakiness, the retro-diffusion of CO_2 from the BSC to the mesophyll cytosol, wasting the energy that was initially used to produce the C_4 metabolites. Under conditions of low-light, C_4 mesophyll processes have been proposed to oversupply the bundle sheath with C_4 acids, exacerbating the loses due to leakiness and decreasing C_4 efficiency in the shade (Evans and Von Caemmerer, 1996). However, this leakiness is reduced by acclimation to low-light conditions (Bellasio and Griffiths, 2014*a*; Sage, 2014). Acclimation is not fully understood, but is proposed to result from either regulation of ATP partitioning between the C_3 and C_4 cell types, or by down regulation of the activity of PEP carboxylase, reducing the flux of CO_2 to the BSC (Bellasio and Griffiths, 2014*b*).

C_4 plants reduced ability to utilise sunflecks is proposed to arise from their more complex photosynthetic metabolism which, on illumination requires a longer period to induce photosynthesis, making the utilisation of short-duration sunflecks inefficient (Evans *et al.*, 1993; Evans and Von Caemmerer, 1996; Tazoe *et al.*, 2008; Sage, 2014). If sunflecks are of a duration that are sufficient to initiate mesophyll processes, but are not sustained long enough for bundle sheath processes to completely use resultant CO_2, leakiness is increased or, at higher light intensities, photosystem II (PSII) activity may be effected (Kubásek *et al.*, 2013; Kromdijk *et al.*, 2014; Sage, 2014; Wang *et al.*, 2014*b*). Under non-steady state illumination, CO_2 uptake and O_2 evolution, the concentration of mesophyll and bundle sheath intermediates, and enzyme activity become uncoordinated between C_3 and C_4 cycles (Usuda, 1985; Laisk and Edwards, 1997; Cousins *et al.*, 2007; Sun *et al.*, 2014). As a result, the cost of increased leakiness or the decreased PSII activity in conjunction with the inability to coordinate metabolite transfer under variable light conditions outweighs the advantage of decreased photorespiration. Depending on C_4 photosynthetic subtype the transfer of metabolites also includes the movement of reductant to the bundle sheath and decreased transport may limit carboxylation due to an inadequacy of reductant. Collectively, these limitations could diminish the C_4 competitive advantage in sunfleck environments, allowing C_3 plants to dominate.

1.3. An evolutionary context

The limitation of these studies explaining the lack of C_4 grasses in the shade is that few make comparisons with their closest C_3 relatives. As majority of C_3 grass species belong to the Pooideae, which shares a common ancestor with the C_4 dominated PACMAD clade, the

role of phylogeny on grass responses to shade becomes important when trying to understand what limits C_4 grass distributions. A study by Edwards and Smith (2010), utilized geospatial and sequence data, along with climate data to create a grass taxon phylogeny of 1 230 species, where this unambiguously phylogenetic approach allowed for the assessment of evolutionary history of climate niches for grasses on a global scale (Edwards and Smith, 2010).

The main findings of this study were that the evolution of C_4 species was not closely linked to temperature but rather that 18 of the 20 transitions correlated with shifts in mean annual rainfall and coincided with the movement of grasses from the understory into open-canopy environments. Of the 21 identified nodes, which represented evolutionary transitions from C_3 to C_4 and thus a pair wise comparison between the two, one third of these showed a climate profile consistent with a shift from closed-canopy tropical moist forests to open tropical savannahs and woodlands. So, what did this decrease in mean annual precipitation mean for the exclusion of C_4 grasses from the shade? As grasses were able to shift their growing season to coincide with the rainy seasons, there was no inherent increase in photorespiration. Rather, there was an indirect effect, where the drier climates would limit canopy growth and ultimately allow for an increase in high light environments (which is accompanied by increases in temperature) (Edwards and Smith, 2010). As such, the creation of these high light environments drove the evolution of the "sun specialist" nature of the C_4photosynthetic pathway.

1.4. Shaded environments are expanding – woody thickening

As shown with the phylogenetic grass study by Edwards and Smith (2010), as well as many others, the evolution of C_4 grasses was accompanied by the shift of closed canopy grasslands into savannah biomes (Givnish, 1988; Klink and Joly, 1989; Pearcy and Yang, 1996; Ehleringer *et al.*, 1997; Ash, 1998; Beerling, 2007; Bond, 2008*a*; Osborne and Freckleton, 2009; Edwards and Smith, 2010; Sage and Zhu, 2011; Way and Pearcy, 2012; Christin and Osborne, 2014; Sage and Stata, 2015). In recent years there has been a shift, where open habitats are being encroached upon by C_3 woody species that are moving into savannah biomes throughout the world, coinciding with increasing global CO_2 concentrations (Bond, 2008*a*; Brantley and Young, 2009).

In the Northern Hemisphere, Kansas Prairies have shown a 69% increase in forests from 1939 to 2002, while in the Southern Hemisphere, forest patches in Queensland have shown a doubling in growth from 1943-1991. In monsoon tropics forests have increase by 42% in the last 5 decades. This has also been shown in conservation areas in South Africa with 12 to 68% increases in woody cover (Bond, 2008*a*). With an increase in woody cover it becomes crucial to understand what limits C_4 growth under the canopy and how increases in canopy cover will affect C_4 savannah biome, as they account for a quarter of the primary productivity on the planet. Therefore it is crucial to understand if light intensity and temperature become the limitations to C_4grass productivity and hence fitness, or if the inability to utilise sunflecks is the mechanism the limits C_4 abundance in understory environments.

1.5. The understory environment – sunflecks

Depending on location underneath the canopy, plants receive sunlight either directly as continuous illumination and intermittent sunflecks or as diffuse light. Nearly two thirds of understory light is long wave-length diffuse light and the majority of this light is below the plants compensation point and plays little role in photosynthesis (Liu *et al.*, 2015). Direct light is much more important for plants and is the major influence of understory temperature, relative humidity and total available *PPFD*(Liu *et al.*, 2015).

Sunflecks are particularly important for understory species where the intensity and duration of sunflecks vary depending on canopy height, leaf phenology, weather conditions and the size and positions of gaps (Pearcy, 1983, 1988; Pearcy *et al.*, 1985; Gendron *et al.*, 2001). A better understanding of the plastic nature of the canopy has lead researchers to focus on the utilization of these flecks, short bursts of direct sunlight, which penetrate to the ground below. It has been shown that variations in photosynthetic photon flux density on as small a scale as seconds, has critical effects on maintaining water balance as well as inducing photosynthesis (Lundegårdh, 1921; Yanhong and Naoki, 1997). The ability of a plant to utilize these flecks is directly related to its ability to keep its photosynthetic apparatus in an active, induced state (Pearcy, 1988).

Sunfleck literature has focused on tropical, forest climates. These studies investigating the diurnal and seasonal patterns in understory light conditions have elucidated that 30-80% of the carbon gain by understory plants is as a result of sunflecks (Lundegårdh, 1921;Chazdon and Fetcher, 1984; Pearcy, 1987; Pearcy, 1988; Pearcy, 1994; Gendron *et al*, 2001). Although forest microclimates vary between forest types, it has been shown that induction of understory plants occurs during flecks and constant light. Carbon gain due to flecks was a result of fleck

intensity, duration of the flecks and the low light periods between them (Pearcy *et al*, 1985; Chazdon and Pearcy, 1986; Chazdon and Pearcy, 1991).

This complexity has meant that investigations using a variety of different sunfleck treatments have measured different responses and come to various conclusions about the ability of C_4 species to utilise sunflecks. The majority of C_3 and C_4 comparisons have used sunflecks of long duration, ranging from 30-sec to 12-min (Chazdon and Fetcher, 1984*b*; Pearcy *et al.*, 1985; Chazdon and Pearcy, 1986*b*; Horton and Neufeld, 1998; Leakey *et al.*, 2005) and under these conditions C_4 plants perform equally as well as C_3 species (Pearcy and Calkin, 1983). Experiments using flecks of ~ 1-sec in duration are rare, but under these conditions maize showed very poor rates of photosynthetic induction (Krall and Pearcy, 1993)

Although the abundance of C_4 species in understories is low, shade adapted C_4species do occur (Pearcy and Calkin, 1983; Smith and Martin, 1987; Kromdijk *et al.*, 2014). The existence of these species and because C_4 canopies frequently self-shade lower leaves, has been used to argue that C_4 photosynthesis offers no fundamental limitation to growth in these environments (Sage, 2014). However, in environments where sunflecks are of short duration, as has been measured in various natural settings including temperate and deciduous forests, crops, thickets and hardwood stands, (Pearcy and Calkin, 1983; Pearcy, 1988; Chazdon and Pearcy, 1991), C_4 species may become fundamentally limited.

1.6. Hypothesis and Research questions

Based on the literature presented throughout this chapter, it is clear that each photosynthetic type utilizes different mechanisms to regulate photosynthesis under changing

light environments, with the C_4 subtype regulation comprising an extra level of control as a result of its dual-cell photosynthetic machinery. As a result I hypothesize that:

(1). The C_3 subtype will be able to outperform the C_4 subtype in response to short duration sunflecks in relatively warm environments; not as a result of quantum efficiency, but rather as a result of a metabolic limitation imposed on the C_4 photosynthetic pathway.

Based on the understanding that the C_4 pathway relies on the production of ATP by cyclic electron transport around PSI in the BSC while NADPH is created by linear electron transport in the MSC around PSII (Wang *et al.*, 2014*a*). As the regeneration of NADPH is essential for the flow of electrons during carbon reduction, its supply in the BSC by PSII is essential for balance between the two cell types. As CO_2 is released into the BSC cytosol at the point of illumination, ATP is consumed in the BSC, resulting in the need of constant energy allocation to the BSC in this pathway. During the exposure to sunflecks, the blue light is preferentially absorbed by the MSC, causing an imbalance in the energy portioning as ATP is over produced in the MSC, limiting the rate of NADPH regeneration, and ultimately inhibiting the reduction of carbon in the carbon reduction cycle (Evans *et al.*, 1993; Bellasio and Griffiths, 2014*a*; Wang *et al.*, 2014*a*,*b*).

(2) C_4 grasses will be excluded from sunfleck rich environments while C_3 grasses will not.

In order to test these hypotheses, the questions posed for investigation in this thesis are as follows:

1. How are C_3 and C_4 photosynthetic gaseous exchange and stomatal conductance affected by short-duration sunflecks? Does this differ and if so, how?

2. Does this translate into effects on growth in simulated sunfleck environment? And,

3. Does this response explain the microhabitat preferences of C_3 and C_4 grasses in a natural understory environment?

1.7. Thesis layout

The questions are addressed in the chapters of this thesis as follows: Chapter 2 will investigate the mechanisms by which C_3 and C_4 *A. semialata* responds to flecking-light as well as the effect of a simulated flecking-light environment on plant growth. Chapter 3 will investigate the flecking-light environment under the canopy in which *Erharta erecta* (C_3) and *Brachiaria chusqueoides* (C_4) are found and determine if differences in light environment limit species distributions under the canopy. This chapter will incorporate the effect of canopy openness on the ability to induce photosynthesis under flecking-light and the role of phylogeny on these responses. The final chapter, Chapter 4, will comprise of an overall synthesis of all the data presented in the thesis.

CHAPTER TWO: PHOTOSYNTHETIC, STOMATAL AND GROWTH RESPONSES OF C_3 AND C_4 GRASSES TO SHORT DURATION SUNFLECKS

2.1. Introduction

C_4 species are not abundant in understory environments and this has been attributed to their high-light requirements, lack of photosynthetic advantage relative to C_3 species in cooler environments, and an inability to adequately utilise sunflecks. Their high-light requirements results from the energetic costs of their CO_2 concentrating mechanism that concentrates CO_2 around Rubisco in the bundle sheath, decreasing rates of photorespiration to very low levels (Pearcy and Calkin, 1983). Although this makes C_4 plants energetically competitive at high temperatures, when C_3 plants have high rates of photorespiration, at lower understory temperatures, rates of photorespiration decrease and the C_4 advantage is reduced (Ehleringer and Björkman, 1977; Ehleringer *et al.*, 1997) However, in tropical and subtropical environments temperatures are not low enough for this phenomenon to explain the low abundance of C_4 plants in forest understories(Sage, 2014).

For both photosynthetic types, rates of photosynthetic induction in response to sunflecks vary according to the intensity of illumination and the length of the dark periods that preceded illumination. RuBP regeneration occurs within 1 to 2 min after exposure to light and Rubisco is only fully activated 5 to 10 min after induction under steady high light, (Bellasio, 2013; Chazdon and Pearcy, 1986*a*, 1991; Sassenrath-Cole and Pearcy, 1992). Compounded on this, the addition of the bundle-sheath (BSC) cells in the C_4 photosynthetic

type adds another level of complexity to the present C_3 processes. C_4 photosynthetic induction is biphasic, where the initial phase of enzyme activation is ≤30-sec while, in the second phase, the build-up of metabolites occurs between 5 to 20 min (Usuda, 1985). Hence, it is not only the duration of the sunfleck, but also the frequency with which these occur in natural habitats, that have important consequences for photosynthetic productivity.

Exploring the adaptive significance of plant photosynthetic type responses has not been well elucidated but is important in understanding what limits plant distributions in the natural environment. The ability of a plant to adapt and acclimate to a variable light environment will depend on the plants genotype which is influenced by the evolutionary environmental history of each species. As a result, the plants acclimation state will be as a result of its life history as well as a combination of current environmental signals in which it is found (Retkute *et al.*, 2015).

As a leaf is exposed to sunflecks after exposure to low light, photosynthesis is induced over several minutes. This induction is affected by biochemical and stomatal limitations (Porcar-Castell and Palmroth, 2012; Way and Pearcy, 2012). In order to increase CO_2 assimilation during flecks, Rubisco activation together with stomatal opening would have to occur rapidly. Leaves of C_3 tree species, commonly found in sunfleck niches, have been shown to increase nitrogen allocation to Rubisco activase as well as having high levels of stomatal conductance. Directly after a sunfleck an 'induction loss' begins, decreasing Rubisco activation and closing the stomata (Porcar-Castell and Palmroth, 2012; Way and Pearcy, 2012). Commonly, this loss occurs more gradually than the activation, 'priming' the photosynthetic machinery to be induced more rapidly by successive flecks (Porcar-Castell and Palmroth, 2012; Way and Pearcy, 2012).

Investigating the physiology associated with sunfleck utilisation has mainly focused on C_3 trees. It has elucidated that in response to a sunfleck, there is a rapid build-up of the metabolites of the Benson-Calvin Cycle (BCC), including RUBP and its precursors. This pool is able to support a few seconds of CO_2 assimilation post exposure to the sunfleck while electron transport becomes uncoupled from CO_2 fixation, but becomes re-coupled after a few seconds as the metabolite pools become filled (Way and Pearcy, 2012). Illumination after short dark periods (<30-sec) results in rapid oxidation of P700 (acceptor in PSI). When the BCC is inactive, the outflow of electrons from PSII to $NADP^+$ is limited, resulting in the over reduction of the NADP pool and the slow consumption of NADPH. Without the efficient activation of the BCC, $NADP^+$ cannot be regenerated and linear electron transport is rendered inefficient (Tikhonov, 2015). As a result, sunfleck duration plays an important role in the ability of a plant to utilize subsequent light efficiently in the BCC.

An extra level of complexity is imposed on the C_4 system when dealing with sunflecks. The dual cell nature of the system adds another level of metabolism for the movement of metabolites between the BCC components of mesophyll (MSC) and bundle sheath cells (BSC). There are circumstances when the supply of C_4 intermediates from the MSC exceeds consumption in the BS and this results in the retro-diffusion of CO_2 from the BSC to the MSC cytosol and has been termed leakiness (Bellasio and Griffiths, 2014*a*). As such, energy that was initially used to produce the metabolites is being wasted. As a result, the C_4 system needs to be able to maximise light capture during sunflecks while minimizing the effects of leakiness (Pearcy, 1983; Sage, 2014; Sage and Stata, 2015).

In this chapter, we look at the ability of closely related C_3 and C_4 subspecies of *Alloteropsis semialata* to utilise short-duration sunflecks, measuring the effect of increasing the period of low light between flecks. We investigated both the role of stomata, BSC leakiness and PSII efficiency as the mechanisms limiting C_4 performance. As both subspecies

of *A. semialata* naturally occur as full-sun grasses, we then use forest understory grasses to determine if observed responses were also evident in plants adapted to understory conditions. Together with the subspecies responses to flecking-light, it is hypothesized that the ability of C_3 and C_4 subspecies of *A. semialata* to grow and establish would differ in sunfleck environments due to their varying abilities to utilize to variable light. I predict that C_3 *A. semialata* growth will be less effected by flecking-light than the C_4 species, while flecking-light will have a negative effect on C_4 *A. semialata* growth.

In order to ascertain if phenotype and time affected the ability of the C_3 and C_4 *A. semialata* subspecies to establish and grow in a sunfleck environment, a natural flecking-light environment was simulated using blue-red LEDs and the growth responses measured. As induction is known to be effected by leaf age, seedlings of both subspecies were germinated at the same time to control for age effects (Pearcy and Way, 2012; Way and Pearcy, 2012; Kubásek *et al.*, 2013).

In order to make these comparisons without having the added complexity of phylogenetic differences, the C_3 and C_4 subspecies of *Alloteropsis semialata* were used. *A semialata* is the only species known to contain a C_3 and a C_4 form. The C_4 subtype of *A. semialata (R. Br.) Hitchc. subsp. semialata*, possesses PEPCK subtype of C_4 photosynthetic pathway and both photosynthetic type grow in full sun environments, (Ueno *et al.*, 2006).

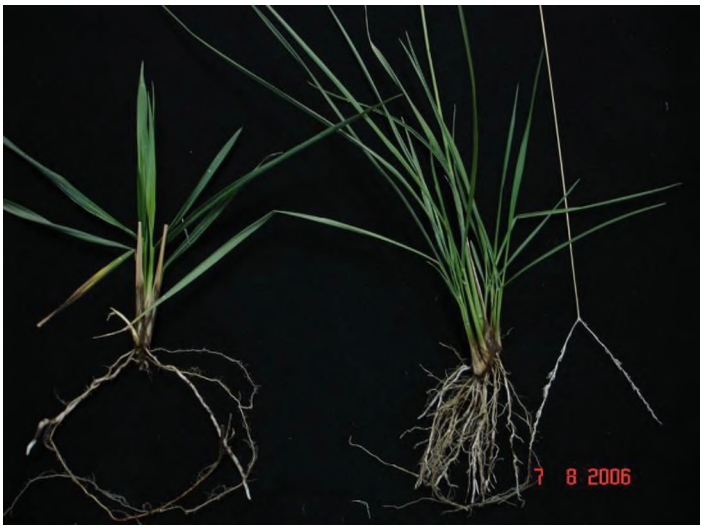

Figure 2-1. Photograph of individual tillers of the C_3 (left) and C_4 (right) subtype of *A. semialata* (Photograph: B.S. Ripley, 2006).

2.2. Materials and Methods

2.2.1. Plants

Tillers were separated from established plants of C_3 and C_4 subspecies *Alloteropsis semialata* and individually potted and maintained in a greenhouse at Rhodes University, South Africa. Each tiller was planted in a 20 L pot, using a 3:1 mixture of topsoil and was watered twice weekly. During the experimental period average day/night tunnel temperatures were 28/21^{o}C with humidity levels ranging from 30 to 50%.

2.2.2. Sunfleck treatments on C_3 and C_4 subspecies of A. semialata

The induction of photosynthesis was measured for the youngest mature leaf on potted plants under three different 20-min light treatments, after initially maintaining leaves at a low

photosynthetically active photon flux density (*PPFD*) of 20 μmol m^{-2} s^{-1} for 20 minutes. The three treatments that the leaves were exposure to were: 1) a constant *PPFD* of 1000 μmol m^{-2} $s^{-1;}$ 2) an intermittent *PPFD* of 6-sec pulses at 1000 μmol m^{-2} s^{-1},interspersed with 6-sec periods of dark; or 3) the same intermittent light pulses interspersed with 30-sec periods of dark (Appendix B). These conditions were generated within the leaf chamber of LI-6400-F Photosynthesis System (Li-Cor Inc. Lincoln, Nebraska, USA), where leaf temperatures were maintained between 23 and 26°C and atmospheric vapour pressure deficit (VPD) at values below 1.7 kPa. Each of the three treatments was applied for 20-min and was conducted on the same leaf, but the order that the treatments were applied was randomised between leaves. By the end of this 20-min, *A* had stabilized and induction was complete. This procedure was repeated on a minimum of three replicate plants from each subspecies.

Gas exchange and chlorophyll fluorescence parameters were recorded at 10-sec intervals and the experiments were repeated at ambient (40 Pa) or saturating (100 Pa) atmospheric CO_2 concentrations supplied by the reference air entering the leaf chamber. The gas exchange parameters were calculated according to von Caemmerer and Farquhar (1981) and von Caemmerer (2000) and the photosynthetic rate at 900-sec was defined as A_{max} for *A. semialata* when photosynthesis reached saturated levels.

2.2.3. Sun fleck treatments on C_3 and C_4 shade understory grasses

C_3 (*Erharta erecta* ((Hochstetter) Pilger) and C_4 (*Brachiaria chusqueoides* (Hack.) Clayton and *Dactylotenium australe*, Steudel) were located in their natural shaded understory environments of a coastal forest, in the Eastern Cape, South Africa (26°44'9.41"E; 33°39'4.66"S). δ13C values were used to confirm these plant photosynthetic type via mass

spectrometry. The same sunfleck treatments as used for *Alloteropsis* were applied to the youngest fully expanded leaves of three replicate plants of each species, except that treatments were applied and induction monitored for 5 min and experiments were not repeated at saturating CO_2. As steady state rates of photosynthesis were attained more rapidly than was the case for *Alloteropsis*, A_{max} was measured at 300 s after the onset of the light treatments.

2.2.4. Leakiness in C_4 leaves

Fully expanded leaves of the C_4 Alloteropsis subspecies were exposed to a sequential decrease in PPFD: 1000, 800, 500, 200, 150, 100, 50μmol m^{-2} s^{-1}, where each light intensity was maintained constant for 2-min, in order to determine their A: PPFD responses. They were then incubated at 500 μmol m^{-2} s^{-1} for 30 min in order to recover from light variations during the light response curve and to ensure that stomatal conductance was not limiting. They were then incubated for a further 12 min at either low (20 μmol m^{-2} s^{-1}) or high (500 μmol m^{-2} s^{-1}) PPFD. The rationale for the two incubations was as follows. The low light incubations simulates background light intensities in forest understories (Pearcy and Calkin, 1983; Pearcy et al., 1985; Chazdon and Pearcy, 1986b,c, 1991; Sharkey et al., 1986; Pearcy, 1988; Krall and Pearcy, 1993; Sims and Pearcy, 1993; Yanhong et al., 1994; Watling et al., 1997a), while the 500 μmol m^{-2} s^{-1}incubation ensures that light dosage prior to, and during the constant or flecking-light treatments, was maintained on average constant. 6-sec of 1000 interspersed with 6-sec dark averages to 500 μmol m^{-2} s^{-1}, equalling the light dosage supplied by the constant light treatment. Following these incubations, leaves were exposed to pulsed-light (6 s on/off) at 1000μmol m^{-2} $s^{-1}$for 12 min (sufficient time for photosynthesis to stabilise to a constant rate). Subsequent to the pulsed light treatment a further three *A: PPFD* curves were

constructed at 0, 15 and 30 minutes after the (Appendix C). For *A: PPFD* curves constructed for the leaves incubated at low light, *PPFD* was decreased as for pre-treatment *A: PPFD* curves, while for leaves treated at 500 μmol m^{-2} s^{-1} the two highest *PPFD*s were omitted. Treatments were repeated at atmospheric conditions of 21 and 1% O_2 by supplying air of these concentrations to the air intake of the LI-6400. Chlorophyll fluorescence measures were made simultaneously with measurements of gas exchange using a LI-6400-F leaf chamber. Initial experiments were conducted to ensure that the modulated light and saturating pulses were optimised according to the LI-6400-F operating instruction manuals (Li-Cor Inc. Lincoln, Nebraska, USA).

As G_{ST} was effected by the light response curves, photosynthetic, leakiness (Φ) and PSII measurements were only calculated from *A: PPFD* curves measured prior to (control), and at 0 min after exposure to a flecking-light treatment. Rates of assimilation and non-photorespiratory CO_2 production were used to calculate leakiness according to Bellasio and Griffiths (2014) (Appendix C).

Leakiness (Φ) is a dimensionless measurement that takes into account the rate at which CO_2 is carboxylated by PEPCase (V_P) in the MSC and the rate at which CO_2 leaks out of the BSC (L) such that:

$$\Phi = \frac{L}{V_P} \qquad (1)$$

Here, leakage rate (L) is defined as the difference between rates of mesophyll CO_2 assimilation and net bundle sheath CO_2 assimilation (A). Mesophyll processes of Pep carboxylation (V_P), respiration in the light (R_M), and activity of bundle sheath reductive pentose phosphate pathway (V_c) and photorespiratory cycle (V_o) are taken into account in the calculation of L such that:

$$A = V_P - L - R_M \quad (2)$$

$$L = V_P - A - R_M \quad (3)$$

Measurements are made under light limiting conditions when V_P is limited by the supply of ATP (J_{ATP}) and in an atmosphere of 2% O_2 when photorespiration is limited. As a result:

$$V_P = \left(\frac{x}{2}\right) \text{ x } J_{ATP} \quad (4)$$

Where x the partitioning factor of J_{ATP} between the C_4 activity (Pep regeneration and Pep carboxylation) and C_3 activity (V_c and V_o) and is a value of 0.4 (von Caemmerer, 2004). J_{ATP} was calculated from gross assimilation (GA) under 2% O_2 where the minimum ATP requirement (3/0.59) is assumed to equal that of the theoretical minimum. As such,

$$J_{ATP} = \left(\frac{3}{0.59}\right) \text{ x } GA \quad (5)$$

If equation (4) is substituted into equation (3), where $GA = R_{Light}$,

$$V_P = \left(\frac{x}{2}\right)\left(\frac{3}{0.59}\right) \text{ x } GA \quad (6)$$

As x = 0.4 and $GA = A + R_{light}$,

$$V_P = \text{ x } A + R_{Light}$$

$$V_P = A + R_{Light} \quad (7)$$

R_{Light} is defined as the total non photorespiratory CO_2 production in the light and is calculated by plotting A against $PPFD.Y\ (II)/3$ (where $Y(II)$ is the yield of $PS(II)$). Once plotted, the y-intercept of the linear regression gives an estimate of $-R_{light}$. In order to calculate L, equation (2) is re-arranged to,

$$L = V_P - A - R_M \quad (8)$$

And where R_M, mesophyll non photorespiratory CO_2 production in the light, is equal to (0.5) X R_{Light}.

2.2.5. Growth in an artificial sunfleck environment

Two separate experiments using newly germinated seedlings or established tillers of C_3 and C_4 subspecies of *A. semialata,* were used to compare the growth of plant under continuous or sunfleck environments. Tillers were separated from established plants of C_3 and C_4 subspecies *A. semialata* and individually potted and maintained in a greenhouse at Rhodes University, South Africa. Each tiller had existing leaves excised at their ligules and was planted in a 2 L pot, using a 3:1 mixture of topsoil and compost, and was watered twice weekly. During the experimental period average day/night tunnel temperatures were 28/21°C with humidity levels ranging from 30 to 50%.

Seeds from established plants of C_4 and C_3 *A. semialata* subspecies were collected from growth plots at Rhodes University, South Africa and Mountain drive (-33.323, 26.533), Eastern Cape, South Africa. Seeds were germinated on filter paper that was wet with distilled water and germinated in a growth cabinet set at 60% RH, 500 *PPFD* and a temperature of 26°C. Once germinated, seedlings were transplanted into 2L pots containing a 3:1 mixture of topsoil and compost and watered 3 times a week in the same growth cabinets until the 3rd leaf had established. Once the third leaf had established, either tillers or seedlings from both the C_3 and C_4 subspecies of *A. semialata* were transferred into a dark room under one of two light regimes controlled under blue-red LEDs (3x1w E27 GU10 MR16 2x red 660nm + 1x blue 445nm plant grow light lamp). The first light regime, referred to as the control treatment, comprised of 4 C_3 and 4 C_4 plants grown under constant light conditions of 12 hours of

daylight (750μmol m^{-2} s^{-1}) and 12 hours of dark, receiving a total daily *PPFD* of 32 mol day^{-1}. The flecking-light treatment comprised of 4 C_3 and 4 C_4 plants grown under intermittent light (1500 μmol m^{-2} s^{-1}) interspersed with 6-sec dark periods (0μmol m^{-2} s^{-1}), again totalling a total daily *PPFD* of 32 mol day^{-1}. The treatments were screened off from one another such that there was no light cross contamination.

2.2.6. Statistics

Photosynthetic, stomatal and leakiness responses

A_{max} extracted from the photosynthetic induction curves for C_3 and C_4 *Alloteropsis* subspecies and of wild grown shade grasses were compared between flecking-light treatments using a Generalised Linear Model (GLM) in R. Analyses of A_{max} for *Alloteropsis* subspecies were repeated separately for each treatment comparing the values from responses at ambient and elevated CO_2.

Apparent quantum efficiencies (ΦCO_2), light saturated photosynthetic rates (A_{sat}), G_{ST}, PSII and leakiness parameters calculated from *A: PPFD* curves were compared within and between responses made prior to, and at 0 minutes after light treatments (control, incubating leaves at low light (20 μmol m^{-2} s^{-1}) or after incubating leaves at a *PPFD* of 500 (μmol m^{-2} s^{-1}) using GLM (Appendix D). In order to determine the combined effect of the light intensity and of the light response curves themselves, an interactive variable was created and run in the model. Once it was determined that the interactive variable had an effect on the extracted measurements, individual Tukey *post-hoc* tests were run for each parameter to determine the extent of the effects.

Leaf growth in an artificial sunfleck environment

The length x breadth of the third leaf, of each plant, was measured weekly (mm^2) over a three week period as an approximate of leaf area (LA). The difference between initial values and values at each weekly time interval were used as a proxy for growth. This is valid over the short period that growth was monitored because the relationship between length x breadth and biomass remained relatively linear. The effects of light treatment (control and flecking) on each subspecies (C_3 and C_4), at each time interval was assessed by running a Generalized Linear Model (GLM) for data with either Poisson or Quasipoisson distributions (R programme. When differences were significant, Tukey *post-hoc* tests were run to determine the extent of the effects (Appendix E).

2.3. Results

2.3.1. Do C_3 and C_4 grasses differ in their response to sunflecks?

When the intensity of the continuous *PPFD* was increased from 20 to 1000 μmol m^{-2} s^{-1}, photosynthesis was induced to steady state rates in both the C_3 and C_4 subspecies (Fig. 2-2 A). Steady state was considered stable at 900 seconds as increasing length of induction to 1500 s, increased *A* between 2 and 4% in both C_3 and C_4 *A. semialata.* Steady state rates were attained faster in the C_3 than C_4 subtype and as would be expected at ambient CO_2 concentrations of 40 Pa, the C_4 plants achieved higher photosynthetic rates. When this induction was repeated using intermittent light interspersed with 6-sec dark periods, the C_3 plants response remained remarkably similar to that of plants illuminated with continuous light (Fig. 2-2 A & C). However, this was not the case for the C_4 plants where intermittent

light induced maximum photosynthetic rates (A_{max}) that were decreased to only 35% of the values recorded under continuous light (Fig. 2-2 A & C). In both subtypes, small 6-sec fluctuations in photosynthetic rate were evident as a result of the intermitted illumination. When the induction was repeated using flashes interspersed with 30-sec dark periods, both subspecies were unable to induce rates as high as were recorded under continuous illumination and values of A_{max} were reduced by only 33% in the C_3, while C_4 rates decreased by 76% (Fig. 2-2 A & E). Again, 30-sec short term fluctuations in A were evident as the intermittent light flashed with this periodicity.

When the continuous light treatment was repeated under saturating CO_2 (100 Pa), steady state rates remained unchanged for C_4 plants while in C_3 plants A_{max} increased by over 50% (Fig. 2-2 D). A large C_3 response would be expected as saturating CO_2 levels increased intercellular CO_2 concentrations by approximately two-fold and would almost eliminate photorespiration and supply Rubisco with saturating substrate concentrations. When this high CO_2 treatment was repeated using intermittent light interspersed with 6-sec dark periods, the C_3 response was little altered, but the A_{max} of C_4 plants decreased by 63% relative to continuous light treatment (Fig. 2-2 E). This was a similar pattern to that observed when these measures were made at ambient CO_2 concentrations. If the length of the dark treatment was increased to 30-sec, as with the ambient CO_2 treatment, the A_{max} of both subspecies were dramatically decreased by 70 and 86% in the C_3 and C_4 plants, respectively, when compared to values measured under continuous light (Fig. 2-2 D & F).

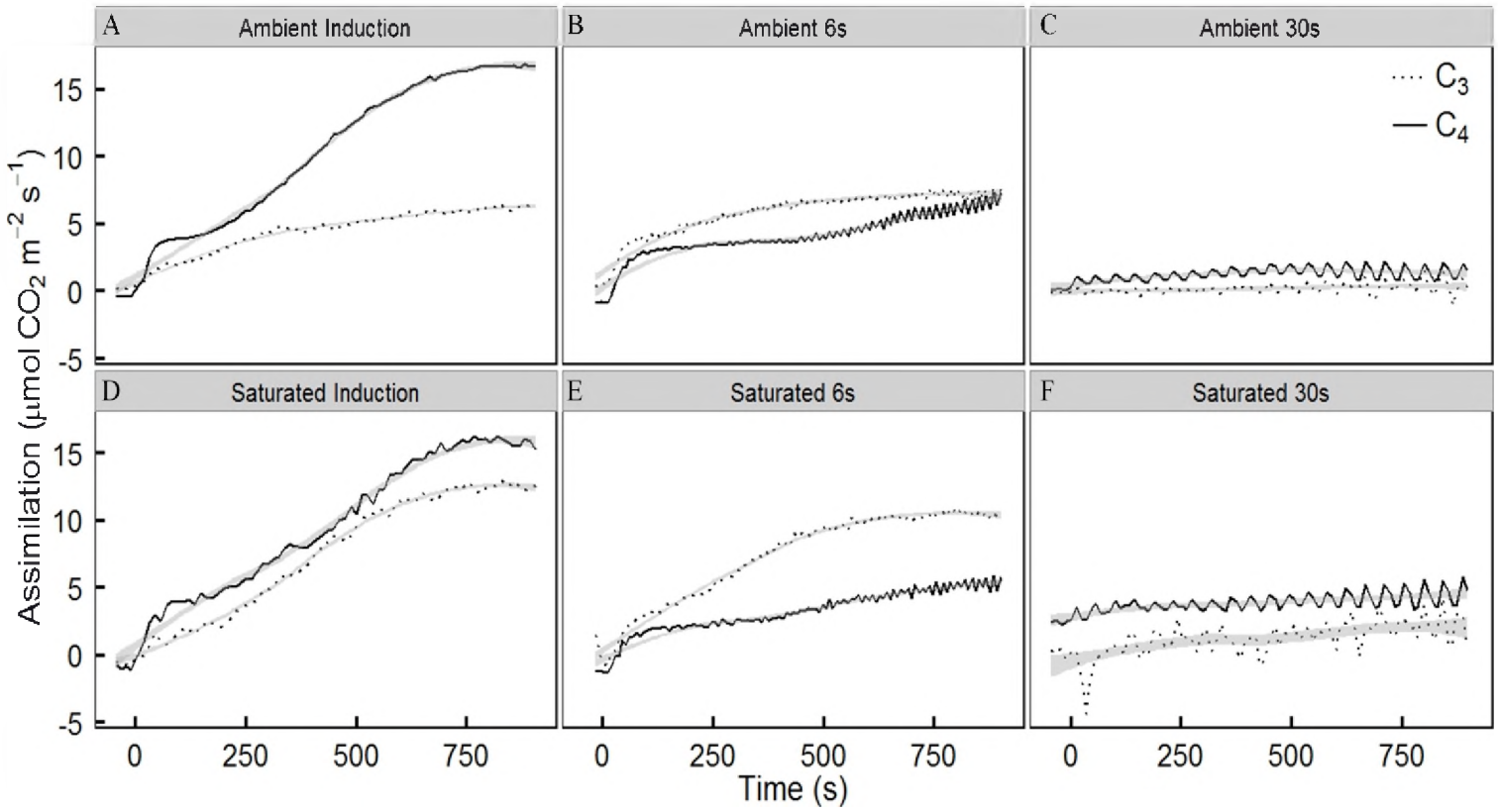

Figure 2-2. Responses of net CO_2 fixation for both C_3 and C_4 subspecies of *Alloteropsis* to light treatments measured at ambient or saturating CO_2 concentrations. All plants were incubated at low light (20 µmol m^{-2} s^{-1}) prior to exposure to each light treatment. Top panel: Photosynthetic responses of both subspecies under ambient CO_2 (40 Pa) conditions. Bottom panel: Photosynthetic responses of both subspecies under saturating CO_2 (1000 Pa) conditions. (A & D) Exposure to continuous high light (1000 µmol m^{-2} s^{-1}); (B & E): exposure to intermittent *PPFD*, interspersed with 6 sec periods of dark; (C & F): exposure to intermittent *PPFD* interspersed with 30 sec periods of dark. For comparative purposes, solid lines represent C_4 *A. semialata*, dotted lines represent C_3 *A.semialata*, light grey lines represent 95% confidence intervals (≥3).

If induced with continuous light the stomata and both C_3 and C_4 plants opened in a pattern that resembled the photosynthetic response (Fig. 2-3 A). If this was repeated with intermittent light and 6-sec dark periods, the C_3 plant's response was little altered (Fig. 2-3 B), although under flecking-light, to achieve comparable photosynthetic rates, the C_3 plants

operated at higher G_{ST} than the C_4 plants. When this G_{ST} response is considered in combination with the observed increased in photosynthetic rates at saturating CO_2, it suggests a tight coupling between the photosynthetic and stomatal responses. However, this relationship between A and G_{ST} was not linear (polynomial fit yielded $r^2 = 0.9$), showing that the relationship between stomatal conductance and photosynthesis was changing over time.

In contrast to the C_3 response under intermittent light, the C_4 plants showed relatively small increases in G_{ST} throughout the experiment (Fig 2-3 B). The patterns of stomatal and photosynthetic response were dissimilar and the relationship between A and G_{ST} was non-linear and biphasic. Initially increasing G_{ST} had little effect on A, but after 450-secs became linear ($r^2 = 0.9$). This shows the long time-lag between the initiation of the flecking-light treatment and the induction of photosynthesis in the C_4 plants. If dark periods between light-flashes were increased to 30-sec, neither subspecies increased their stomatal conductance above 0.02 mol H_20 m^{-2} s^{-1} under ambient CO_2 concentrations (Fig. 2-3 C).

The saturating CO_2 treatment lowered G_{ST} of both C_3 and C_4 plants relative to measurements made at 40 Pa CO_2 in both the continuous and 6-sec flashing treatments (Fig. 2-3 D & E). However besides this reduction, the overall pattern of the induction responses remained similar between ambient and elevated CO_2 treatments suggesting photosynthesis was not limited by the pattern of stomatal responses. The CO_2 treatment had no effect on the G_{ST} induction response of plants subject to the 30-sec intermittent light treatment (Fig. 2- 3 F).

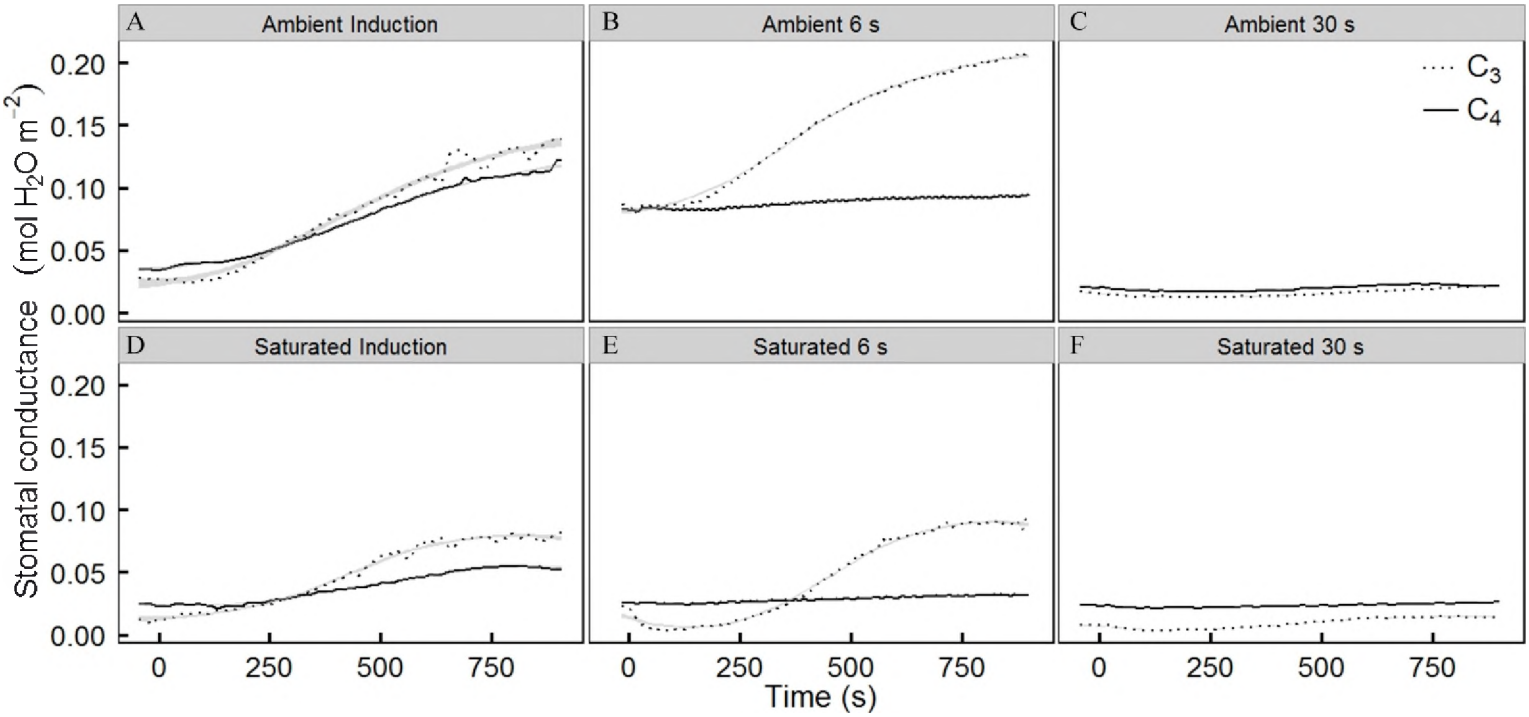

Figure 2-3. Responses of net stomatal conductance (G_{ST}) for both C_3 and C_4 subspecies of *Alloteropsis* to light treatments measured at ambient or saturating CO_2concentrations. All plants were incubated at low light (20 µmol m^{-2} s^{-1}) prior to exposure to each light treatment. Top panel: Stomatal responses of both subspecies under ambient CO_2 (40 Pa) conditions. Bottom panel: Stomatal responses of both subspecies under saturating CO_2 (1000 Pa) conditions. (A & D) Exposure to continuous high light (1000 µmol m^{-2} s^{-1}); (B & E): exposure to intermittent *PPFD*, interspersed with 6-sec periods of dark; (C& F): exposure to intermittent *PPFD* interspersed with 30-sec periods of dark. For comparative purposes, solid lines represent C_4 *A. semialata*, dotted lines represent C_3 *A.*semialata, light grey lines represent 95% confidence intervals (≥3).

2.3.2. Leakiness in C_4 leaves

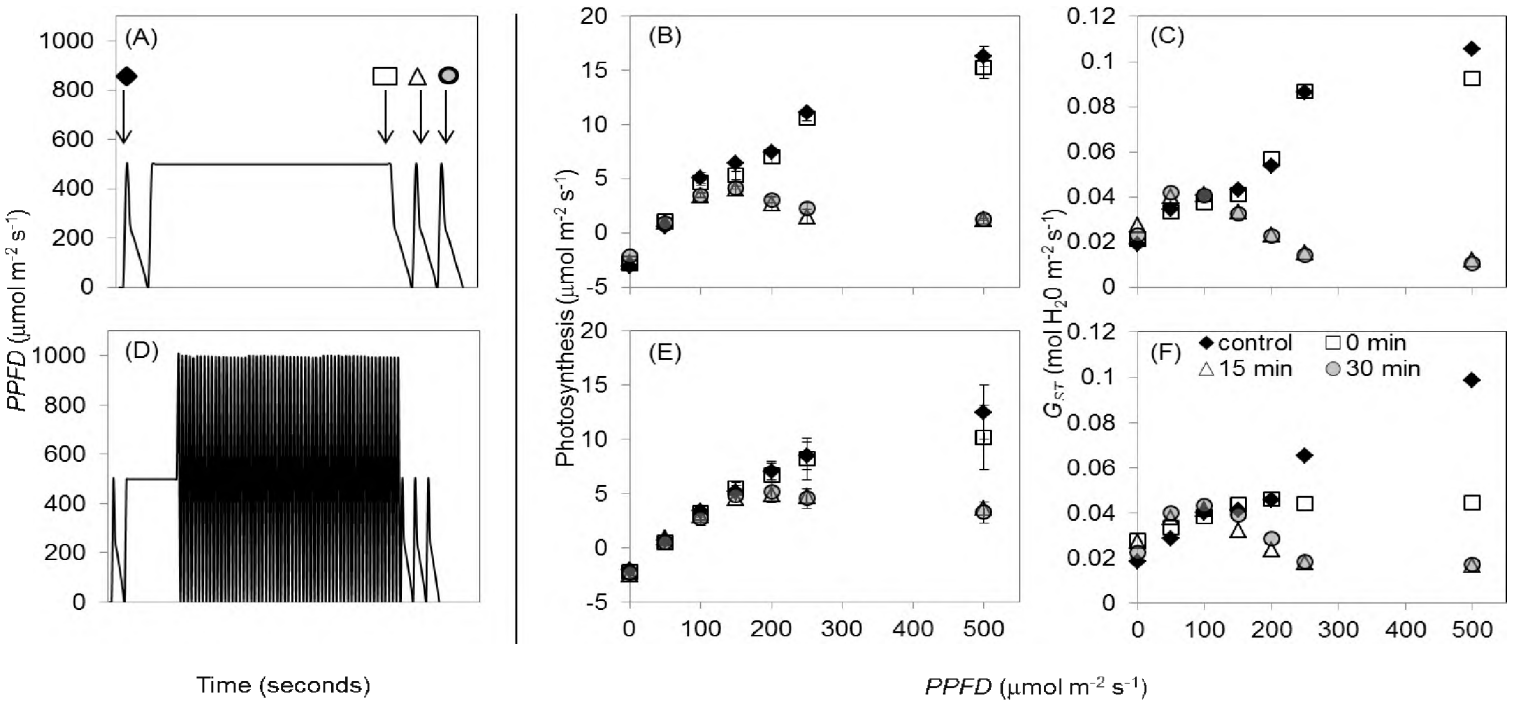

Figure 2-4. Photosynthetic and stomatal conductance responses of C_4 *Alloteropsis. semialata* to increasing light.(A): Schematic representation of the "control" treatment, *A: PPFD* curves were measured prior to (control), and at 0, 15 and 30 minutes after being subjected to constant light at 500 µmol m^{-2} s^{-1} for 720 seconds; (B) Responses of net CO_2 fixation; (C) Response of net stomatal conductance (G_{ST}). Closed diamonds represent measurements made during the control light response curve (prior to treatment), while open squares, open triangles and closed circles represent measurements made during the light response curves made 0 min, 15 min and 30 min after exposure to the treatment. Vertical bars are standard errors (≥3).

All *A: PPFD* curves showed typical patterns increasing linearly at low light intensities (0 – 200 *PPFD*). Curves measured directly before and at 0-min after flecking-light showed typical patterns of becoming saturated at 500 *PPFD* while curves measured at 15 and 30-min after flecking-light followed an atypical pattern, decreasing with increasing light intensities

(Fig. 2-4 B & C). As such, successive *A: PPFD* responses, constructed after the first response, resulted in decreased photosynthetic rate and stomatal conductance (Fig. 2-4 B & C). As photosynthetic responses are effected by stomatal limitation in each successive *A: PPFD* curve, further leakiness calculations were made using only the *A: PPFD* curves constructed prior to and directly after (0 min) the subsequent low light (20 μmol m^{-2} s^{-1}) and high light (500 μmol m^{-2} s^{-1}) flecking-light treatments.

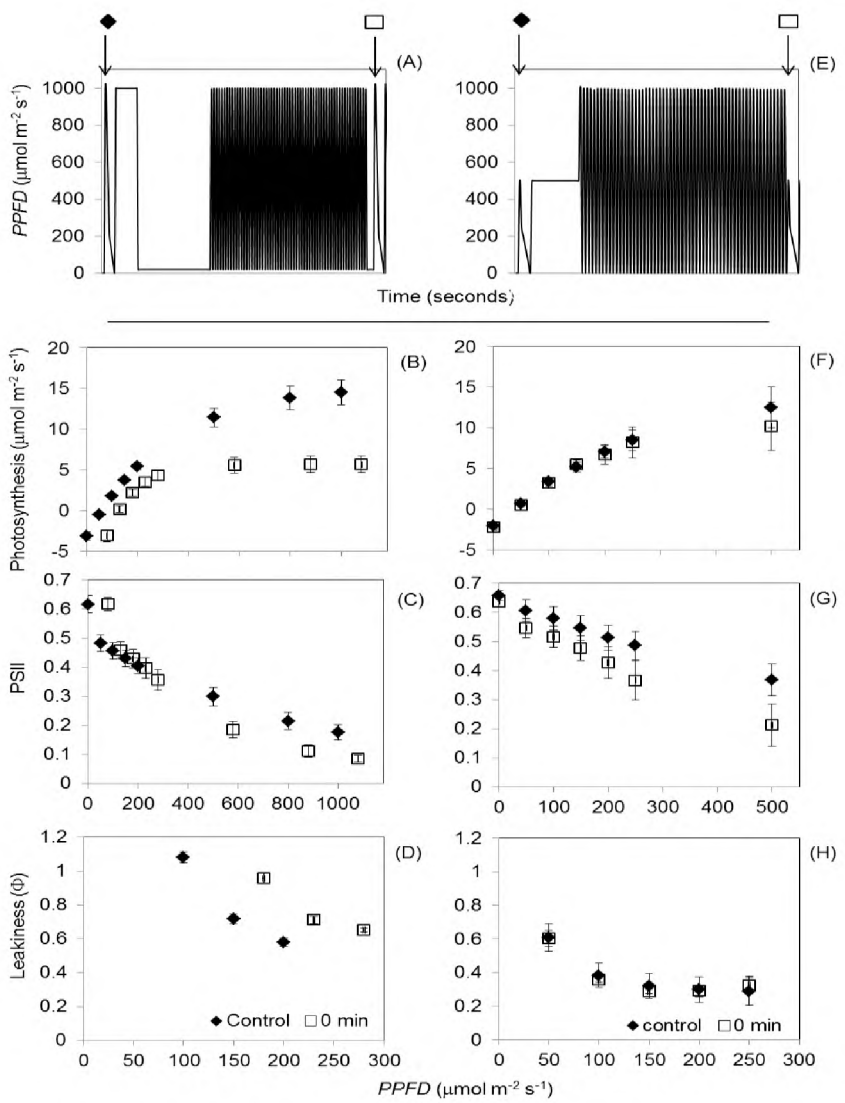

Figure 2-5. Responses of photosynthesis, PSII and leakiness (Φ) of C_4 *Alloteropsis semialata* to increasing light after exposure to flecking-light treatments pre-incubated at either low (20 µmol m^{-2} s^{-1}) or high (500 µmol m^{-2} s^{-1}) light. (A & E): Schematic representation of the "low light" and "high light" treatments, *A: PPFD* curves were measured prior to (control), and at 0 minutes after exposure to flecking-light; (B & F) Responses of net CO_2 fixation to flecking-light; (C & G) Responses of PSII activity to flecking-light; (D & G) Responses of leakiness (F) to flecking-light calculated from the light-limited portions of the light response curves as leakiness can only be calculated when light is not saturating (Bellasio and Griffiths, 2013b). Closed diamonds represent measurements made from the control light response curve (prior to treatment), while open squares represent measurements made from

the light response curves initiated 0 min after exposure to the treatments. Vertical bars are standard errors (≥3).

When simulating background light intensities in forest understories; light curves constructed prior to and after (0 min) the "low light" flecking-light treatment increased linearly at low light intensities (0 – 200 *PPFD*), becoming saturated at levels above 500 *PPFD* (Fig. 2-5 B). As expected, the highest levels of light saturated photosynthesis (A_{sat}) were measured before exposure to flecking light and were decreased by the treatment (Fig. 2-5 B). The initial linear portion of the curves, defining the quantum efficiency of photosynthesis (QE), did not differ significantly before and after the flecking treatments (Fig. 2-5 B). As leakiness is calculated from this linear portion, Φ was not significantly altered by exposure to flecking-light (Fig. 2-5 B, D), showing that the decrease in A could not be explained by an increased leakiness (Fig. 2-5 B, D). Effects of flecking-light on A became apparent when comparing the light saturated portion of the light curves and all values immediately after the flecking-treatment were lower than those of the control (Fig. 2-5 B, D; $F_{3:21}$; $p = 0$). As effects of the flecking-treatment did not affect the quantum efficiency nor stomatal conductance (G_{ST} data not shown here), but rather the A_{sat}, it suggests that energetic limitations of the carboxylation metabolism may underlie the observed responses. This proposed energetic limitation was supported by the response of PSII fluorescence to the flecking treatment (Fig. 2-5 C). The flecking-light treatment decreased ΦPSII relative to the control curve values, where ΦPSII decreased in response to increasing to *PPFD* as is typical of increased non-photochemical energy dissipation at higher light intensities (Fig. 2-5 C).

When ensuring that the light dosage prior to, and during the flecking-light treatment was maintained on average constant, equalling the light dosage supplied by the constant light treatment, both *A: PPFD* curves constructed in the "high light" flecking-light treatment also increased linearly at low light intensities (0 – 200 *PPFD*), becoming saturated at 500 *PPFD*

(Fig. 2-5 F). As in the "low light" treatment, the highest levels of light saturated photosynthesis (A_{sat}) were measured before exposure to flecking light and were decreased by the treatment. But, unlike responses in the "low light treatment", these observed differences were not significantly different (Fig. 2-5 F). As a result, there was no significant effect of the flecking-light on light saturated photosynthesis, PSII or Φ when incubated at 500 *PPFD* prior to exposure to flecking-light (Fig. 2-5 F, G & H). Therefore the energetic limitations of the carboxylation metabolism which may explain a decrease in *A*in response to flecking-light (Fig. 2-5 B), may only take effect when the plant has been exposed to a sufficient period of low light.

2.3.3 Sun fleck treatments on C_3 and C_4 shade understory grasses

Figure 2-6 shows the responses of three shade-adapted grasses, *E. erecta* (C_3), *B. chusqueoides* (C_4) and *D. australe* (C_4) to the same flecking-light treatments applied to the *A. semialata* subspecies. As seen previously for *A. semialata*, when the intensity of the continuous *PPFD* was increased from 20 to 1000 μmol m^{-2} s^{-1}, photosynthesis was induced to steady state rates in both the C_3 and C_4 plants, while steady state rates were highest in the two C_4 species (Fig. 2-6 A, D & G). When this induction was repeated using intermittent light interspersed with 6-sec dark periods, *E. erecta's* (C_3) response remained similar to that of plants illuminated with continuous light (Fig. 2-6 G & H). However, this was not the case for both of the C_4 species, where intermittent light induced maximum photosynthetic rates that were only 55% of the values recorded under continuous light for *B. chusqueoides* and 46% for *D. australe* (Fig. 2-6 A, B & D, E). When the induction was repeated using flashes interspersed with 30-sec dark periods, *E. erecta* showed the smallest reduction in *A* of only 15%, while both C_4 species photosynthetic rates were reduced by 80% (Fig. 2-6 G, H & I).

This shows that C_4 shade adaption allows grasses to utilize sunflecks better than sun adapted C_4 *A. semialata,* but does not allow for the utilization of short sunflecks at the same efficacy as both sun and shade adapted C_3 *A. semialata* and *E. erecta* (Fig. 2-2 & 2-6).

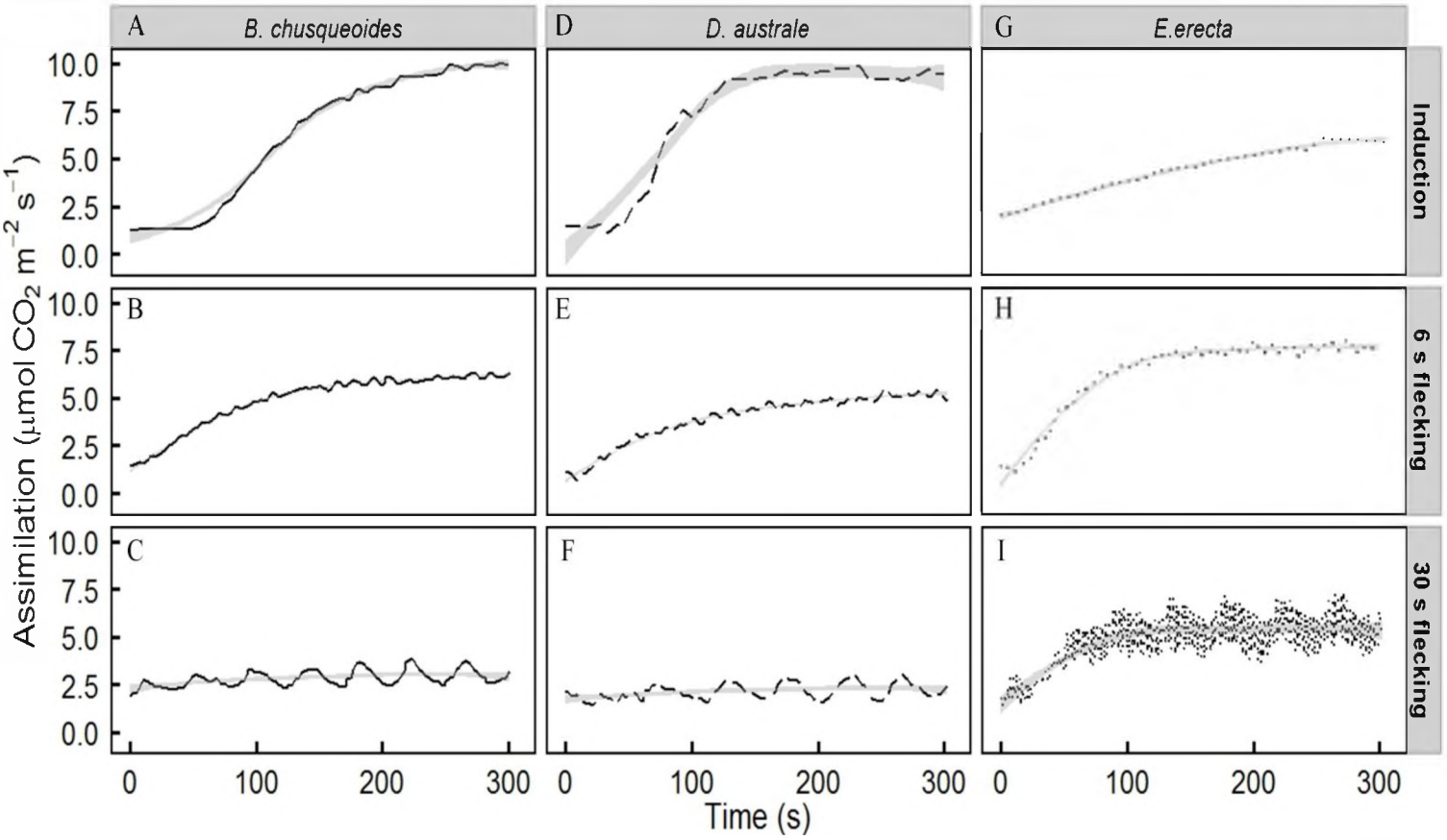

Figure 2-6. Responses of net CO_2 fixation to changes in light duration under ambient CO_2 conditions (40 Pa) for: *Erharta erecta* (C_3), *Brachiaria chusqueoides* (C_4) and *Dactylotenium australe* (C_4). All plants were incubated at low light (20 μmol m^{-2} s^{-1}) prior to exposure to each light treatment. Top panel: Photosynthetic responses of *E. erecta*; Middle panel: Photosynthetic responses of *B. chusqueoides*; Bottom panel: Photosynthetic responses of *D. australe*. (A, B & C) Exposure to continuous high light (1000 μmol m^{-2} s^{-1}); (D, E & F): exposure to intermittent *PPFD*, interspersed with 6 sec periods of dark; (G, H& I): exposure to intermittent *PPFD* interspersed with 30 sec periods of dark. For comparative purposes, solid lines represent *B. chusqueoides*, long-dash lines represent *D. australe*, dotted lines represent *E. erecta*, light grey lines represent 95% confidence intervals (≥3). Results from this figure were obtained from experiments done by Michael D. Cramer, Simon C. Power and Brad S. Ripley.

2.3.1. Growth in an artificial sunfleck environment

Established tillers

The flecking-light treatment had different effects on the growth of C_3 and C_4 *A. semialata* tillers when compared to growth under continuous light (control treatment) (Fig. 2-7 C-D). Under continuous light, C_3 tillers showed minimal re-growth of leaves over the 3 week period while the C_4 tillers showed a linear, non-saturating pattern of growth (Fig. 2-7 C & D). Under the flecking-light treatment, C_3 tillers showed the same pattern of growth that was observed under continuous light, with no significant difference in growth between the continuous and flecking treatments over the three week period (Fig. 2-7 D), with the exception of at 3 weeks, when growth under continuous light was higher than that under flecking conditions. This was not the case for the C_4 subtype, where flecking-light resulted in a hyperbolic pattern of growth, increasing linearly but then stopping after 2 weeks (Fig. 2-7 C), such that by week 3 the leaf dimensions of plants under continuous and flecking-light were significantly different. Therefore it can be seen that the negative effect of flecking-light treatment on the growth of C_4 *A. semialata* occurs after 2 weeks while flecking-light does not negatively affect that growth of the C_3 subtype at any point during the 3 week period.

Seedlings

In order to eliminate the possibility that subspecies growth of tillers was affected by the reallocation of their resources to below ground structures, seedlings of both subspecies were exposed to the same continuous and flecking-light treatments as experienced by the

established tillers. Under continuous light, C_3 seedlings showed a hyperbolic pattern of growth, saturating at 3 weeks. The C_4 seedlings showed minimal growth for the first week, before increasing exponentially after 2 weeks (Fig. 2-7 A & B).

Under flecking-light, the growth of the C_3 subspecies follows a biphasic pattern of growth, increasing initially between the first and second week while the second increase, between the third and fourth week, showed a continued spike in growth (Fig. 2-7 B). Although the pattern of growth is different between the continuous and flecking-light grown C_3 seedlings, the change in growth over the four week period is not significantly different between the treatments (Fig. 2-7 B). As seen with the tillers, the flecking-light had a negative effect on the growth of the C_4 subspecies. An inverted hyperbolic pattern of growth was observed under flecking-light when compared to the exponential pattern observed under the continuous treatment. After two weeks of growth under flecking-light, the C_4 growth saturated. This resulted in a significant difference in growth between the continuous and flecking-light grown C_4 seedlings (Fig. 2-7 A; $p<0.001$).

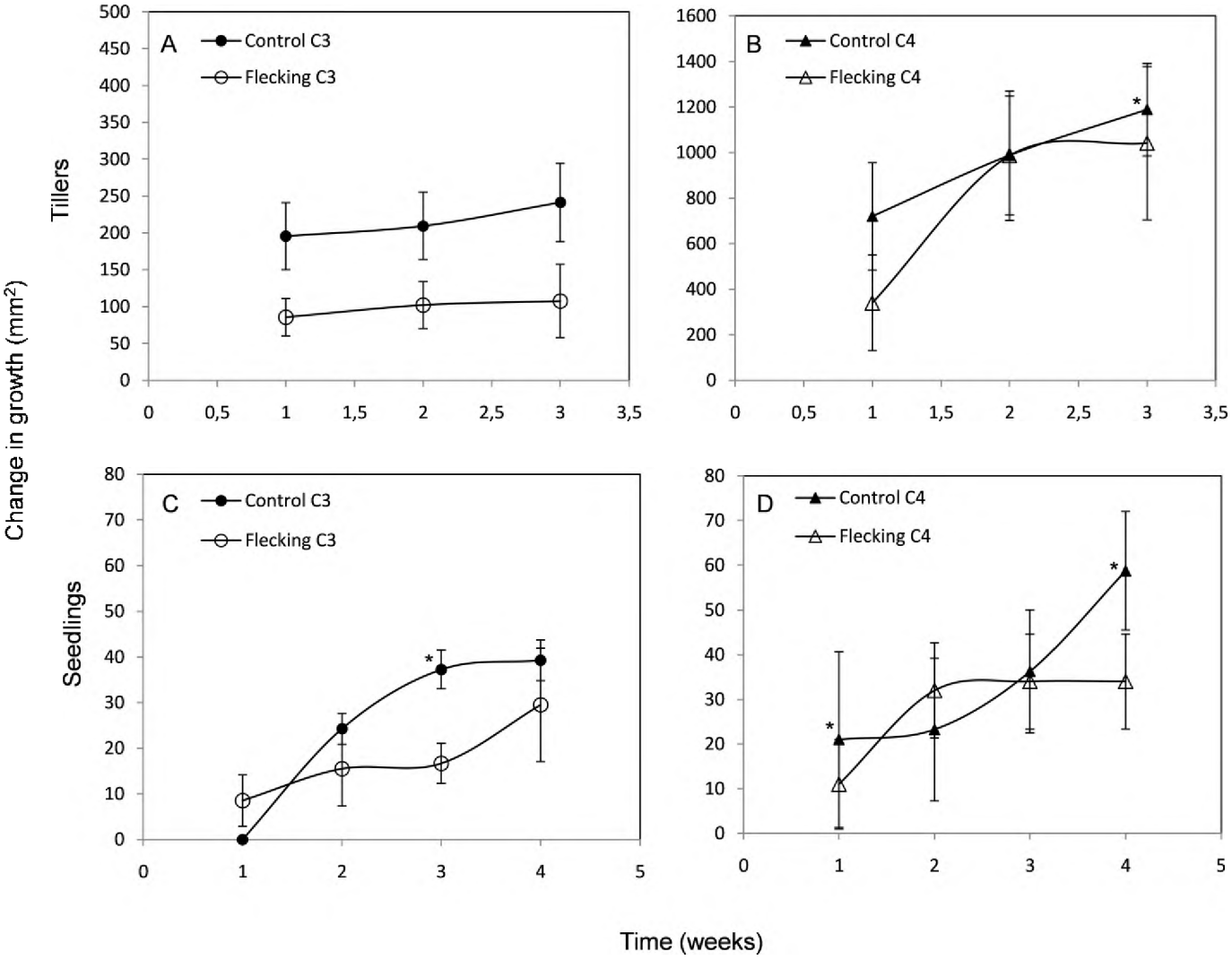

Figure 2-7. Change in growth of C_3 and C_4 *Alloteropsis semialata* seedlings and tillers under environmentally simulated continuous and flecking-light environments over a 3 to 4 week period. Both light treatments were controlled under blue-red LED lights in a controlled dark room environment. Changes in growth were calculated as the difference in (length x breadth) measured at each time interval in comparison to the initial (length x breadth) of each leaf. Top panel (A & B): Comparison of growth between C_3 and C_4 tillers. Bottom panel (C & D): Comparison of growth between C_3 and C_4 seedlings. For comparative purposes, closed symbols represent control treatments while open symbols represent flecking-light treated seedlings and vertical bars are standard errors (≥4). Asterix (*) on panels indicates significant differences at that particular time interval.

2.4. Discussion

The results support the hypothesis that C_3 grasses can outperform the C_4 grasses under sunfleck conditions when comparing photosynthetic responses and growth. The bigger reduction in photosynthetic productivity in the C_4 plants exposed to flecking-light could be attributed to neither stomatal limitation nor increased bundle sheath leakiness and points to direct effects on the carboxylation metabolism when inducing photosynthesis after incubation at low light conditions. This may involve effects on the transfer of metabolites and energy between mesophyll and bundle sheath cells, or on energy production by the light reactions. Results of growth under simulated flecking-light in a controlled dark room environment show that the responses observed for photosynthesis may have translated into differences in growth between the two subtypes.

In response to the fluctuating energy levels created during flecking-light, the phosphorylation or dephosphorylation of the light harvesting complexes (LHCs) during high and low light periods influences the redox state of the intermediate pool, has an important effect on the rate at which the electron transport proceeds (Rochaix, 2011, 2014). In C_4 plants, the addition of the dual cell CCM as well as differences in the biochemistry of the various C_4 photosynthetic sub-types, adds additional levels of complexity, where energy production via linear (LET) and cyclic (CET) electron transport, has to be balanced in both MS and BS cells in order for the carbon reduction cycle to proceed (Rochaix, 2011, 2014).

The C_3 subtype is not affected by the transfer of energy and metabolites between two cell types during carbon reduction. Previous literature has shown that C_3 species allocate more nitrogen to Rubisco activase during exposure to a light-fleck and, as highlighted in this study, have high G_{ST} (Fig. 2-3). As there is a decrease in Rubisco activation and G_{ST} directly after a

sunfleck, known as an "induction loss", the high levels of G_{ST} in C_3 plants would allow for a build-up of the metabolite pools, which would be depleted at a slower rate, allowing for the "priming" of the photosynthetic machinery to be induced by each subsequent light-fleck (Porcar-Castell and Palmroth, 2012; Way and Pearcy, 2012). Therefore high G_{ST} allows the C_3 grasses to maintain carbon gain during the low light periods between flecking as increased G_{ST}, improves carbon gain through an increased carboxylation/oxygenation ratio of the Rubisco reaction and a slower depletion of the metabolic pools (Kirschbaum and Pearcy, 1988; Tinoco-Ojanguren and Pearcy, 1992).

C_4 *A. semialata* utilizes the PEPCK pathway which, along with other C_4 photosynthetic subtypes, have been shown as an inherently "leaky" system (Sage, 2004; Gowik and Westhoff, 2011; Sage *et al.*, 2012 Pearcy *et al.*, 1985; Sage, 2014*b*). Our initial hypothesis was that the biochemical limitation of the C_4 system would occur as a result of increased leakiness, where the C_4 enzymatic reactions would be initiated by the sunfleck but the short duration of the fleck would not be sufficiently long to sustain subsequent BS metabolism. Rather than being incorporated into carbohydrates, the CO_2 resulting from decarboxylation in the bundle sheath would leak out of the system increasing electron consumption and hence decreasing the quantum yield. Surprisingly, immediately subsequent to flashing light treatment after incubation at low light, the quantum efficiency was not decreased, indicating no change to the level of leakiness (Fig. 2-5). An alternative explanation for the decrease in photosynthesis after flecking-light is found when looking at the effect that the light has on PSII function.

In the PEPCK photosynthetic pathway, ATP is produced mainly by cyclic electron transport around PSI in the BS cells while all NADPH is produced in the MS cells via linear electron transport around PSII (Wang *et al.*, 2014*a*). In order for PEPCK to function, NADPH supply via PSII in the BS cells is essential. As PEPCK releases CO_2 into the BS cytosol, ATP

is consumed in the BS cells. As a result, PEPCK requires more energy to be allocated to the BS cells than to mesophyll cells. During light-flecks, the blue wavelengths are preferentially absorbed by the MS cells, creating an imbalance in energy, increasing energy allocation to ATP production in the MS cells (Wang *et al.*, 2014*a*). As a result, NADPH regeneration is limited and CO_2 is unable to be utilized via the carbon reduction cycle. Sun et al (2014) showed a strong positive, linear correlation between total photosynthesis and PSII activation in the MSC under blue light. They attributed the lower PSII levels to (a) imbalance of light absorption between the PSI and PSII in the MSC, (b) differences in light energy absorption between PSII enriched MSC and PSII limited BSC, (c) an increase in non-photochemical quenching or, (d) an overall decrease in the absorption of blue light throughout the leaf (Sun *et al.*, 2014). This offers an explanation as to why the decrease in A in response to flashing light in the C_4 subtype is correlated to the decrease in PSII efficiency (Fig. 2-5).

This explanation holds true when looking at the effect of flecking-light after a pre-incubated under "high light" conditions. When the plant was incubated for 30-min at 500 *PPFD*, there was no significant difference between PSII activity directly before and after flecking-light, which could explain why there was no significant decrease in A_{sat} (Fig. 2-5). A further explanation for this could be attributed to the redox state of the intermediate pools (NADPH and ATP) which, after incubation at "high light", had built-up enough to cope with the energy imbalances caused during the flecking-light treatment.

Limitations under flecking-light of the C_4 subtype were further highlighted in continuous versus flecking-light growth experiments. In response to growth under a flecking-light environment, the C_4 subtype leaf area had a significant decrease after 2 weeks when compared to growth under continuous light (Fig. 2-7 A-D). As it is speculated here that flecking-light imposes a metabolic limitation on PSII in the C_4 photosynthetic pathway (Fig. 2-5 B), the differences in photosynthetic performances translated into differences in leaf

growth. When comparing the growth between the C_4 tillers and seedlings there was no difference in response to growth under flecking-light, and both showed a threefold increase in LA between weeks 1 and 2, before changes in LA saturated in the flashing light treatment (Fig. 2-7 C & D). It has been highlighted in studies by Ripley *et al.*, (2008) that C_3 and C_4 *A. semialata* rely on different growth allocation strategies, whereby the C_3 subtype invests growth into roots and leaves while the C_4 subtype invests biomass into underground storage reserves (Ripley *et al.*, 2008, 2010). This comparison between seedling and tiller growth could highlight that early growth in C_4 tillers was not supported by reallocation of below ground reserves and suggests that this growth allocation strategy does not improve carbon gain under flecking-light.

Although there was no contrast in growth between the C_4 tillers and seedlings, the C_3 subtype showed a difference in LA between the flecking-light grown tillers and seedlings. While the C_3 tillers were able to persist under the flecking-light, the C_3 grown seedlings were able to continue growing after 4 weeks (Fig. 2-7 A & B). This could have implications on the connections between C_3 and C_4 grasses under a changing global environment and establishment of seedlings under encroaching tree canopies if flecking-light is of short enough duration to affect C_4 species.

Differences in the response of photosynthetic induction to variable light was further illustrated by the adaptive ability of different C_3 and C_4 shade grasses to utilize flecking-light (Fig. 2-6). Where shade adaptation resulted in *B. chusqueoides* and *D. australe* having lower levels of reduction in photosynthesis, when compared to C_4 *A. semialata* (±50% compared to 65%), they were still unable to compete with the sun adapted C_3 *A. semialata* and the shade adapted *E. erecta*, which showed no significant reduction in photosynthetic capacity in response to both 6-sec and 30-sec flecking-light treatments (Fig. 2-2 & 2-6). As such, species specific adaptation allows for modification in sunfleck responses but ultimately, the

differences in the ability between C_3 and C_4 subtypes to utilize flecking-light still remains. Ultimately, C_3 grasses were able to induce photosynthesis more effectively than C_4 grass species under these flecking-light conditions.

In contrast to previous literature that has claimed that there is no fundamental limitation imposed on C_4 species ability to utilize sunfleck when compared to C_3 species (Pearcy and Calkin, 1983; Pearcy *et al.*, 1985; Chazdon and Pearcy, 1986*b*,*c*, 1991; Sharkey *et al.*, 1986; Pearcy, 1988; Krall and Pearcy, 1993; Sims and Pearcy, 1993; Yanhong *et al.*, 1994; Watling *et al.*, 1997*a*), our experiments utilized sunflecks of relevant duration and intensity and tight phylogenetic control to test and ultimately dispute this argument.

2.5. Conclusions

- The 6-sec flecking-light treatment had no significant effect on C_3 *A. semialata's* ability to induce photosynthesis while C_4 *A. semialata* A_{max} decreased by 65% compared to values recorded under continuous light. Increasing flecking-light to 30-sec, decreased A_{max} of C_3 and C_4 subtypes by 33% and 76% respectively.

- Again, the 6-sec flecking-light treatment had no significant effect on G_{ST} of C_3 *A. semialata,* relative to measures under continuous light induction. This resembled the same pattern observed under continuous illumination, suggesting a tight coupling between A and G_{ST} in C_3 grass species which changes over time. Alternatively, a biphasic relationship between A and G_{ST} for C_4 *A. semialata* was seen, showing a long lag-time between initiation of the 6-sec flecking-light treatment and the induction of

photosynthesis.

- Light response curves of C_4 *A. semialata* constructed in response to the 6-sec flecking-light treatment showed, that the decrease in *A* is not accompanied by an increase in leakiness (Φ).Differences in *A* are seen in the light saturated portion of the curve, suggesting flecking-light does not affect quantum efficiency, but rather that responses are a result of energetic limitations of the carboxylation mechanism.

- The sunfleck responses observed to *A. semialata* and differences between C_3 and C_4 photosynthetic type were also evident in shade adapted understory grasses. The only difference was that the rate of induction were marginally increased, suggesting some degree of species specific adaptation to changing light.

- When compared to growth under continuous light, both C_4 established tillers and seedlings growth was negatively affected by grown under the 6-sec flecking-light treatment. Both tillers and seedlings showed the same negative response in growth under flecking-light, indicating the presence of underground reserves in the tillers did not improve the C_4 subtypes ability to utilize flecking-light. Although differences were noted, caution must be taken when using LA as a proxy to infer effects on growth. Regardless, the differences between the two under flecking-light has important implications on C_3 and C_4 grass seedling establishment under encroaching tree canopies in a changing global environment.

CHAPTER THREE: DESCRIPTION OF LIGHT MICRO-ENVIRONMENTS FOR CO-OCCURING SHADE ADAPATED C_3 *(Erharta erecta)* and C_4 (*Brachiaria chusqueoides)* GRASSES.

3.1. Introduction

The results of the previous chapter highlight the different responses of C_3 and C_4 subtypes of *A. semialata* to sunflecks. This, coupled with the literature that highlights that a significant proportion (30-80%) of the light received in the understory is in the form of short sunflecks less than 10 seconds in duration, suggests that C_4 grasses should be precluded from these forest environments (Pearcy and Calkin, 1983; Pearcy *et al.*, 1985; Chazdon and Pearcy, 1986*b*,*c*, 1991; Sharkey *et al.*, 1986; Pearcy, 1988; Krall and Pearcy, 1993; Sims and Pearcy, 1993; Yanhong *et al.*, 1994; Watling *et al.*, 1997*a*). However, both C_3 and C_4 grasses are found to co-exist in the understory of coastal forests in the Eastern Cape, South Africa. As a result, this chapter sets out to investigate whether these environments are rich in short duration sunflecks or whether C_3 and C_4 grasses occupy different light micro-habitats in this understory environment. Differences in light micro-habitats might explain why, despite C_4 grasses being less capable at using short-duration sunflecks than C_3 grasses, both types occur in certain forest understories.

All plants have an innate ability to acclimate to their surroundings, but plants which are more phenotypically plastic, have a greater ability to respond to changes in environmental conditions (Sage and McKown, 2006). It is believed that increases in specialization to certain environments leads to a decrease in phenotypic plasticity, "a change in the phenotype

expressed by a single genotype in different environments". It is this plasticity which allows plants to respond to different environmental conditions, affecting the range in which a species can be found (Sage and McKown, 2006; Gratani, 2014). These responses are usually induced changes at the morphological and physiological level, which are crucial to a plants ability to acclimate and survive in heterogeneous, variable conditions. In order to determine the extent to which plasticity allows a plant to survive changing environments, the role of plasticity in specific functional traits in response to these changes has to be determined.

As traits are not independent variables, it is important to view plasticity as an integrated function of morphology, physiology and evolutionary history as different selective pressures play a role in shaping plasticity. One such pressure is the change in light experienced within and below a plant canopy. A noted expression of plant plasticity is the modification of various leaf traits, including photosynthetic induction rate, in response to different concentrations and quality of light received by each leaf (Boardman, 1977; Sage and McKown, 2006).

Plants can be classified as either sun or shade plants depending on their ability to utilize different light intensities experienced in their native environments (Boardman, 1977). Plants that grow in shaded environments exhibit a trade-off between the ability to reach high photosynthetic rates, experienced by high light plants growing under high light intensities, and the ability to perform efficiently enough to outperform other "high light" plants in shaded environments (Boardman, 1977).

Many C_3 grasses have been classified as shade plants. Shade plants are generally exposed to a blend of very weak diffuse light which is enriched with far-red wavelengths as well as direct bursts of saturating light (Anderson *et al.*, 1995). It has been suggested that blue light/UV-A is the main stimuli in light acclimation (Anderson *et al.*, 1995). In an effort to

maximize light capture, the plant has an increase in light harvesting complexes (increased chlorophyll in PSI and PSII). As a result, electron transport and photophosphorylation is compromised, resulting in lower photosynthetic rates which become saturated at lower irradiances (Anderson *et al.*, 1995). When acclimating to low light environments, the plant decreases the amounts of light harvesting complexes (LHCI and LHCII) with increasing light and changes the amount of PS II relative to PS I (photosystem stoichiometry). Shade plants have lower ratio of PSII/PSI of 1-1, 3 due to fewer amounts of PSII with larger light harvesting antennae. Sun plants have ratio of PSII/PSI 1, 8-2, 4 with more PSII units with smaller light harvesting complexes. This stoichiometry is affected by both light quality and intensity; therefore different alterations are made to maintain a balance. Low light intensity causes a decrease in PSII content (chlorophyll basis) while the increase in light that stimulates PSI in deep shade causes an increase in PSII (Anderson *et al.*, 1995).

Alternatively in response to high light, PSII loses functionality but 60% of reaction centres remain open. Therefore the redox state of Q_A is crucial and is regulated by controlling energy distribution between PSII and PSI, electron transport rate, xanthophyll cycling and non-photochemical dissipation of light (Anderson *et al.*, 1995). The rates of electron transfer to oxygen increases transiently in response to light flecks, as there are rapid adjustments in regulation between the processes of the electron transport chain and CO_2 assimilation (Foyer and Noctor, 2000). Light experiences during flecks is greater than that required for metabolism, leading to potential imbalances which are dangerous and could provoke photoinhibitory damage (Foyer and Noctor, 2000). In responses to short term light fluctuations, there are rapid LHCII state transitions, energy dissipation and down-regulation of PSII. As a result, some PSII complexes are rendered non-functional while still allowing efficient capture of light (Anderson *et al.*, 1995). Clearly, PSII is the target for major short

term responses, notably the phosphorylation of LHCII, resulting in state transitions of LHCII. As LHCII acts as a photoreceptor, this allows a direct feedback between electron transport and nuclear encoded photosynthetic genes. As a result, the balance of energy is maintained by controlling the state transitions of the photosystems (Anderson *et al.*, 1995; Kruse, 2001).

As state transitions of LHCII and the extent of phosphorylation induced energy redistribution between PSII and PSI is greatest in plants grown in low light environments, shade plants should be better adapted to deal with the sudden fluctuation of light intensity and quality experienced during sunflecks (Ruban, 2014). As a result, C_4 grass species, which are sun species, should be less competitive than C_3 shade grasses under sunfleck rich environments. Superimposed on the limitation of being a 'sun' species, C_4 grass responses to fluctuating light are further complicated by the dual cell photosynthetic machinery and the extra level of co-ordination needed to maintain the balance of energy between the photosystems and metabolic pools between the mesophyll (MSC) and bundle sheath (BSC) cells which have been shown in the previous chapter.

As a result, it is hypothesized in this chapter that, although both species co-occur in the same forest understory, C_3 *Erharta erecta* will be found in micro-habitats where short duration sunflecks are prevalent while C_4 *Brachiaria chusqueoides* will be found in canopy gaps that allow longer duration sunflecks or in patches of continuous light. In order to explore this hypothesis, the light environment in which both these species were found, underneath a coastal forest canopy, were characterized to determine if the environments differed significantly in 1) The total daily light received, 2) The frequency of sunflecks and 3) The duration of different intervals of sunflecks.

Coupled with these hypotheses is the role that evolutionary history plays in this response. As traits are often shared between taxonomically different species that are exposed

to the same environmental pressures, it is important to determine if the plant response is driven by its evolutionary history (phylogenetic signal) or is rather as a result of species specific acclimation. The role of phylogenetic signal of flammability traits in grasses has been shown by Simpson *et al.*, (2016), whereby closely related grass species tended to show similar re-growth rates of aboveground biomass in response to the average number of times their natural habitats were burnt (Simpson *et al.*, 2016; Ripley *et al.*, 2015).

This evolutionary effect was investigated in this chapter by assessing the role of grass acclimation to sunfleck environments by determining the relationship between the rate at which photosynthesis is turned on (photosynthetic induction) in response to flecking-light as a result of the openness of the location in which it is found below the canopy (percentage canopy openness determined by gap fraction analysis). As such, it is hypothesized that there is a relationship between canopy openness and the rate of photosynthetic induction after exposure to short duration sunflecks. When comparing this response between species, it is hypothesized that phylogenetic signal would play a role in the grasses ability to induce photosynthesis in response to flecking-light and not individual species specific acclimation.

3.2. Methods and Materials

Firstly, the percentage canopy openness (gap fraction) and associated sunfleck environments of a range of *E. erecta* and *B. chusqueoides* were measured to determine if C_3 and C_4 grass species occupy different light niches under the canopy. Secondly, the ability of a range of grasses in different light environments to induce photosynthesis in response to flecking-light was explored as well as the role that canopy openness has on shaping these

responses. Using these responses, the influence of phylogenetic signal (Pagels lambda, λ) was determined by superimposing photosynthetic induction responses to flecking-light as a result of canopy openness on a time-calibrated phylogenetic tree of the selected species as well as the induction responses of *E. erecta* over a range of 'openness' environments to assess single species acclimation.

3.2.1. Sunfleck environments occupied by E. erecta and B. chusqueoides

3.2.1.1. Plants

C_3 (*Erharta erecta* ((Hochstetter) Pilger) and C_4 (*Brachiaria chusqueoides* (Hack.) Clayton and *Dactylotenium australe*, Steudel) were located in their natural shaded understory environments of a coastal forest in Kasouga, in the Eastern Cape, South Africa (26°44'9.41"E; 33°39'4.66"S). Within the canopy, 8 replicates of each species were identified and the light environment was determined for each with the use of Gap Light Analyzer (GLA) software analysis and statistical analysis of light sensor data.

3.2.1.2. GLA (Gap Light Analyzer) analysis for percentage canopy **openness**

At each identified specimen, photographs were taken skyward from the ground using a 180° hemispherical, fisheye camera lens (lens: Sigma 4.5mm F2.8 EX DC HSM Circular Fisheye) which was connected to a levelled Pentax k30 camera with the direction of North recorded in the top right corner of each image. The circular image produced recorded a bitmap image of the size, shape and distribution of gaps in the forest canopy. This image was

analyzed in Gap Light Analyzer (Version 2.0) via the transformation of the binary image pixels into sky and non-sky classes and the subsequent computation of these details into light transmissions, canopy openness, leaf area and sunfleck frequency (Frazer *et al.*, 1999). Canopy openness is calculated by taking into account the percentage of sky versus non-sky class pixels to determine the amount of sky seen from below the canopy by the hemispherical photograph, not taking into account the influence of surrounding topography.

3.2.1.3. Light environment for canopy openness

In order to determine how light was experienced by each *E. erecta* and *B. chusqueoides* specimen, light characteristics where determined via photodiodes placed at each replicate plant. Each sensor was secured into one end of a PVC tube, the other end was sharpened such that it could be inserted into the ground. This was done such that the sensor faced upright, adjacent to, and in the same plane as a fully expanded leaf. During the course of a week, multiple 24 hour light courses were measured for 10 individual leaves of each of the two species. Each photodiode was connected to a Personal Daq/56^{TM} USB Data Acquisition System, which in turn was connected to a laptop running the Personal DaqView software calibrated to record the light frequency of each individual photodiode at a frequency of 1 second (Appendix F). Each photodiode was calibrated prior to measurements using a *PPFD* light sensor connected to a LICOR-6400-F photosynthesis system.

3.2.1.4. Statistics

Canopy openness

Percentage canopy openness was calculated as per section 3.2.1.2 for each individual (8x *Erharta erecta* and 8x *Brachiaria chusqueoides* replicates). Percentage canopy openness was compared between both species using a Single Factor ANOVA, where effects were significant at the 95% confidence level.

Light environment

The three 24 hour data sets were exported into excel and each diode was aligned by their corresponding time stamps to ensure accurate matching of temporal values for statistical comparison using the statistical programme, R. Data presented includes light values recorded between dawn and dusk which was defined as the period when *PPFD* was greater than 50 μmol m^{-2} s^{-1}. If the light was above the threshold, the cumulative light intensity was recorded for that period. The subsequent light data was cut into 1 of 13 bins. Each bin is a time based category into which sunflecks were grouped based on their duration so that an average between probes could be obtained (seconds) (Table. 3-1).

Table 3-1. Duration categories (bins) into which light environment data (seconds)

Number	Bin group [seconds (>) and minutes (-)]
1	>5
2	5 > 20
3	20 > 45
4	45 > 90
5	90 > 180
6	3 - 7
7	7 - 12.5
8	12.5-17.5
9	17.5-30
10	30-60
11	60-120
12	120-240
13	240 -480

Data points for both species were plotted against bin group (i.e. Duration of fleck, s) The light environment was described by the following, 1) The average light intensity (μmol m^{-2} s^{-1}) of flecks falling within each bin category; 2) The percentage daily *PPFD* received as sunflecks of each bin category: Daily *PPFD* (%) and 3) The accumulated light period, summed across all light intervals that fell into a particular duration category: Cumulative *PPFD* (Log, μmol m^{-2}). The code for all programmes run in R is attached (Appendix G).

Coding of data for statistical comparison produced by Michael D. Cramer, Department of Biological Sciences, University of Cape Town.

3.2.2. Phylogenetic vs. acclimatory responses

3.2.2.1. Plants

Species were randomly selected in their natural shaded environments of a coastal forest in Kasouga, in the Eastern Cape, South Africa (26°44'9.41"E; 33°39'4.66"S). Each species was further identified via sequencing and a time calibrated phylogeny of all species was created. Species included: C_4 subspecies Alloteropsis semialata (R.Br.) Hitch. subsp. semialata and C_3 subspecies Alloteropsis semialata (R.Br.) Hitch. subsp. eckloniana (Nees) Gibbs Russ; *Erharta erecta* ((Hochstetter) Pilger); *Brachiaria chusqueoides* (Hack.) Clayton; *Dactylotenium australe*, Steudel; *Cenchrus ciliaris* L.; *Setaria megaphylla* (Steud) Dur.& Schinz and two other *Erharta* species (*Erharta* spp.1 and *Erharta* spp.2).

3.2.2.2. Phylogenetic analysis

(DNA and BEAST procedures carried out by Kimberley Simpson and Pascal-Antoine Christin, Osborne lab group, Department of Animal and Plant Sciences, University of Sheffield).

A phylogeny was constructed using the plastid markers *trnKmatK*, available from the Grass Phylogeny Working Group II (2012) or measured for ten species in this study. *trnKmatK* was amplified via PCR using genomic. PCRs were carried out in a total volume of 25 µl, including *c.* 40–100 ng of gDNA template, 5 µl of 5× GoTaq reaction buffer, 0.1 mM dNTPs, 0.1 µM of each primer, 1 mM of $MgCl_2$, and 0.5 unit of *Taq*polymerase (GoTaq DNA Polymerase, Promega, Madison, WI, USA). Mixtures were incubated in a thermocycler for 3 min at 94°C followed by 36 cycles consisting of 1 min at 94°C, 30 s at 48°C and 1 min at 72°C. This was subsequently incubated at 72°C for 10 minutes. Successful amplifications were cleaned with an Exo-SAP-IT treatment (Affymetrix, Santa Clara, CA, USA) and sequenced using the Big Dye 3.1 Terminator Cycle Sequencing chemistry (Applied Biosystems, Foster City, CA, USA). All sequences were deposited in GenBank.

The new markers were manually aligned to the data set which had been downsized to a smaller subset of species, including all the taxa studied here and representatives of all grass lineages. A time-calibrated phylogenetic tree was obtained through Bayesian inference as implemented in BEAST (Bayesian evolutionary analysis by sampling trees; Drummond & Rambaut, 2007). The initial tree was constructed following the Yule process using the GTR+G+I model. The log-normal relaxed molecular clock was selected. The monophyly of the BEP-PACMAD clade was enforced, leaving *Puelia olyriformis*as the out-group. The calibration prior for the age of the BEP-PACMAD crown was set to a normal distribution, with a mean of 51.2 and a standard deviation of 0.001, setting the age of the root at 51.2 Myr (mean based on Christin *et al.* 2014). Independent runs were conducted for 10 000 000 generations, sampling a tree every 1000 generations. The convergence of the runs and the appropriateness of the burn-in period, set to 1 000 000 generations, were verified using Tracer

(Rambaut A, Drummond AJ (2007) Tracer v1.4, available at http://beast.bio.ed.ac.uk/Tracer). Median ages were mapped on the maximum-credibility tree nodes. Comparative analysis of species relationships were made from this tree. The relationships among the species studied here were extracted from this tree and used for comparative analyses.

3.2.2.3. Sunfleck responses for phylogenetic analysis

The induction of photosynthesis was measured for the third fully expanded leaf, after initially maintaining leaves at a photosynthetically active photon flux density (*PPFD*) of 0 μmol m^{-2} s^{-1} for 30 minutes by attaching dark adapting leaf clips to each leaf. The leaves were exposed to an intermittent *PPFD* of 6-sec pulses at 1000 μmol m^{-2} s^{-1},interspersed with 6-sec periods of dark for a period of 5 minutes. These conditions were generated within the leaf chamber of LI-6400-F Photosynthesis System (Li-Cor Inc. Lincoln, Nebraska, USA), where leaf temperatures were maintained between 23 and 26 ^{o}C, atmospheric vapour pressure deficit (VPD) at values below 1.7 kPa. Gas exchange and chlorophyll fluorescence parameters were recorded at 10-sec intervals and the experiments were repeated at ambient (40 Pa) atmospheric CO_2 concentrations supplied by the reference air entering the leaf chamber. The gas exchange parameters were calculated according to von Caemmerer and Farquhar (1981) and von Caemmerer (2000) and the photosynthetic rate at 300-sec was defined as A_{max} for each selected species.

In order to determine the efficiency at which induction was occurring over time in response to flecking-light, the initial slope of the induction response was compared between species and was termed, induction efficiency (IE).The initial slope (IE) of each flecking-light curve was fitted with the monomolecular equation: A = a(1-EXP(b-cc*Time). Fitted

equations were used to calculate means, standard errors and the parameters a, b and c. These in turn were used to calculate the induction efficiency ($IE=ac^b$) and light saturated photosynthetic rates, *Amax* (*Amax* = a).

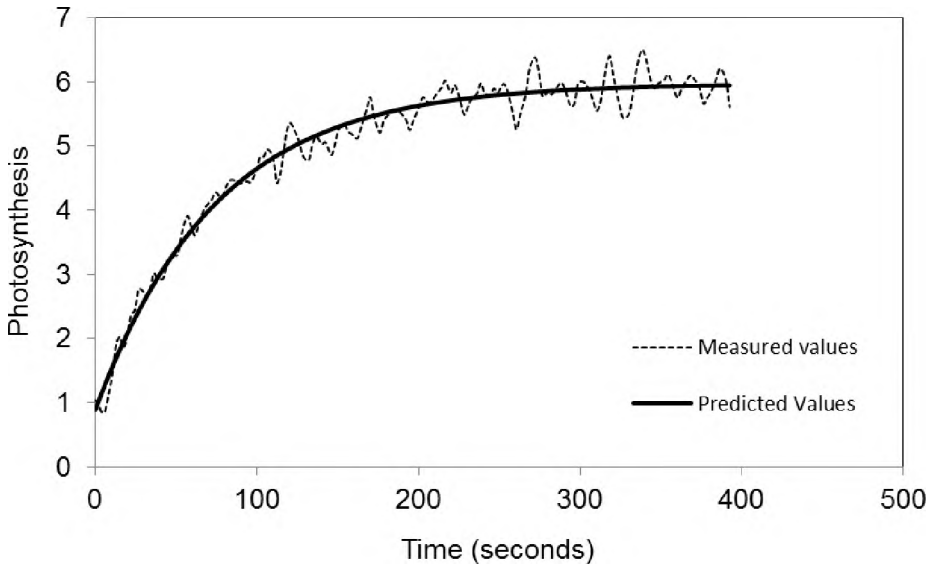

Figure 3-1. Example of curve fitting used to determine the slopes (Induction efficiency, IE) of photosynthetic induction in response to flecking-light interspersed with 6-sec dark periods for various grass species.

3.2.2.4. Statistics

Measurements for multiple replicates of the same species under the same canopy openness were averaged before statistical analysis. Plots of slope vs. canopy openness were created to determine the effect of % canopy openness on the ability of grasses to induce photosynthesis. The significance of this effect was assessed by running a Generalized Linear Model (GLM) for data with a Poisson distributions (R programme) and the strength of the correlation determined via the R^2 value. The effect of phylogeny on this response was determined by testing for the presence of a phylogenetic signal, Pagels lambda (λ). λ measures the similarity of the covariances among species to the covariances expected under Brownian

motion. For $\lambda = 0$, a p-value ≤ 0.05 is significant while for $\lambda = 1$, a p-value ≥ 0.05 is significant. Significant values indicate the presence of a phylogenetic signal. This was done using pgls analysis in the *caper* package (Ormeet *et al.*, 2012) in R.

3.3. Results

3.3.1. Characterization of the light environments of E. erecta and B. chusqueoides

Analysis of canopies above *E. erecta* and *B. chusqueoides* species showed populations were found in areas with 12% and 10% canopy openness respectively with no significant difference in gap light environments between both species (Fig. 3-2; $F_{1,18}= 0.74$; $p= 0.4$).

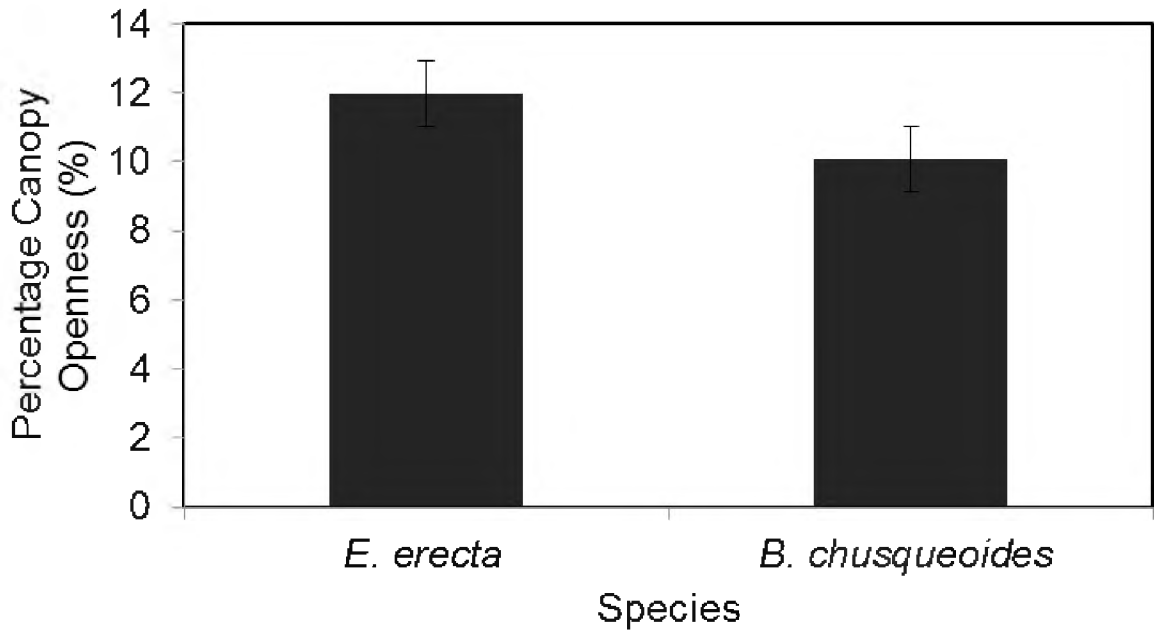

Figure 3-2. Percentage canopy openness in which *E. erecta* and *B. chusqueoides* populations are found within a coastal forest. Vertical bars are standard errors (≥8).

Percentage daily PPFD

The profile of bins (time based category into which sunflecks were grouped) contributing to the daily *PPFD* experienced by *E. erecta* and *B. chusqueoides* were not significantly different between then species ($p = 0.3762$) (Fig.3-3). This indicates that there was no difference between the contribution of short and long duration sunflecks to the total daily light received between species.

Both species received between 37 and 45% of their total *PPFD* in sunflecks ranging between 30-min and 1 hour (long duration). Of the 21% of total *PPFD* received as sunflecks shorter than 3-min, only 8% of flecks were experienced as flashes shorter than 20-sec with only 4% of this value comprising of flecks shorter than 5-sec in duration (Fig. 3-3). Therefore the environments in which *E. erecta* and *B. chusqueoides* were found received the same daily (%) *PPFD* made up mostly of long duration sunflecks and relatively fewer short duration flecks.

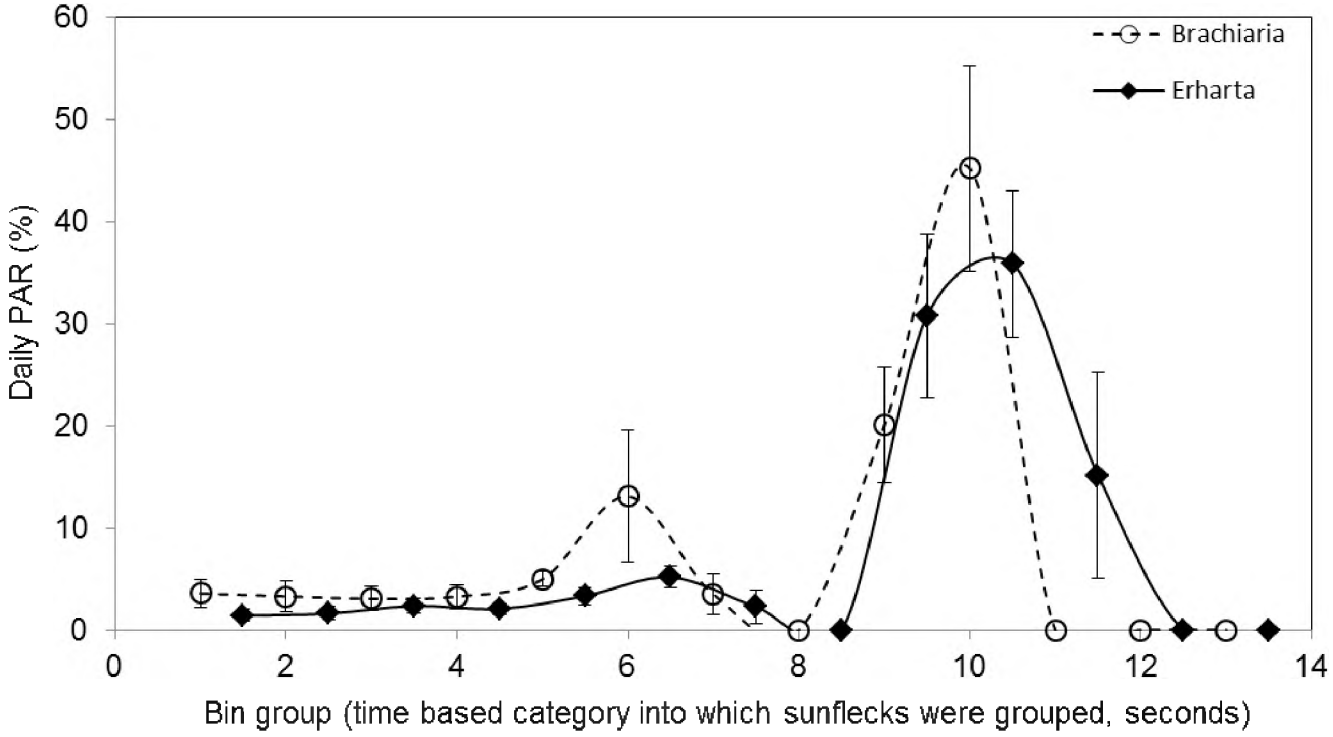

Figure 3-3. The percentage daily *PPFD* received in intervals of different durations (bins) for *E. erecta* and *B. chusqueoides*. Data presented includes light values recorded between dawn and dusk with a threshold *PPFD* value of 50 μmol m^{-2} s^{-1} defining the onset of dawn or dusk. Light data averaged across light sensors was divided into 13 bins (time based category into which sunflecks were grouped, seconds; Table 3-1). Open symbols represent *B. chusqueoides* while closed symbols represent *E. erecta* and vertical bars are standard errors (≥8).

Average light intensity of flecks

The averaged light intensities of the flecks experienced during the day did not differ significantly between *E. erecta* and *B. chusqueoides* (p= 0.28) (Fig. 3-4). Although a staggered pattern emerges across the day, flecks shorter than 3-min ranged in intensity from 65-100 μmol m^{-2} s^{-1} while flecks of 30-min to 1 hour ranged from 85-125 μmol m^{-2} s^{-1} (Fig. 3-4). For both species, flecks that were ≤6 secs in duration average an intensity of 65 μmol m^{-}

^{2} s^{-1}. As sunflecks are defined as "bursts of high, saturating light", it is important to note that the highest sunfleck intensities experienced by both species, before being averaged within each bin group, reached intensities of approximately 2000 μmol m^{-2} s^{-1}.

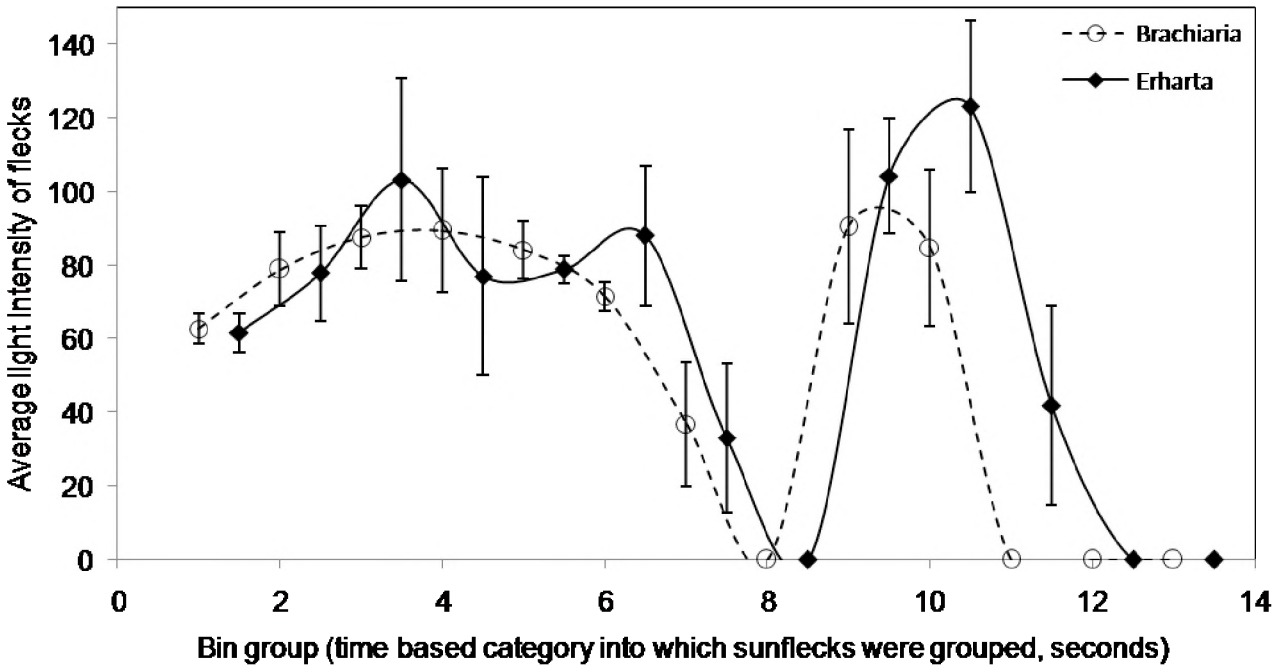

Figure 3-4. The average light intensity of flecks received in intervals of different durations (bins) for *E. erecta* and *B. chusqueoides*. Data presented includes light values recorded between dawn and dusk with a threshold *PPFD* value of 50 μmol m^{-2} s^{-1} defining the onset of dawn or dusk. Light data averaged across light sensors was divided into 13 bins (time based category into which sunflecks were grouped, seconds; Table 3-1). Open symbols represent *B. chusqueoides* while closed symbols represent *E. erecta* and vertical bars are standard errors (≥8).

The accumulated light period

When the *PPFD* over the light period was summed for all the flecks that fell within a particular bin, the staggered light intensity pattern in smoothed (Fig. 3-5). The total amount of

energy received in flecks was uniform over a range of durations up to 3-min, with the highest levels of light coming in between 30-min and 1 hour 'patches' of light (Fig. 3-5). The cumulative *PPFD* of each bin is the same for both species, with no significant difference in the *PPFD* experienced as either short or long duration flecks (p= 0.232) (Fig. 3-5).

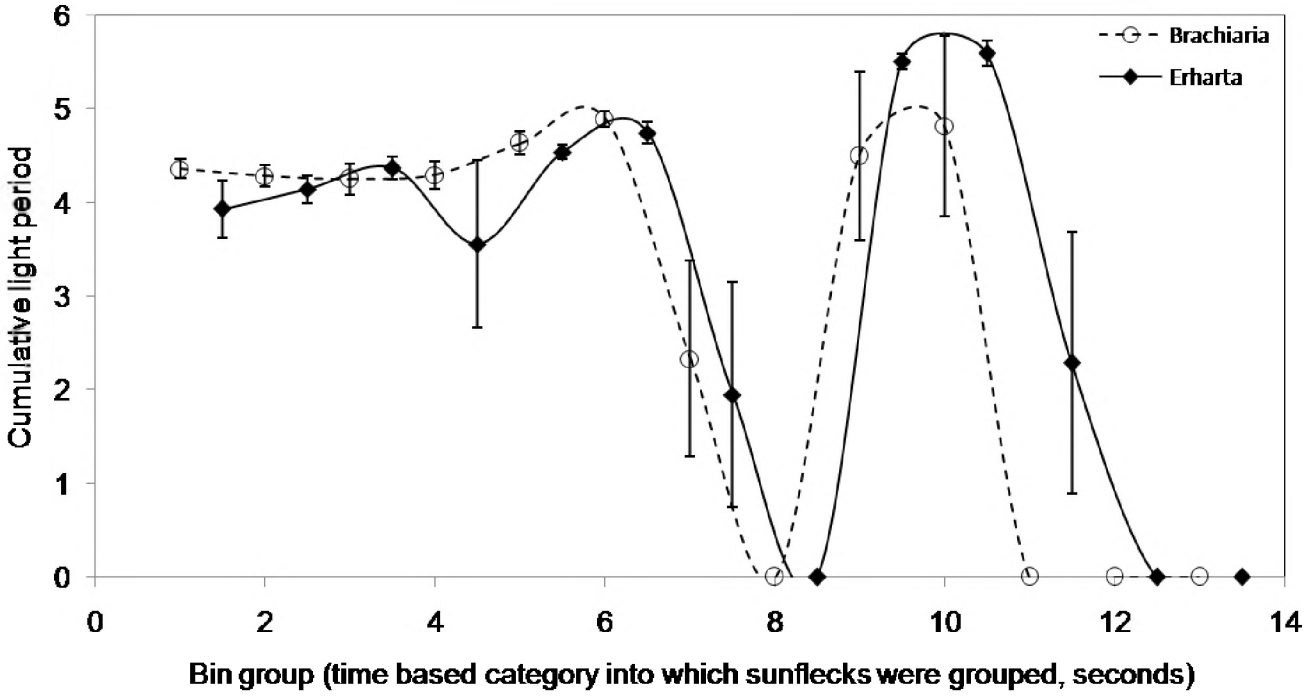

Figure 3-5. The accumulated light period, summed across all light intervals received in intervals of different durations (bins) for *E. erecta* and *B. chusqueoides*. Data presented includes light values recorded between dawn and dusk with a threshold *PPFD* value of 50 μmol m^{-2} s^{-1} defining the onset of dawn or dusk. Light data averaged across light sensors was divided into 13 bins (time based category into which sunflecks were grouped, seconds; Table 3-1). Open symbols represent *B. chusqueoides* while closed symbols represent *E. erecta* and vertical bars are standard errors (≥8).

3.3.2. Photosynthetic induction vs. canopy openness

Figure 3-6 shows the turning on of photosynthesis (photosynthetic induction) in 20 grasses from 10 species groups (C_4 subspecies *A. semialata* (semialata) and C_3 subspecies *A. semialata* (eckloniana); *E. erecta*; *B. chusqueoides*; *D. australe*; *Cenchrus ciliaris*; *Setaria megaphylla* and two other *Erharta* species (*Erharta* spp.1 and *Erharta* spp.2). Induction was calculated via curve fitting in 3.2.2.3 in response to a 6-sec flecking-light treatment as result of individual species percentage canopy openness (Example curve: Fig. 3-1). A significant negative correlation between photosynthetic induction and canopy openness was shown when including both C_3 and C_4 grass species ($F_{1, 14.68} = P<0.01$). As canopy openness increased, the rate at which photosynthesis induced was decreased ($R^2=0.57$; Fig. 3-6 A).

This negative correlation remained when only the data from the C_4 grasses was included ($R^2=0.57$; Fig. 3-6 B). The negative correlation was improved if this was repeated using only the data from the C_3 grasses ($R^2=0.71$; Fig. 3-6 C). This strong positive correlation points to a large effect of canopy openness on the ability of the C_3 grasses to induce photosynthesis after exposure to short duration sunflecks.

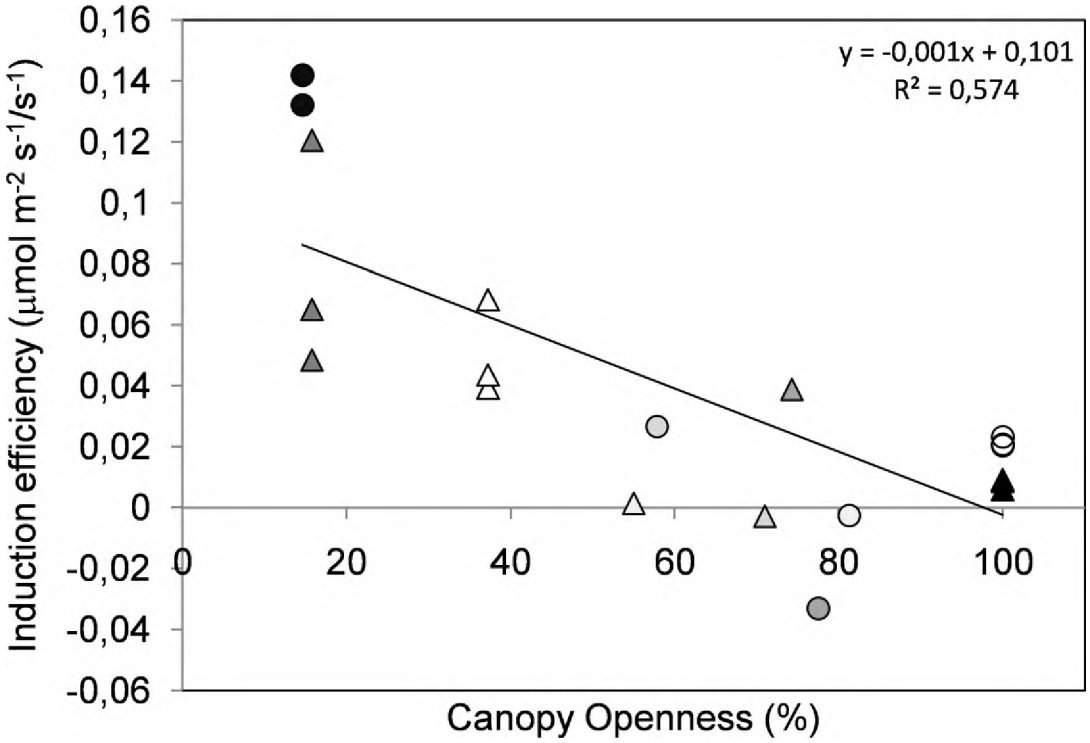

Figure 3-6. Induction efficiency of various grass species under-flecking light in response to canopy openness. Correlation between induction efficiency under flecking-light in response to canopy openness for all measured species; n=20, R^2=0.5748. C_4 grasses represented by closed triangles; n=12, R^2=0.5769. C_3 grasses represented by open circles; n=8, R^2=0.7068. Replicates of species represented by shading. For each individual, the slope (IE) of each flecking-light curve was determined via individual *A* vs. Time curves fitted with the monomolecular equation: *A* = a(1-EXP(b-cc*Time). Slopes were plotted against corresponding canopy openness percentage as determined via GLA.

Although all species showed varied responses of induction in response to canopy openness, there was an overall significantly negative correlation between canopy openness and photosynthetic induction for both C_3 and C_4 grass species ($F_{1,14.68}$ = P<0.01). A significantly negative correlation was also noted between functional type (shade or sun species) and induction efficiency for both C_3 and C_4 grass species ($F_{1,\ 14.34}$ = P<0.001). In order to determine if grass evolutionary history had an effect on these relationships, a time

calibrated phylogeny was created (Fig. 3-7). When the rate of induction to openness correlation was repeated accounting for phylogenetic structure, there was no phylogenetic signal for induction against canopy openness (Pagels $\lambda = 0$; $p = 0.002$ for $\lambda = 1$; $p = 1$ for $\lambda = 0$). This lack of phylogenetic signal was also found for induction against functional type (Pagels $\lambda = 0$; $p = 0.001$ for $\lambda = 1$; $p = 1$ for $\lambda = 0$).

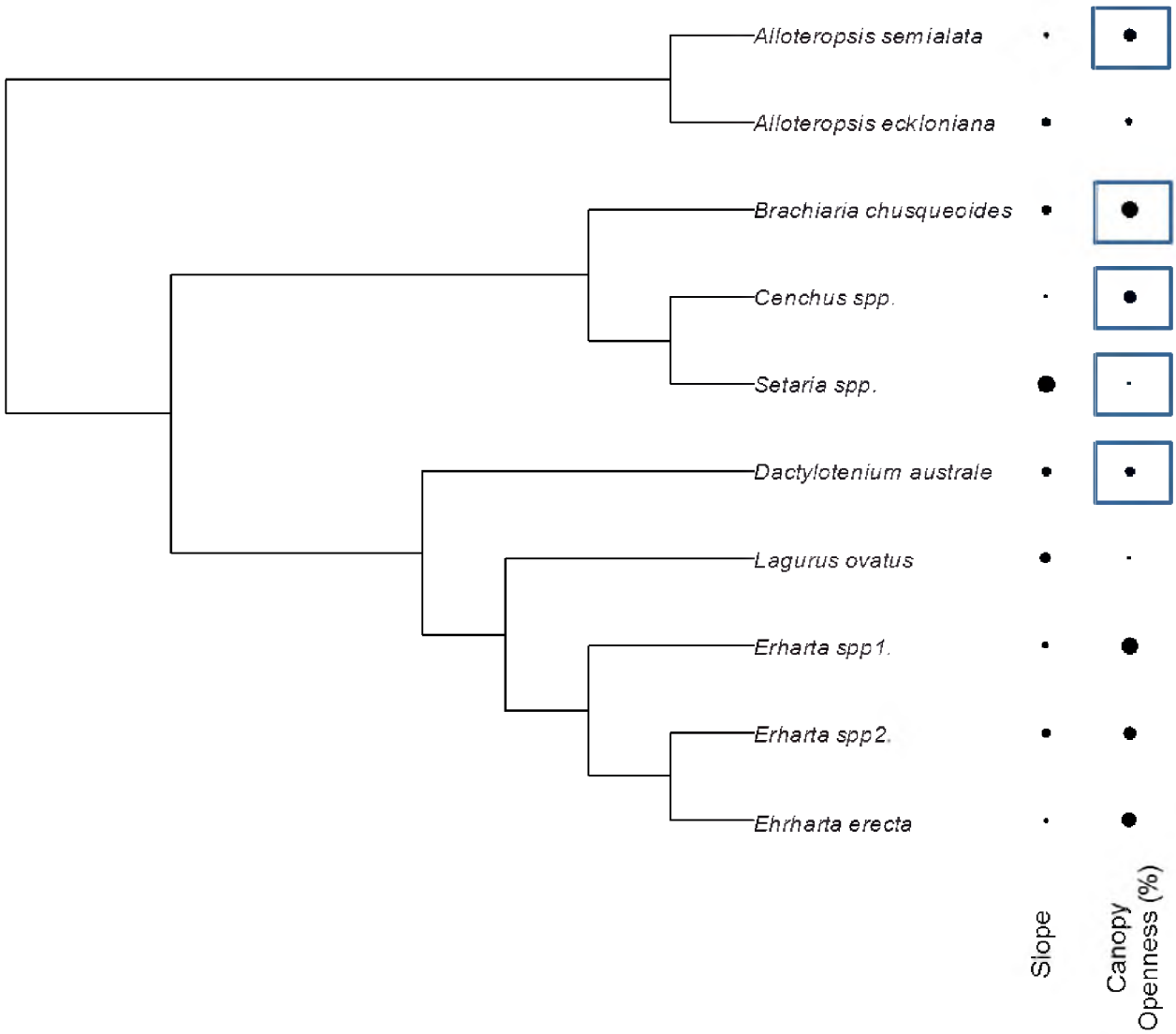

Figure 3-7. Time-calibrated phylogenetic tree showing the evolutionary relationships between species and rates of induction to canopy openness (slope and canopy openness (%)). Blue squares represent C_4 species while no square represents C_3 species. A zero phylogenetic signal was found for slope vs. canopy openness as well as for slope vs. functional type.

As phylogenetic structure could not account for the positive correlation between photosynthetic induction and canopy openness, the role of acclimation was addressed within the single species, *E. erecta* by measuring photosynthetic induction under flecking-light over a range of canopy openness percentages. *E. erecta* photosynthetic induction showed an extremely positive correlation in response to canopy openness (R^2= 0.99; Fig. 3-8). As *E. erecta* is a C_3 shade adapted grass, the higher photosynthetic induction rates reached under closed canopies, where light was limiting, was expected. This is shown where photosynthetic induction rates reached values of 0.14 μmol m^{-2} s^{-1} at 18% canopy openness but decreased to 0.04 μmol m^{-2} s^{-1} and -0.035 μmol m^{-2} s^{-1} at 59 and 78% canopy openness respectively (Fig. 3-8). The positive correlation between canopy openness and photosynthetic induction points to an acclamatory response by *E. erecta* to utilize varying light environments.

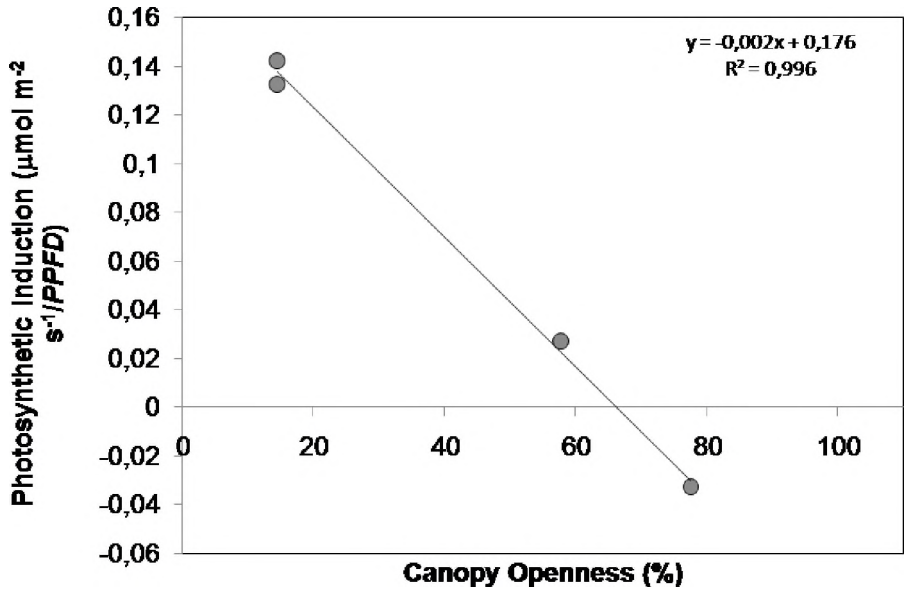

Figure 3-8. Photosynthetic induction of *E. erecta* under-flecking light in response as a result of canopy openness. Correlation between photosynthetic induction under flecking-light in response to canopy openness; n=4, R^2=0.966. For each individual, the slope (IE) of each flecking-light curve was determined via individual *A* vs. Time curves fitted with the monomolecular equation: *A* = a(1-EXP(b-cc*Time). Slopes were plotted against corresponding canopy openness percentage as determined via GLA.

3.4. Discussion

This chapter showed that *E. erecta* and *B. chusqueoides* live in the same light micro-environments. This is possible, despite C_4 compromised photosynthetic productivity in sunfleck environments (Chapter 2), because the daily light dosage is comprised of a significant proportion of long duration sunflecks. GLA analysis of the understory environment, together with the intensity, frequency and accumulated average of the sunflecks received by both species, showed no difference in the light environments between *E. erecta* and *B. chusqueoides*. Further exploration into species induction responses as a result of canopy openness contradicts the hypothesis that phylogenetic history plays a role in these responses, but rather, that individual species acclimation allows survival over a range of variable flecking-light environments.

Photodiode data showed that majority of the light was received in intervals as either short flecks ranging from 0-3-min (21% of daily dosage), or as longer sun-patches ranging from 30-min to 1 hour in duration (37-45% of daily dosage) for both *E. erecta* and *B. chusqueoides* (Fig. 3-3). Only a small proportion (4%) was received as flecks with a duration shorter than 5-sec. There was no difference in accumulated *PPFD* or the light intensity of these flecks ($\leq$ 5-sec) between *E. erecta* and *B. chusqueoides* which, as highlighted in the previous chapter, limited C_4 photosynthetic induction in *A. semialata* to a greater extent than that of C_3 *A. semialata* (Chapter 2, Fig. 2-1). As such, the distribution of the *E. erecta* and *B. chusqueoides* in the same light micro-environment under the canopy cannot be explained by differences in their experience of short duration sunflecks.

Between the two species, the highest accumulated light was experienced in patches ranging from 30-min to 1 hour, with each fleck ranging in intensity from 85-125 μmol m^{-2} s^{-}

[1](Fig. 3-5). In the scale of minutes and hours, induction effects controlled by G_{ST}, Rubisco and cell structure may become second to other limitations such as water stress, leaf temperature and photo-inhibition (Pearcy and Way, 2012; Way and Pearcy, 2012; Smith and Berry, 2013). As *B. chusqueoides* possessesC_4 photosynthetic machinery, it should outcompete *E. erecta* by making use of these longer periods of high light due to its increased carboxylation efficiency (Ehleringer and Björkman, 1977; Ehleringer *et al.*, 1997; Chaffey, 1999). The longer duration and intensity of the flecks, as well as decreased wind movement below the canopy, would result in an increase in leaf temperature (Smith and Berry, 2013). Although a shade plant, *B. chusqueoides* would be less affected by increased temperatures as a result of the heat tolerance of the C_4 enzyme, PEPCase (Boardman, 1977; Ehleringer and Björkman, 1977; Ehleringer *et al.*, 1997).Although there was an apparent difference, there was no significant difference when compared to the short duration flecks both within and between species. As both species are found in an area with many short flecks, favouring *E. erecta* as well as longer patches, favouring *B. chusqueoides* new explanation is posed.

It is speculated that there is a trade-off occurring under the canopy. *E. erecta* is able to flourish in the environment with high levels of G_{ST} and Rubisco coupling to utilize the large percentage of light received as short sunflecks while *B. chusqueoides* is able to outweigh the effects of the sunfleck rich environment by making use of increased quantum efficiency and high levels of heat tolerance during the longer sun patches (Boardman, 1977; Ehleringer and Björkman, 1977; Pearcy and Way, 2012; Porcar-Castell and Palmroth, 2012; Smith and Berry, 2013).

If the C_3 and C_4 plants do in fact use the different duration flecks differently, then the responses are likely to be modified by season. A study of Japanese temperate deciduous forests, receiving 10-20% of its *PPFD* as sunflecks, and coniferous forests, receiving 30-60%

of its *PPFD* as sunflecks, showed high light intensities differed between the growing season of February deciduous forests and May to October for coniferous forests (Way and Pearcy, 2012). Annually, light would be experienced by both species not only as flecks, but as diffuse light transmitted and reflected by the clouds (Smith and Berry, 2013). This adds another level of complexity, not only due to the formation and type of the clouds, but their movement over time and space depending on above and ground winds (Smith and Berry, 2013). Therefore it would be imperative to include seasonality and wind speed effects in order to fully understand how difference photosynthetic types are distributed below the canopy.

Variation in plant traits, which would allow them to respond to changing sunfleck environments, would result from different evolutionary and environmental drivers over various temporal and spatial scales. As such, throughout evolutionary history, grasses would have gained and lost various sun or shade traits, where closely related species would tend to be similar, occupying the same types of environments as their ancestors (Cayssials and Rodríguez, 2013). This is seen where shade characteristics, similar to those of the original grasses found in deep shade environments, are retained today in the earliest diverging grass lineages while the evolution of C_4 photosynthesis allowed grasses to invade and diversify successfully into hot, open climates (Edwards and Still, 2008; Edwards and Smith, 2010; Cayssials and Rodríguez, 2013; Arakaki *et al.*, 2015).

When addressing the role that evolutionary history plays in sunflecks versus canopy openness responses, the lack of phylogenetic signal found in species induction efficiency as a result of canopy openness suggests that evolutionary history cannot explain the correlation between inductions under flecking-light in response to canopy openness (Fig. 3-8). Together with the lack of phylogenetic signal in induction efficiency as a result of functional type (sun versus shade grasses), results point to the conclusion that individual species show the

potential to acclimate to the varying light environments (Fig. 3-9). Caution on the robustness of this conclusion must be taken due to the low statistical power derived from such a small phylogeny(Simpson *et al.*, 2016)

As phylogenetic relatedness is being used more commonly used as a proxy for phenotypes to predict species responses, as closely related species tend to share similar traits in response to a particular niche, it is important to unpack the complexities of environment-trait interactions (Buckley and Kingsolver, 2012). As research into these relationships progresses, it is clear that the linear expectations in these responses needs to be addressed as they cannot fully explain these complexities, including the effects of acclimation as demonstrated in the presented results.

3.5. Conclusions

- Analysis of the light micro-environment under the canopy via the calculation of percentage canopy openness and light profiles determined via photodiodes placed under the canopy showed that there was no significant difference in the total daily light received by *E. erecta* and *B. chusqueoides.*

- No significant difference was found between light experienced as either short duration sunflecks (0-3 min) or longer patches (30-min to 1 hour) between *E. erecta* and *B. chusqueoides* species.

- Induction efficiency after flecking-light was negatively correlated to percentage canopy openness. This was not as a result of phylogenetic evolutionary responses but rather as a result of individual species acclimation or evolutionary variation among individual species.

CHAPTER FOUR: SYNTHESIS

This final chapter attempts to synthesize the results of the pre-ceding two chapters by addressing the theory that there is no fundamental limitation imposed on C_4 species ability to utilize sunflecks when compared to C_3 species (Pearcy and Calkin, 1983; Pearcy *et al.*, 1985; Chazdon and Pearcy, 1986*a*; Sharkey *et al.*, 1986; Pearcy, 1988; Krall and Pearcy, 1993; Sims and Pearcy, 1993; Yanhong *et al.*, 1994; Watling *et al.*, 1997*a*),as well as the commentary of Sage (2014). Sage proposed that, *"Because C_4 plants have a more complex biochemistry, is it possible that they are slower to exploit sunflecks? This is unlikely, as examinations of sunflecks do not reveal any major differences between the photosynthetic pathways...C_4 grasses are also adapted to the shade of forest interiors... examples indicate that there may be no inherent reason for C_4 failure in the shade"*. This resulted in the proposal of various questions relating to the preclusion of C_4 grasses from shaded, understory environments:

- Do light and shade adapted C_4 grasses respond more slowly to short duration sunflecks than C_3 grasses and does this response translate into effects on growth in a simulated sunfleck environment?
- Are these responses seen in the natural environment and are they shaped by past evolutionary history or acclimation?
- What next for C_4 grasses in changing understory environments?

Do light and shade adapted C_4 grasses respond more slowly to short duration sunflecks than C_3 grasses and does this response translate into effects on growth in a simulated sunfleck environment?

Short duration sunflecks had a greater negative effect on C_4 *A.semialata* when compared to the C_3 subtype. Exposure of both subtypes to a 6-sec flecking-light treatment lead to a 65% decrease in C_4 *A. semialata* saturated photosynthesis (A_{max}), which could not be explained by effects on stomatal conductance (G_{ST}). While C_3 *A. semialata* was able to maintain A_{max} levels by increasing G_{ST} rapidly in response to flecking-light. By increasing the dark periods between flashes to 30-sec, A_{max} of C_3 and C_4 subtypes decreased by 33% and 76% respectively (Fig. 2-2; Fig. 2-3). This decreased photosynthetic ability of the C_4 subtype has previously been attributed to their higher energetic requirements in low light environments when compared to C_3 grasses (Ehleringer and Björkman, 1977; Ehleringer, 1978; Ehleringer *et al.*, 1997), but this cannot explain the absence of C_4 grasses in warmer, shaded tropical environments.

It has been proposed that C_4 grasses are not inherently limited from shaded, sunfleck rich environments because of the presence of a few shade adapted C_4 grasses (Chazdon and Pearcy, 1991; Kromdijk *et al.*, 2008, 2010, 2014; Sage, 2014; Sun *et al.*, 2014). Although C_4 shade adapted grasses do exist, results in this thesis highlight that although shade adaptation in *B. chusqueoides* and *D. australe* allowed for an increase in photosynthetic induction in response to short duration sunflecks (Fig. 2-6), these grasses were still not able to induce photosynthesis at as high a rate as either sun or shade adapted C_3 species, *A. semialata* and *E. erecta* (Fig. 2-2; Fig. 2-6). This points to some degree of species specific adaptation to changing light with no a fundamental difference between C_3 and C_4 species, which was also seen when looking at the response of *E. erecta* over varying canopy openness ranges where;

there was a significantly positive correlation between canopy openness and the ability of *E. erecta* to induce photosynthesis under flecking-light (Fig. 3-6).

Differences in leaf growth between C_3 and C_4 *A. semialata* were seen under a simulated flecking-light environment. When compared to growth under continuous light, both C_4 established tillers and seedlings growth was negatively affected by growth under the 6-sec flecking-light treatment over a 2 week period (Fig. 2-7). As seedlings showed the same negative response in growth as tillers under flecking-light, this indicated that the presence of underground reserves, often reported in literature to confer a competitive advantage in various environments, did not improve the C_4 subtypes ability to utilize flecking-light (Ripley *et al.*, 2008). This negative effect on C_4 growth over time is speculated to occur as a direct result of the proposed metabolic limitations on PSII in the CCM. Although, C_3 established tillers and seedlings displayed no significant decrease in growth under 6-sec flecking-light, the differences between the two subtypes under flecking-light has important implications on C_3 and C_4 grass seedling establishment under encroaching tree canopies in a changing global environment.

Commentary by Sage, 2014 was based on examples utilizing either C_3 tree species or by comparing phylogenetically distinct grass species. The use of *A. semialata* in this study allows for a comparison of sunfleck responses without the discrepancies involved when using phylogenetically distinct species. This study also utilized short duration sunflecks which simulated the natural flecking-light environment (Pearcy and Calkin, 1983; Chazdon and Pearcy, 1986*a*, 1991; Sharkey *et al.*, 1986; Pearcy, 1988; Krall and Pearcy, 1993; Sims and Pearcy, 1993; Yanhong *et al.*, 1994; Watling *et al.*, 1997*a*,*b*).The use of *A.semialata* and shorter sunfleck durations highlighted that C_4 grasses are limited under a sunfleck environment. As such, I propose an alternative theory, whereby sunflecks impose a metabolic limitation on the carbon concentrating mechanism (CCM) of the C_4 photosynthetic pathway.

Light response curves of C_4 *A. semialata* constructed in response to the 6-sec flecking-light treatment showed that photosynthesis (*A*) is negatively affected in the light saturated portion of the curve (Fig. 2-5). This possibly links the decrease in *A* in C_4 *A.semialata* to an energetic limitation imposed on the carboxylation mechanism of PSII in the CCM and not to increased levels of leakiness which are often reported when moving plants from continuous high light to continuous low light environments (Sage, 2004; Gowik and Westhoff, 2011; Sage *et al.*, 2012 Pearcy *et al.*, 1985; Sage, 2014*b*). The resultant effect of this proposed carboxylation limitation limited C_4 growth over time (Fig. 2-7), where grasses were unable to cope with the energy imbalances imposed on the carboxylation mechanism of the CCM, whereby the continued lack of NADPH inhibited the reduction of 3-PGA and a down-regulation of PEPCase activity. In contrast, C_3 *A. semialata* was able to utilize their increased stomatal conductance (Fig. 2-3) and less effected Rubisco activity (Porcar-Castell and Palmroth, 2012; Way and Pearcy, 2012; Kirschbaum and Pearcy, 1988; Tinoco-Ojanguren and Pearcy, 1992) to assimilate CO_2.

Is this seen in the natural environment and are these responses shaped by past evolutionary history or acclimation?

As short duration sunflecks had a physiological effect on the ability of C_4 *A. semialata* to utilize short duration sunflecks during photosynthetic induction and growth (Chapter 2), it was expected that C_3 and C_4 grasses would not occupy the same light micro-environment under the forest canopy. Examination of the light micro-environment of shade adapted *E. erecta* (C_3) and shade adapted *B. chusqueoides* (C_4) species under the canopy, showed no significant difference in the total daily light received by both species. Both *E. erecta* and *B. chusqueoides* were found in canopy gaps ranging from 10-12% openness (Fig. 3-2) and

received similar amounts of short (0-3-min) and long (30min- 1hour) duration sunflecks, with light intensities ranging from 60-125 μmol m^{-2} s^{-1} (Fig. 3-3; Fig. 3-4; Fig. 3-5). Sage commented that if shade adapted C_3 and C_4 grasses were as fit as each other in deep shade that the focus should shift from a physiological explanation to one focusing more deeply into non-photosynthetic mechanisms for C_4 preclusion, such as phylogenetic history (Sage, 2014).

When assessing the role that phylogenetic history plays into the ability of C_3 and C_4 grasses to utilize short duration sunflecks to induce photosynthesis over a range of canopy openness percentages, the significant positive correlation between photosynthetic induction and canopy openness ($F_{1, 14.68}$ = P<0.01; R^2=0.57; Fig. 3-6 A) could not be explained by phylogenetic signal (Pagels λ= 0; p= 0.002 for λ= 1; p=1 for λ= 0). This lack of phylogenetic signal was also found for induction against functional type (Pagels λ= 0; p= 0.001 for λ= 1; p=1 for λ= 0).Again, caution must be taken when using such a small phylogeny when inferring a lack of phylogenetic signal.

I speculate that there is a trade-off occurring, which allows both C_3 and C_4 grasses to compete in the same light micro-environment. As seen when comparing sunfleck utilization between *E. erecta* and *B. chusqueoides* in the natural environment, long pulses of light are just as dominant as flecks, which would be able to drive both C_3 and C_4 photosynthesis. As a result, C_3 grasses would be able to thrive, unaffected by the short duration sunflecks, while C_4 grasses would be able to survive the effects of exposure to short sunflecks by utilizing the energy received during the longer periods of high light. As results were obtained from a coastal forest, it is vital to obtain wind and seasonal data to analyse these effects on the light micro-environment.

What next for C_4 grasses in changing understory environments?

In contrast to theories that infer that there is no physiological limitation imposed by the C_4 photosynthetic pathway in response to sunflecks(Ehleringer and Björkman, 1977; Pearcy and Calkin, 1983; Chazdon and Pearcy, 1986*a*; Ehleringer *et al.*, 1997), there is a clear decrease in C_4 photosynthetic induction and growth as a direct result of exposure to short duration to sunflecks (Chapter 2 & 3). These results are also in contrast to the theory that C_3 grasses are able to outperform C_4 grasses in sunfleck environments as a result of their increased QE in cooler, shaded environments. Rather, results in this thesis point to a limitation imposed on the carboxylation mechanism of the CCM at the site of PSII which would negatively affect the balance between the mesophyll and bundle-sheath cells.

As a result of increased anthropogenic activities, global CO_2 concentrations have been on the rise and have resulted in an environmental shift, where C_3 tree species are beginning to encroach into savannah biomes (Bond, 2008*b*; Pearcy and Way, 2012; Way and Pearcy, 2012). As for the future of C_4 grasses, if C_3 tree species become dominant in savannah biomes, denser canopies should ultimately exclude C_4 grasses from their niche environments by increasing the presence of short duration sunflecks (Chapters 2 & 3). Therefore it is crucial to understand how C_4 grasses are excluded from these environments in order to manage the effects of global climate change in savannah biomes. As highlighted by Sage, future studies would still need to incorporate the role of grass life histories as well as other ecological interactions which impact the ability of grasses to thrive under the forest canopy (Sage, 2014).

The question still remains, as C_4 photosynthesis was shown to be physiologically limited by short duration sunflecks but was still found in the same light micro-environment as shade adapted C_3 grass *E. erecta*, what is limiting the expansion of C_4 grasses into the

understory? Various ideas are brought to the table, 1). Temporal effect, whereby light micro-environment results gathered in this thesis only represent days when long periods of high light were prevalent. As such, further experiments would need to be conducted throughout the seasons, as well as taking into account wind speed data that affects spatial and temporal aspects both within and below the canopy. 2). Bottom-up control, whereby light is not the limiting factor, but rather that C_4 grasses are limited as a result of competition, temperature, water and nutrient availability. 3) Acclimation vs. phylogenetic history, whereby species level acclimation allows certain grass species to thrive under shaded, sunfleck environments while the presence of 'sun specialist' traits, which evolved throughout the evolution of C_4 photosynthesis, limit the grasses ability to "reverse" back into shade dwelling species. Although uncommon, reversals from C_4 to C_3 subtypes have been identified, as shown in *A. semialata*. This again highlights the importance of understanding the role that phylogenetic history plays in grass responses (Sinha and Kellogg, 1996; Duvall *et al.*, 2003; Ibrahim *et al.*, 2009). As phylogenetic history has been shown in various studies to play a role in shaping grass response (Lundgren *et al.*, 2015; Simpson *et al.*, 2016) and that the phylogeny in this thesis is too small to make a definite remark on the role of phylogeny in sunfleck responses, future studies need to expand the selection of species and, utilizing similar methodologies, investigate the possibility that C_4 limitations to sunflecks shows a phylogenetic pattern and evolutionary past.

Therefore many questions still remain as to the reason for C_4 preclusion from the understory but results presented in thesis are a step forward into understanding the limitations that short duration sunflecks impose of C_4 photosynthesis. This study also highlights the importance of understanding phylogenetic history when trying to tackle questions about grass responses to a changing environment.

Appendix A

Abbreviation	Definition	Units	Equation
A	Photosynthesis	μmol m^{-2} s^{-1}	
ALA	Alanine		
A_{max}	Maximum photosynthetic rate	μmol m^{-2} s^{-1}	
AMP	Adenosine Monophosphate		
ASP	Aspartate		
ATP	Adenosine Triphosphate		
BCC	Benson Calvin Cycle		
BSC	Bundle Sheath cells		
CA	Carbonic Anhydrase		
CCM	Carbon Concentrating Mechanism		
CET	Cyclic Electron Transport		
CO_2	Carbon dioxide	Pa	
cytb$_6$f	Cytochrome *bf* Complex		
Φ	Leakiness		L / V_p.....*(1)*
ΦCO_2	Quantum Efficiency of photosynthesis		
Fd	ferredoxin		
ΦPSII	Efficiency of PSII		
GA	Gross Assimilation		R_{Light}
GLA	Gap Light Analysis		
GLM	Generalized Linear Model		
G_{ST}	Stomatal Conductance	mol H_20 m^{-2} s^{-1}	
H^+	one proton		

H_2O	Water		
J_{ATP}	Minimum amount of ATP used in electron transport		*3/0.56 XGA....(5)*
L	Leakage rate from BSC to MSC		V_p-*A*-R_M....*(3)*
LET	Linear Electron Transport		
LHC	Light Harvesting Complex		
MSC	Mesophyll cells		
Myr/a	Million years ago		
NAD-ME	NAD-malic enzyme subtype		
NADPH	Nicotinamide Adenine Dinucleotide Phosphate		
NADP-ME	NADP-malic enzyme subtype		
O_2	Oxygen	Pa	
OAA	Oxaloacetic Acid		
PC	Plastocyanin		
PCR	Photosynthetic Chain Reaction		
PEP	Phosphoenylpuruvate		
PEPCase	Phosphoenylpuruvate Carboyxlase		
PEPCK	PEP carboxykinase		
PGA	3-Phosphoglycerate		
PGly	Phosphoglycolate		
P_i	Phosphate		
PPDK	Pyruvate Phopshate Dikinase		
PPFD	Photosynthetically active photon flux density	μmol m^{-2} s^{-1}	

PP_i	Pyrophosphate		
ppm	parts per million		
PQ	Plastoquinone		
PSI	Photosystem I		
PSII	Photosystem II		
R_{Light}	Total non photorespiratory CO_2 production in the light		
R_M	Respiration in the light		
R_M	Mesophyll non photorespiratory CO_2 production in the light		$0.5 X R_{Light}$
Rubisco	Ribulose-1, 5-bisphosphate carboxylase/oxygenase		
RuBP	Ribulose-1, 5-biphosphate		
Tp	Triose Phosphate		
V_C	Reductive Pentose Phosphate Pathway		
V_O	Photorespiratory Pathway		
V_p	PEPCase		$0.5 X J_{ATP}$ ....(4)
VPD	Vapour pressure deficit	kPa	
x	Partitioning factor		0.4

Appendix B

2.2.2 Sunfleck treatments on C_3 and C_4 subspecies of A. semialata

Licor code for induction programme:

The induction of photosynthesis was measured for the youngest mature leaf on potted plants under three different 20-min light treatments, after initially maintaining leaves at a low photosynthetically active photon flux density (*PPFD*) of 20 μmol m^{-2} s^{-1} for 20 minutes. The three treatments that the leaves were exposure to were: 1) a constant *PPFD* of 1000 μmol m^{-2} $s^{-1;}$ 2) an intermittent *PPFD* of 6-sec pulses at 1000 μmol m^{-2} s^{-1},interspersed with 6-sec periods of dark; or 3) the same intermittent light pulses interspersed with 30-sec periods of dark

```
/*

  AutoProgram
  Generated Tue Mar 18 2014 04:17:03
 by AutoProg Builder 1.0b
 Sequence = "GdaCcGdaCcCcGdaCcCcA"

*/

:CHAR defaultFile[] "induction"
:FLOAT
  ctlVal1 20
  wait1 10
  logEvery1 10
  ctlVal2 1000
```

```
  wait2 5
  logEvery2 10
  wait3 20
  logEvery3 10
  ctlVal3 20
  wait4 10
  logEvery4 10
  wait5 20
  logEvery5 10

:PTR user[]
{
 :PTR { ctlVal1 "Light value (æmol/m2/s):" }
 :PTR { wait1 "Wait time (min)" }
 :PTR { logEvery1 "Log every _ (secs)" }
 :PTR { ctlVal2 "Light value (æmol/m2/s):" }
 :PTR { wait2 "Wait time (min)" }
 :PTR { logEvery2 "Log every _ (secs)" }
 :PTR { wait3 "Wait time (min)" }
 :PTR { logEvery3 "Log every _ (secs)" }
 :PTR { ctlVal3 "Light value (æmol/m2/s):" }
 :PTR { wait4 "Wait time (min)" }
 :PTR { logEvery4 "Log every _ (secs)" }
 :PTR { wait5 "Wait time (min)" }
 :PTR { logEvery5 "Log every _ (secs)" }
}

:FCT main
{
 CLEAR
```

```
 defaultFile user LPPrompts2 IF RETURN THEN
 LPPrep
  ctlVal1 2 LampSetNewTarget
   logEvery1 wait1 WaitWithLog
   ctlVal2 2 LampSetNewTarget
   logEvery2 wait2 WaitWithLog
   logEvery3 wait3 WaitWithLog
   ctlVal3 2 LampSetNewTarget
   logEvery4 wait4 WaitWithLog
   logEvery5 wait5 WaitWithLog
  LPCleanup
}
:FCT WaitWithLog
{
  60 * :FLOAT totalSecs
  :FLOAT logEvery
  GETMS totalSecs 1000 * + :LONG stopTimeMs
  LOOP
    stopTimeMs GETMS - :LONG remainingMs
    remainingMs 1000 <= BREAKIF
    remainingMs 1000 / logEvery MIN LPMeasure lpAbort BREAKIF
    LPLog
  ENDLOOP
}
```

Licor code for 6 second dark period programme:

```
/*

  AutoProgram
  Generated Tue Mar 18 2014 07:08:43
```

```
 by AutoProg Builder 1.0b
 Sequence = "GdaCcGdaCcBGdaCcGdaCcAGdaCcA"

*/

:CHAR defaultFile[] "extendedsunfleckstartinglowlight"
:FLOAT
  ctlVal1 1000
  wait1 3
  logEvery1 10
  ctlVal2 20
  wait2 10
  logEvery2 10
  values1 60
  ctlVal3 20
  wait3 0.1
  logEvery3 10
  ctlVal4 1000
  wait4 0.1
  logEvery4 10
  ctlVal5 1000
  wait5 5
  logEvery5 10

:PTR user[]
{
 :PTR { ctlVal1 "Light value (æmol/m2/s):" }
 :PTR { wait1 "Wait time (min)" }
 :PTR { logEvery1 "Log every _ (secs)" }
 :PTR { ctlVal2 "Light value (æmol/m2/s):" }
 :PTR { wait2 "Wait time (min)" }
```

```
 :PTR { logEvery2 "Log every _ (secs)" }
 :PTR { values1 "Loop N times:" }
 :PTR { ctlVal3 "Light value (æmol/m2/s):" }
 :PTR { wait3 "Wait time (min)" }
 :PTR { logEvery3 "Log every _ (secs)" }
 :PTR { ctlVal4 "Light value (æmol/m2/s):" }
 :PTR { wait4 "Wait time (min)" }
 :PTR { logEvery4 "Log every _ (secs)" }
 :PTR { ctlVal5 "Light value (æmol/m2/s):" }
 :PTR { wait5 "Wait time (min)" }
 :PTR { logEvery5 "Log every _ (secs)" }
}

:FCT main
{
 CLEAR

 defaultFile user LPPrompts2 IF RETURN THEN
 LPPrep
  ctlVal1 2 LampSetNewTarget
   logEvery1 wait1 WaitWithLog
   ctlVal2 2 LampSetNewTarget
   logEvery2 wait2 WaitWithLog
   1 :INT i1
  values1 LPRegLoop NLOOP LPLoopStat
    ctlVal3 2 LampSetNewTarget
    logEvery3 wait3 WaitWithLog lpAbort BREAKIF
    ctlVal4 2 LampSetNewTarget
    logEvery4 wait4 WaitWithLog lpAbort BREAKIF
&i1 1 + DROP
  ENDLOOP LPDeregLoop
```

```
    ctlVal5 2 LampSetNewTarget
    logEvery5 wait5 WaitWithLog
  LPCleanup
}
:FCT WaitWithLog
{
  60 * :FLOAT totalSecs
  :FLOAT logEvery
  GETMS totalSecs 1000 * + :LONG stopTimeMs
  LOOP
    stopTimeMs GETMS - :LONG remainingMs
    remainingMs 1000 <= BREAKIF
    remainingMs 1000 / logEvery MIN LPMeasure lpAbort BREAKIF
    LPLog
  ENDLOOP
}
```

Licor code for 30 second dark period programme:

```
/*

  AutoProgram
  Generated Tue Mar 18 2014 07:08:43
 by AutoProg Builder 1.0b
 Sequence = "GdaCcGdaCcBGdaCcGdaCcAGdaCcA"

*/

:CHAR defaultFile[] "extendedsunfleckstartinglowlight"
:FLOAT
```

```
  ctlVal1 1000
  wait1 3
  logEvery1 10
  ctlVal2 20
  wait2 10
  logEvery2 10
  values1 60
  ctlVal3 20
  wait3 0.5
  logEvery3 10
  ctlVal4 1000
  wait4 0.5
  logEvery4 10
  ctlVal5 1000
  wait5 5
  logEvery5 10

:PTR user[]
{
 :PTR { ctlVal1 "Light value (æmol/m2/s):" }
 :PTR { wait1 "Wait time (min)" }
 :PTR { logEvery1 "Log every _ (secs)" }
 :PTR { ctlVal2 "Light value (æmol/m2/s):" }
 :PTR { wait2 "Wait time (min)" }
 :PTR { logEvery2 "Log every _ (secs)" }
 :PTR { values1 "Loop N times:" }
 :PTR { ctlVal3 "Light value (æmol/m2/s):" }
 :PTR { wait3 "Wait time (min)" }
 :PTR { logEvery3 "Log every _ (secs)" }
 :PTR { ctlVal4 "Light value (æmol/m2/s):" }
 :PTR { wait4 "Wait time (min)" }
```

```
 :PTR { logEvery4 "Log every _ (secs)" }
 :PTR { ctlVal5 "Light value (æmol/m2/s):" }
 :PTR { wait5 "Wait time (min)" }
 :PTR { logEvery5 "Log every _ (secs)" }
}

:FCT main
{
 CLEAR

 defaultFile user LPPrompts2 IF RETURN THEN
 LPPrep
  ctlVal1 2 LampSetNewTarget
   logEvery1 wait1 WaitWithLog
   ctlVal2 2 LampSetNewTarget
   logEvery2 wait2 WaitWithLog
   1 :INT i1
  values1 LPRegLoop NLOOP LPLoopStat
    ctlVal3 2 LampSetNewTarget
    logEvery3 wait3 WaitWithLog lpAbort BREAKIF
    ctlVal4 2 LampSetNewTarget
    logEvery4 wait4 WaitWithLog lpAbort BREAKIF
&i1 1 + DROP
  ENDLOOP LPDeregLoop
   ctlVal5 2 LampSetNewTarget
   logEvery5 wait5 WaitWithLog
  LPCleanup
}
:FCT WaitWithLog
{
  60 * :FLOAT totalSecs
```

```
    :FLOAT logEvery
    GETMS totalSecs 1000 * + :LONG stopTimeMs
    LOOP
      stopTimeMs GETMS - :LONG remainingMs
      remainingMs 1000 <= BREAKIF
      remainingMs 1000 / logEvery MIN LPMeasure lpAbort BREAKIF
      LPLog
    ENDLOOP
  }
```

Appendix C

2.2.4 Leakiness in C_4 leaves

Licor code for leakiness programme:

Induction of photosynthesis under either continuous illumination at 500 μmol m^{-2} s^{-1}*PPFD*, or with flecking-light (6-sec dark, 6-sec 1000 μmol m^{-2} s^{-1}*PPFD*) was measured either after incubating leaves at low light (20 μmol m^{-2} s^{-1}) or after incubating leaves at a *PPFD* of 500 μmol m^{-2} s^{-1}.The pre-flecking-light period was maintained for 12-min which was sufficient time for photosynthesis to stabilise to a constant rate. At the end of this period a light response curve (*A: PPDF*) was constructed and plants were subsequently incubated at 500 μmol m^{-2} s^{-1} for 30-min to recover from light variations during the light response curve and to insure that stomatal conductance was not limited. Following this incubation, either the constant or flecking-light treatments were applied for 12-min. Subsequent to treatments; another three successive light response (*A: PPFD*) curves were constructed. Each of these curves took 15 minutes to complete and was hence initiated at 0, 15 and 30 minutes after the end of the constant or flecking-light treatment

Light response curve

```
/*

      Simple Light Curve, with stability checking
        rev 3 3/26/2011

      960822 - Tech Note 14 modification, wait time defaults
      970502 - remembers last time defaults
      980309 - delta-based matching
      020524 - version 5 stability
        110326 - version 6.2
```

```
*/

:INCLUDE "/Sys/Lib/APTools"
:INCLUDE "/Sys/Lib/MatchIF"

:INT  minWaitTime 120
        maxWaitTime 120



:PTR user[]
{
    :PTR { "settings" settingsxml "" "settings" }
    autoprogStability
}

:FCT main
{
    /*      Set program name
    */
    "settings light match" settingsxml InstallMatchIf

    user APPrompts IF RETURN THEN

    APPrep IF APCleanup RETURN THEN

    1 :INT i
    APLampCount LPRegLoop NLOOP LPLoopStat
        /* set the lamp -
        */
        i APSetLamp
```

```
        /* wait for stability
        */
        minWaitTime maxWaitTime LPMeasureTilStable
            lpAbort BREAKIF

        /* match and log
        */
        LPMatchIf
        APLogAction

&i 1 + DROP
    ENDLOOP LPDeregLoop

    APCleanup
}

/* ----------------------------------------
    cosmetics for the front end
---------------------------------------- */

summaryLight
{
        :CHAR &label[]
        DROP
        0 label SETREADY

    APLampCount "%d SetPts for " label SPRINT
    APLampControlGetShort "%s" label SPRINT
}

summaryWait
```

```
{
    :CHAR &label[]
    DROP
    0 label SETREADY

    maxWaitTime minWaitTime "%d to %d s" label SPRINT
}



:XML
settingsxml
{
<settings get=summaryLight disp="Summary" delim=":" >
<lamp addr=apLampControlIndex disp="Lamp control" get=APLampControlGet
edit=APLampControlEdit />
<light get=APShowLampValues edit=APEditLampValues disp="SetPts" delim=":">
<wait get=summaryWait disp="Stability wait" >
<min disp="Minimum (secs)" addr=minWaitTime edit=-1 />
<max disp="Maximum (secs)" addr=maxWaitTime edit=-1 />
</wait>
<match />
<Log addr=apLogType get=APGetLogAction edit=APEditLogAction />
</light>
</settings>
}
```

Licor code for 6 second dark period programme (between curves):

```
/*

  AutoProgram
```

```
  Generated Tue Mar 18 2014 07:08:43
 by AutoProg Builder 1.0b
 Sequence = "GdaCcGdaCcBGdaCcGdaCcAGdaCcA"

*/

:CHAR defaultFile[] "extendedsunfleckstartinglowlight"
:FLOAT
  ctlVal1 1000
  wait1 3
  logEvery1 10
  ctlVal2 20
  wait2 10
  logEvery2 10
  values1 60
  ctlVal3 20
  wait3 0.1
  logEvery3 10
  ctlVal4 1000
  wait4 0.1
  logEvery4 10
  ctlVal5 1000
  wait5 5
  logEvery5 10

:PTR user[]
{
 :PTR { ctlVal1 "Light value (æmol/m2/s):" }
 :PTR { wait1 "Wait time (min)" }
 :PTR { logEvery1 "Log every _ (secs)" }
 :PTR { ctlVal2 "Light value (æmol/m2/s):" }
```

```
 :PTR { wait2 "Wait time (min)" }
 :PTR { logEvery2 "Log every _ (secs)" }
 :PTR { values1 "Loop N times:" }
 :PTR { ctlVal3 "Light value (æmol/m2/s):" }
 :PTR { wait3 "Wait time (min)" }
 :PTR { logEvery3 "Log every _ (secs)" }
 :PTR { ctlVal4 "Light value (æmol/m2/s):" }
 :PTR { wait4 "Wait time (min)" }
 :PTR { logEvery4 "Log every _ (secs)" }
 :PTR { ctlVal5 "Light value (æmol/m2/s):" }
 :PTR { wait5 "Wait time (min)" }
 :PTR { logEvery5 "Log every _ (secs)" }
}

:FCT main
{
 CLEAR

 defaultFile user LPPrompts2 IF RETURN THEN
 LPPrep
  ctlVal1 2 LampSetNewTarget
   logEvery1 wait1 WaitWithLog
   ctlVal2 2 LampSetNewTarget
   logEvery2 wait2 WaitWithLog
   1 :INT i1
  values1 LPRegLoop NLOOP LPLoopStat
    ctlVal3 2 LampSetNewTarget
    logEvery3 wait3 WaitWithLog lpAbort BREAKIF
    ctlVal4 2 LampSetNewTarget
    logEvery4 wait4 WaitWithLog lpAbort BREAKIF
&i1 1 + DROP
```

```
  ENDLOOP LPDeregLoop
   ctlVal5 2 LampSetNewTarget
   logEvery5 wait5 WaitWithLog
  LPCleanup
}
:FCT WaitWithLog
{
  60 * :FLOAT totalSecs
  :FLOAT logEvery
  GETMS totalSecs 1000 * + :LONG stopTimeMs
  LOOP
    stopTimeMs GETMS - :LONG remainingMs
    remainingMs 1000 <= BREAKIF
    remainingMs 1000 / logEvery MIN LPMeasure lpAbort BREAKIF
    LPLog
  ENDLOOP
}
```

Appendix D

2.2.5 Statistics

R code for looking at effects of flecking-light on subtypes: *GLM*

```
> data=read.table(file="clipboard",header=TRUE,sep = "\t")
> attach(data)
> data
> model<- glm(photo~interval*treatment,family="quasipoisson",data=data)
> summary(model)
> anova(model,test="Chisq")
>summary(glht(model, linfct =  mcp(treatment= "Tukey")))
```

R code for looking at leakiness

Photosynthesis

```
> data=read.table(file="clipboard",header=TRUE,sep = "\t")
> attach(data)
> data
> model<-glm(Amax~Curve,family="quasipoisson",data=data)
> anova(model,test="Chisq")
> summary(glht(model, linfct =  mcp(Curve= "Tukey")))
```

PSII

```
> data=read.table(file="clipboard",header=TRUE,sep = "\t")
> attach(data)
> data
```

```
> model<-glm(PSII~Curve,family="quasipoisson",data=data)
> anova(model,test="Chisq")
> summary(glht(model, linfct =  mcp(Curve= "Tukey")))
```

Leakiness (Φ)

```
> data=read.table(file="clipboard",header=TRUE,sep = "\t")
> attach(data)
> data
> model<-glm(FMOD~Curve,family="quasipoisson",data=data)
> anova(model,test="Chisq")
> summary(glht(model, linfct =  mcp(Curve= "Tukey")))
```

Appendix E

3.2.3 Statistics

R code for looking at effect of flecking on growth

Effect of subtype

```
> data$interact <- with(data, interaction(treatment, subtype, sep = "x"))
> model <- glm(area~interact, data= data, family= poisson)
> summary(glht(model, linfct =  mcp(interact= "Tukey")))
```

Effect of time

```
> data$interact <- with(data, interaction(time, subtype, sep = "x"))
> model <- glm(area~interact, data= data, family= poisson)
> summary(glht(model, linfct =  mcp(interact= "Tukey")))
```

AppendixF

3.2.1.3 Light environment for canopy openness

Each sensor was secured into one end of a PVC tube, the other end was sharpened such that it could be inserted into the ground. This was done such that the sensor faced upright, adjacent to, and in the same plane as a fully expanded leaf. During the course of a week, multiple 24 hour light courses were measured for 10 individual leaves of each of the two species. Each photodiode was connected to a Personal Daq/56^{TM} USB Data Acquisition System, which in turn was connected to a laptop running the Personal DaqView software calibrated to record the light frequency of each individual photodiode at a frequency of 1 second. Each photodiode was calibrated prior to measurements using a PPFD light sensor connected to a LICOR-6400-F photosynthesis system

Personal Daq/56^{TM} USB Data Acquisition calibrations

Photodiode #	Slope with intercept =0	Slope	Intercept
1	7093	7295.752	-33.65059
2	7642	7874.797	-35.53051
3	6990	7154.383	-27.69741
4	7677	7849.081	-26.39279

5	7697	7793.496	-15.02143
6	7449	7657.053	-32.73305
7	7420	7586.228	-26.38404
8	6780	6973.612	-33.37004
9	7587	7771.609	-28.56574
10	6701	6925.65	-38.9864
11	8352	8601.657	-35.01366
12	7659	7821.46	-25.07712
13	7583	7776.836	-30.07308
14	6857	6976.812	-20.78001
15	7560	7792.714	-36.04668
16	7064	7306.229	-39.82295
17	7106	7314.662	-34.38171
18	6762	6923.96	-28.10408
19	7121	7386.174	-43.10046
20	7011	7252.154	-39.772

Appendix G

4.2.5 Statistics

R code for photodiode data

```
#Calculation and summary of light fleck data

library(Hmisc) # cut2

library(ggplot2)

library(gtable)

library(scales)

library(maptools)

library(reshape)



theme_set(theme_bw())

theme_mod <- theme(line=element_line(colour = "black", size = 0.5, linetype = 1,lineend = "butt"),

rect = element_rect(fill = "transparent", color = "transparent", size = 0.5, linetype = 1),

                axis.text = element_text(size = rel(1.25), colour = "black"),

                strip.text = element_text(size = rel(1.25), colour = "black"),

                axis.line =         element_blank(),

                axis.text.x =       element_text(colour = "black",size =  rel(1.25), vjust = 1),

                axis.text.y =       element_text(colour = "black",size = rel(1.25), hjust = 1,),
```

```
        axis.ticks =        element_line(colour = "black", size = 1.25),
        axis.title.x =      element_text(colour = "black",size = 24, face = "plain", vjust = 0),
        axis.title.y =      element_text(colour = "black",size = 24, angle = 90, face = "plain", vjust =
.5),
        axis.ticks.length = unit(-0.25, "cm"),
        axis.ticks.margin = unit(0.5, "cm"),
        panel.background =  element_blank(),
        panel.border =      element_rect(fill="NA" ,color="black", size=1.5, linetype="solid"),
        panel.grid.major =  element_blank(),
        panel.grid.minor =  element_blank(),
        panel.margin =      unit(0.25, "lines"),
        panel.margin.x = NULL,
        panel.margin.y = NULL)



#Code to work out dawn dusk sunrise and sunset at Kasouga
Kasouga <- matrix(c( 26.737024, -33.650868), nrow=1)
Kasouga_sp <- SpatialPoints(Kasouga, proj4string=CRS("+proj=longlat +datum=WGS84"))
date_format <- as.POSIXct("2015-11-02", tz="Africa/Johannesburg")
```

```
Sys.timezone(location = TRUE)



## Civil dawn

dawn<- crepuscule(Kasouga_sp , date_format, solarDep=6, direction="dawn", POSIXct.out=TRUE)

dusk<- crepuscule(Kasouga_sp , date_format, solarDep=6, direction="dusk", POSIXct.out=TRUE)



noon<- solarnoon(Kasouga_sp , date_format, POSIXct.out=TRUE)



sunrise<- sunriset(Kasouga_sp , date_format, direction="sunrise", POSIXct.out=TRUE)

sunset<- sunriset(Kasouga_sp , date_format, direction="sunset", POSIXct.out=TRUE)



Times <- data.frame(dawn$time, dusk$time, noon$time, sunrise$time, sunset$time)




night_time <- 10



directory<- "/Users/michaelcramer/Dropbox/Light sensor data/"

data<- read.csv(paste(directory,"Kasouga_light.csv",sep=""))

head(data)

nrow(data)
```

```
data_clean <- data

head(data_clean)

names(data_clean)



#Remove all values where Above.canopy < night_time value

#data_day <- subset(data, data$Above.canopy>night_time)

#Assign all values 0 if they are less than 0

data_clean[,3:20][data_clean[,3:20] < -5] <- NA

data_clean[,3:20][data_clean[,3:20]< 0]<- 1



data_clean$strptime <- strptime(paste(data_clean$Date, data_clean$Time, sep=" "), "%y/%m/%d
%H:%M:%S")



nrow(data_clean)

head(data_clean)

tail(data_clean)

names(data_clean)



#pb <- txtProgressBar(min = 0, max = nrow(data_clean), style = 3)
```

```
#head(data_clean)

#data_clean$strptime_cor <- seq(1:nrow(data_clean))

#data_clean$strptime_cor[i]

#store_current <- as.numeric(data_clean$strptime[1])

#index <- 0

#Assign seconds to the minutes and store in a new varaible called strptime_cor which is also rendered
using POSIXct to time in strptime_correct

#for (i in 2:nrow(data_clean)){

#  if (as.numeric(data_clean$strptime[i]) == as.numeric(store_current)){

#    index<- index + 1

#    data_clean$strptime_cor[i] <-
as.numeric(data_clean$strptime[i])+((index*60)/nrow(subset(data_clean#,
data_clean$date.time==data_clean[i,1])))

#  } else{

#    index<- 0

#    data_clean$strptime_cor[i] <- as.numeric(data_clean$strptime[i])

#  }

#  store_current <- as.numeric(data_clean$strptime[i])

#  setTxtProgressBar(pb, i)

#}

#close(pb)
```

```
#data_clean$strptime_cor <- as.numeric(data_clean$strptime)


#data_clean$strptime_correct <- as.POSIXct(data_clean$strptime_cor, origin = "1970-01-01")

#data_clean$strptime_correct[1] <- as.POSIXct(unlist(data_clean$strptime_cor[1]), origin = "1970-01-01")


head(data_clean)

tail(data_clean)


#names(data_clean)

#save.image("~/Dropbox/Light sensor data/Kasouga_light_workspace.RData")


#Plot a single line at a time and smooth

ggplot(data_clean, aes(x=strptime, y=Erharta_1)) + geom_line(colour="grey") + geom_smooth()+

 scale_x_datetime(breaks=date_breaks("12 hour"), labels=date_format("%H:%M"))+

 theme_mod


#Plot a single line at a time and smooth

ggplot(data_clean, aes(x=strptime, y=Erharta_8)) + geom_line(colour="grey") + geom_smooth()+
```

```
  scale_x_datetime(breaks=date_breaks("12 hour"), labels=date_format("%H:%M"))+
  theme_mod


#Plot a single line at a time and smooth
ggplot(data_clean, aes(x=strptime, y=Brach_1)) + geom_line(colour="grey") + geom_smooth()+
  scale_x_datetime(breaks=date_breaks("12 hour"), labels=date_format("%H:%M"))+
  theme_mod


#Plot a single line at a time and smooth
ggplot(data_clean, aes(x=strptime, y=Brach_8)) + geom_line(colour="grey") + geom_smooth()+
  scale_x_datetime(breaks=date_breaks("12 hour"), labels=date_format("%H:%M"))+
  theme_mod


#Now melt the data so that we can plot all the lines on one graph.
library(reshape)
#Remove dodgy sensors
head(data_clean)
data_clean_good <- data.frame(data_clean[,21], data_clean[,4],data_clean[,7:13],data_clean[,17:19])
names(data_clean_good) <- c("strptime",
names(data_clean)[4],names(data_clean)[7:13],names(data_clean)[17:19])
```

```
data_clean_melt <- melt(data_clean_good, id= "strptime")

data_clean_melt$species <- substr(data_clean_melt$variable, 1, 5)

head(data_clean_melt)



#Plot Brach smooth

ggplot(subset(data_clean_melt, species=="Brach"), aes(x=strptime, y=value, group=variable,
colour=variable)) + geom_line()+

 scale_x_datetime(breaks=date_breaks("12 hour"), labels=date_format("%H:%M"))+

 theme_mod



#Plot Erhar smooth

ggplot(subset(data_clean_melt, species=="Erhar"), aes(x=strptime, y=value, group=variable,
colour=variable)) + geom_line()+

 scale_x_datetime(breaks=date_breaks("12 hour"), labels=date_format("%H:%M"))+

 theme_mod




#Find data only between dawn and dusk

data_day <- subset(data_clean_good, data_clean_good$strptime>Times$dawn.time &
data_clean_good$strptime<Times$dusk.time)
```

```
head(data_day)

ncol(data_day)



#This runs the whole analysis with 3 different threshold levels...

for (k in c(5,20, 50)){

threshold<- k

#Set up an array to store the workings with nrow(data_day). This array has ncol(data_day)-1 columns with 10 "pages".

data_array <- array(nrow(data_day)*(ncol(data_day)-1)*10, dim=c(nrow(data_day), (ncol(data_day)-1), 10))

#Store a NA in every cell to start off. Otherwise values are from a sequence and could get confusing...

data_array[] <- NA



pb<- txtProgressBar(min = 0, max = nrow(data_day), style = 3)

#Initialise values for first element assuming measurements start in the light!

#This one is for photo_runtime

data_array[1, , 1] <-  1

#This one is for dark_runtime

data_array[1, , 6] <- 0
```

```
#This one is for photo_par_cum

data_array[1, , 2] <- as.numeric(data_day[1, 2:12])

#This one is for dark_par_cum

data_array[1, , 7] <- 0



for (j in 1:(ncol(data_day)-1)){

 ld_transient <- 0

 dl_transient <- 0

for (i in 2:nrow(data_day)){

   #Set flag to 1 if the light exceeds the threshold

   photo_flag <- if (as.numeric(data_day[i,j+1]) > threshold)  1 else 0



   #Keep a record of for how long the light has been on in the current light phase

   data_array[i, j, 1] <- if (photo_flag == 1)  data_array[i-1, j, 1] + 1 else 0



   #If the light is above threshold then store the cumulative light intensity for the current period

   data_array[i, j, 2] <- if (photo_flag == 1)    data_array[i-1, j, 2] + as.numeric(data_day[i, j + 1]) else
0
```

```
    #If the light is now below thrreshold but was above in last second, then increment light-dark
transition counter and do
if (photo_flag == 0 & data_array[i-1, j, 1] > 0) {
    ld_transient <- ld_transient + 1
    #store the number of seconds of light on
    data_array[ld_transient, j, 3] <- data_array[i-1, j, 1]
    #store the time of day at the end-point of the period
    data_array[ld_transient, j, 4] <- as.numeric(data_day$strptime[i-1])
    #store cumulative light up to transient
    data_array[ld_transient, j, 5] <- data_array[i-1, j, 2]
   }

   #Keep a record of how long the light has been off in the current dark phase
   data_array[i, j, 6] <- if (photo_flag == 0)  data_array[i-1, j, 6] + 1 else 0
   #If the light is below threshold  store the cumulative light intensity for the current period
   data_array[i, j, 7] <- if (photo_flag == 0)    data_array[i-1, j, 7] + as.numeric(data_day[i,j+1]) else 0
   #If the light is now on but was off last second, then increment dark-light transition counter and do
if (photo_flag == 1 & data_array[i-1, j, 6] > 0) {
    dl_transient <- dl_transient + 1
    #store the number of seconds of light off
```

```
      data_array[dl_transient, j, 8] <- data_array[i-1, j, 6]

      #store the time of day at the end-point of the period

      data_array[dl_transient, j, 9] <- as.numeric(data_day$strptime[i-1])

      #store cumulative light up to transient

      data_array[dl_transient, j, 10] <- data_array[i-1, j, 7]

    }

setTxtProgressBar(pb, i)

 }

close(pb)}



#so we now have a matrix with 10 "pages" of results. so, we need to read the pages we want into
data.frames so as to make it more accesible.



#Get the names from the original data

site_names <- names(data_day)[2:ncol(data_day)]



#This  is for photo conditions to compile a data.frame

photo_time <- data.frame(data_array[, , 3], data_array[, , 4], data_array[, , 5], data_array[, ,
5]/data_array[, , 3])

head(photo_time)
```

```
#Calculate percentage of daily PAR

for (j in 1:ncol(data_day)-1){

 cum_PAR_sum <- sum(data_array[, j, 5], na.rm=TRUE)

 photo_time <- data.frame(photo_time, data_array[, j, 5]*100/cum_PAR_sum)

}

photo_time_names <- c(paste(site_names, "_elapsed", sep=""), paste(site_names, "_strptime", sep=""),
paste(site_names, "_cum_PAR", sep=""), paste(site_names, "_intensity", sep=""), paste(site_names,
"_perc_PAR", sep=""))

names(photo_time) <- photo_time_names

head(photo_time)

names(photo_time)



#This  is for dark conditions to compile a data.frame

dark_time <- data.frame(data_array[, , 8], data_array[, , 9], data_array[, , 10], data_array[, ,
10]/data_array[, , 8])

#Calculate percentage of daily PAR

for (j in 1:ncol(data_day)-1){

 cum_PAR_sum <- sum(data_array[, j, 10], na.rm=TRUE)

 dark_time <- data.frame(dark_time, data_array[, j, 10]*100/cum_PAR_sum)

}
```

```
names(dark_time) <- c(paste(site_names, "_elapsed", sep=""), paste(site_names, "_strptime", sep=""),
paste(site_names, "_cum_PAR", sep=""), paste(site_names, "_intensity", sep=""), paste(site_names,
"_perc_PAR", sep=""))

head(dark_time,20)



#Cut the data into bins so that we can get averages between probes

#What is the maximum elapsed time

max(data_array[, , 3], na.rm=TRUE)



#Set up cuts in a list

cuts_list <- c(0, 10, 30, 60, 120, 240, 600, 900, 1200, 2400, 4800, 9600, 19200, 38400)

cut_points <- c(5, 20, 45, 90, 180, 420, 750, 1050, 1800, 3600, 7200, 14400, 28800)

log10(cut_points)

#Set up a matrix of rows = cuts and columns equal measurements

cut_matrix <- matrix(data= NA, nrow=length(cut_points), ncol=ncol(data_day))

cut_matrix[,1] <- cut_points
```

#In code below the offset on j determines which data is used. e.g. 22 for cumulative, 33 for intensity and 45 for perc_par. The cumulative is the accumulated PAR over a light period summed across all the light intervals that fall into a particular bin (e.g. 0 to 10 s). So this indicates the total amount of energy received in short flashes was pretty uniform over a range of durations.

```
head(photo_time)

names(photo_time)

sum_splits <- list()

for (j in 1:(ncol(data_day)-1))

 {

splits<- split(photo_time, cut2(photo_time[, j], cuts=cuts_list ))

 #Now sum the values in each split section

for (i in 1:length(splits)){

   sum_splits[[i]] <- 0

if (nrow(data.frame(splits[i]))==0) next

   splits_df <- data.frame(splits[i])

   sum_splits[[i]] <- log10(sum(splits_df[j+22], na.rm=TRUE)+1)

   }

 cut_df <- merge(data.frame(seq(1:length(cut_points)), cut_points),
data.frame(rownames(data.frame(unlist(sum_splits))), unlist(sum_splits)),
by.x="seq.1.length.cut_points..", by.y="rownames.data.frame.unlist.sum_splits...")

 cut_matrix[,j+1] <- cut_df[,3]

}



cut_df_brachy <- data.frame(cut_matrix[,1:7], rowMeans(cut_matrix[,2:7], na.rm=TRUE))
```

```
names(cut_df_brachy) <- c("cut", site_names[1:6], "Brach_mean")

cut_df_erharta<- data.frame(cut_matrix[,1], cut_matrix[,7:11], rowMeans(cut_matrix[,7:11],
na.rm=TRUE))

names(cut_df_erharta) <- c("cut", site_names[7:11], "Erharta_mean")

cut_combine <- data.frame(cut_df_brachy[1:7], cut_df_erharta[2:6])

write.csv(cut_combine, paste(directory,"cum_par",threshold,".csv", sep=""))




cut_combine_melt <- melt(cut_combine, id="cut")

cut_combine_melt$species <- substr(cut_combine_melt$variable, 1, 5)



dodge<- position_dodge(width = 0.2)

pdf(paste(directory, "cum_par_",threshold,".pdf",sep=""), width=10,height=10)

ggplot(data=cut_combine_melt, aes(x=log10(cut), y=value, group=species, colour=species))+

  stat_summary(fun.y = 'mean', geom = 'point', size=5, position = dodge)+

  stat_summary(fun.y = 'mean', geom = 'line', size=1, position = dodge )+

  stat_summary(fun.data = mean_cl_normal, geom = "errorbar", mult = 1, aes(width=.2), position =
dodge )+

  #geom_smooth(method="lm")+

xlab("Duration (Log, s)")+
```

```
ylab(bquote("Cumulative PAR (Log, "*mu* 'mol' ~ m^-2*')'))+

  scale_color_manual(labels=c(bquote("Brachylaena "~C[4]), bquote("Erharta "~C[3])),
values=c("#56B4E9", "#D55E00"))+

theme_mod+

  theme(legend.position = c(0.2,0.93), legend.background = element_blank(), legend.title
=element_blank(),legend.key.size =   unit(2, "lines"), legend.key = element_blank(), legend.text =
element_text(size=18))

dev.off()



#This is the percentage of daily PAR received  in the different duration intervals. It indicates that the
largest proprotion of daily light was received in short pulses.

head(photo_time)

names(photo_time)

sum_splits <- list()

for (j in 1:(ncol(data_day)-1))

{

splits<- split(photo_time, cut2(photo_time[, j], cuts=cuts_list ))

  #Now sum the values in each split section

for (i in 1:length(splits)){

    sum_splits[[i]] <- 0

if (nrow(data.frame(splits[i]))==0) next
```

```
    splits_df <- data.frame(splits[i])

    sum_splits[[i]] <- (sum(splits_df[j+44], na.rm=TRUE))

  }

  cut_df <- merge(data.frame(seq(1:length(cut_points)), cut_points),
data.frame(rownames(data.frame(unlist(sum_splits))), unlist(sum_splits)),
by.x="seq.1.length.cut_points..", by.y="rownames.data.frame.unlist.sum_splits...")

  cut_matrix[,j+1] <- cut_df[,3]

}



cut_df_brachy <- data.frame(cut_matrix[,1:7], rowMeans(cut_matrix[,2:7], na.rm=TRUE))

names(cut_df_brachy) <- c("cut", site_names[1:6], "Brach_mean")

cut_df_erharta<- data.frame(cut_matrix[,1], cut_matrix[,7:11], rowMeans(cut_matrix[,7:11],
na.rm=TRUE))

names(cut_df_erharta) <- c("cut", site_names[7:11], "Erharta_mean")

cut_combine <- data.frame(cut_df_brachy[1:7], cut_df_erharta[2:6])

write.csv(cut_combine, paste(directory,"daily_par",threshold,".csv", sep=""))



cut_combine_melt <- melt(cut_combine, id="cut")

cut_combine_melt$species <- substr(cut_combine_melt$variable, 1, 5)



dodge<- position_dodge(width = 0.2)
```

```
pdf(paste(directory, "daily_par_",threshold,".pdf",sep=""), width=10,height=10)

ggplot(data=cut_combine_melt, aes(x=log10(cut), y=value, group=species, colour=species))+

  stat_summary(fun.y = 'mean', geom = 'point', size=5, position = dodge)+

  stat_summary(fun.y = 'mean', geom = 'line', size=1, position = dodge )+

  stat_summary(fun.data = mean_cl_normal, geom = "errorbar", mult = 1, aes(width=.2), position =
dodge )+

  #geom_smooth(method="lm")+

xlab("Duration (Log, s)")+

ylab("Daily PAR (%)")+

  scale_color_manual(labels=c(bquote("Brachylaena "~C[4]), bquote("Erharta "~C[3])),
values=c("#56B4E9", "#D55E00"))+

theme_mod+

  theme(legend.position = c(0.2,0.93), legend.background = element_blank(), legend.title
=element_blank(),legend.key.size =   unit(2, "lines"), legend.key = element_blank(), legend.text =
element_text(size=18))

dev.off()



#This is the sum of the PAR intensity (i.e. the sum of the average intensity over a light pulse) divided
by the number of times that was received. So this indicates the average intensity of pulses of different
lengths... It indicates that short flashes are most intense.....

head(photo_time)

names(photo_time)
```

```
sum_splits <- list()



for (j in 1:(ncol(data_day)-1))

{

splits<- split(photo_time, cut2(photo_time[, j], cuts=cuts_list ))

 #Now sum the values in each split section

for (i in 1:length(splits)){

   sum_splits[[i]] <- 0

if (nrow(data.frame(splits[i]))==0) next

   splits_df <- data.frame(splits[i])

   sum_splits[[i]] <- sum(splits_df[j+33], na.rm=TRUE)/nrow(splits_df[j+0])

 }

 cut_df <- merge(data.frame(seq(1:length(cut_points)), cut_points),
data.frame(rownames(data.frame(unlist(sum_splits))), unlist(sum_splits)),
by.x="seq.1.length.cut_points..", by.y="rownames.data.frame.unlist.sum_splits...")

 cut_matrix[,j+1] <- cut_df[,3]

}



cut_df_brachy <- data.frame(cut_matrix[,1:7], rowMeans(cut_matrix[,2:7], na.rm=TRUE))

names(cut_df_brachy) <- c("cut", site_names[1:6], "Brach_mean")
```

```
cut_df_erharta<- data.frame(cut_matrix[,1], cut_matrix[,7:11], rowMeans(cut_matrix[,7:11],
na.rm=TRUE))

names(cut_df_erharta) <- c("cut", site_names[7:11], "Erharta_mean")

cut_combine <- data.frame(cut_df_brachy[1:7], cut_df_erharta[2:6])

write.csv(cut_combine, paste(directory,"par",threshold,".csv", sep=""))




cut_combine_melt <- melt(cut_combine, id="cut")

cut_combine_melt$species <- substr(cut_combine_melt$variable, 1, 5)




pdf(paste(directory, "par_",threshold,".pdf",sep=""), width=10,height=10)

ggplot(data=cut_combine_melt, aes(x=log10(cut), y=value, group=species, colour=species))+

  stat_summary(fun.y = 'mean', geom = 'point', size=5, position = dodge)+

  stat_summary(fun.y = 'mean', geom = 'line', size=1, position = dodge )+

  stat_summary(fun.data = mean_cl_normal, geom = "errorbar", mult = 1, aes(width=.2), position =
dodge )+

  #geom_smooth(method="lm")+

xlab("Duration (Log, s)")+

ylab(bquote("PAR ("*mu* 'mol'~ m^-2~s^-1*')'))+
```

```
  scale_color_manual(labels=c(bquote("Brachylaena "~C[4]), bquote("Erharta "~C[3])),
values=c("#56B4E9", "#D55E00"))+

theme_mod+

  theme(legend.position = c(0.2,0.93), legend.background = element_blank(), legend.title
=element_blank(),legend.key.size =   unit(2, "lines"), legend.key = element_blank(), legend.text =
element_text(size=18))

dev.off()
```

REFERENCES

Anderson JM, Chow WS, Park Y Il. 1995. The grand design of photosynthesis: Acclimation of the photosynthetic apparatus to environmental cues. Photosynthesis Research **46**, 129–139.

Anderson J, Chow W, Goodchild D. 1988. Thylakoid Membrane Organisation in Sun/Shade Acclimation. Australian Journal of Plant Physiology **15**, 11

Arakaki M, Osborne CP, Edwards EJ, Sciences P, Bank W, Biology E, Christin P, Sciences P, Bank W. 2015. Genetic enablers underlying the clustered evolutionary origins of C4 photosynthesis in angiosperms. Molecular Biology and Evolution **32**.

Ash a. J. 1998. Tree-grass relationships in open eucalypt woodlands of northeastern Australia: Influence of trees on pasture productivity, forage quality and species distribution. Agroforestry Systems **40**, 159–176.

Beerling D. 2007. *The emerald planet: how plants changed Earth's history*. Oxford University Press.

Bellasio C, Griffiths H. 2014*a*. Acclimation of C4 metabolism to low light in mature maize leaves could limit energetic losses during progressive shading in a crop canopy. Journal of experimental botany, 1–12.

Bellasio C, Griffiths H. 2014*b*. Acclimation to low light by C4 maize: Implications for bundle sheath leakiness. Plant, Cell and Environment **37**, 1046–1058.

Boardman NK. 1977. Comparative Photosynthesis of Sun and Shade Plants. Annual Review of Plant Physiology **28**, 355–377.

Bond WJ. 2008*a*. Trees in C4 What Limits and Savannas ? Grasslands. Annual Review of Ecology **39**, 641–659.

Bond WJ. 2008*b*. What Limits Trees in C 4 Grasslands and Savannas? Annual Review of Ecology, Evolution and Systematics **39**, 641–59.

Brantley ST, Young DR. 2009. Contribution of sunflecks is minimal in expanding shrub thickets compared to temperate forest. Ecology **90**, 1021–1029.

Buckley LB, Kingsolver JG. 2012. Functional and Phylogenetic Approaches to Forecasting Species' Responses to Climate Change. Annual Review of Ecology, Evolution and Systematics **43**, 205–26.

Von Caemmerer S, Farquhar GD. 1981. Some relationships between the biochemistry of photosynthesis and the gas exchange of leaves. Planta **153**, 376–387.

Von Caemmerer S. 2000. Biochemical models of leaf photosynthesis. Techniques in Plant Sciences. CSIRO Publishing, 32–145.

Cayssials V, Rodríguez C. 2013. Functional traits of grasses growing in open and shaded habitats. Evolutionary Ecology **27**, 393–407.

Chaffey N. 1999. *C4 Plant biology* (RF Sage and RK Monson, Eds.). Academic Press.

Chazdon RL, Fetcher NED. 1984*a*. Photosynthetic Light Environments in a Lowland Tropical Rain Forest in Costa Rica. Journal of Ecology **72**, 553–564.

Chazdon RL, Pearcy RW. 1986*a*. Photosynthetic responses to light variation in rainforest speciesIInduction under constant and fluctuating light conditions. Oecologia **69**, 517–523.

Chazdon RL, Pearcy RW. 1986*b*. Photosynthetic responses to light variation in rainforest species. Oecologia **69**, 524–531.

Chazdon RL, Pearcy RW. 1986*c*. Photosynthetic Responses To Light Variation in Rain-Forest Species .2. Carbon Gain and Photosynthetic Efficiency During Lightflecks. Oecologia **69**, 524–531.

Chazdon RL, Pearcy RW. 1991. The Importance of Sunflecks for Forest Understory Plants. BioScience **41**, 760–766.

Chollet R, Vidal J, O'Leary MH. 1996. Pyruvate Carboxylase: A Ubiquitous, Highly Regulated Enzyme in Plants. Annual Review of Plant Physiology and Plant Molecular Biology **47**, 273–298.

Christin P-A, Osborne CP. 2014. The evolutionary ecology of C4 plants. New Phytologist **204**, 765–781.

Cousins AB, Baroli I, Badger MR, Ivakov A, Lea PJ, Leegood RC, von Caemmerer S. 2007. The role of phosphoenolpyruvate carboxylase during C4 photosynthetic isotope exchange and stomatal conductance. Plant physiology **145**, 1006–1017.

Duvall MR, Saar DE, Grayburn WS, Holbrook GP. 2003. Complex Transitions between C3 and C4 Photosynthesis during the Evolution of Paniceae: A Phylogenetic Case Study Emphasizing the Position of \emph{Steinchisma hians }(Poaceae), a C3-C4 Intermediate. International Journal Of Plant Sciences **164**, 949–958.

Edwards EJ, Smith S a. 2010. Phylogenetic analyses reveal the shady history of C4 grasses. Proceedings of the National Academy of Sciences of the United States of America **107**, 2532–2537.

Edwards EJ, Still CJ. 2008. Climate, phylogeny and the ecological distribution of C4 grasses. Ecology Letters **11**, 266–276.

Ehleringer JR. 1978. Implications of Quantum Yield and Differences on the Distributions of C3 and C4 grasses. Oecologia **31**, 255–267.

Ehleringer J, Björkman O. 1977. Quantum Yields for CO2 Uptake in C3 and C4 Plants. Plant physiology **59**, 86–90.

Ehleringer JR, Cerling TE, Helliker BR. 1997. C4 photosynthesis, atmospheric CO2,and climate. Oecologia **112**, 285–299.

Evans JR, Von Caemmerer S. 1996. Carbon Dioxide Diffusion inside Leaves. Plant physiology **110**, 339–346.

Evans JR, Jakobsen I, Ogren E. 1993. Photosynthetic light-response curves. Planta **189**, 191–200.

Farquhar GD, Caemmerer S Von, Berry J a. 1980. A Biochemical Model of Photosynthetic CO2 Assimilation in Leaves of C3 Species. Planta **90**, 78–90.

Flowers TJ, Jones HG, Jones MB. 1989. Plants under stress : biochemistry, physiology and ecology and their application to plant improvement. Seminar series (Society for Experimental Biology (Great Britain)).ix, 257 p.

Foyer C., Noctor G. 2000. Tansley Review No . 112 Oxygen processing in photosynthesis : regulation and signalling. Review Literature And Arts Of The Americas **146**, 359–388.

Frazer GW, Canham CD, and Lertzman KP. 1999. Gap Light Analyzer (GLA), Version 2.0: Imagingsoftware to extract canopy structure and gap light transmission indices from true-colour fisheyephotographs, users manual and program documentation. Copyright © 1999: Simon Fraser University,Burnaby, British Columbia, and the Institute of Ecosystem Studies, Millbrook, New York.

Gendron F, Messier C, Comeau PG. 2001. Temporal variations in the understorey photosynthetic photon flux density of a deciduous stand: The effects of canopy development, solar elevation, and sky conditions. Agricultural and Forest Meteorology **106**, 23–40.

Gilmore a M. 1997. Mechanistic aspects of xanthophyll cycle-dependent photoprotection in higher plant chloroplasts and leaves. Physiologia Plantarum **99**, 197–209

Givnish TJ. 1988. Adaptation to Sun and Shade : A whole-plant Perspective. Australian Journal of Plant Physiology **15**, 63–92.

Gowik U, Westhoff P. 2011. The path from C3 to C4 photosynthesis. Plant physiology **155**, 56–63.

Gratani L. 2014. Plant Phenotypic Plasticity in Response to Environmental Factors. Advances in Botany **2014**, 17.

Hofstra J., Aksornkoae S, Atmowidjojo S, Santosa J. B, Sastrohoetomo R., Thu LT. 1972. A study on the occurence of plants with a low CO2 compensation poing in different habitats in the tropics. Annales Bogorienses **5**, 142–157.

Horton JL, Neufeld HS. 1998. Photosynthetic responses of Microstegium vimineum (Trin.) A. Camus, a shade-tolerant, C4 grass, to variable light environments. Oecologia **114**, 11–19.

Horton P, Ruban a. V., Walters RG. 1996. Regulation of Light Harvesting in Green Plants. Annual Review of Plant Physiology and Plant Molecular Biology **47**, 655–684

Hothorn T, Bretz F, Westfall P. (2008). SimultaneousInference in General Parametric Models. Biometrical Journal **50**(3),346--363.

Ibrahim DG, Burke T, Ripley BS, Osborne CP. 2009. A molecular phylogeny of the genus Alloteropsis (Panicoideae, Poaceae) suggests an evolutionary reversion from C4 to C3 photosynthesis. Annals of Botany **103**, 127–136.

Kirschbaum MU, Pearcy RW. 1988. Gas Exchange Analysis of the Fast Phase of Photosynthetic Induction in Alocasia macrorrhiza. Plant physiology **87**, 818–821.

Klink C a, Joly C a. 1989. Identification and distribution of C3 and C4 grasses in open and shaded habitats in Sao Paulo state, Brazil. Biotropica. **21**, 30–34.

Kono M, Noguchi K, Terashima I. 2014. Roles of the Cyclic Electron Flow Around PSI (CEF-PSI) and O2-Dependent Alternative Pathways in Regulation of the Photosynthetic Electron Flow in Short-Term Fluctuating Light in Arabidopsis thaliana. Plant & cell physiology **55**, 990–1004.

Kono M, Terashima I. 2014. Long-term and short-term responses of the photosynthetic electron transport to fluctuating light. Journal of Photochemistry and Photobiology B: Biology **137**, 89–99.

Krall J., Pearcy RW. 1993. Concurrent Measurements of Oxygen and Carbon Dioxide Exchange during Lightflecks in Maize (Zea mays L.). Plant physiology **103**, 823–828.

Kromdijk J, Griffiths H, Schepers HE. 2010. Can the progressive increase of C4 bundle sheath leakiness at low PFD be explained by incomplete suppression of photorespiration? Plant, Cell and Environment **33**, 1935–1948.

Kromdijk J, Schepers HE, Albanito F, Fitton N, Carroll F, Jones MB, Finnan J, Lanigan GJ, Griffiths H. 2008. Bundle sheath leakiness and light limitation during C4 leaf and canopy CO2 uptake. Plant physiology **148**, 2144–2155.

Kromdijk J, Ubierna N, Cousins AB, Griffiths H. 2014. Bundle-sheath leakiness in C4 photosynthesis: a careful balancing act between CO2 concentration and assimilation. Journal of experimental botany **65**, 3443–3457.

Kruse O. 2001. Light-induced short-term adaptation mechanisms under redox control in the PS II-LHCII supercomplex: LHC II state transitions and PS II repair cycle. Naturwissenschaften **88**, 284–292.

Kubásek J, Urban O, Šantrůček J. 2013. C4 plants use fluctuating light less efficiently than do C3 plants: A study of growth, photosynthesis and carbon isotope discrimination. Physiologia Plantarum **149**, 528–539.

Laisk A, Edwards GE. 1997. Post-illumination CO2 Exchange and Light-induced CO2 Bursts during C4 Photosynthesis. Australian Journal of Plant Physiology **24**, 517.

Leakey a. DB, Scholes JD, Press MC. 2005. Physiological and ecological significance of sunflecks for dipterocarp seedlings. Journal of Experimental Botany **56**, 469–482.

Lichtenthaler HK. 1987. Chlorophyll Fluorescence Signatures of Leaves during the Autumnal Chlorophyll Breakdown. Journal of Plant Physiology **131**, 101–110.

Lichtenthaler HK, Meiera D, Buschmann C. 1984. Development of chloroplasts at high and low light quanta fluence rates. Israel Journal of Botany **33**, 185–194.

Liu TY, Lin KC, Vadeboncoeur M a., Chen MZ, Huang MY, Lin TC. 2015. Understorey plant community and light availability in conifer plantations and natural hardwood forests in Taiwan. Applied Vegetation Science **18**, 591–602.

Lundegårdh, H. (1921). Ecological studies in the assimilation of certain forest-plants and shore-plants. *Svensk bot. Tidskr.* **15**, 46

Lundgren MR, Besnard G, Ripley BS, *et al.* 2015. Photosynthetic innovation broadens the niche within a single species. Ecology Letters, 1–9.

Ogren E, Evans JR. 1993. Photosynthetic light-response curves. Planta, 182–190

Osborne CP, Freckleton RP. 2009. Ecological selection pressures for C4 photosynthesis in the grasses. Proceedings. Biological sciences / The Royal Society **276**, 1753–60.

Pearcy RW. 1983. The light environment and growth of C3 and C4 tree species in the understory of a Hawaiian forest. Oecologia **58**, 19–25.

Pearcy RW. 1988. Photosynthetic Utilisation of Lightflecks by Understory Plants. Australian Journal of Plant Physiology **15**, 223.

Pearcy RW, Calkin HW. 1983. Carbon dioxide exchange of C3 and C4 tree species in the understory of a Hawaiian forest. Oecologia **58**, 26–32.

Pearcy RW, Osteryoung K, Calkin HW. 1985. Photosynthetic Responses to Dynamic Light Environments by Hawaiian Trees : Time Course of CO(2) Uptake and Carbon Gain during Sunflecks. Plant physiology **79**, 896–902.

Pearcy RW, Way D a. 2012. Two decades of sunfleck research: Looking back to move forward. Tree Physiology **32**, 1059–1061.

Pearcy RW, Yang W. 1996. A three-dimensional crown architecture model for assessment of light capture and carbon gain by understory plants. Oecologia **108**, 1–12.

Porcar-Castell A, Palmroth S. 2012. Modelling photosynthesis in highly dynamic environments: The case of sunflecks. Tree Physiology **32**, 1062–1065.

Powles SB, Osmond CB. 1979. Photoinhibition of intact attached leaves of c(3) plants illuminated in the absence of both carbon dioxide and of photorespiration. Plant physiology **64**, 982–988.

Rabinowitch EI. 1951. Photosynthesis and Related Processes. Soil Science **72**, 482.

Rajagopalan a V, Devi MT, Raghavendra a S. 1994. Molecular biology of C4 phosphoenolpyruvate carboxylase: Structure, regulation and genetic engineering. Photosynthesis research **39**, 115–35.

R Core Team (2013). R: A language and environment for statisticalcomputing. R Foundation for Statistical Computing, Vienna, Austria.URL http://www.R-project.org/.

Retkute R, Smith-Unna SE, Smith RW, Burgess a. J, Jensen OE, Johnson GN, Preston SP, Murchie EH. 2015. Exploiting heterogeneous environments: does photosynthetic acclimation optimize carbon gain in fluctuating light? Journal of Experimental Botany.

Ripley BS, Abraham TI, Osborne CP. 2008. Consequences of C4 photosynthesis for the partitioning of growth: A test using C3 and C4 subspecies of Alloteropsis semialata under nitrogen-limitation. Journal of Experimental Botany **59**, 1705–1714.

Ripley BS, Donald G, Osborne CP, Abraham T, Martin T. 2010. Experimental investigation of fire ecology in the C3 and C4 subspecies of Alloteropsis semialata. Journal of Ecology **98**, 1196–1203.

Ripley BS, Gilbert ME, Ibrahim DG, Osborne CP. 2007. Drought constraints on C4 photosynthesis: Stomatal and metabolic limitations in C3 and C4 subspecies of Alloteropsis semialata. Journal of Experimental Botany **58**, 1351–1363

Rochaix J-D. 2011. Regulation of photosynthetic electron transport. Biochimica et Biophysica Acta (BBA) - Bioenergetics **1807**, 375–383.

Rochaix J-D. 2014. Regulation and dynamics of the light-harvesting system. Annual review of plant biology **65**, 287–309.

Ruban A V. 2014. Evolution under the sun: optimizing light harvesting in photosynthesis. Journal of Experimental Botany **66**, 7–23.

Ruban a. V, Young a, Horton P. 1994. Modulation of chlorophyll fluorescence quenching in isolated light harvesting complex of photosystem II. Biochimica et Biophysica Acta (BBA) - Bioenergetics **1186**, 123–127.

Russell GEG. 1983. The taxonomic position of C3 and C4 Alloteropsis semialata (Poaceae) in southern Africa. Bothalia **3**, 205–213.

Sage RF. 2004. The evolution of C4 photosynthesis. New Phytologist **161**, 341–370.

Sage RF. 2014. Stopping the leaks: new insights into C4 photosynthesis at low light. Plant, Cell & Environment **37**, 1037–1041.

Sage RF, Kubien DS. 2007. The temperature response of C3 and C4 photosynthesis. Plant, Cell and Environment **30**, 1086–1106.

Sage RF, McKown AD. 2006. Is C4 photosynthesis less phenotypically plastic than C 3 photosynthesis? Journal of Experimental Botany **57**, 303–317.

Sage RF, Sage TL, Kocacinar F. 2012. Photosynthesis. Annual Review of Plant Biology **63**, 19–47.

Sage RF, Stata M. 2015. Photosynthetic diversity meets biodiversity: The C4 plant example. Journal of Plant Physiology **172**, 104–119.

Sage RF, Zhu XG. 2011. Exploiting the engine of C4 photosynthesis. Journal of Experimental Botany **62**, 2989–3000.

Sassenrath-Cole GF, Pearcy RW. 1992. The Role of Ribulose-1,5-Bisphosphate Regeneration in the Induction Requirement of Photosynthetic CO(2) Exchange under Transient Light Conditions. Plant physiology **99**, 227–234.

Sharkey TD, Seemann JR, Pearcy RW. 1986. Contribution of Metabolites of Photosynthesis to Postillumination CO(2) Assimilation in Response to Lightflects. Plant physiology **82**, 1063–1068.

Simpson KJ, Ripley BS, Christin P-A, Belcher CM, Lehmann CER, Thomas GH, Osborne CP. 2016. Determinants of flammability in savanna grass species. Journal of Ecology **104**, 138–148.

Sims D a, Pearcy RW. 1993. Sunfleck frequency and duration affects growth rate of the understorey plant, Alocasia macrorrhiza. Functional Ecology **7**, 683–689.

Singsaas EL, Ort DR, DeLucia EH. 2001. Variation in measured values of photosynthetic quantum yield in ecophysiological studies. Oecologia **128**, 15–23.

Sinha NR, Kellogg EA. 1996. Parallelism and Diversity in Multiple Origins of C 4 Photosynthesis in the Grass Family. Americ **83**, 1458–1470.

Smith WK, Berry ZC. 2013. Sunflecks? Tree Physiology **33**, 233–237.

Smith M, Martin C. 1987. Photosynthetic Responses to Irradiance in Three Forest Understory Species of the C4 Grass Genus Muhlenbergia. Botanical Gazette **148**, 275–282.

Sun W, Ubierna N, Ma J-Y, Walker BJ, Kramer DM, Cousins AB. 2014. The coordination of C4 photosynthesis and the CO2-concentrating mechanism in maize and Miscanthus x giganteus in response to transient changes in light quality. Plant physiology **164**, 1283–92.

Taiz L, Zeiger E. 2002. *Plant Physiology*. Sinauer Associates, Inc., Sunderland, MA, 2002.

Tazoe Y, Hanba YT, Furumoto T, Noguchi K, Terashima I. 2008. Relationships between quantum yield for CO2 assimilation, activity of key enzymes and CO2 leakiness in Amaranthus cruentus, a C4 dicot, grown in high or low light. Plant and Cell Physiology **49**, 19–29.

Tikhonov AN. 2015. Induction events and short-term regulation of electron transport in chloroplasts: an overview. Photosynthesis Research.

Tinoco-Ojanguren C, Pearcy RW. 1992. Dynamic stomatal behavior and its role in carbon gain during lightflecks of a gap phase and an understory Piper species acclimated to high and low light. Oecologia **92**, 222–228.

Ueno O, Sentoku N, Hitchcock RB. 2006. Comparison of leaf structure and photosynthetic characteristics of C3 and C4 Alloteropsis semialata subspecies. Plant, Cell and Environment **29**, 257–268.

Usuda H. 1985. The activation state of ribulose 1,5 bisphosphate carboxylase in maize leaves in dark and light. Plant Cell Physiology **26**, 1455–1463.

Walker RP, Chen Z, Acheson RM, Leegood RC. 2014. Effects of Phosphorylation on Phospho enol pyruvate Carboxykinase from the C 4 Plant Guinea Grass. Plant physiology **18**, 165–172.

Walters RG, Ruban a. V., Horton P. 1994. Higher plant light-harvesting complexes LHCIIa and LHCIIc are bound by dicyclohexylcarbodiimide during inhibition of energy dissipation. European Journal of Biochemistry **226**, 1063–1069.

Wang Y, Bräutigam A, Weber APM, Zhu X-G. 2014*a*. Three distinct biochemical subtypes of C4 photosynthesis? A modelling analysis. Journal of experimental botany **65**, 3567–3578.

Wang Y, Long SP, Zhu X-G. 2014*b*. Elements required for an efficient NADP-malic enzyme type C4 photosynthesis. Plant physiology **164**, 2231–46.

Watling J, Ball M, Woodrow I. 1997*a*. The utilization of lightflecks for growth in four Australian rainforest species. Functional Ecology, 231–239.

Watling JR, Robinson SA, Woodrow IE, Osmond BC. 1997*b*. Responses of Rainforest Understorey Plants to Excess Light during Sunflecks. Australian Journal of Plant Physiology **24**, 17–25.

Way D a., Pearcy RW. 2012. Sunflecks in trees and forests: From photosynthetic physiology to global change biology. Tree Physiology **32**, 1066–1081.

Yanhong T, Hiroshi K, Mitsumasa S, Izumi W. 1994. Characteristics of Transient Photosynthesis in Quercus-Serrata Seedlings Grown under Lightfleck and Constant Light Regimes. Oecologia **100**, 463–469.