The influence of the evolution of anatomical characters on leaf hydraulic capacity

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Abstract

Over geological time, changes in climatic factors have contributed to the evolution of plants. CO₂ is one environmental factor that affects plants, and changes in CO₂ concentration ([CO₂]) in the atmosphere across geological time have been thought to have driven evolutionary changes in plant leaf anatomy, enabling plant leaves to adapt to declining atmospheric [CO₂] over the last 120 million years. This thesis aimed to study the effect varying anatomical characters had on plant leaf function in different taxa. Atmospheric [CO₂] at the crown group age of each species sampled made it possible to link anatomical and physiological variation to the different environmental conditions ([CO₂]) each species (or taxa) evolved under. Several leaf gas exchange and hydraulics parameters were measured and leaves were subjected to light step-change and diurnal regimes to ascertain their gas exchange and hydraulic responses over prolonged periods.

Results showed significant variation between species in leaf anatomical characteristics (stomatal and vein density). There was also a significant difference between species in their hydraulic responses to changes in light. No significant linear relationships were found between leaf anatomical characteristics and gas exchange parameters and leaf hydraulics (hydraulic conductance or water flow into the leaf). However, significant relationships were found among gas exchange parameters with leaf hydraulics, with species exhibiting higher photosynthetic capacities also displaying higher leaf water flow. The general difference was between angiosperms and other taxa, with angiosperms showing higher values for anatomical and functional characters. Under dynamic light (step-change or diurnal change), angiosperms had more coordination among gas exchange parameters with leaf hydraulic flow. Even though no significant relationships were found between variables measured and [CO₂] at the time of taxa divergence, the higher values shown by angiosperms, which evolved under declining atmospheric [CO₂], still suggests towards a [CO₂] effect on leaf evolution.
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Abbreviations

- **A**: Carbon assimilation rate.
- **auc**: Area under the curve.
- **Ci**: CO₂ concentration inside the leaf.
- **Ds**: Stomatal density per unit area.
- **Dv**: Vein density per unit area.
- **e**: Leaf transpiration rate.
- **E**: Water flow into the leaf per unit time and area.
- **gs**: Stomatal conductance of the leaf.
- **Gs max**: Maximum stomatal conductance of the leaf.
- **mya**: Million years ago.
- **ppm**: Parts per million.
- **Ss**: Stomatal aperture size.
- **Kleaf**: Hydraulic conductance of the leaf.
- **WUE**: Water use efficiency.
- **Vc max**: Maximum carboxylation rate.
- **Ψleaf**: Leaf water potential.
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Chapter 1: Introduction and Literature Review

Introduction

Climatic conditions have played a significant role in the evolution of life on earth (Matthew 1915; Grenfell et al. 2010), with life responding to changing climate over geological times and influencing climate in return (Berner 1997; Beerling & Berner 2005; Franks et al. 2014). Plants, and their counterparts across marine systems like green algae, are active in this atmosphere-biosphere interaction (Kasting & Siefert 2003). This is mainly because of their dependence on photosynthesis, which requires atmospheric carbon dioxide (CO$_2$) and releases oxygen (O$_2$), with this exchange impacting the global CO$_2$ and O$_2$ cycles significantly.

The gas exchange rates of CO$_2$, O$_2$ and water vapour between plants and the atmosphere greatly influence local weather and hydrology (Berner 1997; Gedney et al. 2006; Keenan et al. 2013), and combined with their influence on terrestrial primary productivity, plants provide the fulcrum of the ecological structure on which terrestrial ecosystems function. Climatic conditions, on the other hand, have a large effect on plant growth, with changes in atmospheric CO$_2$ concentrations ([CO$_2$]), water availability, amount of sunlight and temperature fluctuations all important environmental factors directly influencing plant performance (Rosenzweig & Parry 1994; Wagner et al. 1996; Medlyn et al. 2001; Medrano et al. 2002; Bernacchi et al. 2002; Flexas et al. 2007; Ward & Gerhart 2010; Scoffoni et al. 2011; Franks et al. 2013; Donohue et al. 2013; Lawson & Blatt 2014). This interconnectivity between plants and the environment means that changes in either aspect of this relationship (plant productivity or environmental factors) is bound to influence the other aspect. For example, changes in atmospheric CO$_2$ levels affect plant gas exchange and this feeds back onto the ecosystem as plants will change their stomatal conductance and thus the amount of water returning to the ecosystem through evapotranspiration.
Furthermore, this is highlighted over evolutionary history (Beerling & Woodward 1993; Beerling 1996; Ward & Kelly 2004; Brodribb et al. 2009; Grenfell et al. 2010; Leakey & Lau 2012; Assouline & Or 2013). Environmental factors that vary over geological time periods elicit an adaptive (and eventually, an evolutionary) response from plants in response to environmental variation, and chief among those environmental variations is fluctuations in atmospheric [CO\textsubscript{2}] (Ackerly et al. 2000; Beerling & Berner 2005; Royer 2006; Ward & Gerhart 2010; Blankenship 2010; Leakey & Lau 2012; Boyce & Zwieniecki 2012).

This aims of this introductory chapter is to highlight drivers of change in atmospheric [CO\textsubscript{2}] across geological history, and consequently the influence of changes in atmospheric [CO\textsubscript{2}] on plant ecophysiology. This chapter will explore evolutionary adaptations in plants driven by climatic change, such as the evolution of leaf anatomical characters (specifically venation and stomatal characteristics), and how the evolution of those anatomical characters influenced leaf physiology, and the resulting increase in photosynthetic capacity and plant productivity due to these evolutionary improvements. The effect of changing [CO\textsubscript{2}] on plant anatomy and physiology will be linked to the leaf’s hydraulic capacity, represented by variables like \( E \), the flow of water into the leaf, and \( K_{\text{leaf}} \), which is the hydraulic conductivity of the plant leaf, which over evolutionary timescales helped shape plant photosynthetic and hydraulic performance.

**Atmospheric CO\textsubscript{2} interactions across geological time and the role of plants in the carbon cycle**

CO\textsubscript{2} concentration in the atmosphere is regulated through long term and a short term natural cycles of carbon (Berner 1998; Berner & Kothavala 2001). The short term cycle, taking place on a decades-to-millennia time scale, concerns carbon moving between the biosphere (terrestrial or oceanic) and the atmosphere (Berner 1998;
Berner & Kothavala 2001). This cycle involves ecological processes like photosynthesis, in which plants fix CO$_2$ from the atmosphere into organic components. Carbon is transferred to the soil when plants die or leaves fall to the ground where it is broken down by soil microbiota. Carbon returns to the atmosphere during this cycle mainly due to the action of respiration (microbial, floral or faunal) as well as release of volatile carbon compounds like methane and isoprene as well as CO$_2$ that are the result of metabolic reactions or material breakdown.

The long-term carbon cycle centres on weathering. Weathering is the chemical reaction of atmospheric elements with rocks and deep earth material, and CO$_2$ is one of the atmospheric gases that contribute to rock weathering. This cycle occurs over a multi-million year time scale and involves the reaction between CO$_2$ and silicate rocks (usually magnesium silicate or calcium silicate), producing carbonates that are buried in sediment (Berner et al. 1983; Berner 1998; Berner & Kothavala 2001). This process reduces [CO$_2$] in the atmosphere, however this is compensated for by processes like rock metamorphism, oxidative weathering and tectonic plate movement that force CO$_2$ back to the surface, or a process like magmatism driven by thermal pressure that can lead to volcanic eruptions which release large amounts of CO$_2$ back into the atmosphere increasing [CO$_2$] in the atmosphere (Berner et al. 1983; Berner & Canfield 1989; Berner 1998; Berner & Kothavala 2001).

The long-term carbon cycle was heavily affected by the emergence of plants (Berner 1997; Berner 1998; Beerling & Berner 2005), especially vascular plants (Berner & Kothavala 2001), as plants affect rock weathering (Berner 1997; Bormann et al. 1998; Beerling & Berner 2005), and weathering is one of the main components of the long-term carbon cycle. Plants, especially trees, have deep, burrowing roots, which penetrate rocks and sediment exposing them to more contact with atmospheric CO$_2$. Release of organic acids from plant roots containing carbon also increases contact between rocks and carbon (as well as other minerals), and hence plants accelerate
the rate of weathering due to increased contact time between carbon compounds (including CO\textsubscript{2}) and exposed rocks. In addition, the exchange of water between plants and the atmosphere through transpiration recirculates water in the ecosystem causing extra rainfall, and extensive rooting system can also hold more water in the soil and sediment. Thus, the emergence of plants (around Cambrian-Ordovician boundary) resulted in creating a more humid atmosphere around the earth surface, favouring an increase in weathering rate as rocks are more susceptible to weathering under moisture (Beerling & Berner 2005).

The relationship between plants and the long-term carbon cycle over geological time formed a coupling between plants and atmospheric [CO\textsubscript{2}], and consequently between plants and climate (Beerling & Berner 2005). The Devonian (420-360 million years ago) saw the first radiation of vascular land plants, which ultimately lead to an increase in chemical weathering, while it also lead to an increase in CO\textsubscript{2} uptake by plants which caused a decrease in atmospheric [CO\textsubscript{2}] in the short term as well (Berner 1998; Beerling & Berner 2005). However, it is thought that atmospheric [CO\textsubscript{2}] was already declining before the emergence of plants due to the evolution of the sun, and the rise in earth’s temperatures associated with this (Berner & Kothavala 2001; Beerling & Berner 2005). The rise in earth’s temperature provoked a counterbalancing action that included increased weathering (Berner 1998; Berner & Kothavala 2001), as weathering rate itself increases at high temperature. Plus, the explosion of land plants into the scene can also be considered as a “cooling” response from the earth as land plants helped reduce atmospheric [CO\textsubscript{2}] in the air (through weathering or CO\textsubscript{2} uptake), and thus minimize the greenhouse effect on temperature. The impact of plants on [CO\textsubscript{2}] in the atmosphere highlights the importance of plants in regulating climate, as well as how they can be affected by it (Beerling & Berner 2005). Plants evolved more complex features after [CO\textsubscript{2}] decreased after the Devonian, with plants developing leaves 25 times as big as they
were pre-Devonian (Beerling et al. 2001; Osborne et al. 2004). However, it is thought that this increase in leaf area might have affected plants negatively later. During the Triassic-Jurassic boundary (220-180 Mya), a sharp rise in atmospheric [CO₂] occurred (reaching several thousand ppm) elevated temperatures due to the greenhouse effect. Evaporative cooling in large leaves was constricted during this period lead to impairment of leaf function, resulting in the loss of many plant taxa during this period (Beerling et al. 2001; Beerling & Berner 2005). Leaf evaporative cooling depends on increasing water loss, with the water removing latent heat from the leaf. The water escapes through the stomatal pores, further highlighting the influence of atmospheric [CO₂] on plants, as the evolution of complex anatomical, specifically stomatal, characteristics is considered one of the main aspects of the [CO₂] effect on plant evolution (Beerling & Chaloner 1993; McElwain & Chaloner 1995; Brodribb et al. 2009; Franks & Beerling 2009a; Franks & Beerling 2009b).

Stomata are small pores on leaf surface, facilitating gas exchange, including uptake of CO₂ and loss of water. Previous work has shown that plants that emerged in low [CO₂] environments had smaller but higher densities of stomata, with the opposite found in plants that evolved in high CO₂ environments (see latter sections of this review). These alterations in stomatal size and density had implications for plant productivity and water use, because these anatomical characters have on stomatal conductance and therefore photosynthetic rate, as well as the leaf’s water relations and hydraulic capacity. Also, stomatal properties have been the most prominent and conspicuous effect of fluctuating [CO₂] on plant evolution. Changes in stomatal densities and size have been used to estimate paleo-[CO₂] in fossilized leaves (McElwain & Chaloner 1995; Berner 1998; Royer 2001; Royer et al. 2004; Fletcher et al. 2007; Steinthorsdottir & Vajda 2013). The effect of changing atmospheric [CO₂] on stomatal characters would affect leaf function, instigating further adaptations in the leaf.
Atmospheric [CO$_2$] levels in the geologic record

Reconstructing [CO$_2$] levels of the past provides a gateway towards understanding the contribution of current rise in atmospheric [CO$_2$] to current climate change (Yapp & Poths 1992). Climatic paleo-proxies, which are natural records that provide climatic information (e.g. tree rings, ice cores) have been increasing in prominence in the past 2 decades as a method of estimating past climatic conditions (Royer 2006). These proxies are usually an earth system component that varies or responds to [CO$_2$] change and is quantified and then applied to the past (Royer 2006). Among these proxies are paleosols, stratified sedimentary soils that formed during past geological eras. Carbonate in those soils usually comes from atmospheric CO$_2$ precipitating in the soil, and thus analysis of these soils for climatic markers (such as stable isotopes) has proved useful in constructing past atmospheric [CO$_2$]. Cerling (1984; 1991; 1992) formulated a model of isotopic mass balance to calculate atmospheric [CO$_2$] in composition of soils inferred from calcite. Yapp & Poths (1992, 1996) used a modelling proxy but inferred from the natural mineral goethite (iron mineral containing carbon). Ekart et al. (1999) also built a geochemical model from data based on pedogenic carbon (carbon from calcite precipitated during soil formation). Rothman (2002) used strontium isotopes abundance ratio in marine sedimentary rocks, associating this ratio with weathering rates and thus linking CO$_2$ ocean uptake to weathering, with this study confirming strong dependence of CO$_2$ cycle on chemical weathering. Other paleo-proxy methods include using the stomata-CO$_2$ relationship described earlier, with fossilized leaf material studied to infer atmospheric [CO$_2$] from the inverse relationship between stomatal density and [CO$_2$] (McElwain & Chaloner 1995; Retallack 2001; Royer 2001; Royer et al. 2001; Royer 2003; for review see McElwain 1998; Beerling & Royer 2002). However, the stomatal approach has limitations, as the CO$_2$-stomata relationship is species specific, and such relationships are generally assumed rather than calculated due to
use of fossilized material. Other paleo-proxy techniques include using isotopic discrimination of $^{13}$C in photosynthesis to infer isotopic ratios from phytoplankton (Freeman & Hayes 1992; Pagani et al. 1999; Pagani et al. 2005), however this approach has a problem with differentiating the cause of isotopic fractionation as phytoplankton growth rate can cause this kind of isotopic discrimination instead of changes in $[\text{CO}_2]$ (Royer 2014). This approach can also be applied to terrestrial equivalents of phytoplankton, bryophytes, which are typically astomatous (Fletcher et al. 2005; Fletcher et al. 2007). Other studies (Pearson & Palmer 2000; Hönisch & Hemming 2005) have used the differences in response of Boron isotopes to pH change, linking the variation in isotopic composition of marine carbonate to past changes in pH, with pH change ultimately used to estimate dissolved CO$_2$ and then atmospheric [CO$_2$].

However, biogeochemical modelling based reconstruction of past atmospheric CO$_2$ concentrations have been more prominent (Royer et al. 2004; Royer 2006; Royer 2014). These efforts consider the long-term carbon cycle and formulate modelling that incorporates long-term carbon cycle processes, such as weathering and degassing, into a long term but low resolution reconstruction of paleo-[CO$_2$]. The GEOCARB model (Berner et al. 1983; Berner & Canfield 1989; Berner 1994; Berner & Kothavala 2001; Berner 2006; Berner 2008) describes paleo-[CO$_2$] fluctuations in a model that depends on this long term carbon cycle. The model was later updated (GEOCARBSULF) to describe the isotopic mass balance of carbon and sulfur in the earth system surface, relating the isotopic composition of carbon at a given time in the past to the flux of carbon moving into and out of the earth surface (Berner 2006). Several factors are considered in this model, like the impact of rock age, continental relief and most relevant to this project, the impact of the evolution and development of plants being incorporated into the model along its different manifestations. There are other geochemical models that follow similar modelling principles as GEOCARB.
(Tajika (1998) is an example), while some models expanded on the CO$_2$/O$_2$/sulfur framework to include a large biogeochemical feedback model that infers past changes of several elements, with the COPSE model (Bergman et al. 2004) a prominent example. These long-term models have low time resolutions, usually caused by the way data is inputted as these models depend on long term geological processes to formulate.

Fig 1.1 below shows atmospheric [CO$_2$] fluctuations across the phanerozoic. The Fig. presents data from GEOCARBSULFvolc (Berner 2008), COPSE (Bergman et al. 2004) and an amalgamation of paleo-proxy estimates collated by Royer (2014). GEOCARB and COPSE are biogeochemical cycling models, while the paleo-proxy data include estimations from paleosols, stomatal indices as well as other proxies discussed earlier. 2 versions of GEOCARBSULFvolc are presented, based on the updated version of Berner (2008). The 2 versions are the maximum and minimum estimations that result from the updated model output which added different granite/basalt weathering rates. Proxies are based on earth material and so do not provide [CO$_2$] estimations for time periods not represented by the fossilized material, and so proxy based [CO$_2$] estimations are scattered across geological time. However, Royer (2014) collated different paleo-proxy indicators and averaged their estimations to present a plot that runs across most of the Phanerozoic. Fig.1.1 shows that these atmospheric [CO$_2$] estimations have some differences. COPSE does not show significant fluctuation in atmospheric [CO$_2$] levels during the pre-Devonian period like GEOCARBSULFvolc does, and COPSE also has a higher atmospheric [CO$_2$] estimation than the other models during the Carboniferous and Permian. Post-Permian, COPSE and GEOCARBSULFvolc (maximum) generally exhibit similar estimations, while the paleo-proxy estimation shows a higher peak during the Triassic-Jurassic boundary (200 mya), that might be explained by the mass extinction event that occurred during that period that included the loss of large
leafed taxa (Beerling & Berner 2005). Paleo-proxy displayed lower atmospheric [CO₂] levels during the Cretaceous than GEOCARBSULFvolc (maximum) and COPSE, while also showing a peak at around 50 mya.

**Figure 1.1.** Atmospheric [CO₂] values (ppm) over the Phanerozoic (approx. last 500 million years). The figure is a representation of the GEOCARBSULFvolc long term carbon cycle reconstruction model (Berner 2008). The aforementioned study updates the GEOCARB model to include basalt and granite weathering rates. The figure presents the minimum CO₂ values presented in the study (blue dots, weathering rate=10, NV=0, fB(0)=0.75), and the maximum (orange dots, Weathering rate=2, NV=0.015, fB(0)=5). The COPSE model is also presented (grey dots). Paleo-proxy data is presented based on the data presented by Royer (2014, Fig. 4), in which data from several studies using varying paleo-proxy methods was collated (yellow dots).

In this thesis GEOCARBSULFvolc will be used as the predictor of past atmospheric [CO₂] as most previous research that utilised past atmospheric [CO₂] to deduce conclusions into botanical and paleo-botanical research such as attempted in thesis have used the GEOCARB model (Royer 2001; Franks & Beerling 2009b; Franks & Beerling 2009a; Haworth et al. 2011; de Boer et al. 2012; Franks et al. 2013; Elliott-Kingston et al. 2016). It is also the most well-known of paleo-CO₂ models.
Biogeochemical model estimations present a better alternative to paleo-proxies due to the already discussed features of paleoproxies (scatter of data, uncertainty of certain methods like Boron isotopes usage (Royer 2014)). These models also have the advantage of producing atmospheric [CO$_2$] estimations spanning the entire phanerzoic. GEOCARBSULFvolc (Fig. 1.2) was also preferred to COPSE as it is the most recent version of GEOCARB, which is an older and more attuned and updated model (Berner & Canfield 1989; Berner 1994; Berner & Kothavala 2001; Berner 2006; Berner 2008). Also, it incorporates vegetative feedbacks among the earth system components.

**Figure 1.2.** Average estimations of atmospheric [CO$_2$] values (ppm) over the Phanerozoic according to the GEOCARBSULFvolc (Berner 2008) model. The model (red line) is the averaged estimation as calculated by Royer (2014) from the model by Berner (assuming basalt/granite weathering rate of 5 (between 2 and 10, the maximum and minimum model estimations). The likely range of model input parameters represent the error estimation presented by the 2 outer lines (in black) as originally presented by Berner & Kothavala (2001) and then Royer (2014).
The influence of vein and stomatal characteristics on leaf productivity and leaf hydraulics

The previous section highlighted the relationship between changing atmospheric [CO₂] and plant evolution and ecology, highlighting the impact of changing atmospheric [CO₂] on leaf anatomy and hydraulics. This section will probe the mechanisms that illustrate the effect of anatomical features on plant leaf hydraulics and productivity. Stomatal pores are controlled by two cells called the guard cells, and the action of these cells controls the size of the stomatal pore and thus determine the gas exchange rate. Thus, the behaviour of stomatal pores has gained an immeasurable importance in studying leaf physiology (Jarman 1974; Cowan & Farquhar 1977; Farquhar 1978; Morison 1985). Since stomata are the major route for CO₂ uptake into the leaf, the size of their aperture can represent a limitation on carbon assimilation rate. The size of the stomatal pore is determined by changes in guard cell turgor (Buckley 2005) driven by changes in water potential as a result of uptake and release of solutes (Cowan & Farquhar 1977; Mott 1988).

Stomata have been shown to respond to environmental variables like humidity, light and most importantly atmospheric [CO₂] (Cowan & Farquhar 1977; Woodward & Bazzaz 1988; Mott 1988; Morison 1998; Buckley 2005; Franks & Farquhar 2007). Stomatal conductance (gₛ), which is a measure of how much H₂O is leaving (or CO₂ entering) through the stomatal pore per unit time, changes in response to atmospheric [CO₂] fluctuations due to changes in pore size. Stomatal aperture decreases in response to increasing [CO₂], hence decreasing gₛ, while the opposite response occurs with decreasing [CO₂] with gₛ increasing (Wagner et al. 1996; Medlyn et al. 2001; Ainsworth & Rogers 2007; Franks & Farquhar 2007; de Boer et al. 2011). Water use efficiency (WUE) is the ratio of carbon assimilation (A) to transpiration (i.e. carbon gain to water loss), and stomata have evolved to respond to these environmental cues in a way that enhances the leaf’s WUE (Farquhar 1978;
Mott et al. 1997; Buckley 2005; Pou et al. 2008; Brodribb et al. 2009; Lawson & Blatt 2014; Franks et al. 2015). The decrease in $g_s$ under high atmospheric [CO$_2$] results in plant leaves reducing water loss through transpiration. Since CO$_2$ assimilation remains high (due to high atmospheric [CO$_2$] increasing internal CO$_2$ concentrations ($C_i$)), an improvement in WUE can be obtained. When atmospheric [CO$_2$] decreases, stomata open to increase CO$_2$ flux into the leaf to maintain photosynthetic assimilation rate. The close relationship between $g_s$ and $A$ has led to assumptions that $C_i$ is the driver behind the stomatal [CO$_2$] response (Mott 1988; Morison 1998; Ainsworth & Rogers 2007), linking stomata to the mesophyll demand for CO$_2$. Thus, stomata are heavily interlinked with photosynthetic capacity, with leaves that have higher $g_s$ usually having higher $A$ due to the greater availability of CO$_2$ inside the leaf.

Stomatal conductance can also be altered through changes to the anatomical characteristics of the stomata (Jones 1977; Woodward & Bazzaz 1988; Beerling & Chaloner 1993; Wagner et al. 1996). Plants growing under conditions favouring high conductance (low atmospheric [CO$_2$]), for example, will usually have leaves with high stomatal density and smaller sized pores, which results in reduced stomatal pore depth enhancing $g_s$ (Franks & Farquhar 2007). Woodward pioneered the research into the effect of atmospheric [CO$_2$] on stomatal density ($D_s$), starting with his 1987 study (Woodward 1987) where he collected herbarium leaves collected over the last 200 years and measured their $D_s$, showing that $D_s$ has decreased as atmospheric [CO$_2$] has increased over the same time period. A subsequent study, Woodward & Bazzaz (1988), compared $D_s$ and carbon assimilation ($A$) in species over an altitude gradient, as elevated regions have lower atmospheric [CO$_2$] than regions at sea level, and the study found increasing $D_s$ as altitude increased (and atmospheric [CO$_2$] decreased). Additional studies (Woodward & Kelly 1995; Ainsworth & Rogers 2007; Franks & Beerling 2009a; Brodribb & Jordan 2011; Doheny-Adams et al. 2012) only highlighted further the response of $D_s$ to changes in atmospheric [CO$_2$],
and how this change alters $g_s$, influencing plant productivity and growth. For example, a recent study, Doheny-Adams et al. (2012), used genetic manipulation to test the effects of change in $D_s$ and stomatal pore size ($S_s$) on plant function, finding that plants with low $D_s$ and high $S_s$ had lower $g_s$, and thus lost less water. These plants also tolerated water stress better. However, there is evidence that increase in $D_s$ does not manifest in an increase in $g_s$ or $A$ (Zhao et al. 2015), with the cited study showing the increase in $D_s$ as a response to low soil water content, still resulting in a minor increase in $WUE$. Tanaka et al. (2013) did show a positive impact of $D_s$ on photosynthetic capacity but it did not result in biomass/yield changes, so it remain to be seen how important stomatal anatomy can be towards solving food security problems.

Other studies have concentrated on the effect changes in stomatal characters and behaviour can have on the wider ecosystem. Medlyn et al. (2001) collated data from 13 long term studies about the effect of elevated atmospheric [CO$_2$] on European forests. The meta-analysis indicated that $g_s$ has decreased by 21% in response to elevated atmospheric [CO$_2$], prompting a decrease in evapotranspiration rates, which leads to increasing moisture levels in the soil due to the plant not absorbing more water, ultimately leading to more soil runoff. Keenan et al. (2013) analysed long term measurements of ecosystem carbon and water exchange in boreal forests of the northern hemisphere, and found that $WUE$ has increased, with this increase attributed to the rise in atmospheric [CO$_2$]. The increase in $WUE$ is brought about by stomatal closure due to high atmospheric [CO$_2$], reducing the evapotranspiration rate while $C_i$ is maintained owing to the increase in atmospheric [CO$_2$]. This change in gas rates across the stomatal pore would have an impact on ecosystem hydrology similar to that outlined in the Medlyn et al. (2001) study previously. A study by de Boer et al. (2011) also developed models that showed similar observations on the response of stomata to increased atmospheric [CO$_2$] over the past 200 years, which
highlighted the effect of reduced $g_s$ on ecosystem hydrology, as it showed ecosystem transpiration flux will decrease by 60 W.m$^{-2}$ if current atmospheric [CO$_2$] levels are doubled. Lee & Boyce (2010) also showed that changes in transpiration rates over ecosystem wide scales can alter rainfall patterns.

The full impact of stomatal behaviour on leaf function cannot be fully appreciated without taking WUE into consideration. As previously mentioned, stomata respond to atmospheric [CO$_2$] in a way that maximizes WUE, which at high atmospheric [CO$_2$] means reducing the conductance to decrease transpiration rate, even if less air is diffusing in as that air would have a higher [CO$_2$]. Therefore, stomata are also driven by hydraulic signals as it senses changing water status (Jarman 1974; Whitehead 1998; Buckley 2005; Brodribb & Jordan 2008; Brodribb & Jordan 2011; Sack & Scoffoni 2012; Locke & Ort 2014). Guard cell turgor, which itself controls stomatal aperture, is dependent on leaf osmotic and water potential (Buckley 2005), with guard cells being directly affected by the leaf’s hydraulic supply. The association between the response of stomatal aperture and leaf hydraulic status has been confirmed before. A number of experiments (Rufelt 1963 and Raschke 1970, reported in Buckley 2005; Comstock & Mencuccini 1998) in which soil water content or water potential was altered via changing humidity levels, resulting in changes to leaf water balance, have reported the impact it has on stomatal behaviour. These studies often report an initial stomatal opening with decreasing humidity followed by closing afterwards, in what is termed the “wrong-way” response. This response is not new and has been known for decades as “Ivanov effect” (Ivanov et al. 1950) and has been associated with stomata for a long time (Huber 1923 and Stalfelt 1944 cited in McAdam & Brodribb 2014). Interestingly, Xu & Zhou (2008) found that Leymus chinensis plants that were grown under different soil water content replicated the Ivanov effect but through changes in $D_s$. $D_s$, which can be an indicator of $g_s$, was found to increase as leaf water potential ($\Psi_{\text{leaf}}$) decreased (a result of drying or low
humidity). $D_s$ then decreases as leaf water potential declined further, thus forming a response that mimics the Ivanov effect exhibited by individual stomatal apertures.

Changing vapour pressure deficit (VPD) has also been shown to cause changes in epidermis turgor and this affecting the behaviour of nearby stomata (Mott et al. 1997; Mott & Franks 2001). Mott & Parkhurst (1991) attribute stomatal response to humidity to water loss rates from the leaf not to stomatal sensing of VPD changes. Subsequent studies reported similar findings (Monteith 1995; Franks et al. 1997), thus confirming the link between leaf hydraulics. However not all species in those studies conformed, and some reports have added a temporal and diurnal elements to stomatal response to hydraulic signals (Franks et al. 1997; Mencuccini et al. 2000). Furthermore, Lake & Woodward (2008) linked changes in $D_s$ (and ultimately $g_s$) to changes in humidity triggered through Abscisic acid signal transduction pathways. Hence, stomatal behaviour is linked to feedback from hydraulic signals, and this itself affects the leaf’s water relations and especially hydraulic capacity, represented by factors like $K_{leaf}$, which is the flow of water through the leaf divided by $\Psi_{leaf}$. $K_{leaf}$ and $g_s$ have been shown to have a positive relationship (Hubbard et al. 2001; Meinzer 2002; Brodribb & Holbrook 2004; Nardini & Salleo 2005; Franks 2006; Brodribb & Jordan 2008), and this interaction helps improve leaf hydraulic viability, by restoring leaf water potential (Whitehead 1998) or preventing xylem cavitation under high conductive pressure (Brodribb & Holbrook 2004; Nardini & Salleo 2005).

The relationship between the stomata and the leaf’s water relations status would mean that stomata would have a connection with leaf venation. Leaf veins are an extension of the xylem, and they distribute water around the leaf. Differences in vein architecture or structure affect leaf function (Mott & Buckley 1998; Brodribb et al. 2005; Sack & Holbrook 2006; Noblin et al. 2008), with high vein densities ($D_v$) shown to correlate with higher $A$ as well as $D_s$ (Brodribb & Jordan 2011) as well as higher $K_{leaf}$. Sack & Frole (2006) conducted a study on 10 tropical tree species, showing
that $K_{leaf}$ increases with xylem conduit diameter. They also divided the species into sun and shade adapted plants, and found that sun plants had larger xylem conduits and higher $D_v$ than shade plants, with larger conduits meaning lesser resistance and thus higher conductance of water. This indicates that sun species, due to the availability of more light, will have higher $A$ and so their leaves are adapted to service this high $A$ rate with increased $K_{leaf}$, while shade plants, since their photosynthesis is limited due to lesser amount of light reaching those leaves, increase hydraulic resistance to preserve water status. Sack et al. (2008) showed that leaves with higher $D_v$ had better tolerance to damage of xylem conduits enabling them to withstand any damage that might hamper hydraulic performance. The effect of veins on $K_{leaf}$ changing the water supply to the leaf and thus leaf water status, can ultimately affect stomatal behaviour, with $g_s$ shown to respond to $K_{leaf}$ and vice versa.

To illustrate the relationship between veins and stomata, Mott & Buckley (1998) found that areas of leaf surface with high $D_s$ align with veins. Veins have a high water drawdown and so surrounding them with stomata can provide hydraulic signals quickly to the stomata to respond to, with one mechanism being that changes in vein water drawdown can affect epidermis cell turgor and this can consequently affect guard cell turgor (Mott & Buckley 1998). To provide further evidence that increased $D_v$ is associated with improved stomatal function, Noblin et al. (2008) conducted an experiment where they used an artificial leaf consisting of parallel polymeric channels acting as veins. They showed that the distance between vein edges and the evaporative surface (which is the sub-stomatal air cavity in plant leaves (Pieruschka et al. 2010)) and $D_v$ have a strong negative relationship. The negative relationship indicates that as $D_v$ increases, water will travel a smaller distance to the evaporative surface before exiting the leaf, providing the necessary water supply to the stomata to sustain a higher transpiration rate. Boyce et al. (2009) further established the relationship between stomata characters and vein density by
showing that $g_s$ and $D_v$ have a positive relationship in a selection of angiosperms, conifers and ferns, with angiosperms having the highest $g_s$ and $D_v$ (Fig. 2 in Boyce et al., 2009). Brodribb et al. (2013) also illustrated a close positive relationship between $D_v$ and $D_s$ in a sample of 48 tree species (Fig. 2 in Brodribb et al., 2013). This highlights the close link between stomatal and leaf hydraulic characteristics. High $g_s$, inflicting higher transpiration, forces the leaf to conduct more water (high $K_{leaf}$) in order to sustain the high transpiration pressure and keep $\Psi_{leaf}$ balanced across the leaf, with more sophisticated veinal structure being a key innovation in doing that.

With both veinal and stomatal characteristics responding and influencing leaf hydraulics as well as productivity, it is logical to suggest that leaf hydraulics and productivity are strongly correlated (Brodribb & Feild 2000; Sack et al. 2005; Brodribb et al. 2007; Locke & Ort 2014). A couple of studies have measured leaf photosynthetic efficiency (estimated by chlorophyll fluorescence) and $K_{leaf}$ and found a strong correlation between the two (Brodribb & Feild 2000; Brodribb et al. 2005). In another study, carried out across a vast taxonomic range, Brodribb et al. (2007) found that $K_{leaf}$ and $A$ are positively correlated, and are both influenced by $D_v$ and vein proximity to stomata. To summarize, there is a correlation between stomatal ($D_s$, $S_s$ and $g_s$) and veinal ($D_v$, vein proximity to stomata) characteristics in plants aimed at tolerating high hydraulic demand that is needed to facilitate higher $A$, with this network generally reacting to the environment in a way that maximizes $WUE$ (as suggested by the strong stomatal response that attempts to maximize $WUE$).

Maximizing $WUE$ is done either by reducing water loss for the least reduction in photosynthetic rate manageable or increasing the photosynthetic rate enough to overcome any extra water loss. Subsequently, this interaction between $A$ and anatomical characteristics has changed over geological time as plants responded to changing climate, and the changes to this hydraulic-photosynthetic coupling,
manifested by changes in stomatal and veinal characteristics, can be seen over evolutionary history.

**The evolution of leaf anatomical characteristics in response to changing atmospheric \([\text{CO}_2]\) over geological timescales**

The previous section described how stomatal anatomy and leaf vein characters influence each other and how these leaf characteristics responded to contemporary \([\text{CO}_2]\) changes, with changes to vein and stomatal characteristics subsequently affect leaf gas exchange and hydraulic capacity. Since these anatomical changes \((D_s, S_s, D_v)\) can be instigated by changes in atmospheric \([\text{CO}_2]\) and variation in atmospheric \([\text{CO}_2]\) change over geological time as outlined above, this section will centre on the effect of varying atmospheric \([\text{CO}_2]\) over geological time on plant leaf anatomy, specifically the stomata and veins of plants leaves. Over geological time, stomata adapted to changing atmospheric \([\text{CO}_2]\) in a similar direction to the short term response (Franks et al. 2012; Franks et al. 2013), with reduced conductance at high \([\text{CO}_2]\) to reduce transpirational water loss, or increased conductance at low \([\text{CO}_2]\) to keep up photosynthetic assimilation. Already cited reports (Woodward 1987; Woodward & Bazzaz 1988; Woodward & Kelly 1995) first showed decreased \(D_s\) in herbarium leaves over the past 200 years, coinciding with rising atmospheric \([\text{CO}_2]\) levels. Beerling & Chaloner (1993) followed up by showing similar findings with preserved *Quercus robur* leaves collected over the last 200 years. Beerling (1996), using data from *Salix herbacea* leaves sampled over the same period to a model of stomatal response and showed a 60% decrease in \(D_s\) while also showing increased rates of carbon assimilation \((A)\) due to the increase in atmospheric \([\text{CO}_2]\) over the selected period. Beerling (1996) also included data from *Pinus flexilis* leaves that were preserved in packrat middens spanning the last 30000 years (to the last glacial maximum). Atmospheric \([\text{CO}_2]\) has been fluctuating since the last glacial maximum with a slight increasing trend, and the results from the *P. flexilis* data show \(A\)
increasing with increasing atmospheric [CO₂], and gs decreasing correspondingly. Beerling & Woodward (1993) have also reported the trend of increasing A and decreasing gs since the last glacial maximum. Beerling & Kelly (1997) measured Da changes in woodland flora and compared it with the data in Salisbury (1928), and found that Da has decreased as CO₂ levels continued to rise during that time. Franks et al. (2012) expanded on these findings by observing changes in guard cell nucleus and DNA size in response to changes in atmospheric [CO₂] in leaf samples from different taxa, highlighting that plants adapt extensively to facilitate changes in Da or Sa to respond to change in atmospheric [CO₂]. However, this stomatal response, specifically the Da response, to atmospheric [CO₂] change has been shown to be species dependent (Knapp et al. 1994), dependant on adjacent change in environmental factors (Lake & Woodward 2008), or taxa dependent (De Boer et al. 2016).

Over geological time, particularly over the last 150 million years, anatomical changes in response to atmospheric [CO₂] change were key in leaf evolution, particularly in angiosperms. Angiosperms became the dominant plant form over the past 150 million years (Brodribb & Feild 2010), overlapping with a general trend of decreasing atmospheric [CO₂], and the resulting [CO₂] shortage, affecting the leaf’s photosynthetic rate, initiated adaptive feedback responses from plants through changes in leaf anatomical characteristics (Franks et al. 2013). There is now solid evidence that angiosperms have developed to have the highest gs among plants as they dominate the CO₂ starved atmospheres (McElwain & Chaloner 1995; Brodribb et al. 2009; Franks & Beerling 2009b; Haworth et al. 2011; de Boer et al. 2012; Assouline & Or 2013). The high gs occurred through the combination of decreasing Sa and increasing Da. Franks & Beerling (2009b) sampled the stomatal characters of fossil and extant leaves from species that emerged or evolved throughout geological history under different atmospheric [CO₂]. The findings of their study illustrated that
$S_s$ increases with atmospheric [CO$_2$], while $D_s$ decreases. The study also found a negative relationship between $g_s$ and rising atmospheric [CO$_2$]. This means that $S_s$ is inversely proportional to $g_s$ (see Fig. 4 in Franks & Beerling (2009b) for plots). Therefore, increases in $g_s$ as atmospheric [CO$_2$] declined over the last 100 million years was due to increasing $D_s$ combined with decrease in $S_s$. Assouline & Or (2013) explained that smaller stomata result in resistance forces around the pore being reduced, therefore allowing for more gas diffusion into the stomatal pore, while the smaller $S_s$ results in shorter pore depth leading to a shorter diffusional path length for CO$_2$ to cross. This anatomical response to atmospheric [CO$_2$] change is linked to leaf attempts to optimize $WUE$ (Assouline & Or 2013). Decreasing atmospheric [CO$_2$] means that to reach a fulfilling photosynthetic activity, plants would require an increase in CO$_2$ uptake. However, increasing CO$_2$ uptake through the stomata comes at the expense of allowing more water to escape. The extra uptake of CO$_2$ and increased $A$, as well as some biochemical improvements that were also the result of leaf adaptation to decreasing atmospheric [CO$_2$], can help leaves improve $WUE$ levels (Franks et al. 2013), with angiosperms (with higher $D_s$, $g_s$ and $A$ than any other plant taxa) having the highest capacity to optimize $WUE$ ratios (Brodribb et al. 2009).

To facilitate increased CO$_2$ uptake, water supply to sites of evaporation also had to increase in angiosperms through evolutionary adaptations in their hydraulic architecture. Increased transpirational demands due to increased $g_s$ means that the leaf requires greater supply of water (Franks & Beerling 2009a), and consequently this led to the development of more complex venation patterns in the angiospermous leaf (Roth-Nebelsick et al. 2001). $D_v$ increased significantly in angiosperms along with the matching increase in $g_s$ (Boyce et al. 2009; Brodribb & Feild 2010; Boyce & Zwieniecki 2012; de Boer et al. 2012). A study by de Boer et al. (2012) proposes a mechanism that illustrates how angiosperms revolutionized leaf hydraulic
architecture based on inner-leaf geometrical physics. The core of their hypothesis is: since higher $D_s$ and lower $S_s$ meant CO$_2$ crosses shorter path lengths increasing conductance, this required a high $D_v$ and elaborate veinal structure to keep greater water supply to the sites of evaporation, but this venation structure is also designed in a way to reduce water loss through the stomata. This is because the stomatal pore is most resistant to the substance with the shortest transport length. This means that increasing $D_v$ to match the high $D_s$, increasing veinal networking and reducing the post-venous distance between vein endings and the pore would lead to water vapour traversing a shorter distance and thus encountering more resistance. de Boer et al. (2012) concluded that there is a critical $D_v$ that most modern and more complex angiosperms reach that results in the post-venous water distance to the pore being shorter than the distance CO$_2$ has to cross thus tipping the conductance rate towards CO$_2$. Zwieniecki & Boyce (2014) showed that angiosperm leaves contain a high level of precision in placing their veins by measuring the ratio of the distance between veins to the distance between vein edge and stomata, and found that apart from basal clades, most angiosperms have a vein to vein distance/vein to stomata distance ratio that is close 1, stressing the importance of balanced water transport around the leaf that goes with sustaining the high hydraulic demands of the complex angiosperm leaf. These adaptations are facilitated by increasing $D_v$, and developed to limit water loss that occurs with the increased $g_s$ that angiosperms developed through the past 100 million years due to decreasing atmospheric [CO$_2$] (Boyce & Zwieniecki 2012).

Decreasing atmospheric [CO$_2$] has put pressure on plants to select for those with high levels of $C_i$, through higher $g_s$ (and thus higher CO$_2$ uptake), leading to higher $A$, with angiosperms achieving the highest $g_s$ values allowing them to achieve higher $A$ (Brodribb & Feild 2000; Franks & Beerling 2009a). Hence, the evolution of more complex anatomical traits in angiosperms have allowed them to evolve higher $A$.
capacities than other plant taxa (Boyce et al. 2009; Brodribb & Feild 2010; McElwain et al. 2016). Brodribb & Feild (2010), for example, estimated that high $D_v$ had improved angiosperm assimilation capacity at low atmospheric $[\text{CO}_2]$ by 270% compared with other plant groups, while Boyce & Zwieniecki (2012) have determined that productivity had increased during the period of angiosperm ascendancy through geological history basing this on the amount of charcoal available from that period. Similarly, McElwain et al. (2016) also confirmed the relationship between increasing $D_v$ and increasing $g_s$, which conferred a competitive advantage for angiosperms over other taxa and lead to their diversification, by increasing the plasticity of their gas exchange by maintaining higher photosynthetic rates with declining atmospheric $[\text{CO}_2]$ due to their high $g_s$ and $D_v$. However, Franks & Beerling (2009a) have developed a model that estimates stable levels of productivity over the last 100 million years due to the gradual decrease in atmospheric $[\text{CO}_2]$, which suggests that even though photosynthetic capacity in plants increased in response to declining atmospheric $[\text{CO}_2]$, the low atmospheric $[\text{CO}_2]$ levels might still have kept ecosystem wide-productivity at a constant. Ultimately, the innovation in angiosperms is that they kept a constant relative gradient for $\text{CO}_2$ diffusion ($C_i$/atmospheric $[\text{CO}_2]$) through leaf anatomical changes, specifically in stomata (Franks et al. 2013). The increase in $g_s$ (due to anatomical tweaking like changes in $D_s$ and $S_s$) maintained the $\text{CO}_2$ gradient favourable to more conductance, and thus angiosperms improved their own competitive advantage ahead of other plants groups. Even though it shows stable productivity throughout geological time, the model by Franks & Beerling (2009a) still shows an increase in $g_s$ over geological time as well as greater photosynthetic capacity in plants. Boyce & Zwieniecki (2012) also illustrated that there is no direct photosynthetic advantage with $D_v$ greater than 8 mm mm$^{-2}$ to high, but there is a hydraulic advantage. Thus, increased hydraulic capacity due to higher $D_v$ quenches
the high stomatal transpirational demand, and keeps the stomata open, preserving CO₂ conductance levels.

Overall, the key response to low atmospheric [CO₂] is the preservation of a high WUE (Franks et al. 2013), as water availability is always one of the limiting environmental requirements for plants. So, the described changes in anatomical characteristics allowed plants to keep a high level of CO₂ conductance, but to also adapt to the increased water demand that comes with the high conductance, and thus plants, mainly angiosperms, have evolved higher photosynthetic capacities to balance the high water demands. Low atmospheric [CO₂] intervals throughout history have coincided with the emergence of some key taxa (Haworth et al. 2011), and this illustrates the impact of atmospheric [CO₂] as a driver of plant function & evolution. Other aspects of plant response to atmospheric [CO₂], like the biochemical reaction centring around the Rubisco enzyme, may have had a role to play in plant response to changing atmospheric [CO₂], and the reported study by Franks & Beerling (2009a) incorporated the carboxylation efficiency of Rubisco, \( V_{c \text{ max}} \), into their model and showed that this variable responded to atmospheric [CO₂] over geological time. Finally, anatomical evolution of the plant leaf also impacted leaf hydraulics, and the hydraulic aspect of plant leaves has not been investigated in an evolutionary perspective in response to ancient changes in atmospheric [CO₂]. Generally, species with better hydraulic capacities are more successful (Haworth et al. 2011), and the anatomical adaptations reported by previous literature as outlined in this chapter point towards a higher hydraulic capacity, with angiosperms already shown to have higher \( K_{\text{leaf}} \) values than other plant taxa (Brodribb & Feild 2000) to match their more sophisticated anatomical characteristics. Changing atmospheric conditions are expected to lead to more droughts and water shortages and thus understanding response of plant leaf water use to changing environment is becoming more important. Future work must focus on the response of plant hydraulic capacity to
atmospheric [CO₂] fluctuations over geological time and try and reconcile it with the responses of other plant leaf characters to atmospheric [CO₂].

Conclusions and Aims

The driver of plant response to atmospheric [CO₂] is to preserve WUE as this has been a feature of plant colonization of the terrestrial environment, and so future work should focus on how plant adaptation has influenced the leaf’s hydraulic capacity. The subsequent chapters will concentrate on determining plant hydraulic capacity in leaves of different taxa, but mainly in angiosperm species. Decreasing atmospheric [CO₂] over the last 100-120 million years means angiosperms have had to adapt constantly, and some of the available data does show constant changes in Ds and Ss throughout geological history (Franks & Beerling 2009b; unpublished data), along with similar trends in venation, and these anatomical changes affecting the hydraulic demand of the leaf. This thesis will look at how those anatomical changes affected the leaf’s hydraulic capacity by determining the leaf’s hydraulic capacity, through E (the water flow into the leaf per unit time and area) or K_leaf and the response of those parameters to certain environmental stimuli. Species from different taxa or clades that emerged under different atmospheric [CO₂] through geological history will be sampled, thus providing an inkling into how leaf hydraulic capacity contributed to plant response to changing atmospheric [CO₂], linking changes in hydraulic capacity to precursor changes in leaf anatomy like Dv or Ds.
Chapter 2: Variation in leaf anatomical characters because of changing atmospheric CO₂ concentrations in species of different taxa

Introduction

Plants depend on CO₂ uptake for energy, changes in atmospheric [CO₂] concentrations throughout earth’s history have had a great evolutionary effect on plants compared to other environmental factors (Berner 1997; Royer et al. 2004). Stomata facilitate the uptake of CO₂ and exit of H₂O, and atmospheric [CO₂] change has been shown to affect these pores as well as assimilation rate (Cowan & Farquhar 1977; Morison 1998; Buckley et al. 2003). Exposing plant leaves to real-time, in situ incremental change in atmospheric [CO₂] leads to an increase in carbon assimilation levels and a decrease in stomatal conductance (gₛ). The decrease in gₛ is caused by closer of the aperture, leading to a decrease in the rate of H₂O exit. A decrease in gₛ will also affect the rate of CO₂ uptake, but since the air taken in will contain a higher concentration of CO₂, carbon assimilation rate (A) remains high because of the increased concentration of CO₂ inside the leaf (Cᵢ), and this combination leads to a higher water use efficiency (WUE). On the other hand, subjecting the leaf to a reduction in atmospheric [CO₂] decreases A due to the decrease in Cᵢ, while gₛ increases as stomata open to increase the CO₂ flux into the leaf and keep a high Cᵢ and hence maintaining A.

Plants growing under different atmospheric [CO₂] or experience change in atmospheric [CO₂] over several years respond in a similar way to plant leaves experiencing a direct, in situ change (like the one described above), and this is achieved by modifying the anatomical characters of the stomata over time (Woodward & Kelly 1995; Lake et al. 2001; Long et al. 2004; Ainsworth & Rogers 2007; Lake & Woodward 2008). Under low atmospheric [CO₂], leaves develop with a
higher stomatal density ($D_s$; number of stomata per unit area), with this usually corresponding with a decrease in the size of the individual stomatal pore ($S_s$) (Woodward & Bazzaz 1988; Miller-Rushing et al. 2009). High atmospheric [CO$_2$] stimulates the development of lower $D_s$ and high $S_s$. Increase in $D_s$ combined with low $S_s$ due to low atmospheric [CO$_2$] increases the evaporative surface area of the leaf and results in higher $g_s$, thus increasing CO$_2$ uptake. On the other hand, high atmospheric [CO$_2$] stimulates low $D_s$/high $S_s$ and this reduces water loss due to decreased $g_s$. Lake et al. (2001) demonstrate that mature leaves in Arabidopsis thaliana detect changes in atmospheric [CO$_2$] and transmit this information to younger leaves, allowing the younger names to change stomatal development to adjust to the new [CO$_2$] environment, and this mechanism is thought to occur within plant communities to adjust to environmental change. The leaf’s ability to alter stomatal characters in response to atmospheric [CO$_2$] was shown to heavily affect leaf transpiration, rosette growth and tolerance to water shortage (Doheny-Adams et al. 2012), and this enables the leaf to maintain WUE. Atmospheric [CO$_2$] change through glacial to evolutionary time-scales has a similar effect on plant leaves. First of all, rising atmospheric [CO$_2$] over the past 200 years caused by the industrial revolution has been shown to affect the stomatal characters of leaf samples (Woodward 1987; Beerling & Chaloner 1993; Beerling 1996), and the reported data generally conform to the expected pattern of decreasing $D_s$ with some increase in $S_s$. This was expanded to investigating fossil leaves from the period that includes the last glacial maximum and up to the Miocene (Beerling & Woodward 1993; McElwain & Chaloner 1995; Kurschner et al. 2008), with similar observations reported. This change in stomatal characters was eventually also linked to the leaf’s attempt to improve WUE like it does in short term responses to changing atmospheric [CO$_2$] (Beerling & Woodward 1993; Franks et al. 2012).
Significant differences in stomatal characters between taxa have been established for a long time (Salisbury 1928). This prompted interest into the evolutionary origins of different taxa and their anatomical characters, and how the response of these characters to changing atmospheric \([\text{CO}_2]\) over geological time and the impact that had on the evolution of the plant leaf. Work by Franks & Beerling (Franks & Beerling 2009a; Franks & Beerling 2009b) showed that \(D_s\) is higher in fossilized leaves from periods of low atmospheric \([\text{CO}_2]\) compared to fossils from high atmospheric \([\text{CO}_2]\) periods, with \(S_s\) responding in concert with high \(S_s\) matching the low \(D_s\) and low \(S_s\) matching the high \(D_s\), with this most prominent in the angiosperms (Brodribb et al. 2009). Decrease in atmospheric \([\text{CO}_2]\) over the past 100 million years initiated a shift to low \(S_s/\)high \(D_s\) that enabled leaves to have lower diffusive gas exchange resistance around the pore and thus gas exchange sensitivity increased (Assouline & Or 2013). Species that evolved under low atmospheric \([\text{CO}_2]\), and thus develop these stomatal characters, can decouple gas exchange rate (the rate of \(\text{CO}_2\) uptake) from leaf evaporative area (Assouline & Or 2013), as low \(S_s/\)high \(D_s\) leads to reduced leaf evaporative area as a proportion of leaf surface area, leading to a gain in photosynthetic surface area. Improved thermal regulation and evaporative cooling capacities also be the result of optimised stomatal characters (Beerling et al. 2001; Osborne et al. 2004).

These evolutionary changes in stomatal characters that led to increase in \(g_s\) means a further hydraulic demand is imposed on the leaf. Hence, the leaf’s hydraulic features are thought to have also played a role in the evolution of the leaf in response to atmospheric \([\text{CO}_2]\). Similar to the evolution of stomatal characters, increased vein densities \((D_v)\) have been shown to be more prominent in angiosperms (Boyce et al. 2009; Brodribb & Feild 2010; Boyce & Zwieniecki 2012), with high \(D_v\) also shown to correlate with higher assimilation rate in those studies. The distinction in anatomical features acquired by angiosperms is generally ascribed to declining atmospheric
[CO₂] during the period of angiosperm emergence (mid to late-Cretaceous) with low atmospheric [CO₂] imposing a selection pressure that drove the evolution of the angiosperm leaf (Franks & Beerling 2009b; Brodribb & Feild 2010; Boyce & Zwieniecki 2012; Franks et al. 2013). McElwain et al. (2016) scaled the relationship between anatomical traits \( (D_s, D_v) \) and gas exchange capacity in different taxa, and they found that the acquisition of higher \( g_s \) in angiosperms conferred a competitive advantage for them by increasing their gas exchange plasticity and expanding their ecophysiological niche.

The aim of this research was to elucidate the effect of atmospheric [CO₂] change over geological time on plant leaf anatomical characters. Species from different taxa were selected, and the diversification age of their crown group was used as a temporal variable. Selecting species based on crown group age each species to correspond to the atmospheric [CO₂] level during the emergence of that species ancestors, thus linking each species’ anatomical characters with the atmospheric [CO₂] level during the emergence of each taxa. \( D_s \) and \( D_v \) were measured in the species selected and then compared to illustrate patterns of difference between taxa, group and crown group ages. Ultimately, results presented here would further clarify the influence of atmospheric [CO₂] on \( D_s \) and \( D_v \) and to confirm the already discussed patterns of declining atmospheric [CO₂] stimulating improvements in these characters that allowed certain taxa to prosper over others. A portion of the work presented here was conducted prior to the start of the thesis, but it was included to illustrate the full breadth of the data at hand and to solidify the base on which the rest of this thesis is built around, as the anatomical variation between species is key to unlocking the hydraulic and photosynthetic characteristics of the species investigated in the upcoming chapters. Finally, this effort can add to the understanding of how plants would respond to changing atmospheric [CO₂] due to climate change and provide more areas for investigation (namely manipulating plant leaf anatomy) that
can help in the endeavour to improve plant productivity and response to climate change.

**Materials and Methods**

**Plant material**

Species of varying taxa and evolutionary history were selected. The main selection factor to picking those species is the age of their crown group (the group that contains the species along with its relatives back to their most recent common ancestor; i.e. a species and all its descendants). Crown age is used here to link the species to environmental conditions that its group diversified under. This would link the evolution of species to environmental factors, specifically atmospheric [CO$_2$], allowing for the testing of one of this thesis’ hypotheses that changes in atmospheric [CO$_2$] influenced the evolution of the plant leaf. Variation in leaf characteristics between the species will be attributed to different levels of [CO$_2$] in the atmosphere at the time of the crown group or species evolution, however the ecological and phylogenetic factors will also be highlighted. Species are listed below in alphabetical order, with information summarised in table 2.1 below.

**Butia capitata**

*B. capitata* is a palm that belongs to the *Arecaceae* family (Subfamily *Arecoideae*), which is the largest group of woody monocots. This group is native to South America where they grow in humid, warm, neo-tropical conditions (Cornwell et al. 2014).

*B. capitata* is known to be a tough species that can withstand uncharacteristically low temperatures for this group of palms (Up to -10°C). Observed cells in the vascular systems of these palms remain metabolically active for a long time, with substantial thickening and secondary thickening occurring around the vascular system, allowing palms to have sustained growth and indefinite vascular functioning (Tomlinson 2006), giving it significant competitive advantage in tropical regions. Crown group
*Arecales*, which is the order that *B. capitata* belongs to, has been estimated to have diversified 130 mya (Magallón & Castillo 2009). *Areaceae* diverged later, with its crown group age being estimated at 100 mya ago (Couvreur et al. 2011) up to 120 mya (Tank et al. 2015). Crown group *Arecoideae* is thought to have emerged around 73 mya (Couvreur et al. 2011), and the subfamily *Cocoseae* (which belongs to *Arecoideae*) estimated to be 63 mya old (Meerow et al. 2015). The crown age of the genus *Butia* has been estimated to be about 7 million years old (Couvreur et al. 2011; Baker & Couvreur 2013; Meerow et al. 2015).

**Drimys winteri**

*D. winteri* is an evergreen tree native to the temperate montane forests of southern Argentina and Chile. It belongs to the subfamily *Winteroideae* of the family *Winteraceae*, a group of vessel-less, tracheid-using trees that occur in similar wet ecological conditions as *D. winteri*. These trees are considered primitive angiosperms due to their xylem not containing vessel elements (Feild et al. 2000). The leaves of *Winteraceae* have wax-covered, cutin-plugged stomata, thought to help the leaves constrict gas exchange to relief excess conductive pressure on their under-developed xylem (Feild et al. 1998). These families belong to order *Canellales* which has been estimated to have diversified in the early to mid-Cretaceous, with most studies pinning its age at around 125-127 mya (Wikström et al. 2001; Magallón & Castillo 2009; Bell et al. 2010; Magallón et al. 2013; Thomas et al. 2014; Muller et al. 2015; Massoni et al. 2015; Tank et al. 2015). Crown group *Winteraceae* is thought to have diversified in the late cretaceous (62 or 90 mya according to Muller et al. (2015) and Thomas et al. (2014) respectively). Consequently, those studies pin crown group *Winteroideae* at around 45 or 70 mya respectively. The genus *Drimys* is thought to have diversified 12 mya (Thomas et al. 2014) or 6 mya (Muller et al. 2015).
**Ginkgo biloba**

*G. biloba* (The maidenhair tree) is a deciduous tree native to China that is the only extant species of the gymnospermous division *Ginkgophyta*, with leaves that show dichotomous venation. Fossil *Ginkgo* have been associated with riparian, disturbed environments with ample water supply and drainage (Royer et al. 2003), with the extant *G. biloba* found to have leaves that are almost identical to fossil *G. adiantoides* leaves that were associated with these habitats (Zhou 2009). This gave indications that *Ginkgo* represents an ancient form of competitive strategy, as current *G. biloba* traits (like long life span, late sexual maturity) are not competitive in modern disturbed habitats (Zhou & Zheng 2003; Royer et al. 2003) as they would be outcompeted by angiosperms. Thus, *Ginkgo* are considered to be an example of pre-angiospermous life strategy (Royer et al. 2003), as extant *G. bilboa* occupies temperate woodlands (Zhou & Zheng 2003) as opposed to what the Cretaceous and pre-Cretaceous fossils indicate (Zhou & Zheng 2003). Zheng & Zhou (2004) attribute this change in *Ginkgo* ecology to changing climatic conditions during the Cretaceous and Palaeocene. Family *Ginkgoaceae* has been estimated to have a stem group age as far back as 265 mya (Tank et al. 2015). Clarke et al. (2011) put the age of crown group *Ginkgo* diverging with *Coniferae* at 165 mya. Crane (2013) Mentions an estimated diversification age for *Ginkgo* at around 160 mya – 100 mya.

**Laurus nobilis**

Bay laurel (or bay tree), *L. nobilis*, belongs to the *Laurus* genus of evergreen trees of the *Lauraceae* family (Order *Laurales*). It is native to the Mediterranean basin. Crown group *Laurales* has been given varying estimates of crown ages, between 130-140 mya (Wikström et al. 2001), ≈120 mya (Magallón & Castillo 2009; Bell et al. 2010; Michalak et al. 2010; Magallón et al. 2013; Tank et al. 2015), to ≈110 (Renner 2004; Massoni et al. 2015). Naumann et al. (2013) strays from these estimates to claim an
average stem group age of 77 mya for Laurales, however they do have a maximum estimate of up to 120 mya. Crown group Lauraceae has been estimated to have diversified around 60-70 mya (Michalak et al. 2010; Naumann et al. 2013), 93 mya (Tank et al. 2015), 104 mya (Magallón et al. 2013). Crown group Laureae has also been estimated to have diverged from Cinnamomumae around 55 mya (Huang et al. 2016).

**Nageia nagi**

*N. nagi* is a coniferous, evergreen tree native to China and nearby parts of far east Asia belonging to the Podocarpaceae family (order Pinales). Podocarps are known as shade tolerant species with a diversity of leaf morphologies (Biffin et al. 2012), with examples of distichous leaf morphology or leaf flattening, which can be strategies to develop leaves capable of capturing light under overcompetitive conditions. Crown group Pinales has an age of around 260 mya (Clarke et al. 2011; Leslie et al. 2012; Magallón et al. 2013) up to 230 mya (Quiroga et al. 2016). Crown group Podocarpaceae has an age of 150-160 mya (Biffin et al. 2012), while Leslie et al. (2012) puts it at a slighter older age (between 150-200 mya). Quiroga et al. (2016) puts the diversification age of this group even higher at 230 mya, while Magallón et al. (2013) estimates the opposite by pinning the age of Podocarpaceae at 101 mya. Biffin et al. (2012) and Quiroga et al. (2016) also provide an age for crown group Nageia at around 48-50 mya.

**Osmunda regalis**

The royal fern (*O. regalis*) is a deciduous fern that grows in dark, humid, water saturated conditions that is native to most of the old world. It belongs to the family Osmundaceae, the only extant family of order Osmundales (Metzgar et al. 2008). Ferns are primitive vascular plants, thought to have diversified as far back as the Devonian (Clarke et al. 2011; Magallón et al. 2013). They have been used to
compare evolutionary changes in plant leaf function across geological time (Brodribb & Holbrook 2004; Brodribb & McAdam 2011; McAdam & Brodribb 2012; McAdam & Brodribb 2015; Martins et al. 2015), as these evolutionary changes are thought to have caused a big competitive dent for ferns when angiosperms diversified in the Cretaceous (Schneider et al. 2004). Crown group Osmundaceae has been estimated to have diversified between 300 mya (Schneider et al. 2004; Schuettpelz & Pryer 2009; Schneider et al. 2015) and 250 mya (Carvalho et al. 2013; Grimm et al. 2015). Crown group Osmunda has an estimated age of 199 mya (Schuettpelz & Pryer 2009), 133-143 mya (Grimm et al. 2015) and 120 mya (Schneider et al. 2015).

**Passiflora caerulea**

Blue Passionflower (*P. caerulea*) is a tendril vine, deciduous species native to tropical South America. *Passiflora* are the largest genus of the *Passifloraceae* family (Muschner et al. 2012), and this family belongs to the diverse order Malpighiales (APG 1998; APG 2016), which is an ecologically important group as 40% of understory tropical tree species belong to Malpighiales (Xi et al. 2012), and almost 6% of all angiosperms (Davis et al. 2005). Thus, *P. caerulea* can be a good representative of a competitive angiosperm from high productivity environments, and would provide good comparison to species used in this thesis from other taxa. Crown group Malpighiales has an age of 100-120 mya (Davis et al. 2005; Xi et al. 2012; Tank et al. 2015). Wikström et al. (2001) had a younger estimate of 85 mya, similar to Magallón & Castillo (2009) (89 mya), while Bell et al. (2010) pins Malpighiales diversification to the mid-Cretaceous as well (90-100 mya). *Passifloraceae* has an age of 110 mya according to Davis et al. (2005), which is older than a number of age estimates for Malpighiales. A slightly younger age (99 mya) is given by (Tank et al. 2015), but this a stem group age. Xi et al. (2012) provide an estimation for *Passifloraceae* of 50 mya (stem-group) and 26 mya (crown-group). Muschner et al. (2012) present ages for different clades and crown groups that diverged from
Passifloraceae (65 mya), including estimating the diversification and radiation of crown group Passiflora at around 17 mya.

Vitis vinifera

Common Grapevine (V. vinifera) is a species of deciduous climbers that is heavily cultivated, native to areas in southern Europe and the middle and near east. It belongs to the family Vitaceae, the only family of order Vitales. Vitis have wide vessels (Lovisolo & Schubert 1998) that can withstand huge conducting pressure as observed during spring refilling of vessels (Sperry et al. 1987), making the investigation of its hydraulic capacity interesting. Crown group Vitaceae has estimated ages of 117-108 mya in some studies (Wikström et al. 2001; Bell et al. 2010; Magallón et al. 2013; Tank et al. 2015), down to 95-90 mya in others (Magallón & Castillo 2009; Wen et al. 2013; Wan et al. 2013; Liu et al. 2016). Crown group Vitis has a diversification age of 28 mya (Wan et al. 2013), up to 32-38 mya (Wen et al. 2013; Liu et al. 2016).

Vicia faba & Phaseolus vulgaris

Fava beans (V. faba) is a leguminous crop that's part of the pea family, Fabaceae (order Fabales). French bean (P. vulgaris) is a herbaceous leguminous crop that is part of the same family as V. faba, both belonging to subfamily Faboideae. Those species were picked to represent modern, productive crops. Crown group Fabales has an estimated age of 80 mya (Wikström et al. 2001) to ≈ 90 mya (Bell et al. 2010; Koenen et al. 2013), with (Magallón & Castillo 2009) putting the stem age more at 110 mya. Crown group Fabaceae diversified around 60 mya (Wikström et al. 2001; Lavin et al. 2005) or 70 mya (Tank et al. 2015) up to 90 mya (Hohmann et al. 2015). Hohmann et al. (2015) estimated subfamily Faboideae (both V. faba and P. vulgaris belong to it) to have diversified 73 mya down, but other estimates are 59 mya (Koenen et al. 2013) or ≈ 50 mya (Cannon et al. 2015). Lavin et al. (2005) offer
estimation for diversification ages for the crown group of *Vicia* & *Pisum* at 17 mya, while also estimating the age for crown group of *Phaseolus* & *Vigna* at 10 mya.

**Zea mays**

Corn (*Z.mays* varieties) is a grain crop plant that is the result of human breeding of its ancestor teosinte, native to the new world (mainly Latin America). *Zea* belongs to the group of C₄ grasses in the tribe *Andropogoneae* (*Panicoideae*), family *Poaceae*, order *Poales*, giving it a high rate of productivity that is known in C₄ grasses. Age estimations for crown group *Poales* vary but mostly is pinned around 100-110 mya (Paterson et al. 2004; Magallón & Castillo 2009; Magallón et al. 2015) or 80-100 mya (Merckx et al. 2008; Mennes et al. 2013; Mennes et al. 2015). Crown *Poaceae* diversified in the late Cretaceous, with estimates from ≈ 80 mya to 68 mya (Bouchenak-Khelladi et al. 2009) & Bouchenak-Khelladi et al. (2014) respectively). The younger age of 59 mya has been estimated by (Magallón et al. 2015). The crown group of the BOP/PACMAD clade that contains *Z.mays* as well as species like *Oryza sativa* has been estimated to have emerged at around 60-70 mya (Paterson et al. 2004) or later at around 50 mya (Vanneste et al. 2014). Subfamily *Panicoideae* diversified during the early Miocene, 23-20 mya (Cotton et al. 2015). However Bouchenak-Khelladi et al. (2009) pin *Panicoideae* divergence (tribe *Andropogoneae*) to an earlier date, 29 mya.

**CO₂ concentration at age of crown group diversification**

The GEOCARBSULF model (Fig.1.1 and 1.2) adapted from Berner (2006, 2008) will be the template used to infer past atmospheric [CO₂]. Table 2.1 below shows the species sampled with their crown group age and the corresponding atmospheric [CO₂] at the time of crown group diversification. GEOCARBSULFvolc maximum and minimum values are given, plus the average of those values, with the average value being used for the rest of the thesis. Atmospheric [CO₂] values from COPSE and
paleo-proxy (Fig. 1.1) are also presented for comparison. To give a standardised age group for the species, the crown age of the genus was used during data analysis and presentation throughout the thesis. Hence, species like *L. nobilis*, that has no genus crown group age estimation, had the crown group age of its genus estimated based on its subfamily age estimation (55 mya), with an average atmospheric [CO\(_2\)] level taken for the period after subfamily origin, and so on. Average age estimates are presented to compensate for lack of exact consensus in the literature.

Table 2.1. Range of species sampled for this species with estimations of subfamily and genus crown group age and the corresponding atmospheric [CO\(_2\)] according to the models presented in Fig. 1.1.

<table>
<thead>
<tr>
<th>Species</th>
<th>Mean crown age (mya) for subfamily/tribe</th>
<th>Mean crown age (mya) for genus</th>
<th>Atmospheric [CO(_2)] at crown genus (ppm)</th>
<th>Atmospheric [CO(_2)] at crown genus volc maximum (ppm)</th>
<th>Atmospheric [CO(_2)] at crown genus volc minimum (ppm)</th>
<th>Atmospheric [CO(_2)] at crown genus volc average (ppm)</th>
<th>Atmospheric [CO(_2)] at crown genus Volc (ppm) based on COPSE model</th>
<th>Atmospheric [CO(_2)] at crown genus paleoproxies</th>
</tr>
</thead>
<tbody>
<tr>
<td>Butia capitata</td>
<td>63</td>
<td>7</td>
<td>285</td>
<td>140</td>
<td>140</td>
<td>210</td>
<td>405</td>
<td>280</td>
</tr>
<tr>
<td>Drimys winteri</td>
<td>57</td>
<td>9(6-12)</td>
<td>285</td>
<td>140</td>
<td>140</td>
<td>210</td>
<td>405</td>
<td>280</td>
</tr>
<tr>
<td>Ginkgo biloba</td>
<td>165</td>
<td>160-100</td>
<td>600-1500</td>
<td>200-400</td>
<td>750</td>
<td>1000-1200</td>
<td>1100-650</td>
<td>1100</td>
</tr>
<tr>
<td>Laurus nobilis</td>
<td>55</td>
<td>&lt;55</td>
<td>450</td>
<td>130</td>
<td>290</td>
<td>750</td>
<td>500</td>
<td>500</td>
</tr>
<tr>
<td>Nageia nagi</td>
<td>NA</td>
<td>49</td>
<td>520</td>
<td>150</td>
<td>335</td>
<td>860</td>
<td>880</td>
<td>880</td>
</tr>
<tr>
<td>Osmunda regalis</td>
<td>NA</td>
<td>199-120</td>
<td>750-1300</td>
<td>150-375</td>
<td>650</td>
<td>700-1100</td>
<td>1600-700</td>
<td>1600</td>
</tr>
<tr>
<td>Passiflora caerulea</td>
<td>NA</td>
<td>17</td>
<td>285</td>
<td>115</td>
<td>200</td>
<td>475</td>
<td>270</td>
<td>270</td>
</tr>
<tr>
<td>Vitis vinifera</td>
<td>NA</td>
<td>33</td>
<td>330</td>
<td>105</td>
<td>215</td>
<td>725</td>
<td>725</td>
<td>725</td>
</tr>
<tr>
<td>Vicia faba</td>
<td>17*</td>
<td>&lt;17</td>
<td>285</td>
<td>115</td>
<td>200</td>
<td>435</td>
<td>260</td>
<td>260</td>
</tr>
<tr>
<td>Phaseolus vulgaris</td>
<td>10**</td>
<td>&lt;10</td>
<td>285</td>
<td>135</td>
<td>210</td>
<td>400</td>
<td>280</td>
<td>280</td>
</tr>
<tr>
<td>Zea mays</td>
<td>25</td>
<td>&lt;25</td>
<td>300</td>
<td>100</td>
<td>200</td>
<td>480</td>
<td>280</td>
<td>280</td>
</tr>
</tbody>
</table>

*Age of divergence for Vicia-Pisum clade. **Age of Phaseolus-Vigna clade.

Stomatal density (\(D_s\))

Stomatal density (\(D_s\)) was estimated by taking the impression of the abaxial side of leaves using Xantopren polysiloxane-based precision dental material (Hanau, Germany), as suggested by (Weyers & Johansen 1985) along with the accompanying activation liquid that caused the dental material to harden. A mixture of the dental
material with the activation liquid was prepared in a clear plastic petri dish. The leaf was then cut and its abaxial surface was immediately placed on the mixture and left until the mixture completely hardened (few seconds to a min, depending on how much activation liquid is added). The leaf needed to be placed on the mixture quickly so that the mixture does not solidify before an impression of the leaf was indented into the dental material. After the mixture solidifies, ensuring a leaf impression was produced, the leaf was removed and nail varnish was applied to the negative xantropen impression and left for about 30 seconds to dry. A sellotape was then placed on the dry nail varnish, and when the sellotape is removed the nail varnish layer was transferred to the surface of the sellotape. The positive nail varnish impression was stuck to a microscope glass slide, and was studied under an Olympus BX60 microscope. The position on the leaf where stomatal counts were taken was chosen randomly. A photograph of the position chosen was taken using an eye piece camera (Bresser MicroCam 5.0MP, Rhede, Germany) in conjunction with the BX60 microscope and the photograph then transferred to a computer. The stomata were counted \textit{in silico}. The area of the photography was estimated using a 1 mm graticule slide so that $D_v$ could be measured accurately and converted into mm$^2$.

**Vein density ($D_v$)**

A leaf clearing technique (adapted from original protocol in Scoffoni et al. 2011) was used to remove epidermal tissue and stain the vein structure of each leaf, with veins becoming clearer and easier to see. Leaves were chemically cleared using an aqueous solution of 5 NaOH. Leaves were left in the solution until they appeared transparent, this varied between 2 and 7 days depending on the species (tougher tissues taking considerably more time). Once leaves were transparent they were rinsed with $\text{H}_2\text{O}$ and set aside for 30 min to dry. Ethanol (EtOH) dilution series was used to bring specimens up to a level for staining. Leaves left in each dilution (20\%, 40\%, 80\%, 100\%) for approximately 5 min. After the 100\% dilution each leaf was
covered in 1% Safranin (1g safranin/100ml of 100% EtOH) and left to soak for 5 to 15 min (dependant on tissue delicacy). After being rinsed with 100% EtOH, the leaves were covered in 1% Fast green (1g fast green/100ml of 100% EtOH) for seconds and then rinsed again with 100% EtOH. Reverse dilution series (100%, 80%, 40%, 20%) was used before placing specimens in H₂O to allow excess stain to be removed. A photograph of the position chosen was taken using an eye piece camera (Bresser MicroCam 5.0MP, Rhede, Germany) in conjunction with the BX60 microscope and the photograph then transferred to a computer. \( D_v \) was estimated in silico using ImageJ (Schneider et al. 2012). The area of the photograph/image was estimated using a 1 mm graticule slide so that \( D_v \) could be measured accurately and converted into mm².

**Analysis of data**

Figures and statistical analysis was conducted in R software (R Core Team (2014). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. URL http://www.R-Project.org/). Normality was checked by plotting a generalized linear model and inspecting residual plots. Differences between species in \( D_v \) and \( D_s \) were analysed via a linear mixed-effects model (package lme4 and nlme), with the \( D_v \) or \( D_s \) as the variable, Species as the factor and the individual as the source of random variation. The model took the following form:

\[
F_{vs} = \mu + S_p + I_s + \epsilon_{ivs}
\]

Where \( F_{vs} \) is the either the variable \( D_v \) or \( D_s \), \( S_p \) is the treatment, \( I_s \) is the random effect of the individual, and \( \epsilon_{ivs} \) represents the residuals. Regression analysis was carried in R using linear modelling (lm). The model was formulated to predict the significance of a linear relationship between the two variables in the plot in the form:

\[
y = mx + c
\]
Where \( y \) (predicted) and \( x \) (predictor) are the y-axis and x-axis variables respectively, and \( m \) is the slope of the relationship, and \( c \) is the y-axis intercept. \( m \) represents the direction of the relationship (negative or positive). \( R^2 \) values and \( p \) values were obtained from the analysis output and used to interpret significance of relationships.

**Results**

\( D_s \) was significantly higher in *P.caerulea* and *L.nobilis* than all other species (\( p<0.05 \)), while *P.roebellennii* and *B.capitata* also had \( D_s \) values significantly higher than all other species (\( p<0.05 \)) except *L.nobilis* and *P.caerulea* (Fig. 2.1). There was no significant difference between the rest of the species (\( p>0.05 \)). \( D_v \) increases in species through geological time and the increase sharpens as \([\text{CO}_2]\) started to decrease from about 150 mya onwards (Fig. 2.2). However, there is a drop in \( D_v \) in *V.faba* and especially *Z.mays* afterwards. *P.caerulea*, *L.nobilis* and to a lesser extent *D.winteri* all had significantly higher \( D_v \) than all other species (\( p<0.05 \)). *G.biloba*, *N.nagi*, *O.regalis* and *V.vinifera* had significantly lower \( D_v \) values than the other species (\( p<0.05 \)).
Figure 2.1. The heterogeneity $D_s$ (Stoma mm$^{-2}$) between species with different crown ages (in brackets). Species crown age is used to link the evolution of the species to the [CO$_2$] in the atmosphere at that specific time (see Fig. 2.1). 3 Leaves were sampled for each species. An impression was taken off the abaxial side of the leaf and put onto a microscope slide enabling, after which an image of the impression was taken under the microscope. The ANOVA statistical test was applied to a linear mixed effects model of the data to ascertain statistical differences in $D_s$ between species. The Fig. helps illustrate the statistical differences between the species, with species sharing the same letter above them having no significant differences in $D_s$ values.
Figure 2.2, The heterogeneity $D_v$ (mm mm$^{-2}$) between species with different crown ages (in brackets). Species crown age is used to link the emergence/evolution of the species to the concentration of CO$_2$ in the atmosphere at that specific time. 3 Leaves were sampled for each species. Using a protocol adapted from (Scoffoni et al. 2011), the leaf was cleared and stained with dye, then put under a microscope where an image was taken, with $D_v$ calculated in silico. The ANOVA statistical test was applied to a linear mixed effects model of the data to ascertain statistical differences in $D_v$ between species. The Fig. helps illustrate the statistical differences between the species, with species sharing the same letter above them having no significant differences in $D_v$ values.
Figure 2.3. The relationship between $D_s$ (stoma per mm$^2$) and $D_v$ (mm mm$^{-2}$) with atmospheric [CO$_2$] concentration (ppm) at the time of species crown age. Relationships in plot were investigated using linear regression ($n=3$, error bars=standard error). An impression was taken off the abaxial side of the leaf and put onto a microscope slide enabling, after which an image of the impression was taken under the microscope. Using a protocol adapted from (Scoffoni et al. 2011), the leaf was cleared and stained with dye, then put under a microscope where an image was taken. $D_s$ and $D_v$ were calculated in silico. Atmospheric [CO$_2$] are taken from Table 2.1. (a) $D_s$ vs. atmospheric [CO$_2$] concentration at the time of taxa divergence ($R^2$=0.27, $p$>0.05, $D_s = (-0.1525)*CO_2 + 162.2784$). (b) $D_v$ vs. atmospheric [CO$_2$] concentration at the time of taxa divergence ($R^2$=0.004331) $CO_2 + 4.981841$). (c) $D_s$ vs $D_v$ ($R^2$=0.54, $p$<0.05, $D_s = (30.86)D_v - 2.994$).
There was no significant relationship between $D_s$ ($R^2=<0.2$, $p>0.05$) and $D_v$ ($R^2=0.27$, $p>0.05$) with corresponding atmospheric [CO$_2$] levels assigned per crown group age (Fig. 2.3). A significant correlation ($R^2=0.54$, $p<0.05$) was found between $D_s$ and $D_v$, strengthening the assumed relationship and dependence between these two leaf characters. Further analysis (Fig. 2.4) showed that higher $D_s$ and $D_v$ is an angiospermous characteristic.

**Figure 2.4.** The relationship between $D_s$ (stoma per mm$^2$) and $D_v$ (mm mm$^{-2}$) with atmospheric [CO$_2$] concentration (ppm) at the time of species crown age. Different coloured dots represent the plant division each species belongs to. The figure aims to illustrate variation between Angiosperms, Gymnosperms and Ferns in the measured parameters. (a) $D_s$ vs. atmospheric [CO$_2$] concentration at the time of taxa divergence ($R^2=<0.2$, $p>0.05$, $D_s = (-0.1525) \text{CO}_2 + 162.2784$). (b) $D_v$ vs. atmospheric [CO$_2$] concentration at the time of taxa divergence ($R^2=0.27$, $p>0.05$, $D_v = (-0.004331) \text{CO}_2 + 4.981841$). (c) $D_s$ vs $D_v$ ($R^2=0.54$, $p<0.05$, $D_s = (30.86) D_v - 2.994$).
Discussion

Generally, species that emerged under lower atmospheric [CO$_2$] have leaves that exhibit higher $D_s$ and $D_v$, but this was not consistent. There was a significant drop in $D_s$ for *D.winteri* even though it emerged later than species with high $D_s$ like *P.caerulea*, however atmospheric [CO$_2$] was slightly higher (210 ppm) when *D.winteri* emerged compared with earlier periods in the Miocene (200 ppm at around 17 mya). This reduction in $D_s$ might be a mechanism to reduce water uptake into the leave via restricting transpiration rate through the stomata, as this can be associated with the assumed function of the cutinous stomatal plugs found on *D.winteri* leaves, believed to be a mechanism to compensate for the lack of water conducting vessels in *D.winteri* wood (Feild et al. 2000). However, the role of those plugs has been challenged (Feild et al. 1998), with the high $D_v$ of *D.winteri* shown here (Fig. 2.2) goes against the assumption of reduced leaf hydraulic capacity in *D.winteri* due to lack of vessels in the woody parts. A previous data set from our lab does show *D.winteri* exhibiting higher $D_s$ values (Matthews & Lawson, unpublished data), further alluding the possibility of maintained higher hydraulic conductivity by the leaf. Low $D_s$ noticed in *V.vinifera* is also perplexing as it is comparable with $D_s$ values in non-angiosperms, however it can be speculated the use of *V.vinifera* as a crop throughout human history, leading to selective breeding, would have changed original characteristics of the *V.vinifera* leaf. The low $D_v$ values of *V.faba* and *Z.mays* can also be to human-inflicted changes that might have occurred to them as crop species. Reducing $D_s$ (and hence $g_s$), coupled with increasing productivity, can be a characteristic of breeding for higher WUE which is an important crop trait that breeders/farmers select for (Ainsworth & Rogers 2007; Doheny-Adams et al. 2012).

Results for $D_v$ (Fig. 2.2) show a slightly leaner response as species with a younger crown group age usually accompanied by low atmospheric [CO$_2$] and higher $D_v$. The exception is seen towards the end in *Z.mays* and *V.faba*, but again it can be
attributed to breeding practices in those species trying to lessen crop water consumption and achieve more effective use of scarce water resources, or due to the C₄ photosynthetic pathway in *Z.mays* which means the *Z.mays* leaf is more adapted to low atmospheric [CO₂] biochemically. *V.vinifera* is known as a species with high hydraulic capacity, yet here it has significantly lower $D_v$ than other species. However, *V.vinifera* has very wide vessels (Lovisolo & Schubert 1998) which might compensate for the low $D_v$.

Angiosperms tend to exhibit higher densities of veins and stomata compared to the other taxa (Fig. 2.4), and this is consistent with literature (Boyce et al. 2009; Boyce & Zwieniecki 2012; Zwieniecki & Boyce 2014; McElwain et al. 2016). The significant correlation between $D_s$ and $D_v$ in Fig. 2.3(c) suggests that these two anatomical traits influence each other, with higher conductance as a result of increased $D_s$ requiring a high $D_v$ to match its demand (Fiorin et al. 2015). *P.caeurlea*, which is a good example of a species from high productivity environments (Xi et al. 2012), and the data show that it has the highest $D_s$ and $D_v$ from the sampled species, indicating higher productive and hydraulic capacities. *N.nagi*, despite emerging in low [CO₂] environments compared to other non-angiosperms, has low densities of veins and stomata, but it is characterized as a shade plant (Biffin et al. 2012), and hence the lower productivity associated with low light environments can explain the low anatomical densities of *N.nagi*.

**Conclusion**

Decreasing atmospheric [CO₂] over the last 100 million years or so has put a selection pressure on plants to maintain productivity (Bateman et al. 1998), leading to anatomical innovations (like increase in $D_s$) which maximized CO₂ intake to keep productivity up. High $g_s$ (due to high $D_s$) increases water demand by the leaf (Buckley 2005), which instigated an increase in $D_v$ and thus improved water supply, enabling the plant leaf to maintain a healthy *WUE* (Brodribb et al. 2009; Franks & Beerling.
2009a; Assouline & Or 2013). The co-evolution of vein and stomatal characters, as plants adapted to low atmospheric [CO$_2$], spurred plant evolution (Fig. 2.4) as it permitted plant leaves to overcome CO$_2$ starvation by increasing CO$_2$ uptake and to handle the repercussions of higher CO$_2$ uptake like higher water demand, leading to the emergence of angiosperms, the most productive and dominant taxa of plants on the planet (Feild et al. 2009; Boyce & Zwieniecki, 2012). Increased water demand would manifest in an increase in the hydraulic capacity of the leaf, which would be investigated in the following chapters among other functional characteristics that would be effected by improvements in anatomical characters, like flexibility of stomatal behavior and the dynamic responses of gas exchange parameters.
Chapter 3: Functional analysis of leaf hydraulics and gas exchange for species with different crown ages

Introduction

Most previous work on the influence of past climatic changes on plants has centred on the response of plant leaf anatomy to change in atmospheric [CO$_2$] (Woodward 1987; McElwain & Chaloner 1995; Franks & Beerling 2009b). The anatomical evolution of the plant leaf, especially in angiosperms, reflects a tendency towards improving leaf gas exchange through the stomata to achieve higher WUE (Beerling & Woodward 1993; Beerling 1996; Brodribb et al. 2009; Boyce & Zwieniecki 2012). Over geological time, stomata have responded in a similar fashion to long term change in atmospheric [CO$_2$]. Plants that evolved under low atmospheric [CO$_2$] increased their $g_s$ via increasing their stomatal density ($D_s$), with increasing $D_s$ also linked with a corresponding reduction in stomatal size ($S_s$) (Woodward & Kelly 1995; McElwain & Chaloner 1995; Franks & Beerling 2009b). Whereas in plants that evolved during periods of high [CO$_2$], the reverse is observed with these species having low $g_s$ due to them retaining a high $S_s$/low $D_s$ stomatal characters that lead to a reduction in $g_s$. The development of high $D_s$/low $S_s$ characteristics was mostly prominent in angiosperms leading to their high $g_s$ values compared to other taxa (Feild et al. 2004), and which subsequently explains their higher photosynthetic capacities (Brodribb et al. 2005; McElwain et al. 2016).

Impact of stomatal acclimation affects the water status of the leaf (because changing $g_s$ alters the rate of H$_2$O exchange which changes hydraulic demand on the leaf), and so an evolutionary trend in leaf hydraulic features is observed alongside this stomatal change, manifested by modifications to leaf vein characteristics. The increase in $g_s$ in angiosperms was matched by an increase in vein density ($D_v$), in order to facilitate the expected increase in hydraulic demand imposed by high $g_s$. 

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(Beerling et al. 2001; Brodribb et al. 2007; Noblin et al. 2008; Boyce et al. 2009; de Boer et al. 2012; Zwieniecki & Boyce 2014; Fiorin et al. 2015). Chapter 2 elucidated the relationship between stomatal and veinal characters between each other and atmospheric [CO₂], and found a general trend of increasing densities in low [CO₂] environments. It also found a strong correlation between the two anatomical variables, \( D_s \) and \( D_v \).

This indicates the strong relationship, and probable correlation, between leaf gas exchange and hydraulic capacity (Brodribb et al. 2005; Brodribb et al. 2007). Brodribb & Feild (2010) showed that increased \( D_v \) led to an increase in photosynthetic capacity in plants over geological times, while there is data showing strong correlation between \( D_s \) (i.e gas exchange) and \( D_v \) (i.e hydraulic capacity) (Brodribb et al. 2013) with other studies (Brodribb & Feild 2000; Brodribb et al. 2005) confirming this relationship in different types of plant taxa, confirming the findings in Chapter 2. Leaf hydraulic conductance (\( K_{leaf} \)) was shown to be higher in angiosperms than any other taxa (Brodribb & Holbrook 2004; Brodribb et al. 2005; Sack & Holbrook 2006), and shown to be associated with anatomical characteristics of the leaf mesophyll and epidermis (Aasamaa et al. 2001). Changing stomatal size and density have also been assumed to change stomatal behaviour and speed of response, with Drake et al. (2013) demonstrating that the stomatal opening response is negatively correlated with \( S_s \) but positively correlated with \( D_s \). Thus, high \( D_s \)/low \( S_s \) species are more equipped to achieve higher photosynthetic gas exchange rates through a faster \( g_s \) in response to environmental variables, with Raven (2014) showing how stomatal size can affect guard cell function. However this does not always lead to higher WUE (Drake et al. 2013).

The relationship between smaller stomata and quicker opening/closing responses is not always consistent and can depend on other environmental conditions. Angiosperms have been shown to differ in their \( g_s \) response to other taxa (Brodribb &
Holbrook (2004) under dry conditions, showing a delay in their stomatal closing response, with this behaviour thought to be a mechanism to maximize gas exchange capacity, relying on xylem investment and complex venation to withstand hydraulic demand (Brodribb & Holbrook 2004). This is evidenced by higher $D_s$ found in angiosperms, and its correlation with higher assimilation rates. Lawson & Blatt (2014) also show that under water stress, the advantage of the high $D_s$/low $S_s$ characteristics can be reduced. A study has also found an increase in $g_s$ response with increase $S_s$ in an Arabidopsis ecotype (Monda et al. 2016), so the high $D_s$/low $S_s$ mechanism does not always result in higher $g_s$ values. Franks & Farquhar (2007) also found stomatal responses to be correlated with guard cell and aperture shape rather high $D_s$ or low $S_s$. McAusland et al. (2016) also found no correlation between $D_s$ and stomatal response speed in elliptical shaped guard cells, but the correlation was observed in dumbbell shaped stomata. The differences in response speeds has been attributed to climate, with most species that have shown faster response speeds in those studies being graminoid grasses that thrive in dry climates (Vico et al. 2011). Elliott-Kingston et al. (2016) also found no strong correlation between low $S_s$/high $D_s$ pattern and stomatal response in varying taxa. Hence, relationship of stomatal anatomy with stomatal behaviour is still unclear and requires further investigation.

This chapter aims to quantify the hydraulic capacity of species from varying taxa in response to environmental stimulus. Also, leaf stomatal and photosynthesis response to light will also be assessed. The chapter aims to link leaf hydraulic and gas exchange capacities in species of varying taxa, and subsequently link the variation in functional capacity to leaf anatomical characters illustrated in Chapter 2. The species were selected based on crown group ages as per Chapter 2. Species were picked to represent a wide geological time frame and that incorporated different atmospheric [CO$_2$] during these periods. This study involved measurement of
hydraulic flow ($E$) or $K_{leaf}$, as well as gas exchange parameter like $A$ and $g_s$. The results of this research will provide insight into how plant species will respond to future climatic changes in atmospheric [CO$_2$]. The research also aims to expand the knowledge of the relationship between leaf stomatal behaviour and leaf hydraulic and productive capacities.

**Materials and Methods**

**Plant material**

Species that were sampled for this study were picked based on crown group age and taxa as outlined in Chapter 2. They were: *Butia capitata*; *Drimys winteri*; *Ginkgo biloba*; *Laurus nobilis*; *Nageia nagi*; *Osmunda regalis*; *Passiflora caerulea*; *Vitis vinifera*.

**Measurement of $E$ and $K_{leaf}$**

Sack & Scoffoni (2012) outlined a set-up to measure plant leaf hydraulics via the evaporative flux method (also see Flexas et al. (2013)), and a variation on the protocol and methods of Sack & Scoffoni (2012) was used (Fig. 3.1). A leaf was cut under water at the petiole and then connected to water filled tubing whilst maintaining the cut petiole and tubing under water. This is to ensure a continuous column of water inside the leaf petiole with no air bubbles infiltrating. To make sure the leaf-tubing connection was sealed, water proof oil grease (Dow Corning High Vacuum Grease, USA) was applied around the petiole-tube connection to prevent air entering the tubing and losing the water column. The tubing draws water from a cylinder or small beaker that was placed on a recording sensitive balance from Sartorius (Sartorius CP | Gem$^+$ Series, Sartorius, Goettingen, Germany). The balance was linked to a computer that records balance measurements at 30 seconds interval. The leaf, after it was connected to tubing, was placed inside a chamber (Plant cuvette NPH 1000, Campbell Scientific, Logan, Utah, USA) where the
environmental conditions were controlled. \([\text{CO}_2]\) around the leaf was controlled via a mass flow controller (Bronkhorst, Newmarket, UK) connected to 0% \(\text{CO}_2\) air source and a 10% \(\text{CO}_2\) air source; temperature was controlled using a water bath connected to the chamber. The chamber has a clear top through which photosynthetically active radiation (PAR) is provided by white LEDs (Luxeon Star LEDs, Brantford, Ontario, Canada), with light levels controlled by computer software (TLC application, Technologica, Frating, UK). As the leaf transpires, water is drawn from the beaker on the balance as the leaf takes in water. The water lost from the beaker is recorded and is used to estimate water uptake by the leaf per unit time. Wet tissue was placed inside the balance chamber in and around the water filled beaker to increase the humidity and reduce evaporation from the beaker, further ensuring that the water lost from the beaker (and recorded by the balance) is attributed to leaf conductance. This setup allows for the manipulation of environmental conditions (light, \([\text{CO}_2]\), temperature) to induce different functional responses from the leaf.
Using the setup described above, light response experiment was conducted. The light response experiment involved supplying different light levels to the leaf and measuring the hydraulic flow and conductance of plant leaves. (a) outlines a horizontal cross-section used to measure leaf hydraulic capacity: at the centre the Campbell chamber contains the leaf. The chamber has fans underneath the leaf which keep the air moving inside the chamber and try to reduce the effect of boundary layer around the leaf. The leaf is connected through tubing to a beaker on a balance, which itself connected to a computer that records balance readings. The environment inside the chamber is controlled via a water bath for temperature and a mass flow controller to control [CO₂]. Photosynthetically active radiation is provided through an LED sheet placed vertically above the chamber (the Campbell chamber has a clear top) and the LEDs are connected to a computer that allows for the control of the light level that the LEDs provide. Fig. 1(b) provides a vertical cross-section of the Campbell chamber showing the input and output to and from the chamber.

Hydraulic response to light

Using the setup described above, light response experiment was conducted. The light response experiment involved supplying different light levels to the leaf and
measuring $E$ at each level. The leaf was given an average of 30 minutes under each light level to stabilise, with the last 10 to 15 balance readings being used to calculate an average $E$ value for that specific light level. Four light levels were applied: 100, 500, 1000 and 1500 $\mu$mol m$^{-2}$ s$^{-1}$. Temperature was kept at 23°C and CO$_2$ at 400 ppm. After each regime ends the leaf was removed and placed inside a plastic bag with a wet tissue put inside it, and the bag is then exhaled in and sealed. The leaf is left in the bag for about 20 minutes as leaf stabilizes to prepare the leaf for water potential, $\Psi_{leaf}$, measurement, which was conducted using a psychrometer (PSYPRO water potential datalogger, Wescor Inc., Logan, Utah, USA). To calculate $K_{leaf}$, the ratio of $E$ to $-\Psi_{leaf}$ is obtained and further normalized by leaf area (Sack & Holbrook 2006; Sack & Scoffoni 2012). For each light level, the corresponding $E$ measurement was divided by $-\Psi_{leaf}$ to find $K_{leaf}$ at that light level. Species used for the light experiment are: *G.biloba, N.nagi, O.regalis, B.capitata, V.vinifera, D.winteri, L.nobilis, P.caerulea*.

**Hydraulic response to CO$_2$ and temperature**

Using the setup described above, the response of $E$ to temperature and CO$_2$ was measured. The leaf was cut under water and connected to water filled tubing that draws water from a beaker on a recording balance, and then the leaf was put in a chamber where the environment surrounding the leaf (light level, [CO$_2$] and temperature) can be controlled. Light was kept constant at 500 $\mu$mol m$^{-2}$ s$^{-1}$. [CO$_2$] and temperature of the air around the leaf were manipulated instead using the mass flow controller and water bath respectively. Once the leaf is put through the system, it is first subjected to an increase in air [CO$_2$] while temperature was kept constant, then [CO$_2$] was stabilised and temperature was then increased, as follows: the leaf is put in the chamber with temperature at 23°C, and is kept at that level while [CO$_2$] is incrementally increased to induce a response. [CO$_2$] starts at 100 ppm and then raised to 400 and then 800 ppm. Just like the light response experiment, the leaf was
kept under each [CO₂] level for about 30 minutes, and after each [CO₂] level the average of the last 10 balance readings is taken as the \( E \) value for that [CO₂] level. After the [CO₂] treatment is finished, [CO₂] was reduced back to the ambient concentration of 400 ppm and the leaf is left for 30-35 minutes to stabilize under 400 ppm with temperature and light kept constant as well. Then, temperature is increased to 35°C and then 50°C, with the leaf kept under each temperature condition for about 30 minutes, after which \( E \) was calculated after each condition like it was for the [CO₂] response above. Due to equipment failure, sampling was restricted to only \( P.\) caerulea for this experiment.

**Effect of light on \( A \) and \( g_s \)**

The response of leaf gas exchange to a change in light was investigated using an Infra-Red Gas Analyser (Li-Cor 6400, Licor Biosciences, Lincoln, Nebraska, USA). The leaf was equilibrated at 100 \( \mu \)mol m\(^{-2}\) s\(^{-1}\) of light for 20 minutes. Light was then notched up to 1000 \( \mu \)mol m\(^{-2}\) s\(^{-1}\) for 90 minutes, after which light was returned to 100 \( \mu \)mol m\(^{-2}\) s\(^{-1}\) for 60 more minutes before the leaf was removed. During the experiment, leaf chamber [CO₂] was maintained at 400 ppm, vapour pressure deficit (VPD) maintained at around 1. Species sampled for this experiment were: \( G.\) biloba, \( N.\) nagi, \( O.\) regalis, \( B.\) capitata, \( V.\) vinifera, \( D.\) winteri, \( L.\) nobilis, \( P.\) caerulea.

**Data analysis**

Figures and statistical analysis was conducted in R software (R Core Team (2014). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. URL http://www.R-Project.org/). The effect of light on \( K_{\text{leaf}} \) across the different species was investigated via a mixed effects model, LME (package lme4 and nlme) of the form:

\[
W_{ipk} = \mu + S_i + L_p + I_s + (SL)_p + e_{ipk}
\]
Where \( W_{ipk} \) is the variable \( K_{leaf} \), \( \mu \) is the overall mean, \( S_i \) is the Species effect on \( K_{leaf} \) variation, \( L_p \) is the effect of light levels on \( K_{leaf} \) variation, \( I_s \) is the random effect of the individuals, \((SL)_{ip}\) is the Species*Light interaction effect and \( \varepsilon_{ipk} \) represents the residuals. Normality was checked by plotting a generalized linear model (GLM) and inspecting residual plots. A GLM was also used to analyse the difference in \( K_{leaf} \) between Species in each light level. In a similar vein, the temperature/CO\(_2\) experiment was also analysed via a LME, with the treatment applied (e.g. 23°C with 400 ppm) as the environmental factor and the individual as the source of random variation. The model took the following form:

\[
F_{itk} = \mu + T_t + I_s + \varepsilon_{itk}
\]

Where \( F_{itk} \) is the variable \( E \), \( T_t \) is the treatment, \( I_s \) is the random effect of the individual, and \( \varepsilon_{itk} \) represents the residuals.

To analyse data from the light step-change study, the maximum \( A \) and \( g_s \) values were used and taken from the end of the high light (1000 \( \mu \)mol m\(^{-2}\) s\(^{-1}\)) period after the \( A/g_s \) response had stabilized. The area under the curve (\( auc \)) was also calculated for both the \( A \) and \( g_s \) response, calculating the area under the \( A \) or \( g_s \) response curve for each sample, with this providing an indication of each Species total assimilation of conductance capacity over a prolonged response. Statistical differences in maximum \( A \) or \( g_s \) values or in \( auc \) values were obtained in a similar method to that used to obtain differences in \( E \) between different temperature and CO\(_2\) treatment (see above), with \( A \) or \( g_s \) or \( auc \) as the variable and Species as the factor.

Furthermore, Vico et al. (2011) provide an equation describing the response time of stomata to a step change in light, in the form of:

\[
g(t) = g(\phi) + [g_0 + g(\phi)] \exp(-k/t_0)
\]

Where \( g(\phi) \) is the maximum, stable \( g_s \) value at the high light period, \( g_0 \) is the initial starting conductance, \( t_0 \) is the time necessary to get to 63% of the variation between
\( g_0 \) and \( g(\phi) \), while \( k \) is the exponential time evolution variable used to estimate the speed of response of the stomata. This \( k \) value was obtained using non-linear squares modelling in R. Regression analysis was carried in R using linear modelling (lm). The model was formulated to predict the significance of a linear relationship between the two variables in the plot in the form:

\[
y = mx + c
\]

Where \( y \) (predicted) and \( x \) (predictor) are the y-axis and x-axis variables respectively, and \( m \) is the slope of the relationship, and \( c \) is the y-axis intercept. \( m \) represents the direction of the relationship (negative or positive). \( R^2 \) values and \( p \) values were obtained from the analysis output and used to interpret significance of relationship.

**Results**

**Hydraulic response to light**

Measurement of hydraulic response to increase in light intensity is summarised in Fig. 3.2 below. *P.caerulea* and *D.winteri* had higher \( K_{\text{leaf}} \) values than all other species. *O.regalis* and *N.nagi* showed no clear response to increasing light intensity. *G.biloba* showed the lowest \( K_{\text{leaf}} \) values while *P.caerulea* showed the highest. *L.nobilis* appears to be an outlier, as its \( K_{\text{leaf}} \) values are similar to species that evolved earlier like *V.vinifera* and *O.regalis*. *L.nobilis* also did not display an increase in \( K_{\text{leaf}} \) as light increased. Statistically, there was a significant interaction between species and light (\( p<0.0001 \)) influencing the \( K_{\text{leaf}} \) response, meaning that \( K_{\text{leaf}} \) in respond differently in different species to light. This is evidenced in species such as *P.caerulea* and *D.winteri* that showed strong increase in \( K_{\text{leaf}} \) in response to increasing light intensity, while species such as *N.nagi* and *G.biloba* showed little or no response to change in irradiance. Variation between species were further highlighted as significant variation between species in \( K_{\text{leaf}} \) value within each light
level was found (p<0.05).

Figure 3.2, the response of $K_{leaf}$ (mmol m$^{-2}$ s$^{-1}$ MPa) to incremental change in light intensity (µmol m$^{-2}$ s$^{-1}$). Measurements were conducted via the hydraulic set up described in Materials and Methods (Fig. 3.1), under 23°C temperature and 400 ppm CO$_2$ concentration. The leaf was first cut under water and attached to tubing that connected to a water filled beaker on a balance. A computer connected to the balance recorded the water loss from the beaker. The rate of water loss from the beaker allows for the estimation of $E$, and combined with $\Psi_{leaf}$, $K_{leaf}$ can be calculated. Each species sampled corresponded to a specific crown age (in brackets after species name in the legend). (For G. biloba, N. nagi, L. nobilis, P. caerulea, V. vinifera, n=6. For B. capitata, O. regalis, n=4. For D. winteri, n=3). The asterisks signify that there was significant difference between species in $K_{leaf}$ values at that light level. (error bars=standard error).

Hydraulic response to CO$_2$ and temperature

The response of $E$ to temperature and [CO$_2$] in P. caerulea was measured and shown in Fig. 3.3. As [CO$_2$] is increased, $E$ increases as well. The increase in $E$ is then exacerbated with rising temperatures. There was a significant difference in $E$ between treatments, with $E$ significantly at a [CO$_2$] of 400 & 800 ppm than at 100
ppm (p<0.05). There was a larger increase in $E$ when temperature rose, with $E$ being significantly different at 35°C (p<0.05) than at any of the different CO$_2$ concentrations. $E$ at 50°C was significantly higher than any of other the temperature or [CO$_2$] values (p<0.05) as well.

Figure 3.3. The response of $E$ (mmol m$^{-2}$ s$^{-1}$) to temperature (°C) and [CO$_2$] (ppm) in *P. caerulea*. [CO$_2$] was increased under a constant temperature of 23 °C, after which [CO$_2$] was stabilised at 400 ppm while temperature was increased, hence the effect of each of the 2 factors was observed separately. Using the setup highlighted in Fig. 3.1, the leaf was cut under water and attached to tubing linked to a beaker on a balance that records water loss from the beaker on a computer. The leaf is put into a chamber were the environmental conditions surrounding the leaf are controlled, allowing for the manipulation of those environmental conditions for this experiment. Light was kept constant at 500 μmol m$^{-2}$ s$^{-1}$ throughout. Statistical analysis highlighted the difference in $E$ between the different conditions applied, and the Fig. illustrated those differences by showing the same letter above the conditions that produce $E$ values that were not significantly different. (n=6).
Effect of light on $A$ and $g_s$

The response of $A$ and $g_s$ to the increase in light intensity from 100 to 1000 μmol m$^{-2}$ s$^{-1}$ seemed to varied among species (Fig. 3.4). All species had a rise $A$ to the increase in light, except $N.nagi$, with $A$ ultimately reaching steady state in the species. $g_s$ increased in response to the increase in light intensity in all species, with $N.nagi$ again being the exception. However, compared to the $A$ response, the response of $g_s$ was not as rapid or as directional as the $A$ response, with $A$ reaching steady-state earlier than $g_s$ does in all the species. Despite the lack of synchrony between $g_s$ and $A$, the maximum steady state values of $A$ and $g_s$ were correlated among the species, with species reaching high $g_s$ values also having high $A$ values (Fig. 3.5 and 3.6) except for $O.regalis$ and $L.nobilis$, with the former having higher $g_s$ values but low $A$, while $L.nobilis$ experienced the opposite. Significant differences were observed between the steady state $A$ maximum of $G.biloba$, $D.winteri$, $L.nobilis$ and $P.caerulea$ and the steady state $A$ maximum of $N.nagi$ (p<0.05). In general, however, there was no significant difference in the maximum steady state $A$ values between most species (Fig. 3.5). The maximum steady-state value of $g_s$ was also not significantly different between most species (Fig. 3.6), with the only significant difference observed between the steady state $g_s$ maximum of $G.biloba$ and $D.winteri$ and the steady state $g_s$ maximum of $N.nagi$ (p<0.05).
Figure 3.4. The response of $A$ ($\mu$mol m$^{-2}$ s$^{-1}$) and $g_s$ (mol m$^{-2}$ s$^{-1}$) to a step increase in light intensity in species with different crown ages. The leaf was put inside a Li-6400 Infra-Red Gas Analyser chamber under 100 $\mu$mol m$^{-2}$ s$^{-1}$ of light for 20 minutes (0-20 mins on the y-axis), before light was increased to 1000 $\mu$mol m$^{-2}$ s$^{-1}$ for 90 minutes (20-110 mins on the y-axis). Light intensity was then diminished back to 100 $\mu$mol m$^{-2}$ s$^{-1}$ for 60 minutes (110-170 mins on the y-axis). $[CO_2]$ was kept at 400 ppm throughout. The 8 species are presented above in the order of their crown age, with the oldest, $O$.regalis, first and the youngest, $B$.capitata last. Standard error bars were excluded from the figure for clearer presentation (check appendix for individual figures with error bars). (n=2-3).
Figure 3.5. The maximum steady state $A$ (µmols m$^{-2}$ s$^{-1}$) value reached by a species after step increase in light, taken towards the end of the high light period when the response of $A$ had stabilised. Light during this period was 1000 µmols m$^{-2}$ s$^{-1}$.

Measurement was carried out using a Li-6400 from Licor, with [CO$_2$] in the leaf chamber maintained at 400 ppm. Species are laid out in order of oldest to youngest in terms of crown age (see Table. 2.1). Species that share the same letter above have no significant difference between them. (n=2-3).
Analysis of the exponential time evolution variable, \( k \), which was used to examine the relative speed of stomatal response, showed no significant difference between the species in the speed of response of \( g_s \) to increase in light intensity. At the other end of the light regime, when light was returned to 100 µmols m\(^{-2}\) s\(^{-1}\), species also show some variation in response of \( A \) and \( g_s \). The decline in \( A \) was quickest in

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**Figure 3.6.** The maximum steady state \( g_s \) (mol m\(^{-2}\) s\(^{-1}\)) value reached by a species after step increase in light, taken towards the end of the high light period when the response of \( A \) had stabilised. Light during this period was 1000 µmols m\(^{-2}\) s\(^{-1}\). Measurement was carried out using a Li-6400 from Licor, with [CO\(_2\)] in the leaf chamber maintained at 400 ppm. Species are laid out in order of oldest to youngest in terms of crown age (see Table. 2.1). Species that share the same letter above have no significant difference between them. (n=2-3).
G. biloba, D. winteri, L. nobilis and P. caerulea, but for G. biloba the $g_s$ decreasing response (stomatal closing) is slower than the 3 other species, in which $g_s$ declines with the same rapidity as the decline in $A$. Reducing the light intensity did not result in any noticeable decline in $g_s$ in O. regalis and N. nagi, while V. vinifera and B. capitata decreased in $g_s$ to match their decreasing $A$, although the reduction in $g_s$ was still slower than that of species such as D. winteri or P. caerulea.

Relationship between gas exchange and hydraulic parameters with leaf anatomical characters and atmospheric [CO$_2$] concentration at time of taxa divergence

The hydraulic ($E$) and gas exchange ($A$, $g_s$) parameters measured were plotted with atmospheric [CO$_2$] at the time of taxa divergence and leaf anatomical data from Chapter 2 to infer the relationship between leaf function, leaf anatomy and the effect of CO$_2$ levels on leaf characteristics and function. There was generally very weak ($p=0.07$ in Fig.3.7(a)) to no correlation ($p>0.05$ in Fig.3.7(b) and (c)) between hydraulic and gas exchange parameters with [CO$_2$] at time of crown group age. The relationship between the functional parameters themselves was also not significant (Fig. 3.7(d) and (e), $p>0.05$) except for the significantly positive relationship between $A$ and $g_s$ ($R^2=0.6$ and $p<0.05$, Fig. 3.7(f)). There were no significant correlations between the functional parameters and leaf anatomical parameters presented in Chapter 2 (Fig. 3.8), except for Fig.3.8(e), which shows a weak correlation ($p=0.07$) between $E$ and $D_v$. Fig. 3.9 and 3.10 show the same correlation plots shown in the previous 2 figures but with the data points sorted per plant division, with the plots showing angiosperms generally exhibiting higher $D_v$ and $D_s$ combined with higher hydraulic and gas exchange variables.
Figure 3.7. The relationship between leaf hydraulic and gas exchange parameters for each species sampled with each other and with atmospheric [CO$_2$]. The plots display the maximum recorded value for each species for the respective variable (E, A and $g_s$) against atmospheric [CO$_2$] at the time of taxa divergence (Table 2.1), as well as linear regression trends. Coloured dots represent the species sampled. (a) E (mmol m$^{-2}$ s$^{-1}$) vs. atmospheric [CO$_2$] (ppm) ($R^2$=0.43, p=0.07, $E = (-0.0017953) \text{CO}_2 + 1.4934854$). (b) A (µmol m$^{-2}$ s$^{-1}$) vs. atmospheric [CO$_2$] (ppm) ($R^2$=0, p>0.05, $A = (0.0001597) \text{CO}_2 + 5.7196408$). (c) $g_s$ (mol m$^{-2}$ s$^{-1}$) vs. atmospheric [CO$_2$] (ppm) ($R^2$=0.1, p>0.05, $g_s = (0.00005078) \text{CO}_2 + 0.03327$). (d) E (mmol m$^{-2}$ s$^{-1}$) vs. A (µmol m$^{-2}$ s$^{-1}$) ($R^2$=0.1, p>0.05, $E = (0.06436) A + 0.47989$). (e) E (mmol m$^{-2}$ s$^{-1}$) vs. $g_s$ (mol m$^{-2}$ s$^{-1}$) ($R^2$=0.1, p>0.05, $E = (4.4130) g_s + 0.6247$). (f) A (µmol m$^{-2}$ s$^{-1}$) vs. $g_s$ (mol m$^{-2}$ s$^{-1}$) ($R^2$=0.6, p<0.05, $A = (64.532) g_s + 2.458$). (error bars=standard error, n=3-6)
Figure 3.8. The relationship between leaf hydraulic and gas exchange parameters for each species sampled with leaf anatomical variables measured in Chapter 2. The plots display the maximum recorded value for each species for the hydraulic and gas exchange variables (E, A and gs) against the anatomical variables (Ds and Dv) as presented in Chapter 2. The figure also shows linear regression trends in the plots. Coloured dots represent the species sampled. (a) A (μmol m$^{-2}$ s$^{-1}$) vs. Ds (stoma per mm$^2$) ($R^2$=0.2, p>0.05, $A = (0.01336)D_{s} + 4.11476$). (b) E (mmol m$^{-2}$ s$^{-1}$) vs. Ds (stoma per mm$^2$) ($R^2$=0.42, p>0.05, $E = (0.00499)D_{s} + 0.23072$). (c) gs (mol m$^{-2}$ s$^{-1}$) vs. Ds (stoma per mm$^2$) ($R^2$=0.2, p>0.05, $gs = (-0.00003468)D_{s} + 0.05574$). (d) A (μmol m$^{-2}$ s$^{-1}$) vs. Dv (mm per mm$^2$) ($R^2$=0.36, p>0.05, $A = (0.9831)D_{v} + 2.3768$). (e) E (mmol m$^{-2}$ s$^{-1}$) vs. Dv (mm per mm$^2$) ($R^2$=0.43, p>0.07, $E = (0.22463)D_{v} + 0.02189$). (f) gs (mol m$^{-2}$ s$^{-1}$) vs. Dv (mm per mm$^2$) ($R^2$=0.2, p>0.05, $gs = (0.004726)D_{v} + 0.037757$). (error bars=standard error, n=3-6)
Figure 3.9. The relationship between leaf hydraulic and gas exchange parameters for each species sampled with each other and with atmospheric \([\text{CO}_2]\). The plots display the maximum recorded value for each species for the respective variable (\(E\), \(A\) and \(g_s\)) against atmospheric \([\text{CO}_2]\) at the time of taxa divergence (Table 2.1), as well as linear regression trends. Coloured dots represent the group that the sampled species belongs to. (a) \(E\) (mmol m\(^{-2}\) s\(^{-1}\)) vs. atmospheric \([\text{CO}_2]\) (ppm) \((R^2=0.43, p=0.07, E=\left(-0.0017953\right) \text{CO}_2 + 1.4934854)\). (b) \(A\) (\(\mu\)mol m\(^{-2}\) s\(^{-1}\)) vs. atmospheric \([\text{CO}_2]\) (ppm) \((R^2=0, p>0.05, A=\left(0.0001597\right) \text{CO}_2 + 5.7196408)\). (c) \(g_s\) (mol m\(^{-2}\) s\(^{-1}\)) vs. atmospheric \([\text{CO}_2]\) (ppm) \((R^2=0.1, p>0.05, g_s=\left(0.00005078\right) \text{CO}_2 + 0.03327)\). (d) \(E\) (mmol m\(^{-2}\) s\(^{-1}\)) vs. \(A\) (\(\mu\)mol m\(^{-2}\) s\(^{-1}\)) \((R^2=0.1, p>0.05, E=\left(0.06436\right) A + 0.47989)\). (e) \(E\) (mmol m\(^{-2}\) s\(^{-1}\)) vs. \(g_s\) (mol m\(^{-2}\) s\(^{-1}\)) \((R^2=0.1, p>0.05, E=\left(4.4130\right) g_s + 0.6247)\). (f) \(A\) (\(\mu\)mol m\(^{-2}\) s\(^{-1}\)) vs. \(g_s\) (mol m\(^{-2}\) s\(^{-1}\)) \((R^2=0.6, p<0.05, A=\left(64.532\right) g_s + 2.458)\).
Figure 3.10. The relationship between leaf hydraulic and gas exchange parameters for each species sampled with leaf anatomical variables measured in Chapter 2. The plots display the maximum recorded value for each species for the hydraulic and gas exchange variables ($E$, $A$ and $g_s$) against the anatomical variables ($D_s$ and $D_v$) as presented in Chapter 2. The figure also shows linear regression trends in the plots. Coloured dots represent the group that the sampled species belongs to. (a) $A$ (μmol m$^{-2}$ s$^{-1}$) vs. $D_s$ (stoma per mm$^2$) ($R^2$=0.2, $p$>0.05, $A = (0.01336) \ D_s + 4.11476$). (b) $E$ (mmol m$^{-2}$ s$^{-1}$) vs. $D_s$ (stoma per mm$^2$) ($R^2$=0.42, $p$>0.05, $E = (0.00499) \ D_s + 0.23072$). (c) $g_s$ (mol m$^{-2}$ s$^{-1}$) vs. $D_s$ (stoma per mm$^2$) ($R^2$=0.2, $p$>0.05, $g_s = (-0.00003468) \ D_s + 0.05574$). (d) $A$ (μmol m$^{-2}$ s$^{-1}$) vs. $D_v$ (mm per mm$^2$) ($R^2$=0.36, $p$>0.05, $A = (0.9831) \ D_v + 2.3768$). (e) $E$ (mmol m$^{-2}$ s$^{-1}$) vs $D_v$ (mm per mm$^2$) ($R^2$=0.43, $p$=0.07, $E = (0.22463) \ D_v + 0.02189$). (f) $g_s$ (mol m$^{-2}$ s$^{-1}$) vs. $D_v$ (mm per mm$^2$) ($R^2$=0.2, $p$>0.05, $g_s = (0.004726) \ D_v + 0.037757$).
Discussion

This study aimed to characterize some physiological and hydraulic variables of leaves from taxa that evolved under different atmospheric [CO₂] throughout geological time. The evolution of anatomical traits, mainly complex venation and higher stomatal densities, especially in angiosperms, that facilitated functional improvement in plant leaves (Franks & Beerling 2009a; Franks et al. 2013). Higher $D_s$ was accompanied by a reduction in $S_s$, and this increased $g_s$ (see Assouline & Or 2013), leading to increased carbon assimilation capacity. The rise in $g_s$ means the leaf would require an increase in water supply to keep up with the increase in transpiration rate brought about by increase in $g_s$.

The results showed that *P.caerulea* and *D.winteri* had greater and more responsive hydraulic capacities to light (Fig. 3.2). *P.caerulea* is a species endemic to high productivity tropical forests, and tropical species, especially trees, have been shown to have high hydraulic capacity (Sack & Frole 2006). *P.caerulea* also exhibited the higher $D_s$ and $D_v$ than other species, and several studies have shown high productivity tropical species exhibiting more complex anatomical characters to match their high hydraulic capacity (Sack et al. 2005; Sack & Frole 2006). The response of $E$ in *P.caerulea*, was higher under high temperatures than at high [CO₂] (Fig. 3.3), and the response of $E$ to increased temperature was quicker than the response to increased atmospheric [CO₂]. Under high atmospheric [CO₂] stomata close, reducing the evaporative demand on the leaf; however, increased [CO₂] increases $A$, and $A$ has been shown to correlate with higher hydraulic conductivity (Brodribb et al. 2007; Scoffoni et al. 2016), and this is manifested by the rise in $E$. A rise in temperature (depending on the magnitude) can cause an increase in photosynthetic rate (Farquhar et al. 1980; Kobza & Edwards 1987), mainly due to optimal enzymatic activity, and this can eventually result in increased hydraulic activity. Increased hydraulic conductance has been shown to increase with temperature (Sellin &
Kupper 2007), however higher temperatures have also been observed to cause a
decrease in $D_s$ (Beerling & Chaloner 1993) which would mean reduced transpiration
and thus reduced hydraulic demand. Beerling & Chaloner (1993) only focused on
growth temperatures, however, and on more mild temperature variation than used in
this experiment. Evaporative cooling can be one mechanism to explain the
significant rise observed in $E$ (Fig. 3.3), as a substantive rise in temperature can
cause over-heating and damage the leaf. Evaporative cooling, which is an increase
in transpiration rate aimed at getting the leaf to lose heat energy, can protect the leaf
from this damage, and accompanied by higher leaf conductance of water can protect
the xylem from embolism that might be brought upon by higher transpiration rate
(Schymanski et al. 2013). On the whole, the author of this chapter is not aware of
many studies that measured leaf hydraulic conductivity in response to a change in
temperature. Hu et al. (2014) investigated the effect of varying growth temperatures
on tobacco leaf characteristics, and observed leaves growing under higher
temperatures exhibiting higher $K_{leaf}$ as well as higher $D_c$ and $D_s$ than leaves growing
under lower temperatures. Changing temperatures over geological time are bound to
have played a role in shaping plant evolution, and coupled with the effect of
temperature on membrane fluidity and permeability (Cochard et al. 2000), plus the
reported observation from Hu et al. (2014) that growing temperature can affect leaf
anatomy, would mean leaves with different anatomical characteristics would respond
differently to temperature. This chapter had a minor aim of investigating the reported
response in Fig 3.3 in different species, but equipment failure and time constraints
thought otherwise.

$D.winteri$ belongs to a family of woody, vessel-less trees, and this trait has been
thought to impact their hydraulic capacity as they have been assumed to be an
ancestral, angiosperm relic, but this idea has been challenged (Feild et al. 2000).
$D.winteri$ exhibited higher $K_{leaf}$ values than other angiosperms, except for $P.caerulea$. 

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This does support the growing notion that Winteraceae is not a static, unchanging group and that it might have adapted throughout its history to overcome its anatomical restrictions, as also evidence by its higher $A$ and $g_s$ values (Fig. 3.5 and 3.6).

There was no significant trend in correlation between atmospheric [CO$_2$] and functional parameters measured ($E$, $A$, $g_s$). However, there was a general trend of angiosperms leaning towards displaying higher hydraulic and gas exchange values coupled with higher vein and stomatal densities (Fig. 3.8 and 3.10). Angiosperms diversified under decreasing atmospheric [CO$_2$] in the mid-Cretaceous, (Wing & Boucher 1998; Boyce et al. 2009; Brodribb & Feild 2010; Boyce & Zwieniecki 2012; Brodribb et al. 2013), and so the impact of atmospheric [CO$_2$] change on plant evolution cannot be discounted. Angiosperms have been shown to have higher $K_{leaf}$ than other taxa (Brodribb & Holbrook 2004; Brodribb et al. 2005). Most recently, a study by Scoffoni et al. (2016) found a correlation between $A$ and $K_{leaf}$ in different species of the genus *Viburnum*, finding that this correlation diverged depending on the climate the species is accustomed to, with this influencing the venation architecture of the leaf. This highlights the importance of leaf anatomical features in the ecological success of a species, as anatomical features, specifically vein and stomata, develop in tandem to achieve a higher photosynthetic rate, while keeping up high hydraulic supply to the leaf.

The response of $A$ and $g_s$ of the sampled species to a step increase in light followed by a decrease in light was conducted to give an insight into the variation in gas exchange capacity and stomatal behaviour between the species. Significant differences between species were rare, and this was attributed to limited replication ($n=2-3$), as well as timing of the experiment, which was conducted in the summer of 2015, with this time expected to be optimum for species. Instead, some species (*V.vinifera*, *G.biloba*) did not look at their optimum and were either losing leaves or
have just initiated leaves again. Having said this, some species had higher $A$ than others (like $P.\text{caerulea}$, $L.\text{nobilis}$ and $D.\text{winteri}$), and those were angiosperms that evolved under lower atmospheric $[\text{CO}_2]$, agreeing with observations in the literature (Franks & Beerling 2009a; Boyce & Zwieniecki 2012) that declining atmospheric $[\text{CO}_2]$ conferred a selection pressure for higher photosynthetic capacity. $G.\text{biloba}$ was an outlier, however, having high $g_s$ and $A$ values despite being a gymnosperm with an old crown group age. $Ginkgos$ are thought to have emerged ecologically in riparian, disturbed environments (Zhou 2009) which is an environment that requires adaptability. Still, some studies have reported lower gas exchange values for $Ginkgo$ (McAusland et al. 2016; Elliott-Kingston et al. 2016), so the values displayed here by $Ginkgo$ might not be representative of its actual capacity as a species.

Upon further observation, there is a difference in how species $g_s$ behaves in response to the light regime and how the $g_s$ response differs with $A$ response. Species that had higher $A$ also had a quicker decline in $g_s$ after light was decreased after the high light period. This is most probably a water loss restricting mechanism (Lawson & Blatt 2014), allowing the stomata to respond rapidly and in synchrony with mesophyll demand for $\text{CO}_2$, which would decrease with decreasing light and thus sustaining high $g_s$ would not be beneficial to the leaf and would only incur further water loss. A quick closing response time of stomata has been considered an important trait that correlates with higher $\text{WUE}$ (Pou et al. 2008; Lawson & Blatt 2014; McAusland et al. 2016), and to see it exhibited in species that 1) achieve higher $A$ values and 2) have a higher $D_s$ and $D_v$ values (see chapter 2) than species that lack this closing response adds to the possible contributions that these anatomical characters ($D_v$, $D_s$) provide to the plant leaf. Drake et al. (2013) already provided a report that confirms this (see Introduction). Furthermore, Dow et al. (2014) used different $\text{Arabidopsis thaliana}$ genotypes that produces leaves with different $D_s$, and they reported results that show that as $D_s$ increases within the
genotypes the quicker the stomatal closing response in that genotype. It needs to be said, however, that Dow et al. (2014) use change in [CO$_2$] to stimulate stomatal response as opposed to the use of light in the present study. Also, some reports have contradicted this conclusion regarding higher $D_s$ correlation with stomatal speed of response (Monda et al. 2016; Elliott-Kingston et al. 2016).

In an evolutionary context, the slight response of $O.regalis$ and also $N.nagi$ can be explained by the difference in passive vs. active stomatal control. Basal plant lineages, like ferns, do not exhibit significant changes in stomatal behaviour in response to environmental or chemical (ABA) stimulus (Brodribb & McAdam 2011) and this can be shown by the minimal change in $g_s$ for $O.regalis$ in response to light (Fig. 3.4). McAdam & Brodribb (2014) also found a similar tendency in a conifer ($Metasequoia glyptostroboides$) that can also explain the lack of response from $N.nagi$, another conifer. Angiosperms have an active stomatal control that depends on osmotic and ionic changes that propel changes in stomatal aperture, with this being an adaptation at maximizing CO$_2$ uptake under drought stress (Brodribb & McAdam 2011), another indication of angiosperm innovation and evolution compared to their plant ancestors.

Conclusion

The coordinated evolution of anatomical characters ($D_s$, $D_v$ and $S_s$) is thought to be a driver of the improvement in hydraulic capacities and stomatal responses in the species that evolved under low atmospheric [CO$_2$], specifically angiosperms. Data presented here does support the superiority of angiosperms of in displaying higher gas exchange and hydraulic values. However, differences between species, whether in crown group age and atmospheric [CO$_2$] were rare and only taxonomic differences were noticed.
Chapter 4: Simultaneous diurnal responses of leaf gas exchange and hydraulics in species with different crown ages

Introduction

The current rise in CO\textsubscript{2} concentration ([CO\textsubscript{2}]) in the atmosphere presents an opportunity to observe the effects of changing atmospheric [CO\textsubscript{2}] on plant and ecosystem function (de Boer et al. 2011). Stomata respond to changes in the environment, especially to changes in atmospheric [CO\textsubscript{2}] (Cowan & Farquhar 1977; Mott 1988; Franks & Farquhar 2007), and hence changing atmospheric [CO\textsubscript{2}] will affect plant leaf’s gas exchange rates with the environment. Abrupt changes in $g_s$ can destabilize internal water potential and lead to xylem cavitation (Buckley 2005; Locke & Ort 2014); On the other hand, an increase in $g_s$ increases the transpiration rate, leading to an increase in hydraulic demand on the plant leaf which leads to the cavitation of the xylem if supply and demand are not in coordination (Medrano et al. 2002; Tixier et al. 2013; Schymanski et al. 2013). The link of stomatal performance to hydraulic signals in the leaf (Jarman 1974; Whitehead 1998; Buckley 2005; Brodribb & Jordan 2008; Brodribb & Jordan 2011) is key understanding the bottleneck stomata represent to water movement in the soil-plant-atmosphere continuum (Meinzer 2002). Individual stomates respond to nearby changes in leaf turgor and water potential (Buckley et al. 2003; Buckley 2005 for an extensive review), and so stomata both respond to and subsequently influence hydraulic supply and demand, as changes in $g_s$ influence leaf hydraulic demand (Addington et al. 2004; Locke & Ort 2014; Ocheltree et al. 2014), while changes in leaf water status also elicit a change in stomatal behaviour (Raschke 1970; Maier-Maercker 1998; Medrano et al. 2002; Grassi & Magnani 2005; Bunce 2006). The correlation between stomatal behaviour and hydraulics has been confirmed in a number of studies that showed a positive relationship between $g_s$, and subsequently
carbon assimilation ($A$), with leaf hydraulic conductance, $K_{\text{leaf}}$ (Hubbard et al. 2001; Meinzer 2002; Brodribb & Holbrook 2004; Nardini & Salleo 2005; Hernandez-Santana et al. 2016). Atmospheric [CO$_2$] fluctuations over geological timescales have affected stomatal and vein characteristics (Royer 2001; Franks & Beerling 2009a; Boyce et al. 2009; Ward & Gerhart 2010; Brodribb & Feild 2010). The past 120 million years have seen a gradual decrease in atmospheric [CO$_2$], coinciding with the emergence of angiosperms (which dominate terrestrial ecosystems), with angiosperms generally exhibiting higher $D_s$ (and thus higher $g_s$) (Brodribb et al. 2009) and $D_v$ (Brodribb & Feild 2010). High $D_v$ enable the angiosperm leaf to supply the high hydraulic demand required by higher $g_s$, and Chapter 2 has already illustrated that higher vein densities correlate with higher $D_s$ (and thus $g_s$).

The interlinking between stomatal characters and hydraulic signals through evolutionary history, as suggested by the increase in $D_v$ with $D_s$ (see Chapter 2), indicate that there is a synchrony in hydraulic and gas exchange parameters response to rapid changes in the environment. The evolution of anatomical characters in angiosperm leaves clarifies this trend. Observations reported by de Boer et al. (2012) and Zwieniecki & Boyce (2014), outlined in previous chapters, show that leaf venation and stomata have developed in concert to achieve optimal control over water exchange between the leaf and atmosphere, to effectively supply stomatal demand for water under high transpirational conditions, and to attune the stomata’s ability to detect changes in leaf water status more quickly. Most of these improvements are achieved by manipulating leaf architecture. The functional relationship between stomatal responses (and subsequently carbon assimilation) and leaf hydraulic capacity, however, has not been examined, especially when considering leaf anatomical differences. Investigating the effect of varying leaf anatomical differences on the dynamic response of leaf hydraulics and gas exchange can give insight into limiting factors among the leaf’s ecophysiological
response parameters. Consequently, illustrating the temporal relationship between the hydraulic and gas exchange parameters can further highlight the impact of changing environmental conditions on plant leaf response.

Stomata are known to have different response speeds in different species and can be, for example, much slower to open in response to light compared to the leaf’s biochemistry (Buckley et al. 2003; Franks & Farquhar 2007; Drake et al. 2013; Dow et al. 2014; Lawson & Blatt 2014; McAusland et al. 2016). This lag in the stomatal opening response means the leaf fails to capitalize fully on the light increase as its stomata responds slowly while $A$ increases in response to light. On the other hand, the stomatal closing response represents a bigger disadvantage, as the stomata have been shown to also lag behind the leaf’s biochemistry when light levels drop, causing excess water loss without any benefit in photosynthetic rate, hence decreasing leaf’s $WUE$ (Lawson & Blatt 2014; McAusland et al. 2016). This indicates that the leaf’s ability to coordinate the response of its physiological parameters to environmental change is a key adaptation to environmental change. Since plant evolution has been greatly influenced by changing atmospheric $[CO_2]$, it is intriguing to explore variation in coordinated response of hydraulic and gas exchange parameters in different species and lineages that evolved under different atmospheric $[CO_2]$. Chapter 3 has already highlighted some differences in stomatal responses between different species, as well as highlighting differences in hydraulic capacity. Previous studies have also highlighted differences in $A$ and $g_s$ in species instigated by changes in atmospheric $[CO_2]$ over geological time (Brodribb et al. 2005; Boyce et al. 2009; Franks & Beerling 2009a; McElwain et al. 2016), as well as differences in hydraulic capacity (Scoffoni et al. 2016).

The aim of this chapter is to further investigate the effect of the evolution of plant leaf anatomical characteristics on leaf function. With plants playing a key role in ecosystem function and geo-processes (Field et al. 1995; Hetherington & Woodward
changing atmospheric [CO₂] over geological time, which drove these anatomical changes, would have resulted in evolutionary adaptations which have changed the ecological structure of ecosystems, as well as influencing geo-hydrological processes (Kasting & Siefert 2003; Beerling & Berner 2005; Feild et al. 2009). It is hypothesized that species that have higher $D_s$ and $g_s$, as well as higher $D_v$, would have higher and more responsive and synchronized hydraulic and gas exchange capacities compared to species that have lower $D_s$ and $g_s$. It is projected that atmospheric [CO₂] will keep increasing throughout this century, and thus understanding how past atmospheric [CO₂] change affected plant response can be of importance to understanding the effect of future atmospheric [CO₂] change on plant and ecosystem function, especially as effect of atmospheric [CO₂] on plant anatomy has been shown and explored in the literature as well as this thesis earlier.

Combining the hydraulic conductance setup illustrated in Chapter 3 with an Infra-Red Gas Analyser chamber allows for the measurement of gas exchange and hydraulic parameters simultaneously. This provided a chance to explore the synchrony in response between hydraulic and gas exchange leaf variables in response to environmental stimulus. Since a step-change experiment was conducted earlier, exposing the leaf to a diurnal light regime was thought to provide an interesting and fresh angle to explore leaf ecophysiological responses in species from different taxa, ecological habitat and different anatomical characters. Using a diurnal regime would subject the leaf to a more natural fluctuating environment and would offer a way to link the prevalence of leaf characteristics, whether anatomical ($D_s$ or $D_v$) or physiological ($A$, $g_s$), to the species’ ecology, taxonomic relationships or to the effect of past environmental change (atmospheric [CO₂]) on its function. All these factors (ecology, taxonomy, past [CO₂] change) shape the leaf’s ecological suitability and
efficient leaf function, and using a diurnal regime, with frequent light variation, would highlight differences between species in leaf function along those lines.

Material and Methods

Plant Material

Species that were sampled for this study were picked based on crown group age and taxa as outlined in Chapter 2. They were: *Drimys winteri; Nageia nagi; Passiflora caerulea; Vitis vinifera*. *Phaseolus vulgaris*, a modern crop species, was also sampled. Only 5 species were used due to time constraints, initial uncertainty about methodology, and problems with species losing leaves and health (*G. biloba* and *L. nobilis*). Still, the 5 species encompassed different taxonomic groups and different crown group ages and ecological habitats, allowing for ample comparison points. Three leaves were sampled off each species.

Setup to simultaneously measure leaf gas exchange and hydraulic flow

A system like that described in Chapter 3 was used here to combine measurement of leaf hydraulic flow and gas exchange parameters. The gas exchange chamber of the Infra-Red Gas Analyser (IRGA) was used to control the environment surrounding the leaf, similar to the way the Campbell chamber was used in Chapter 3, and enabling measurement of gas exchange parameters (stomatal conductance, $g_s$; carbon assimilation, $A$; Transpiration, $e$) while simultaneously water flow into the leaf ($E$) was determine from changes in water loss measured from water uptake from a beaker placed on a balance. Before placing the leaf in the chamber, a branch is cut from the individual plant, and then a leaf off the branch is cut under water and attached, also under water, to tubing that contains a water column drawn from a cylinder placed on a sensitive balance (Sartorius CP | GemPlus Series, Sartorius, Goettingen, Germany). To make sure the leaf-tubing connection was sealed, water proof oil grease (Dow Corning High Vacuum Grease, USA) was applied around the
petiole-tube connection to prevent air entering the tubing and losing the water column. The balance was connected to a computer used to record balance readings every 30 seconds. Wet tissue was placed inside the balance chamber to increase humidity and reduce evaporation from the cylinder. This controlled for any change in the balance reading from evaporation; and thus, all variation in balance readings will be assumed to have been instigated by leaf conductance.

The leaf was placed inside the gas exchange chamber of the IRGA (LCPro SD Portable Photosynthesis System, ADC Bioscientific, Hoddesdon, Hertfordshire, UK). The gas exchange cuvette has a clear glass top, to allow for the passage of light from an LED light source (Iso Light 400, Technologica, UK) placed above the chamber. The diurnal response was elicited by programming a light regime into a computer software (TLC application, Technologica, Frating, UK). The light regime was a sinusoidal wave pattern that replicates field-like light conditions based on light measurements from the field, with Fig. 4.1 below showing the light values throughout the wave. \([\text{CO}_2]\) inside the chamber was set at 400 ppm. \([\text{H}_2\text{O}]_{\text{ref}}\) generally varied between 18 and 25 mmol m\(^{-2}\) s\(^{-1}\). Since the IRGA chamber can only cover a limited surface area of the leaf, the area of the leaf outside the IRGA chamber was covered with oil grease (Dow Corning High Vacuum Grease, USA) to prevent any exchange through the leaf surface that might affect the leaf’s hydraulic activity, and only the leaf area inside the chamber was considered to be “active” in terms of gas exchange, with measurements controlled for the area inside the chamber. After the leaf is put into the chamber the light was switched on starting at 20-40 μmol m\(^{-2}\) s\(^{-1}\) and the light regime was run for about 10 hours, with the plant experiencing the light variation highlighted in Fig. 4.1.
Statistical Analysis

Figures and data representation was done in R (R Core Team (2014), R Foundation for Statistical Computing, URL http://www.R-project.org). To statistically analyse the data, the maximum observed value of each parameter measured, as well as the area under the response curve (auc) of each parameter, was calculated for each sample and compared statistically between the species. Normality was checked by plotting a generalized linear model (GLM) and inspecting residual plot. A linear mixed effects (LME) model was applied to the data, with the model taking the following form:

$$F_s = \mu + S_s + I_s + \varepsilon_{ls}$$

Where $F_s$ is the variable considered ($A$, $E$, $g_{st}$, $e$ or $auc$), $S_s$ is the effect of different Species, $I_s$ is the random effect of the individual, and $\varepsilon_{ls}$ represents the residuals. Analysis of Variance test was then applied to the model to deduct statistical differences between species. Regression analysis was carried in R using linear
modelling (lm). The model was formulated to predict the significance of a linear relationship between the two variables in the plot in the form:

\[ y = mx + c \]

Where \( y \) (predicted) and \( x \) (predictor) are the y-axis and x-axis variables respectively, and \( m \) is the slope of the relationship, and \( c \) is the y-axis intercept. \( m \) represents the direction of the relationship (negative or positive). \( R^2 \) values and \( p \) values were obtained from the analysis output and used to interpret significance of relationship.

Results

The response of the variables (\( A, g_s, e \) and \( E \)) measured to diurnal light change. \( E \) and \( e \) show similar responses to each other in response to light for all the species, as \( E \) increases with \( e \) and vice versa (Fig. 4.2). \( P.vulgaris \) achieved the highest values of \( E \) (5.5 mmol m\(^{-2}\) s\(^{-1}\)) during the period of high light between 4 and 5 hours while \( e \) was highest in \( P.caerulea \) (2.3 mmol m\(^{-2}\) s\(^{-1}\)), also observed during the same period. \( g_s \) and \( A \) also show similar responses, as \( A \) increases as \( g_s \) increases (Fig. 4.3). However, \( g_s \), in all species, still showed a “lag” in response to light change behind \( A \), similar to what was observed in the step change responses in Chapter 3 (Fig. 3.4). For example, for \( D.winteri \), when light intensity increased at around 2.5 hours a quick \( A \) response was elicited, reaching a stable high of 3 \( \mu \)mol m\(^{-2}\) s\(^{-1}\), while \( g_s \) kept increasing with time and did not stabilise. \( P.caerulea \) on the other hand, had an \( A \) and \( g_s \) responses that matched each other more closely compared to the other species, but \( g_s \) still showed what looks like a linear increase during the first 4-5 hours when \( A \) was a more responsive to the occasional light intensity drop during that 4-5 hour period, so there was still some mismatch. There was also a similarity in the responses of \( A \) and \( E \) within all species, with an increase in one is matched by an increase in the other, and both responses showing a solid match throughout the
diurnal regime. $g_s$ and $e$ also show a matched response, but that is mainly because $g_s$ itself is estimated by the IRGA through estimating the $e$ rate.

Figure 4.2. The response of $E$ (mmol m$^{-2}$ s$^{-1}$) and $e$ (mmol m$^{-2}$ s$^{-1}$) to diurnal light variability for the 5 species sampled. The leaf is cut and connected to tubing under water, and then placed inside the chamber of an Infra-Red Gas Analyser. The leaf draws water through the tubing from a beaker placed on a balance. CO$_2$ concentration inside the chamber was kept at 400 µmol m$^{-2}$ s$^{-1}$. $E$ was estimated via change in water loss from a balance and normalized for the stomatal ratio and leaf area, while $e$ was measured by the Infra Red Gas Analyser. The unit for both $E$ and $e$ is mmol m$^{-2}$ s$^{-1}$, hence the "E" label on the y-axis is not supposed to represent $E$ only. The grey shading represents the error margins (standard error). (n=3)
The maximum $E$ value achieved during the diurnal light period for $P. vulgaris$, $P. caerulea$ and $V. vinifera$ was significantly higher ($p<0.05$) than the values achieved by $D. winteri$ and $N. nagi$ (Fig. 4.4a). $P. vulgaris$ had the highest average maximum $E$ (6.1 mmol m$^{-2}$ s$^{-1}$) while $N. nagi$ had the lowest (1.03 mmol m$^{-2}$ s$^{-1}$). For $e$, a similar pattern was observed (Fig. 4.4b); however, $P. caerulea$ achieved the highest maximum $e$ value (3.17 mmol m$^{-2}$ s$^{-1}$), while $N. nagi$ was significantly lower than all the other species ($p<0.05$). For $g_s$ (Fig. 4.4c), $P. vulgaris$ had significantly higher maximum than any of the other species (1.73 µmol m$^{-2}$ s$^{-1}$; $p<0.05$). Similarly,
*P. vulgaris* had significantly higher maximum value for $A$ (15 µmol m$^{-2}$ s$^{-1}$; p<0.05) than all the other species (Fig. 4.4d). Almost all the samples realised their maximum values during the period of high light between 2.5 and 5 hours into the diurnal regime, usually towards the end of that period as the plant leaf response stabilizes.

The area under the curve (*auc*) was calculated from the response curves shown in Fig 4.2 and 4.3. As opposed to showing the maximum values achieved by each species, the *auc* values (Fig. 4.5) would highlight the ability of each species to maintain higher gas exchange or hydraulic capacity levels over the whole period of the light regime. For *E* (Fig. 4.5a), *P. vulgaris, P. caerulea* and *V. vinifera* had significantly higher *auc* than both *D. winteri* and *N. nagi* (p<0.05). For *e* (Fig. 4.5b), *N. nagi* had significantly lower *auc* than the rest of the species (p<0.05), while *P. caerulea* had the highest *auc* for *e* (15.02 mmol m$^{-2}$). The *auc* for *g*$s$ (Fig. 4.5c) was highest for *P. vulgaris* followed by *P. caerulea* (3.83 and 2.87 µmol m$^{-2}$ respectively), with those species being significantly higher than the other species (p<0.05). The *auc* for $A$ (Fig. 4.5d) had a similar pattern to the *auc* for $g$s, with *P. vulgaris* and *P. caerulea* having the significantly higher *auc* (p<0.05). Basically, for each parameter, the species that achieved the higher maximum values were the ones showing higher *auc* values, and *vice versa*, and so the two sets of data follow similar patterns between the species.
Figure 4.4. Variation in the maximum observed value between the species sampled for the 4 variables measured: (a) $E$ (mmol m$^{-2}$ s$^{-1}$); (b) $e$ (mmol m$^{-2}$ s$^{-1}$); (c) $g_s$ (mol m$^{-2}$ s$^{-1}$); (d) $A$ ($\mu$mol s$^{-1}$). The Fig. also illustrates statistical difference between the species, with the species sharing the same letter above their panel having NO significant statistical difference between them. The highest value for each sample was picked for each variable, with these values generally observed during the high light period of the light regime between 2.5 and 5 hours. Data was collected by excising a leaf under water and attaching it to tubing to draw water from a beaker on a balance, with the leaf then placed in an Infra Red Gas Analyser (IRGA) chamber. This combination allows for the measurement of gas exchange parameters ($A$, $g_s$, $e$) through the IRGA chamber and hydraulics ($E$) through water loss difference from the balance. (n=3)
Figure 4.5. Variation in the area under the curve ($\text{auc}$) between the species sampled for the 4 variables measured: (a) $\text{auc}_E$ (mmol m$^{-2}$); (b) $\text{auc}_e$ (mmol m$^{-2}$); (c) $\text{auc}_g_s$ (mol m$^{-2}$); (d) $\text{auc}_A$ ($\mu$mol m$^{-2}$). The $\text{auc}$ was calculated from each sample response curves for each variable, of which the mean responses were shown in Fig. 4.2 and 4.3. The $\text{auc}$ was calculated in order to give a holistic view into each species' capacity over the diurnal period, as opposed to individual points showing maximums like in Fig. 3.4. The Fig. illustrates statistical difference between the species, with the species sharing the same letter above their panel having NO significant statistical difference between them. Data was collected by excising a leaf under water and attaching it to tubing to draw water from a beaker on a balance, with the leaf then placed in an Infra Red Gas Analyser (IRGA) chamber. This combination allows for the measurement of gas exchange parameters ($A$, $g_s$, $e$) through the IRGA chamber and hydraulics ($E$) through water loss difference from the balance. (n=3)
There was variation between the species in their dynamic response to diurnal light variation in each of the 4 variables. Increasing light intensity elicited an increase in all variables, and *vice versa*, but different species had different speeds of response to light increase and different relationships within variables (Fig. 4.6). Such variation between species can be observed in the response of A to light change (Fig. 4.6A). When light intensity increased quickly around 1.5 hrs into the light regime, A increased to maximum levels in *P. vulgaris* and *D. winteri*, while *V. vinifera* and *P. caerulea* also increase but to a fraction of their maximum. A for *P. vulgaris* reached its maximum during this period much earlier than the A response of *D. winteri*, which seems to lag behind a little, with the A response of *V. vinifera* and *P. caerulea* similar to that of *D. winteri*, also increasing at lower rate than *P. vulgaris* to light increase. During the same period of light increase (after 1.5 hrs), *P. vulgaris* showed a sharper slope of increase in $g_s$ compared to the other species (Fig. 4.6C). *V. vinifera* did not show a significant increase in $g_s$ in response to light increase during this period, while *D. winteri* showed an increase in $g_s$ but at a slower rate compared to *P. vulgaris*, with this slow increase in $g_s$ for *D. winteri* observed again when light increases again after 2.5 hrs. *V. vinifera*, however, did have a higher increase in $g_s$ when light increased again after 2.5 hrs. *P. caerulea* had an increase in $g_s$ that continued throughout the first few hours of the light regime, with the increase in $g_s$ becoming slower when light levels decrease, until *P. caerulea* reached its maximum in both A and $g_s$. *P. vulgaris*, over the length of the light regime, displayed quicker $g_s$ response to light change, with $g_s$ values increasing and decreasing sharply with light. *D. winteri* did not display a similar magnitude of change in $g_s$, with $g_s$ in *D. winteri* remaining within 50% (0.5) or more of the maximum and changing with that limit in response to light change. The response of $E$ throughout the diurnal period was matched more closely to the response of A than $g_s$ for each species (Fig. 4.6B), while e followed $g_s$ (Fig. 4.6D). *N. nagi*, alone among the species, showed very little interaction with light variations.
level change across variables, with the only noticeable activity is an increase in $g_s$ in the first 2 hrs of the regime and $g_s$ then stays the same through the regime, with $E$ and $e$ also following the same pattern in *N.nagi*.

**Figure 4.6**, Diurnal responses of (a) carbon assimilation ($A$), (b) water flow into the leaf ($E$), (c) stomatal conductance ($g_s$) and (d) transpiration ($e$) in response to a diurnal light regime for the 5 species sampled normalized between 0 and 1. This is to eliminate the disparity in values between species and to highlight the variation in the dynamic responses between the species to light change. Data was collected by excising a leaf under water and attaching it to tubing to draw water from a beaker on a balance, with the leaf then placed in an Infra Red Gas Analyser (IRGA). This combination allows for the measurement of gas exchange parameters ($A$, $g_s$, $e$) through the IRGA and hydraulics ($E$) through water loss difference from the balance. The species sampled are shown in the legends accompanied by the crown age of each species. (n=3)
Figure 4.7, The relationship between leaf hydraulic and gas exchange parameters for each species sampled with each other and with atmospheric [CO$_2$]. The plots display the maximum recorded value for each species for the respective variable ($E$, $A$ and $g_s$) against atmospheric [CO$_2$] at the time of taxa divergence (Table 2.1), as well as linear regression trends. Coloured dots represent the species sampled. 

(a) $E$ (mmol m$^{-2}$ s$^{-1}$) vs. atmospheric [CO$_2$] (ppm) ($R^2=0.5$, $p>0.05$, $E = (-0.02561) \text{CO}_2 + 9.49350$). 

(b) $A$ ($\mu$mol m$^{-2}$ s$^{-1}$) vs. atmospheric [CO$_2$] (ppm) ($R^2=0.65$, $p=0.09$, $A = (-0.07972) \text{CO}_2 + 25.57543$). 

(c) $g_s$ (mol m$^{-2}$ s$^{-1}$) vs. atmospheric [CO$_2$] (ppm) ($R^2=0.34$, $p>0.05$, $g_s = (-0.006671) \text{CO}_2 + 2.205810$). 

(d) $E$ (mmol m$^{-2}$ s$^{-1}$) vs. $A$ ($\mu$mol m$^{-2}$ s$^{-1}$) ($R^2=0.84$, $p<0.05$, $E = (0.3379) A + 1.1584$). 

(e) $E$ (mmol m$^{-2}$ s$^{-1}$) vs. $g_s$ (mol m$^{-2}$ s$^{-1}$) ($R^2=0.71$, $p=0.07$, $E = (2.6892) g_s + 1.7816$). 

(f) $A$ ($\mu$mol m$^{-2}$ s$^{-1}$) vs. $g_s$ (mol m$^{-2}$ s$^{-1}$) ($R^2=0.9$, $p<0.05$, $A = (8.169) g_s + 1.705$). (n=3, error bars=standard error)
Regression analysis showed no significant correlation between the maximum values of each parameter measured and atmospheric [CO$_2$] at crown group age (Fig. 4.7(a)-(c)). On the other hand, there was a significant correlation between the maximum values of the functional parameters measured (Fig. 4.7(d)-(f), p<0.05), with R$^2$ values as high 0.9, strengthening the hypothesis that leaf hydraulics and gas exchange are linked.

**Discussion**

This chapter concentrated on examining the responses of hydraulic and gas exchange parameters in response to a dynamic light regime mimicking what would be observed under natural conditions in species with different crown group ages (and different leaf anatomical characteristics; see Chapter 2). Results from this chapter conform to the trend that species from taxa that diversified under lower atmospheric [CO$_2$], like *P. vulgaris*, had higher $A$ values than species that evolved under higher [CO$_2$] like *V. vinifera*. Species that evolved under low atmospheric [CO$_2$] also generally had higher $E$ and $g_s$ values as well (Fig. 4.4). *V. vinifera* showed higher $E$ values than it did in Chapter 3, probably because it was producing newer leaves at the time of this experiment compared to the *Kula* one in Chapter 3. *V. vinifera* are known for their highly conductive vessels (Sperry et al. 1987) and the $E$ observed here is expected. Chapter 3 already outlined the difference in photosynthetic and hydraulic capacity between several species of different crown ages, including ones used in this chapter, and the causes of these differences in capacity. These differences in ecophysiological and functional responses were linked to anatomical changes in the leaf over geological time, and this links to the variation in the dynamic response over the diurnal period explored in this chapter. Anatomical change over geological time has been linked to changing atmospheric [CO$_2$], highlighting the possible effect of environmental change in shaping the evolution of plant leaf performance and characteristics. Still, the highest values for these variables where
exhibited by *P. vulgaris* (which is a high productivity leguminous crops) and *P. caerulea* (a species from high productivity tropical forests), while the lowest were exhibited by *N. nagi*, which is a gymnosperm accustomed to low light environments (Biffin et al. 2012), so the species’ habitat and ecology does play a role in its performance.

A step-change response like the one used in Chapter 3 is the standard protocol to examine stomatal behaviour, but the diurnal regime experienced by the leaves here offers insight into how the leaf behaves to constant variation over a prolonged period, however it is more difficult to evaluate or quantify the closing and opening responses. Nonetheless, enough variation was observed between species in the dynamic responses of the parameters measured to gain an idea into ecophysiological responses of the species selected. *P. vulgaris*, a modern crop species, was selected for this study to provide a comparison to the older species selected throughout this thesis in terms of ecophysiological capacity. Indeed, *P. vulgaris* had a more responsive \( g_s \) to changes in light over the diurnal period, with \( g_s \) increasing quickly when light increases and \( g_s \) also decreasing quickly when light levels decrease. This quick response by the stomata can explain the sharp A response of *P. vulgaris*, with A in *P. vulgaris* not “lagging” when light increases like the other species when light levels rise around 1.5 hrs into the regime. *P. vulgaris*, across the variables measured, generally had a synchronized response, with light causing a decrease in A with \( g_s \) and \( E \) decreasing with A. A quick decrease in \( g_s \) in response to low light means that *P. vulgaris* is more equipped to limit excess water losses, as a delayed \( g_s \) decrease permits more \( H_2O \) leaving the leaf with no \( CO_2 \) benefit to the plant as A levels are already diminishing due to low light. At the other end, a quick increase in \( g_s \) in response to light level increase means *P. vulgaris* could make the most of the light increase by increasing \( CO_2 \) uptake.
Older species, like *D. winteri*, *P. caerulea* and *V. vinifera* had more variation in response between them, with no specific dynamic pattern distinguishing between them. The constant increase, irrespective of light levels, in $g_s$ during the first 5 hrs of the light regime in *P. caerulea* suggests that *P. caerulea* aims to maximize photosynthetic gain over water loss. Interestingly, *P. caerulea* had the highest $e$ among all the species sampled, including *P. vulgaris*, indicating that *P. caerulea* might have less of a control on water exit from the stomata compared to *P. vulgaris* or even *D. winteri* or *V. vinifera*. *P. caerulea*, as mentioned, is endemic to tropical forests (Xi et al. 2012), which are wet and water saturated, and thus *P. caerulea* might not prioritise water retentions, with the high $E$ of *P. caerulea* might be a mechanism by which the leaf keeps the water supply high in order to match the transpirational demand and keep the stomata opened. *P. caerulea* also had more of a match between the $g_s$ and $A$ response compared to *D. winteri*, signifying that the *P. caerulea* leaf is indeed more built to maximize photosynthetic gain compared to *D. winteri*. Compared to *P. vulgaris*, *P. caerulea* seems to have sharp $g_s$ responses to light increase, stimulating $A$ increase, while decreasing light levels elicit less of a response from $g_s$. On the other hand, compared to *D. winteri*, *P. caerulea* also had more of an adventurous opening response, with $g_s$ increasing with light at a quicker rate compared to the $g_s$ response of *D. winteri*, but *D. winteri* had more tangible closing responses. Thus, *P. caerulea* seems to possess the $A$ maximizing tendencies of *P. vulgaris*, while *D. winteri* seems to exhibit the water-loss limiting tendencies of *P. vulgaris*, as $g_s$ decreases in *D. winteri* in response to light level decrease. However, findings by Feild et al. (1998) have shown that the waxy stomatal plugs characteristic to *D. winteri* function to increase transpiration rate, with the authors of the study concluding that the plugs are mechanisms to minimize the effect of water films on leaves preventing $\text{CO}_2$ entry, thus keeping assimilation rates high. However, these plugs have long been theorized to be water loss constricting mechanisms until those
findings (Feild et al. 2000). In any case, the results presented here adds to the
debate regarding the evolutionary history of Winteraceae.

*V. vinifera* also had a good match between the *A* and *g*ₚ response, except for
approximately the first 2.5 hrs of the regime, with *g*ₚ increasing sharply when light
increases at 2.5 hrs. *V. vinifera* had a higher hydraulic values (*E* and *auc_E*) than
*D. winteri*, despite *D. winteri* achieving higher *g*ₚ. However, *V. vinifera* had a less
limiting *g*ₚ response when light increased at 2.5 hrs. The higher hydraulic values of
*V. vinifera* might also be connected to the climates that *V. vinifera* grows in, hotter
Mediterranean climates (Lavee 2000), that would select for leaves that better control
their temperature through higher water flow into the leaf (Schymanski et al. 2013; Hu
et al. 2014), as well as *V. vinifera*’s specialised vessels mentioned earlier (Sperry et
al. 1987; Lovisolo & Schubert 1998). Generally, the response of *E* was more
synchronized with *A* than *g*ₚ for all species, with a clear example in *D. winteri*, with its
*g*ₚ response lagging when light increases at around 2.5 hrs (Fig 4.6C), while the *A*
and *E* response of *D. winteri* (Fig. 4.6A and B respectively) increase sharply together.
Even though transpirational demand can drive leaf hydraulic conductance, the
importance of water to the leaf’s biochemistry would mean that other factors in the
leaf also determine the leaf’s hydraulic behaviour, and higher photosynthetic rates do
require high water supply to drive electron transport.

Finally, the lack of activity of *N. nagi* is not particularly surprising, as previous
measurements from our lab show that *N. nagi* has low photosynthetic and stomatal
response capacities, a result of their very primitive leaf anatomical characters, that
include low *Dₚ*, *Dᵥ* and high *Sₚ*. The negative *A* values of *N. nagi* are attributed to the
IRGA machine not picking the low assimilation levels usually exhibited by *N. nagi*.
The low light environment from which gymnosperms like *N. nagi* come from does not
encourage the development of higher gas exchange capacities. Also, these
environments are similar to those ferns occupy, and ferns have been shown to
exhibit the passive stomatal response that lacks the ability for dynamic change in response to stimulus (Brodribb & McAdam 2011; McAdam & Brodribb 2012; McAdam & Brodribb 2014). This was also mentioned in Chapter 3 and can explain the lack of hydraulic and gas exchange response from N. nagi in this experiment.

Conclusion

This study reported data that emphasised the relationship between leaf gas exchange and hydraulic parameters over a diurnal period. Variation in response between species to diurnal light change is attributed to their evolutionary history and ecological niche that allowed for the development of leaf anatomical characters that allow to leaf more flexibility in controlling their physiological responses. The variation in auc and maximum values (Fig. 4.4 & 4.5) do suggest that these different species exhibit different capacities over a diurnal period, and thus the evolution of those species would have influenced ecosystem function and productivity. The variation in the matching of responses to light change between the different parameters (gₛ with A, or A with E) means that the evolution of those species might also have contributed to changing ecosystem ecology as species acquired different ecophysiological capacities and ultimately different niches.
Chapter 5: Summary and future directions

Examining plant response to climatic factors could benefit our understanding of ecosystem response as well as helping in the exploitation of areas for improving crop performance. Over geological times, changes in atmospheric [CO₂] have influenced the evolution of plants (Ward & Kelly 2004; Leakey & Lau 2012), with this influence manifested in changes in leaf anatomy aimed at minimized the limitations imposed by declining atmospheric [CO₂] over the past 100 million years (Boyce et al. 2009; Brodribb & Feild 2010; Franks et al. 2013; McElwain et al. 2016). Probing the effect of past climatic changes on plant leaf physiology can act as indicator for the response of flora to future climatic changes, and can provide a glimpse into how different species and taxa evolved their different physiological capacities.

This thesis was devised to build on previous work done in the lab that aimed to understand the effect of atmospheric [CO₂] change over geological time in shaping anatomical variation between taxa. It was interesting to see how such anatomical characters influenced leaf function. Extant species, belonging to different taxa that diverged at different points through geologic history (crown group age was used), were picked to represent a gradient of atmospheric [CO₂] across geological time. The variation in anatomical characters was investigated previously, with some of this data presented in Chapter 2. Despite the lack of significant regressions (Fig. 2.3) presented in chapter 2, the anatomical data does follow findings in previous studies that showed changes in leaf anatomical characters can be a response to changing atmospheric [CO₂] over geological time. Increase in stomatal density ($D_s$) under low [CO₂] is also accompanied by decreasing $S_s$ (Franks & Beerling 2009b; Matthews and Lawson, Unpublished). The combination of high $D_s$/low $S_s$ is thought to contribute to increasing $g_s$ and subsequently gas exchange rates at the leaf surface (Konrad et al. 2008; Assouline & Or 2013). The increase in gas exchange capacity, including the increase in transpiration rates, means an increase in water demand by
the leaf, and the corresponding increase in $D_v$ means leaves that evolved under low atmospheric [CO$_2$] increased their hydraulic supply to match the demands of the higher $g_s$ required to respond to low atmospheric [CO$_2$].

Most of the experimental work in this thesis was dedicated to demonstrating the variation in the leaf’s ecophysiological and hydraulic capacities in species with different $D_s$ and $D_v$. Generally, higher hydraulic capacities were observed in species that evolved under lower atmospheric [CO$_2$], with all being angiosperms, than older species that had crown ages in high atmospheric [CO$_2$] periods, which were non-angiosperms (Fig. 3.9). Hydraulic capacity increased with increased $D_s$ and $D_v$ (Fig. 3.8). Increased $g_s$ due to high $D_s$ (and low $S_s$) means an accompanying increase in transpiration, putting an extra hydraulic demand on the leaf. Thus, a limited leaf capacity to uptake water would limit the leaf’s ability to sustain stomatal opening, and under low atmospheric [CO$_2$] reduced stomatal opening would affect a species’ productivity. Increased $D_v$ was already a signal that species under selection pressure from low atmospheric [CO$_2$] had a hydraulic adaptation, and previous research (Boyce et al. 2009; de Boer et al. 2012) has shown that innovation in vein characteristics has allowed angiosperms to leap over other taxa and achieve higher $g_s$, and subsequently higher $A$, due to their better supply of water to and around the leaf.

Changes in $D_s$ and $S_s$ do not only affect bulk $g_s$ values, but they can have an impact on the behaviour and the responsiveness of the stomatal aperture. A few studies (Drake et al. 2013; Lawson & Blatt 2014; Raven 2014) have shown that, in some cases, high $D_s$/low $S_s$ stomatal ratio results in a faster response by the stomatal aperture to environmental changes, allowing the leaf to match the responses of $g_s$ and $A$ much more efficiently and thus making the most of increases in light, while also minimizing excess water loss when $A$ decreases and extra CO$_2$ uptake is not needed. This is not set in stone and those same studies (Lawson & Blatt 2014;
McAusland et al. 2016), plus some others (Franks & Farquhar 2007; Monda et al. 2016; Elliott-Kingston et al. 2016) have argued against the extent of influence high \( D_s / S_s \) has on stomatal behaviour. Data presented in Chapter 3 (Fig. 3.4) did not show any significant variation in the \( g_s \) response to an increase in light. However, there was more of an observable difference in the \( g_s \) response to decreasing light, with species that evolved under lower atmospheric \([CO_2]\) (with high \( D_s / low S_s \)) showing a sharper decrease in \( g_s \) when light diminishes, thus limiting excess water loss through the stomatal pore. Water is always a limiting resource in plants, and thus increasing hydraulic supply without taking into consideration water availability is disadvantageous to plants, hence developing mechanisms to limit excess water usage are beneficial to a species’ ecophysiological success. However, the current ecological characteristics of a species’ habitat cannot be discounted, with the impact of previous environments (\( CO_2 \) change) not representing a full explanation for leaf function of the different species. For example, the species that exhibited this decrease in \( g_s \) are endemic to high humidity forests (\( P. caerulea, D. winteri \)), and so might not be water constrained. However others like \( L. nobilis \) are endemic to the more dry Mediterranean environment. \( B. capitata \), which also exhibited this decrease in conductance, has been known to have extensive vascular thickening that can be a mechanism of more efficient water supply (Tomlinson 2006).

\( P. vulgaris \), a crop species, was used in Chapter 4 to compare its responses with those of species which have crown group ages further back across geological history. \( P. vulgaris \) displayed faster \( g_s \) responses across a diurnal period compared to the other species sampled, highlighting its capacity to make the most of favourable photosynthetic conditions, while limiting losses under less favourable conditions. It provided a contrast to the responses of the other species across the diurnal period. A diurnal light regime subjects the plant to different rates of light change, similar to what a plant would experience in the field, and thus the species ability to adapt and
coordinate its physiological processes is put under pressure. There was some variation in stomatal speeds of response and in the matching of the $A$ and $g_s$ responses, with species that generally achieve more of a match between the $A$ and $g_s$ response also achieving higher $A$ and $g_s$ values. What the data from the diurnal responses highlighted was that hydraulic capacity might be at least as influenced by photosynthesis as by stomatal activity, as the $E$ and $A$ responses were closer in shape to each other than $E$ and $g_s$. Fig. 4.7 did show a stronger positive correlation between $E$ and $A$ than between $E$ and $g_s$. Also, in chapter 3, Fig. 3.3 did show $E$ responding to increasing atmospheric [CO$_2$], which brings back the point highlighted earlier that water remains an important resource for plants for a variety of intra-leaf processes, and increasing photosynthetic capacity through higher $g_s$ would mean extra amounts of water is needed not just to sustain transpiration but to also supply water for biochemical reactions and processes.

The diurnal responses shown also enabled the estimation of photosynthetic and hydraulic capacities across a prolonged period, and the results (Fig. 4.5) showed a general trend of increasing total (over the whole diurnal period) photosynthetic and hydraulic capacity (represented by calculating the auc) in species that emerged under lower atmospheric [CO$_2$], despite the not very discernible variation in dynamic responses across the diurnal regime. However, those species, like *P. vulgaris* and *P. caerulea*, are part of taxonomic groups that are known for higher productivity rates (a leguminous crop and a tropical forest species respectively), recollecting the effect of species ecological niche on their exhibited capacity. Also, while there is some evidence that some species do achieve some control over stomatal response to light, it seems that leaf control and balance of carbon gain to water loss, or in other words WUE, developed later. WUE and how its coordinated is complicated to elucidate (Vialet-Chabrand et al. 2016), but a difference was found between basal plants and modern angiosperms in the way the stomata responds, the termed
“passive vs. active” control, and this is thought to be a mechanism to coordinate carbon gain with water loss under environmental change (Brodribb & McAdam 2011; McAdam & Brodribb 2014).

Suggestions for future investigation

While leaf anatomical characters like $D_s$, $S_s$ and $D_v$ have been investigated already, it is interesting to probe further and find intra-leaf variation in leaf anatomy. A number of studies have already highlighted evolutionary responses in leaf form and size (Beerling et al. 2001; Osborne et al. 2004), and so one area for further examination could be intra-leaf anatomical variation that can affect the leaf’s photosynthetic or hydraulic capacity. Examining leaf vein size and diameter, intra-leaf distances between mesophyll, veins and sub-stomatal air spaces, concentration of air spaces and other anatomical aspects that help limit or restrain water loss can give an idea into how the leaf is equipped to its ecophysiological functions. Putting these findings in an evolutionary context as a response to changing environmental conditions across geological time can bring about a holistic picture of the plant evolution.

Measuring functional parameters in extant plants can help link anatomical changes to leaf function, however more species need to be used to correspond to different crown ages, as this study assumed that species that share crown ages would evolve similar characters, but of course, drivers of natural variation are not that straightforward. Finally, highlighting the relationship between leaf hydraulics and gas exchange and pinning down the main drivers of coordination of leaf hydraulics with leaf gas exchange can have a huge impact on understanding plant evolution, ecological function as well as providing ideas for exploitation in terms of improving plant traits for agriculture, especially under current climatic predictions that project increased drought and water unavailability.
References


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Supplementary material

Appendix 1

This appendix is supplemented to Fig. 3.4, as that figure excluded error bars for clearer presentation. Here the responses of $A$ and $g_s$ are separated into two separate graphs with error margins added. The figures show the step change response for each variable, with the grey shading around the curve representing the error (standard error). Figures are presented in order of species crown age (oldest to youngest):

1.1 *O. regalis*
1.2 *G. biloba*

1.3 *N. nagi*
1.4 *V.* *vinifera*

![Graph 1.4](image1)

1.5 *L.* *nobilis*

![Graph 1.5](image2)
1.6 *P. caerulea*

![Graph showing data for *P. caerulea* over time](image1)

1.7 *D. winteri*

![Graph showing data for *D. winteri* over time](image2)
1.8 *B. capitata*
Appendix 2

This appendix is supplemented to Fig. 4.3, which showed diurnal response of $A$ and $g_s$ to light change. As with Appendix 1, this Appendix is to separate the $A$ and $g_s$ responses, as well as $E$ and $e$, and show each curve with the margin of error associated with it (the grey shaded area around the curve; standard error was used). This is presented for the 5 species sampled.

2.1 *N.nagi*
2.2 *V. vinifera*
2.3 *P. caerulea*
2.4 *D. winteri*
2.5 *P. vulgaris*
Appendix 3

Presented here is the full output of the regression analysis shown in Chapters 2, 3 and 4. Below is each figure with the accompanying statistical output and regression equation.

<table>
<thead>
<tr>
<th>Figure number</th>
<th>Statistical output</th>
<th>Regression equation</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.3(a)</td>
<td>$F(1,8) = 1.727$, $p=0.2253$, $R^2 = 0.1775$</td>
<td>$D_s = (-0.1525) \text{CO}_2 + 162.2784$</td>
</tr>
<tr>
<td>2.3(b)</td>
<td>$F(1,7) = 2.533$, $p=0.155$, $R^2 = 0.2657$</td>
<td>$D_v = (-0.004331) \text{CO}_2 + 4.981841$</td>
</tr>
<tr>
<td>2.3(c)</td>
<td>$F(1,7) = 8.347$, $p=0.02335$, $R^2 = 0.5439$</td>
<td>$D_s = (30.86) D_v - 2.994$</td>
</tr>
<tr>
<td>3.7(a)</td>
<td>$F(1,6) = 4.544$, $p= 0.07704$, $R^2 = 0.4309$</td>
<td>$E = (-0.0017953) \text{CO}_2 + 1.4934854$</td>
</tr>
<tr>
<td>3.7(b)</td>
<td>$F(1,6) = 0.0008726$, $p= 0.9774$, $R^2 = 0.0001454$</td>
<td>$A = (0.0001597) \text{CO}_2 + 5.7196408$</td>
</tr>
<tr>
<td>3.7(c)</td>
<td>$F(1,6) = 0.6817$, $p= 0.4406$, $R^2 = 0.102$</td>
<td>$g_s = (0.00005078) \text{CO}_2 + 0.03327$</td>
</tr>
<tr>
<td>3.7(d)</td>
<td>$F(1,6) = 0.6459$, $p= 0.4522$, $R^2 = 0.09719$</td>
<td>$E = (0.06436) A + 0.47989$</td>
</tr>
<tr>
<td>3.7(e)</td>
<td>$F(1,6) = 0.4227$, $p= 0.5397$, $R^2 = 0.06582$</td>
<td>$E = (4.4130) g_s + 0.6247$</td>
</tr>
<tr>
<td>3.7(f)</td>
<td>$F(1,6) = 8.994$, $p= 0.02404$, $R^2 = 0.5998$</td>
<td>$A = (64.532) g_s + 2.458$</td>
</tr>
<tr>
<td>3.8(a)</td>
<td>$F(1,6) = 0.9096$, $p= 0.377$, $R^2 = 0.1316$</td>
<td>$A = (0.01336) D_v + 4.11476$</td>
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<tr>
<td>3.8(b)</td>
<td>$F(1,6) = 4.538$, $p= 0.07692$, $R^2 = 0.4312$</td>
<td>$E = (0.00499) D_v + 0.23072$</td>
</tr>
<tr>
<td>3.8(c)</td>
<td>$F(1,6) = 0.03721$, $p= 0.8534$, $R^2 = 0.006163$</td>
<td>$g_s = (-0.00003468) D_v + 0.05574$</td>
</tr>
<tr>
<td>3.8(d)</td>
<td>$F(1,5) = 2.822$, $p= 0.1538$, $R^2 = 0.3608$</td>
<td>$A = (0.9831) D_v + 2.3768$</td>
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<tr>
<td>3.8(e)</td>
<td>$F(1,5) = 3.617$, $p= 0.1156$, $R^2 = 0.4197$</td>
<td>$E = (0.22463) D_v + 0.02189$</td>
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<tr>
<td>3.8(f)</td>
<td>$F(1,5) = 0.3232$, $p= 0.5942$, $R^2 = 0.06072$</td>
<td>$g_s = (0.004726) D_v + 0.037757$</td>
</tr>
<tr>
<td>4.7(a)</td>
<td>$F(1,3) = 2.941$, $p= 0.1849$, $R^2 = 0.495$</td>
<td>$E = (-0.02561) \text{CO}_2 + 9.49350$</td>
</tr>
<tr>
<td>4.7(b)</td>
<td>$F(1,3) = 5.576$, $p= 0.09929$, $R^2 = 0.6502$</td>
<td>$A = (-0.07972) \text{CO}_2 + 25.57543$</td>
</tr>
<tr>
<td>4.7(c)</td>
<td>$F(1,3) = 1.541$, $p= 0.3027$, $R^2 = 0.3393$</td>
<td>$g_s = (-0.006671) \text{CO}_2 + 2.205810$</td>
</tr>
<tr>
<td>4.7(d)</td>
<td>$F(1,3) = 16.03$, $p= 0.02793$, $R^2 = 0.8424$</td>
<td>$E = (0.3379) A + 1.1584$</td>
</tr>
<tr>
<td>4.7(e)</td>
<td>$F(1,3) = 7.554$, $p= 0.07084$, $R^2 = 0.7157$</td>
<td>$E = (2.6892) g_s + 1.7816$</td>
</tr>
<tr>
<td>4.7(f)</td>
<td>$F(1,3) = 25.69$, $p= 0.01483$, $R^2 = 0.8954$</td>
<td>$A = (8.169) g_s + 1.705$</td>
</tr>
</tbody>
</table>