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Stomatal Responses to Biotic and Abiotic Stress

Implications of elevated atmospheric carbon dioxide and drought conditions on the *in situ* stomatal responses of grassland species

Aidan Holohan

*Doctor of Philosophy*

University College Dublin

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Supervisor: Prof Jennifer McElwain

Submitted to University College Dublin, December 2015
Declaration

I hereby certify that the submitted work is my own work, was completed while registered as a candidate for the degree stated on the Title Page, and I have not obtained a degree elsewhere on the basis of the research presented in this submitted work.

Aidan Holohan
Chapter contributions

Each individual data chapter presented in this thesis was solely written and compiled by the author under the guidance of the principle supervisor. Additional contributions were also made by collaborating individuals and their contributions are outlined in Table 1.

Table 1: Research collaborators and individual contributions per chapter

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AH = Aidan Holohan, JME = Prof Jennifer McElwain (principle supervisor), CM = Prof Christoph Müller (co-supervisor), EH = Eamon Haughey (collaborator), JF = Dr John Finn (collaborator), MC = Prof Margaret Collinson (collaborator), Dr Lutz Kunzmann (collaborator), MS = Dr Margret Steinhorsdottir (collaborator), AP = Amanda Porter (collaborator)

In addition to the data chapters and published material presented in this thesis, a further 18 month experiment aimed at assessing the combined influence of light intensity and atmospheric carbon dioxide concentrations on stomatal initiation and functioning was carried out on a range of woody angiosperms differing in xylem architectures. Data for this experiment has been compiled and analysed and draft manuscripts are currently being prepared for peer review.
Summary

Diversity in the ecological distribution of plant species across a vast array of environments and/or ecological niches is indicative of the adaptive capacities of individual species to maintain optimal biological functioning through the exploitation of evolved competitive advantages. This ability to exploit particular environments through morphological and/or physiological adaptation is fundamental to the ecological range of species and of particular relevance in predicting the expansion/contraction or fundamental altering of ecosystems with predicted environmental change.

Elevated atmospheric carbon dioxide concentrations have been shown to alter physiological, morphological and developmental traits of extant plant species to varying degrees and often provoke acclamatory responses where plants are grown under sustained enrichment regimes over extended time periods. Despite this, contemporary evolutionary responses amongst extant plant species that coincide with modern, anthropogenically driven CO₂ rise have not, as yet, been demonstrated.

This thesis combines a long term free-air carbon dioxide enrichment experiment with short term growth chamber and diversity trials in order to assess the implications of moderate levels of atmospheric carbon dioxide enrichment in a semi natural European grassland system. Complementary use of these experimental protocols allows for the isolation of significant environmental effects and an assessment of their true ecological implications.

Results presented here demonstrate a persistent alteration to certain key physiological traits amongst the offspring of plants exposed to long term [CO₂] enrichment. Significantly these observations are not uniform across all species which indicates a differential capacity to demonstrate a heritable response to increasing atmospheric CO₂ between species in this
particular grassland community. Findings in this instance may have serious implications for biodiversity, ecosystem dynamics and productivity in temperate grasslands. Further, the incorporation of a diversity trial as part of this research demonstrated that investigations of evolutionary responses in the context of their biotic communities are essential although considerably more complex than studying responses to abiotic factors in isolation.
Acknowledgements

First and foremost I would like to thank Professor Jennifer McElwain for her supervision, guidance and advice without which the completion of this thesis would not have been possible. I also thank Professor Christoph Müller for providing me a home away from home at the Justus Liebig University of Giessen, as well as for his guidance and instruction in the fieldwork elements of this thesis.

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Chapter 1
Introduction - Part 1

The case for anthropogenic climate change has become increasingly compelling with the currently observed temperatures of the terrestrial surface, free atmosphere and ocean systems being in excess of anything detectable over the last 1.3 kyr (Osborn & Briffa 2007). Recent reports have determined that the dominant cause of warming over the past several decades has been the result of anthropogenically derived greenhouse gas forcing and that the probability of this warming being due to any other factor, or natural variability inherent within the climate system, being extremely unlikely as responses to climate forcing have been detected on all continents, with the exception of Antarctica, and certain sub-continental regions (IPCC 2014).

Significantly, the rate of global climate change seen in recent history is likely to advance over the coming century with probable implications including increased intensity in precipitation events (Meehl, Arblaster & Tebaldi 2005), increased and prolonged drought events (Dai 2011, 2013), increased nitrogen deposition (Vitousek et al. 1997; Dentener et al. 2006) and increased atmospheric CO₂ concentrations. Principally, these changes are the result of increasing CO₂ emissions from fossil fuel combustion and land use change (IPCC 2014).

Predicting the ecological impacts of future climate change on plant functioning and biogeographical distributions remains one of the most significant challenges in global change biology (Guisan & Thuiller 2005; Maalouf et al. 2012; Eskelinen & Harrison 2015) as it is probable that plant communities will see major shifts in composition and structure with long-term changes in key environmental factors, particularly atmospheric CO₂ concentrations and drought events (Klironomos et al. 2005a; Thuiller et al. 2008; Dijkstra et al. 2010; Hoover,
Knapp & Smith 2014). Globally, grassland ecosystems are amongst the largest habitat types in the world covering an estimated area of 52.5 million km$^2$ or approximately 40.5% of the earth’s total landmass (Suttie J. M., Reynolds S. G. 2005). In a European context, grasslands account for 30% of the total area and 50% of the utilised agricultural land. They are often highly species rich habitats with calcareous grasslands being Europe’s most species-rich plant community containing up to 80 plant species per m$^2$ (Wallis De Vries, Poschlod & Willems 2002).

![Spatial distribution of permanent grassland as a percentage of utilized agricultural land (average of 1995–2004) in Europe](image)

Figure 1: Spatial distribution of permanent grassland as a percentage of utilized agricultural land (average of 1995–2004) in Europe (Smit, Metzger & Ewert 2008).

The impacts of elevated atmospheric CO$_2$ concentrations and the implications of increasing drought events, which are key to species function and proliferation, are therefore of significant importance and consequently the principle topic of research in this PhD Thesis. Additionally, although the effects of climate and atmospheric changes might not directly
interact (additive response), and consequently be predictable based on single factor experiments (Zavaleta et al. 2003; Tilman, Reich & Knops 2006), it is perhaps more likely that the effects of simultaneously changing environmental factors will act synergistically (amplifying) (Reich et al. 2001) or antagonistically (canceling or damping) (van der Kooi et al. 2016) producing larger or smaller responses then may be expected based on single factor results. To that end we further aim to assess plant responses under additional, potentially confounding, environmental factors and in particular through the assessment of increasing inter-specific competition.

**Leaf level gas exchange and stomatal responses to [CO₂]**

![Diagram of stomatal responses to CO₂](image)

Figure 2: Points of resistance to the diffusion of CO₂ from outside the leaf to the chloroplasts (Taiz 2010)
Stomatal pores occurring on the leaf surface allow for the exchange of gases (particularly water vapour and CO₂) between the plant and atmosphere, regulating both transpiration and photosynthesis. Despite the large body of research into the environmental regulators of guard cell function, understanding is still limited in terms of how exactly environmental cues are detected by guard cells, how they influence photosynthetic signalling processes (Kim et al. 2010; Brodribb & McAdam 2013; Chater et al. 2015) as well as those associated the regulation of stomatal development (Casson & Gray 2008; Pillitteri & Torii 2012; Engineer et al. 2014). Mature leaves sense atmospheric changes in CO₂ concentrations and relay any alterations in concentration to the developing, juvenile leaves (Brownlee 2001; Lake, Woodward et al. 2002; Hetherington and Woodward 2003). Despite a lack of understanding in terms of long distance signalling pathways, recent advances have demonstrated that a number of plant hormones may be involved in the process including abscisic acid, ethylene and certain Jasmonic acid’s (Hetherington and Woodward 2003).

Although it is considered that [CO₂] is a major contributor to stomatal initiation responses, the fact that hormones involved in a number of other important plant functions including abscission of leaves, senescence, ripening of fruit and opening of flowers etc are also involved in the [CO₂] response processes. This has led to the theory that a specific plant response mechanism to elevated CO₂, separate from other environmental stresses such as drought or light intensity may be unlikely and is probably complicated by a number of additional short and medium distance signalling responses including alterations in cytosolic free calcium concentration, apoplastic and cytoplasmic pH gradients and photosynthetically derived ATP and protein phosphorylation or dephosphorylation (Ainsworth and Rogers 2007). The lack of any definitive understanding in this regard necessitates a great deal more study.
In terms of short distance signaling, the *HIC* (high carbon dioxide) gene has been demonstrated to encode coenzyme (3-ketoacyl-CoA synthase) negatively inhibiting stomatal development (Lake, Woodward et al. 2002; Hetherington and Woodward 2003; Bergmann and Sack 2007). These Co-A synthases are involved in the production of long-chain fatty acids which are constituents of the waxy cuticle on the outer surface of the epidermis, the composition of which is considered fundamental to the movement of signals in the apoplast of the leaf epidermis.

Presumed inhibitory factors are secreted into the apoplast where they suppress differentiation of adjacent epidermal cells into guard cells and are thus involved in controlling the stomatal density response to elevated CO$_2$. The diffusion of these signals is considered to be dependent on the composition of wax esters encoded in the cell wall or cuticle. This was demonstrated by exposing strains of *Arabidopsis* deficient in the *HIC* gene to elevated concentrations of CO$_2$. The contradictory response in stomatal density observed (i.e. the increase in stomatal density) was considered to be due to the fact that the defective, *HIC* mutants were incapable of reducing the effective range over which any inhibitory signals may be transported and therefore were incapable of reducing stomatal density in response to environmental pressure (Gray 2005).

Over a shorter period the CO$_2$-sensing mechanism in guard cells that is responsible for the alteration in the size of the stomatal aperture remains largely unknown although it is generally considered, at least in the case of modern angiosperm species (Brodribb & McAdam 2013), that increasing CO$_2$ interacts with guard cell membrane channels, via a Ca$^{2+}$ dependent signalling pathway (Hashimoto *et al.* 2006; Israelsson *et al.* 2006; Hu *et al.* 2010).
FACE studies have highlighted that stomatal conductance consistently decreased in both C3 and C4 plant species, largely independently of alterations in stomatal density (Bettarini, Vaccari et al. 1998; Engloner, Kovacs et al. 2003; Reid, Maherali et al. 2003; Ainsworth and Rogers 2007). As a plants ability to make changes to the size of the stomatal pore is likely to influence any long term response in terms of stomatal density further investigation into these cellular responses to climate change is also considered fundamental. These observations suggest that the majority of taxa studied in FACE experiments have reduced their stomatal conductance under elevated [CO₂] via partial closure of stomatal pores. This is in sharp contrast with the majority of historical herbarium studies of woody C3 taxa which show decreased stomatal density and index under elevated [CO₂] (McElwain & Chaloner 1995a; Woodward & Kelley 1995; Royer 2001; Beerling & Berner 2005; Haworth, Elliott-Kingston & McElwain 2011a)

Photosynthesis-dependent stomatal responses to [CO₂]

In addition to the direct effects of [CO₂] on guard cell membrane channels, there is some evidence for additional, photosynthesis-dependent stomatal responses. These have been derived from the responses of stomata from isolated samples of leaf epidermis which contain no photosynthesising tissue with stomata from fully intact leaves (Messinger, Buckley & Mott 2006; Sibbernsen & Mott 2010; Fujita, Noguchi & Terashima 2013). Diminished stomatal responses in the photosynthetically isolated epidermal samples were consistent in these cases although the precise mechanism affected was not distinguished and remains unknown. Based on these findings however, it is considered that some signal must be generated by photosynthesising mesophyll cells which is then transmitted to the guard cells in the apoplast (Mott 2009; Brodribb & McAdam 2013; Lawson et al. 2014).
Stomatal acclimation to elevated [CO₂]

Accurately predicting plant function and global biogeochemical cycles under future climate scenarios will be complicated if \( g_s \) (stomatal conductance to water vapour) acclimates to elevated [CO₂]. By far the majority of studies have focused on photosynthetic acclimations which have been widely observed in response to elevated [CO₂] (Moore et al. 1999; Rogers & Humphries 2000; Long et al. 2004; Ainsworth & Rogers 2007). These photosynthetic acclimations are often characterised by an increase in Rubisco activation as determined from an increase in the ratio of \( V_{c,max} \) (maximum rate of ribulose bisphosphate (RuBP) carboxylation) to overall Rubisco content, in the absence of nitrogen fertilisation (Long et al. 2004). In addition there is often observed little, or highly inconsistent alterations to \( J_{max} \) (maximum potential linear electron flux through photosystem II) which is an increasingly significant factor at elevated [CO₂] where photosynthesis becomes less limited by Rubisco and increasingly limited by RuBP regeneration. In contrast to photosynthetic acclimation, physiological stomatal acclimation to elevated [CO₂] is less considered. As acclimation is often described as a growth [CO₂] effect on stomatal conductance values measured under identical environmental conditions (Ziska & Bunce 2006) few studies have demonstrated or have been able to distinguish that stomata acclimate to elevated [CO₂] independently of a photosynthetic acclimation. Furthermore, because stomatal conductance is tightly coupled with changes in photosynthesis, lower conductance in plants, as a consequence of reduced photosynthetic capacity, is generally expected. Consequently it is considered that stomata do not acclimate to [CO₂] without a corresponding acclimation in photosynthesis (Long et al. 2004). However, as there are potentially multiple and distinct mechanisms through which plants sense and respond to [CO₂] it is possible that these mechanisms can be altered.
independently. In terms of field studies however, relatively few examples of independent stomatal acclimation exist (Drake, Gonzalez-Meler & Long 1997; Anderson et al. 2001; Bunce 2001)

**Water-use efficiencies**

Broadly, water-use efficiency is defined as the ratio of water lost to carbon assimilated by plants and is a key characteristic of ecosystem function and plays a pivotal role in global hydrological, energy and carbon cycles (Keenan et al. 2013). The generally observed effects of elevated [CO₂] on plant physiological functioning (specifically increasing photosynthetic capacity and decreasing stomatal conductance) necessitate an increase in plant water use efficiencies. Although large scale carbon dioxide enrichment trials tend to be quite variable, certainly in the magnitude of plant responses to [CO₂] enrichment, they are almost ubiquitous in describing increases in various forms of water-use efficiency metrics from leaf to biome scales (Long et al. 2004; Ainsworth & Long 2005; Rogers, Ainsworth & Kammann 2006; Ainsworth & Rogers 2007; Bernacchi & VanLoocke 2015).
Table 2: Water use efficiency metrics. Table taken from Bernacchi & VanLoocke (2015)

<table>
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<th>Integrated WUE</th>
<th>Intrinsic WUE</th>
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<td>$A/Tr$</td>
<td>$\Sigma A/\Sigma Tr$</td>
<td>$A/gs$</td>
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<tr>
<td>Canopy</td>
<td>$GPP/Tr_{(can)}$</td>
<td>$NPP/\Sigma ET$</td>
<td>$GPP/gc$</td>
</tr>
<tr>
<td>Ecosystem</td>
<td>$NEE/ET$</td>
<td>$NEP/\Sigma ET$</td>
<td>$NEE/\surf$</td>
</tr>
<tr>
<td>Harvest</td>
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<tr>
<td>Biome</td>
<td></td>
<td>$NBP/\Sigma ET$</td>
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Abbreviations: $A$, leaf net carbon assimilation ($\mu$mol m$^{-2}$ s$^{-1}$); ET, evapotranspiration (mm H$_2$O); $g_c$, canopy conductance (mol m$^{-2}$ s$^{-1}$); GPP, gross primary production (g m$^{-2}$ s$^{-1}$); $g_s$, stomatal conductance (mol m$^{-2}$ s$^{-1}$); $g_{surf}$, surface conductance (mol m$^{-2}$ s$^{-1}$); NBP, net biome productivity (g C m$^{-2}$); NEE, net ecosystem exchange (g C m$^{-2}$ s$^{-1}$); NEP, net ecosystem productivity (g C m$^{-2}$ s$^{-1}$); NPP, net primary production (g C m$^{-2}$ s$^{-1}$); $T_r$, leaf transpiration (mmol m$^{-2}$ s$^{-1}$); $T_{r(can)}$, canopy transpiration (g m$^{-2}$ s$^{-1}$); yield, harvested biomass (g dry matter m$^{-2}$); time integrated. Note: 1 mm H$_2$O over 1 m$^2$ area = 1 L H$_2$O = 1 kg H$_2$O.

Although there are a multitude of different WUE metrics (Table 1), they can, and often are, considered in parallel to assess the water costs of ecosystem services and plant functioning as a whole (Bernacchi & VanLoocke 2015). In assessing WUE as regulated by plant stomata perhaps the most appropriate measurement, particularly in terms of prolonged exposure to elevated [CO$_2$], is the quantity of $A/g_s$ (intrinsic or physiological water-use efficiency) as it is indicative of the potential WUE based on physiological components uninfluenced by environmental conditions, particularly VPD (leaf to air vapour pressure deficit) (Franks et al. 2013). Leaf-level measurements of intrinsic water-use efficiency (iWUE) using gas exchange measurement systems are straightforward to acquire however, iWUE values recorded in this
way should be interpreted with caution due to the direct influence of VPD on stomatal conductance. In situations where it is not possible to maintain VPD to within an adequate range, such that it would not substantially influence stomatal conductance values, the alternative metric, instantaneous water use efficiency, which is dependent on overall transpiration rates and consequently incorporates leaf to air VPD, is typically used despite this method being less sensitive to the role of guard cell resistance. For example, In a situation where the relative humidity of the air is 100% and relative humidity of the leaf interior is also 100% (as it is generally considered to be) then transpiration will be extremely low despite stomatal resistance (as the reciprocal of stomatal conductance) also being very low. At the leaf scale, rising [CO$_2$] generally lowers transpiration through reduced stomatal conductance; however it is important to account for the fact that conductance and transpiration may not be perfectly in sync due to the fact that changes in transpiration are directly linked to changes in VPD.

Assessing stomatal responses to [CO$_2$] enrichment

Over the past several decades plant responses to elevated and sub-ambient [CO$_2$] concentrations have been extensively studied in response to a wide variety of experimental protocols. Over time, these protocols have become increasingly sophisticated and many of the unintended artefacts of earlier studies (e.g. pot effects, pulsed vs continuous nutrient supply etc) have been eliminated or accounted for.

Free-air carbon dioxide enrichment experiments (FACE) have gained significance as a methodology in determining plant responses to elevated [CO$_2$] in the sense that they may potentially yield more peremptory results as plants are exposed to more realistic
environmental conditions. They differ principally from other forms of exposure studies in that they are conducted in fully intact floral communities on a scale large enough to be comparable to agronomic trials without limiting growing space, or altering microclimate, precipitation, etc (Ainsworth et al. 2008).

In terms of findings from FACE studies, perhaps the most significant are associated with vegetative production. Initial, observations from early [CO₂] enrichment studies describe that prolonged exposure often led to a down regulation in photosynthetic rates which generally coincided with a decline in Rubisco, leaf nitrogen and protein content. This long-term down-regulation was often linked with sink limitations (the capacity to invest the products of photosynthesis in other parts of the plant) imposed by restrictions in the available rooting space of plants grown in pots (Long et al. 2004) Summary results from FACE studies however, demonstrated that although reductions in Rubisco content were observed a corresponding reduction in N per unit leaf area was not. Subsequently, acclimation to growth at elevated [CO₂] did not necessarily result in the reduction in photosynthetic resources but rather a redistribution of those resources amongst the enzymatic constituents of the carbon reduction cycle often resulting in an increased nutrient and water use efficiency and, significantly, a general increase, in overall photosynthetic rates (Paul & Foyer 2001; Ainsworth et al. 2004; Long et al. 2004; Kirschbaum 2011).

Where sink limitations were not a factor however, [CO₂] enrichment experiments often displayed an enhancement of photosynthetic rates (Long et al. 2004) Based on these early experiments an average 32% increase in net photosynthetic rates was predicted in response to a doubling of [CO₂], a significant finding as it has serious implications in terms of global crop resources and food security and the amount of land that will be required to meet the
demands of an exponentially increasing population. Currently however, and in contrast to closed environment studies, FACE experiments report a significantly lower increase in photosynthetic rates, ca. 20% across a range of C3 plants (Ainsworth & Long 2005).

Although FACE sites have provided an invaluable contribution, in terms of increasing our knowledge of the potential implications of climate change, they have demonstrated a lack of consistency in results in terms of both physiological (functional process) and morphological (structure and form) stomatal responses. For example, the expected decrease in stomatal stomatal density and stomatal index clearly demonstrated by fossilised plant species, has not been demonstrated as strongly from results both between and amongst FACE sites with some plant species showing a decline in numbers, others showing no change and some even showing an increase (Bettarini, Vaccari et al. 1998; Reid, Maherali et al. 2003; Marchi, Tognetti et al. 2004).

The reasons for this lack of uniformity of response are unclear but may be the result of a number of confounding factors. Stomatal density, for example, is not only influenced by elevated CO$_2$ but also by a number of additional environmental variables such as water availability, light intensity and temperature all of which may act upon the plant in varying degrees. The combination of stresses causes the plant to adjust its number of stomata (or the size of stomatal apertures) in order to maximize photosynthetic ability while reducing water loss through the leaf surface. Specific signaling pathways and/or the interaction of those pathways influencing stomatal development within the plant is little understood although recent studies have begun to shed light on how precisely environmental factors affect the functioning of mature stomata as well as the initiation and patterning of stomata on juvenile leaves (Bergmann and Sack 2007).
Although of substantial benefit in assessing stomatal responses to [CO$_2$] enrichment, FACE studies are not without flaws of their own. Often plant communities within FACE experiments are exposed to quite extreme, stepped increases in [CO$_2$] which may impose a strong perturbation, influencing plant responses in a manner that may not be the same as exposure to gradual of minor changes. Real replication in FACE studies is costly and difficult to achieve in a natural setting as additional and unconsidered variables may act independently on individual experimental blocks. Potentially confounding experimental artefacts may be quantifiable and accounted for as chamber effects in controlled environment studies (Porter et al. 2015) but may be more difficult to assess under FACE conditions. Lack of adequate replication may result in difficulty detecting small but important differences responses of plants and/or ecosystems to rising [CO$_2$] due to limited statistical sensitivity. Enrichment systems themselves may also cause issues particularly in situations where CO$_2$ enriched air is blown into or across treatment rings. Increased air velocity may artificially increase factor such as vapour pressure deficits and may consequently affect plant physiological functioning, particularly transpiration rates and stomatal resistances. Increasing [CO$_2$] concentrations in an open air environment provides further restrictions and it is often the case that FACE studies are limited in assessing plant responses up to a general maximum enrichment level of 700 ppm. In that case we may currently only assess plant responses to the predicted [CO$_2$] concentrations of the coming century and so are limited in terms of how far into the future we can project findings. Neither can plants be assessed in terms of the impacts of below ambient [CO$_2$] concentrations in the field as scrubbing the gas from the atmosphere is not currently feasible.

Despite some potential shortcomings FACE studies have, and continue to make, an invaluable contribution to our understanding of the effects of [CO$_2$] on plants and
communities and are arguably the most appropriate currently available method. Due to the limitations to inherent to both chamber trials and FACE experiments this thesis will aim to reconcile both experimental protocols in order to resolve the potential uncertainty in findings based on chamber study or FACE results in isolation.

**Thesis Aims [Part 1]**

Predicting the ecological impacts of future climate change is one of the most significant challenges in global change biology. Plant species responses to climate change are highly individualistic and context dependent and consequently predictions relating to plant function and ecosystem structure remain contentious particularly in natural or semi-natural systems. The critical role that plant gas exchange processes play in global climate has made it a crucial but often contentious topic in plant research. This thesis aims to assess if responses in particular leaf traits critical to leaf level gas exchange rates (stomatal density, stomatal conductance, leaf level photosynthetic rate and water-use efficiencies) are detectable at marginal levels of $[\text{CO}_2]$ enrichment and whether or not those responses, if any, reflect the generally observed trends of plants that are typically exposed to larger stepped increases in $[\text{CO}_2]$.

Specifically part one of this thesis will address three principle aspects;

1. Does long term, moderate $[\text{CO}_2]$ enrichment effect morphological, developmental or physiological responses of pant species in a semi-natural grassland community?
2. Do all species show uniform acclimation to long term (17 years) of moderate $[\text{CO}_2]$ enrichment at 20% above ambient levels?
3. Is there heritability of long term acclimation responses implying contemporary evolution/adaptation under moderate CO$_2$ rise

Introduction - Part 2

Abiotic factors – Resource Competition and Plant-Plant Interactions

Competition is a key process in the structure and composition of plant populations and communities. Therefore, to accurately predict the responses of ecological systems to environmental change, a comprehensive and mechanistic understanding of how plant competition may influence species responses to a changing environment is key.

By definition, competition may be considered the direct or indirect interaction between individuals of the same or differing species that vie for a common, limiting resource which ultimately leads to an increase in the fitness of one species or individual at the expense of another (Case, T.J. 1974). In vegetative communities, exploitative competition (where two species compete indirectly through the consumption of a limited resource) is most prevalent and is potentially a significant driver in the promotion of functional diversity which in turn may be considered key to the successful coexistence amongst species.

The mechanism through which functional diversity promotes biological multiplicity is often referred to as the competitive exclusion principle (Gause 1935) and is based on the principle that in order to coexist, two species occupying the same ecological niche space would require the same resources at the same time and in the same proportion and thus would be in direct competition. In this instance the term niche refers to the specific requirements of a
particular individual or species necessary to survive and reproduce. Consequently, the strongest competitor would exclude rival species implying that large species diversity necessitates a significant range of functional diversity.

As environmental conditions determine the structure of fundamental ecological niches, any significant changes in the environment will likely alter the specific niche space which particular species occupy, forcing adaptations to the new set of environmental conditions through morphological, anatomical or physiological adjustments. The ability to maintain optimal growth and reproduction rates in the face of environmental change considering the constraints imposed by competing individuals is an important consideration in predicting plant responses and community structure. However, although functional complementarity/diversity may impose restrictions on species responses to abiotic stress and supported by some studies (Onipchenko *et al.* 2009; Bermúdez & Retuerto 2014), inter-specific complementarity (Loreau & Hector 2001) and its implications have not been extensively investigated using mechanistic approaches.

**Intra/Inter-specific Trait Variability**

Assessing species responses to abiotic stress has, and is primarily conducted through the observation of alterations to mean/median values of particular functional traits and generally ignores the extensive range of intraspecific variation commonly displayed for a host of traits, particularly physiological traits (Violle *et al.* 2012). More recently there has been a renewed interest in the contribution of intraspecific trait variability to trait-based coexistence theory which is often less considered in ecological studies ((Bolnick *et al.* 2011; Courbaud, Vieilledent & Kunstler 2012). It may be considered a failing then, to utilise mean trait values
only when assessing species responses to environmental stress as it has been
demonstrated that this approach may underestimate the ability of species to coexist
alongside other members of a diverse community, may misrepresent the fraction of
resources that the population can use, and underestimates the degree of niche and trait
overlap between species (Violle et al. 2012).

A study by Clarke (2010) carried out in forests of the south-eastern United States highlighted
that the variation in growth rates among the individuals within populations varied in their
distributions while the mean values for the corresponding populations did not differ
significantly. Further, a study by Jung et al. (2014) described how intraspecific trait variability
was a significant driver in the short-term functional responses in leaf dry matter content,
specific leaf area, leaf nitrogen concentration and leaf carbon concentration of subalpine
grassland communities. These responses were not strongly reflected by changes in mean
trait values and highlighted how a reliance trait averages only could seriously underestimate,
or even wrongly estimate the response of communities to extreme drought events.

The typical reliance on mean/median values and the assumptions that these values
adequately capture the full range of species responses to environmental stress, despite the
prevalence of significant Intra/Inter-specific variability in multiple measured trait values is
indicative of a potential flaw in the foundations of community ecology and eco-physiology.

Both model predictions and theory incorporating intraspecific variability predict higher local
diversity than those based on population averages alone (Lichstein et al. 2007; Yamauchi &
Miki 2009; Clark 2010) it is therefore considered that a full appraisal of the distribution of
functional trait values, within and between species, may help provide more meaningful
insights into the structuring of plant communities.
Thesis Aims [Part 2]

A key limitation of climate envelope models (which aim to project species' geographical range expansion/contraction under future climate change scenarios) is the parameterization of ecological interactions between species and the potential implications of those interactions on physiological function. Because experiments have shown that responses of individual plants grown in pots or in monocultures are not good predictors of the same species responses grown in competition within a diverse plant community due to competition restrictions and because investigations have shown that species diversity can affect community stability/structure by influencing the responses of individual species to changes in their environment (Loreau et al. 2001; Tilman et al. 2006)) part two of this project aims to decouple the inherent responses of species to abiotic stress from the responses of plant–plant interactions. In particular changes in the physiological performance ($g_s$, $A$ and IWUE) of target species with environmental stress are assessed, in this instance drought treatment, is the same in the absence of inter-specific competition (i.e. abiotic stress effects only) as it would be in competitive mixtures (competitor mediated effects). Part two of this thesis therefore aims to build on the outcomes of part one by disentangling the influence of competitive interactions of neighbouring species which potentially impose additional restrictions on essential resources (Belote, Weltzin & Norby 2004).
Chapter 2
Interactive effects of long-term, moderate atmospheric CO$_2$ enrichment and soil moisture content on morphological stomatal traits in a semi-natural grassland community

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Abstract

Free air carbon dioxide enrichment experiments (FACE) have become an increasingly popular alternative to growth chamber studies in assessing plant developmental responses to environmental variables because they potentially yield more peremptory results as plants are exposed to more realistic environmental conditions. However, results obtained from FACE studies thus far have demonstrated that developmental stomatal responses to CO$_2$ among extant plant species are not always detectable while those that have been detected are often contradictory. The disparity in stomatal developmental responses observed under FACE conditions requires further in depth study because of the important role stomata play in controlling carbon uptake and water loss.
Principally, this study is focused on the effects of both soil moisture content and elevated CO$_2$ as discrete factors in stomatal development. Detection of environmental effects on stomatal development are investigated via the analysis of stomatal density (SD = number of stomata per area of leaf surface) and stomatal index (SI = number of stomata as a percentage of epidermal cells per area of leaf surface) analysis of four grassland community species; *Arrhenatherum elatus*, *Triestum flavescens*, *Gallium mollugo* and *Geranium pretense* grown at the University of Giessen’s long term free air carbon dioxide enrichment facility.

Results have demonstrated that CO$_2$ elevation (480 ppm) as an independent factor was not sufficient to affect any significant response in terms of an overall change in stomatal initiation at this particular site, a finding which is in keeping with the majority of FACE studies but contradictory to a significant number of herbarium studies which describe significant alterations in stomatal morphology with similar increases in atmospheric carbon dioxide levels (~100 ppm)

Keywords: Stomatal density, stomatal index, elevated CO$_2$, soil moisture gradient, FACE, grasslands.
Introduction

The crucial association between plant life and atmospheric carbon dioxide is perhaps the most fundamental relationship in nature and constitutes an intrinsic aspect of environmental research. Variation in CO$_2$ availability directly impacts respiratory and photosynthetic processes, provoking a wide range of physiological and morphological responses in plants a thorough understanding of which is a considerable advantage in predicting the effects of future climate change on extant species.

One notable, and well documented, consequence of elevated levels of [CO$_2$] is manifest in the ability, and general tendency, of plant species to reduce the number of stomatal pores on the leaf surface, particularly when subjected to a sustained increase over extended periods of time (McElwain & Chaloner 1995b; Woodward & Kelley 1995; Royer 2001; Lammertsma et al. 2011). However, although this response is often observed, relatively little is understood in terms of a precise CO$_2$ response mechanism as plants are capable of modulating any reaction, in terms of stomatal development, to a combination of additional and often conflicting environmental factors, including; light intensity, water availability, temperature and vapor pressure deficits (Hetherington & Woodward 2003; Gray 2005). In addition, the response of plant species is further complicated by the dynamics of the broader, natural environment in which they occur. Competitive interactions, endemic to specific plant community structures, cause conflict amongst species over resources that may be both spatially and temporarily heterogeneous (Belote et al. 2004), as a consequence, those species which demonstrate greater functional plasticity may be more likely to survive novel environmental conditions.
Stomatal indices, specifically stomatal density (SD = number of stomata per area of leaf surface) and stomatal index (SI = number of stomata as a percentage of epidermal cells per area of leaf surface), are frequently exploited as a method of approximating prehistoric atmospheric CO₂ concentrations (McElwain & Chaloner 1995b; Woodward & Kelley 1995; McElwain 1998; Royer et al. 2001; Beerling & Royer 2002; McElwain, Mayle & Beerling 2002; Konrad, Roth-Nebelsick & Grein 2008; Beerling, Fox & Anderson 2009), it is crucial that we continue to develop an understanding of the effects of combined environmental pressures, which through their often antagonistic effects on stomatal initiation, conspire to affect any overall change.

As a modern approach to CO₂ enrichment, the FACE experimental technique is considered to be at the cutting edge in analysing plant responses to variable concentrations of atmospheric CO₂. It has become an increasingly popular alternative/complimentary to controlled environment studies (greenhouses, growth rooms and open topped chambers (OTC)) because FACE experiments are conducted in situ in the field and theoretically have the potential to yield more realistic results under [CO₂] enrichment as plants are exposed to natural climate or weather variability. However, results obtained thus far have demonstrated that responses to elevated CO₂ among extant plant species are not always detectable, while those that have been observed may be an increase or a decrease in stomatal initiation dependent on the parameters of the experiment (Woodward & Kelly 1995). The dichotomy of results observed across FACE studies, as well as between FACE and fossil/herbarium studies suggests that either the FACE system is flawed, in that it does not truly reflect the reality of a CO₂ enriched environment in some aspect, or that there are additional factors exerting an influence on stomatal density which interact with elevated CO₂, which weakens the treatment effect. It has been proposed by Haworth et al (2013) for example, that the
disparity in stomatal developmental responses observed in FACE and growth chamber studies are likely due to key differences in the type of species investigated and the method by which these different species predominantly control gas exchange.

Although changes in stomatal initiation rates have been well described across a number of FACE sites (Ainsworth & Long 2005; Ainsworth et al. 2008), responses have never been assessed in a semi-natural grassland community over such a protracted time period. The duration of [CO₂] enrichment (beginning in 1998 and currently ongoing) makes this the longest running, continuously enriched FACE site of its type currently in existence and affords a unique opportunity to assess stomatal responses to chronic [CO₂] enrichment.

Why there is general consistency in terms of fossil and herbaria studies that elevated [CO₂] concentrations are linked with a reduction in stomatal densities (Woodward & Kelley 1995; Woodward, Lake & Quick 2002), Results thus far obtained from FACE studies are out of step showing no overall response, in terms of modern plant taxa, to an average doubling of current ambient [CO₂] levels (Ainsworth & Long 2005).

It has been demonstrated that certain species may be limited in their ability to alter stomatal densities (Haworth, Elliott-Kingston & McElwain 2013; Haworth et al. 2015). An observed non-linearity of stomatal density adjustments to increasing [CO₂] concentrations and demonstration of limitations in the minimum obtainable stomatal densities of several species implies genetic constraints to stomatal initiation rates. However the role of genetic diversity in plant responses amongst species is not often considered, despite it being shown to potentially have stronger links with stomatal density rates then [CO₂] concentration (Zhang et al. 2012).
Potentially, genotypic variation in stomatal responses to [CO₂] within natural populations could affect the composition and structure of plant communities over time and should stomatal density be a genetically constrained trait, natural plant communities may require extensive time periods to show clear anatomical responses to [CO₂] enrichment as responsive species, or those with lower stomatal densities, are filtered out of the natural plant populations. In this case, and despite the relatively minor increase in [CO₂], it may be possible to determine a significant reduction in stomatal initiation rates where none have been none have previously been detected.

Although interactive effects between factors influencing SD/SI are not unconsidered in FACE experiments, they are little understood in terms of their effects on long term stomatal responses particularly in the context of a broader, competitive environment. It is therefore the focus of this study to elucidate what precisely the effect of more than one potentially influential factor, specifically soil moisture content, in conjunction with elevated carbon dioxide is likely to have on stomatal initiation when applied to a species rich grassland ecosystem.
Materials and Methods

Site Description

The study site (°32'N and 8°41.3'E at an elevation of 172m above mean sea level) is located on the outskirts of Leihgestern, close to the city of Giessen in the federal state of Hesse, Germany. Situated on a flood plain of the Lückenbach rivulet, the site covers an area of 4.5 ha.

As of 1997, six of the most ecologically similar plots, from an original set of 16 previously monitored 100m² plots were selected as the locations for three ring pairs (three control rings and three CO₂ enriched rings, with each treatment being assigned to one ring per block at random. Blocks cover a slight soil moisture gradient caused by the variation in depth of a soil clay layer and ground water table.

The site is characterized by a mean annual temperature of 9.4°C and a mean annual precipitation of 575 mm (observation period: 1996 - 2005) Vegetation is classified according to the British National Vegetation Classification as an *Arrhenatheretum elatioris* Br.Bl. *Filipendula ulmaria* sub-community.

In total there are approximately 69 species present at the GiFACE site, the most numerous of them being grass species (Poaceae). These are followed by 40 non-leguminous herbs, five legumes, one species of rush (*Luzula campestris*) and one moss species (*Rhytidiadelphus squarrosum*).

*A. elatus, T. flavescens, G. mollugo* and *G. pretense* were examined from the total species pool for stomatal response (SI/SD) to elevated CO₂
Figure 2.1: Arial view of the university of Gissens Free-air CO₂ enrichment experiment denoting ambient/control rings (A) and elevated/treatment rings (E). Due to the presence of a slight soil moisture gradient at this particular site rings A1 and E1 are designated as dry (39% soil moisture content), A2 and E2 are designated as intermediate (41% soil moisture content) and A3 and E3 are designated as wet (46% soil moisture content).

CO₂ enrichment system

The installation of the FACE system was carried out in 1997 and CO₂ enrichment began in May 1998. CO₂ is elevated year-round during daylight hours (2 hours after sunrise - 2 hours before sunset) to +20 % of ambient conditions.

The FACE technique is based on the system developed by Brookhaven National Laboratory's in the late 1980’s and early 1990’s. The Giessen PlumeX system is characterized by a number of features including;

- A circular active plenum (CAP), incorporating 44 individually controlled intake and exhausting vent pipes
- A symmetrical layout, autonomous of any external blower enabling wind-direction independent CO₂ enrichment control

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• Regulation of the target CO₂ concentration with an electronic controller (PID), which leads to CO₂ concentrations of 20% above ambient at a height of 40 cm above ground level.

After installation CO₂ concentration was gradually increased to 20% above ambient in order to avoid a sudden step-increase and thus avoid any non-natural ecosystem response.

Since its inception the Giessen FACE experiment has provided continuous carbon dioxide enrichment at approximately 470 ppm for all but five days.
Sampling and Laboratory processing

Plant specimens were randomly harvested from the six FACE rings on the 21st of May 2010. Once harvested plants were dried and pressed before being stored in paper envelopes in a fume hood (extraction rate of 450m³/hour) at room temperature until samples could be processed. Five counts for each of ten leaves were counted for all four species at each of three ambient rings and three treatment rings. In total 1,100 counts were made for stomatal density and stomatal index at the Giessen FACE site. Stomatal index could not be determined for G. pretense and results presented in this chapter are of stomatal density values only for this species.
In the case of both *A. elatus* and *T. flavesens* leaves were placed in test tubes containing approximately 20mls of a 1:1 solution of CH$_3$COOH (Ascetic acid) and 30% H$_2$O$_2$ (Hydrogen peroxide). These tubes were then placed in a Koffermann labortechnik (TYP 3047) water bath at 70˚C for a period of approximately 2 hours or until leaf pigment was destroyed.

Once bleached, leaf samples were removed from test tubes, rinsed thoroughly in a petri dish with deionised water and placed on a slide where the abaxial epidermal layer was separated by carefully scraping away unwanted leaf material using a razor blade.

The lower epidermal layer was isolated, stained with the copper phthalocyanin dye C$_{47}$H$_{52}$CuN$_{14}$O$_6$S$_3$ (astra blue), separated in to five sections between the base and tip of the leaf and mounted in Kaisers glycerol-gelatin (Merck) on glass slides.

In the case of both *Gallium mollugo* and *Geranium pretense* nail varnish impressions were taken from abaxial leaf surfaces using clear nail varnish (Manhattan Cosmetics GMBH, Germany).

Nail polish was applied to the entire abaxial surface of *Gallium mollugo* leaves and left to dry for a period of approximately four hours before being removed, mounted on clear glass slides and covered with cover slips. Dried specimens of *Geranium pretense* were less amenable to nail varnish impressions due to distortions of the leaf incurred during pressing and drying of samples. Subsequently, it was necessary to soak leaf samples in glycerol and water overnight. On removal, the surfaces of the leaves were blotted dry using blotting paper and nail varnish was applied to five separate areas of the leaf surface. Impressions were removed after approximately one hour and again mounted on clear glass slides.
All prepared samples were observed using an Olympus BHS (BH2) light microscope. Stomatal density counts were made at X200 and Stomatal index was determined at X400. The area of the field of view was obtained in both cases using a Carl Zeiss optical scale slide (1/100mm) and counts were taken directly from the microscope using a Carl Zeiss cross hair ocular lens dividing the area of view into four sections.
Figure 2.3: Microscope Images of *A. elatius* (A1 and A2), *T. flavescens* (B1 and B2), *G. mollugo* (C1 and C2) and *G. pretense* (D1). 1 = x200 magnification. 2= x400 magnification. *A. elatius* and *T. flavescens* are stained and mounted cuticles prepared using chemical maceration. *G. pretense* and *G. mollugo* are nail polish impressions.
Results

Analysis of variance was performed on linear mixed effects models in R (R Core Team 2012). Stomatal densities and stomatal indexes were recorded for each species from three rings of ambient (400 ppm [CO₂]) concentrations and three enriched rings 480 ppm [CO₂] concentrations). Consequently the following model was applied:

\[ X_{ijkl} = \mu + \text{Treatment} + \text{Ring(Treatment)}_{k(i)} + \epsilon_{ikl} \]  

(Eq. 2.1)

Where \( X \) = measured variable (stomatal density or stomatal Index) recorded for each plant (l) per ring (k) under treatment (i), \( \mu \) is the overall mean and \( \epsilon \) represents the variability in either stomatal density or stomatal Index for plants within the same ring. In the above equation, ‘Ring’ is included as a random factor nested within ‘Treatment’.

The presence of a slight soil moisture gradient across the three ring pairs (Fig 2.1) may potentially be a confounding factor. However, insufficient replication at each level of soil moisture concentration did not allow for this additional, interactive factor to be comprehensively tested and results presented in this regard are indicative of the potential effects of soil moisture content only.

Interactive results presented were obtained using the following model;

\[ X_{ikl} = \mu + \text{Treatment} \times \%SMC + \text{Ring(Treatment)}_{k(i)} + \epsilon_{ikl} \]  

(Eq. 2.2)

All confidence intervals were set at the 0.95% level.
Linear mixed effects models revealed no significant effect of elevated [CO$_2$] on the stomatal densities or stomatal indices of any species (p > 0.05) (Fig 2.4, Table 2.1 and 2.2). Similarly, soil moisture content had no significant effect on either SD or SI (Fig 2.5, Table 2.3 and 2.4). Statistically, there was no significant interaction between [CO$_2$] treatment and soil moisture content (Fig 2.6, Table 2.5 and 2.6) although interaction plots indicated a tendency for both *A. elatius* and *T. flavescens* to reduce stomatal density in response to elevated [CO$_2$] under dry soil moisture conditions (39% SMC) and increase stomatal density in response to elevated [CO$_2$] under wet soil moisture conditions (46% SMC). This was not the case for the forb species where graphing indicated a tendency to increase SD/SI in response to elevated [CO$_2$] regardless of soil moisture content (Fig 2.6).
Figure 2.4: Stomatal density and stomatal index responses of all species to [CO$_2$] enrichment. In each case boxplots describe the median, first and third quartiles and standard deviations for all species grown under both ambient and elevated [CO$_2$]. Black dots indicate measured values which are significantly removed from the typical range of values.
Table 2.1: Mean and median values of stomatal density obtained for plants grown under ambient or elevated conditions at the Giessen FACE site. Standard errors are recorded in parenthesis alongside mean values and standard deviations are recorded in parenthesis alongside median values. \( F \) and \( P \) values are the results of a linear mixed effect model where stomatal density is a function of \([\text{CO}_2]\) expressed in ppm.

<table>
<thead>
<tr>
<th>Stomatal Density (mm(^{-2}))</th>
<th>Ambient ([\text{CO}_2] – 400 \text{ ppm})</th>
<th>Elevated ([\text{CO}_2] – 480 \text{ ppm})</th>
<th>([\text{CO}_2])</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean (SE) Median (SD)</td>
<td>Mean (SE) Median (SD)</td>
<td>( F )-value ( P)-value</td>
</tr>
<tr>
<td>A.elatius</td>
<td>37.6 (0.7) 37.0 (9.6)</td>
<td>34.7 (0.6) 35.6 (7.1)</td>
<td>0.9102 0.4408</td>
</tr>
<tr>
<td>T.flavescens</td>
<td>54.8 (1.5) 47.9 (18.6)</td>
<td>46.9 (1.2) 45.2 (14.2)</td>
<td>0.52484 0.5441</td>
</tr>
<tr>
<td>G.pratense</td>
<td>291.2 (4.0) 296.8 (49.4)</td>
<td>309.5 (4.8) 307.5 (59.2)</td>
<td>1.4508 0.3516</td>
</tr>
<tr>
<td>G.mollugo</td>
<td>155.1 (2.5) 152.4 (30.5)</td>
<td>155.9 (2.2) 155.1 (27.7)</td>
<td>0.0066 0.9426</td>
</tr>
</tbody>
</table>

Table 2.2: Mean and median values of stomatal index obtained for plants grown under ambient or elevated conditions at the Giessen FACE site. Standard errors are recorded in parenthesis alongside mean values and standard deviations are recorded in parenthesis alongside median values. \( F \) and \( P \) values are the results of a linear mixed effect model where stomatal index is a function of \([\text{CO}_2]\) expressed in ppm.

<table>
<thead>
<tr>
<th>Stomatal Index</th>
<th>Ambient ([\text{CO}_2] – 400 \text{ ppm})</th>
<th>Elevated ([\text{CO}_2] – 480 \text{ ppm})</th>
<th>([\text{CO}_2])</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean (SE) Median (SD)</td>
<td>Mean (SE) Median (SD)</td>
<td>( F)-value ( P)-value</td>
</tr>
<tr>
<td>A.elatius</td>
<td>11.6 (0.3) 11.6 (3.2)</td>
<td>10.4 (0.2) 10.5 (3.0)</td>
<td>0.67706 0.4971</td>
</tr>
<tr>
<td>T.flavescens</td>
<td>13.8 (0.3) 13.3 (4.0)</td>
<td>12.3 (0.3) 11.8 (3.6)</td>
<td>0.79203 0.4674</td>
</tr>
<tr>
<td>G.mollugo</td>
<td>14.8 (0.3) 14.3 (3.7)</td>
<td>15.9 (0.3) 15.9 (3.4)</td>
<td>0.8256 0.4595</td>
</tr>
</tbody>
</table>
Figure 2.5: Stomatal density and stomatal index responses of all species to soil moisture content. In each case boxplots describe the median, first and third quartiles and standard deviations for all species grown under both ambient and elevated [CO₂]. Black dots indicate measured values which are significantly removed from the typical range of values.
Table 2.3: Mean and median values of stomatal density obtained for each of the three ring pairs covering the soil moisture gradient at the Giessen FACE site. Standard errors are recorded in parenthesis alongside mean values and standard deviations are recorded in parenthesis alongside median values. $F$ and $P$ values are the results of a linear mixed effect model where stomatal density is a function of soil moisture content expressed in percentage terms (%SMC).

**Stomatal Density (mm$^{-2}$)**

<table>
<thead>
<tr>
<th>Plant</th>
<th>Dry [39%]</th>
<th>Intermediate [41%]</th>
<th>Wet [46%]</th>
<th>%SMC F-value</th>
<th>%SMC P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean</td>
<td>Median</td>
<td>Mean</td>
<td>Median</td>
<td>F-value</td>
<td>P-value</td>
</tr>
<tr>
<td><strong>A.elatius</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>40.3 (0.9)</td>
<td>39.7 (9.1)</td>
<td>33.8 (0.8)</td>
<td>34.2 (7.9)</td>
<td>34.3 (0.7)</td>
<td>35.6 (7.1)</td>
</tr>
<tr>
<td><strong>T.flavescens</strong></td>
<td></td>
<td></td>
<td></td>
<td>1.5688</td>
<td>0.3370</td>
</tr>
<tr>
<td>59.9 (1.9)</td>
<td>57.5 (19.6)</td>
<td>42.6 (0.9)</td>
<td>41.8 (8.8)</td>
<td>50.1 (1.6)</td>
<td>47.9 (16.1)</td>
</tr>
<tr>
<td><strong>G.pratense</strong></td>
<td></td>
<td></td>
<td></td>
<td>0.19512</td>
<td>0.7019</td>
</tr>
<tr>
<td>315.8 (5.1)</td>
<td>318.2 (51.6)</td>
<td>288.4 (4.4)</td>
<td>295.5 (44.9)</td>
<td>296.8 (6.4)</td>
<td>286.1 (64.3)</td>
</tr>
<tr>
<td><strong>G.mollugo</strong></td>
<td></td>
<td></td>
<td></td>
<td>0.5027</td>
<td>0.5518</td>
</tr>
<tr>
<td>162.3 (3.1)</td>
<td>171.1 (31.5)</td>
<td>146.2 (2.5)</td>
<td>147.1 (25.4)</td>
<td>158.1 (2.8)</td>
<td>160.4 (27.9)</td>
</tr>
</tbody>
</table>

Table 2.4: Mean and median values of stomatal index obtained for each of the three ring pairs covering the soil moisture gradient at the Giessen FACE site. Standard errors are recorded in parenthesis alongside mean values and standard deviations are recorded in parenthesis alongside median values. $F$ and $P$ values are the results of a linear mixed effect model where stomatal index is a function of soil moisture content expressed in percentage terms (%SMC).

**Stomatal Index**

<table>
<thead>
<tr>
<th>Plant</th>
<th>Dry [39%]</th>
<th>Intermediate [41%]</th>
<th>Wet [46%]</th>
<th>%SMC F-value</th>
<th>%SMC P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean</td>
<td>Median</td>
<td>Mean</td>
<td>Median</td>
<td>F-value</td>
<td>P-value</td>
</tr>
<tr>
<td><strong>A.elatius</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>13.1 (0.3)</td>
<td>13.8 (3.0)</td>
<td>10.1 (0.3)</td>
<td>9.9 (2.9)</td>
<td>9.9 (0.2)</td>
<td>9.8 (2.4)</td>
</tr>
<tr>
<td><strong>T.flavescens</strong></td>
<td></td>
<td></td>
<td></td>
<td>2.48978</td>
<td>0.2553</td>
</tr>
<tr>
<td>14.2 (0.4)</td>
<td>13.9 (4.4)</td>
<td>12.0 (0.3)</td>
<td>11.8 (3.0)</td>
<td>13.0 (0.4)</td>
<td>12.3 (3.9)</td>
</tr>
<tr>
<td><strong>G.mollugo</strong></td>
<td></td>
<td></td>
<td></td>
<td>0.12449</td>
<td>0.7579</td>
</tr>
<tr>
<td>15.9 (0.3)</td>
<td>16.0 (3.3)</td>
<td>14.2 (0.3)</td>
<td>14.0 (3.1)</td>
<td>16.0 (0.4)</td>
<td>15.7 (4.1)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.2018</td>
<td>0.6973</td>
</tr>
</tbody>
</table>
Figure 2.6: Interaction plots for [CO₂] and soil moisture concentration. Individual species plots describe the response in terms of both stomatal density (A) and stomatal index (B) to elevated [CO₂] at each level of soil moisture concentration (Dry = 39%, Intermediate = 41%, wet = 46%). %SMC values are annual means in this case.
Table 2.5: Mean values of stomatal density obtained for each treatment replicate. K1, K2 and K3 are the three ambient FACE rings and P1, P2 and P3 are the three elevated FACE rings at the Giessen field site. Standard errors are recorded in parenthesis alongside mean values. F and P values are the results of a linear mixed effect model where stomatal density is the consequence of the interaction between soil moisture content (%SMC) and [CO₂] with ring (replicate) considered as a random factor nested within treatment.

<table>
<thead>
<tr>
<th>Stomatal Density (mm⁻²)</th>
<th>Ambient [CO₂] – 400 ppm</th>
<th>Elevated [CO₂] – 480 ppm</th>
<th>[CO₂]* %SMC</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>K1</td>
<td>K2</td>
<td>K3</td>
</tr>
<tr>
<td>A. elatius</td>
<td>44.9 (1.1)</td>
<td>33.2 (0.9)</td>
<td>36.7 (1.0)</td>
</tr>
<tr>
<td>T. flavescens</td>
<td>17.8 (0.4)</td>
<td>12.2 (0.4)</td>
<td>12.3 (0.4)</td>
</tr>
<tr>
<td>G. pratense</td>
<td>311.2 (3.4)</td>
<td>287.6 (6.1)</td>
<td>290.3 (3.2)</td>
</tr>
<tr>
<td>G. mollugo</td>
<td>165.8 (3.6)</td>
<td>158.6 (3.1)</td>
<td>149.0 (3.4)</td>
</tr>
</tbody>
</table>

Table 2.6: Mean values of stomatal index obtained for each treatment replicate. K1, K2 and K3 are the three ambient FACE rings and P1, P2 and P3 are the three elevated FACE rings at the Giessen field site. Standard errors are recorded in parenthesis alongside mean values. F and P values are the results of a linear mixed effect model where stomatal index is the consequence of the interaction between soil moisture content (%SMC) and [CO₂] with ring (replicate) considered as a random factor nested within treatment.

<table>
<thead>
<tr>
<th>Stomatal Index</th>
<th>Ambient [CO₂] – 400 ppm</th>
<th>Elevated [CO₂] – 480 ppm</th>
<th>[CO₂]* %SMC</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>K1</td>
<td>K2</td>
<td>K3</td>
</tr>
<tr>
<td>A. elatius</td>
<td>14.4 (0.3)</td>
<td>9.8 (0.2)</td>
<td>11.1 (0.4)</td>
</tr>
<tr>
<td>T. flavescens</td>
<td>17.8 (0.4)</td>
<td>12.2 (0.4)</td>
<td>12.3 (0.4)</td>
</tr>
<tr>
<td>G. mollugo</td>
<td>15.8 (0.5)</td>
<td>14.9 (0.6)</td>
<td>13.8 (0.5)</td>
</tr>
</tbody>
</table>
Discussion

FACE experiments, with typical $[\text{CO}_2]$ enrichment levels of approximately double ambient, tend to show little or no changes in stomatal traits despite what are often prolonged treatment times. Results typically range from -10% to +10% in this regard and are usually highly species specific (Ainsworth & Rogers 2007). It has been considered that a minor increase in stomatal numbers as a response to stress maybe due to the fact that smaller stomata offer increased dynamic control over the gas exchange process particularly in response to drought conditions (Drake, Froend & Franks 2013). Despite there being some criticism as to whether smaller stomata are functionally superior to larger stomata (Raven 2014), it is at least conceivable that species may adopt a strategy of increasing stomatal numbers and deceasing stomatal size in order to increase functional control in response to moderate environmental stress.

Although we found no significant effect of soil moisture content on SD or SI for any of the species included in this study (Fig 2.5), there was observed a slight increase in median stomatal density and Index with decreasing soil moisture content (between 46% and 39%). The soil moisture gradient at this particular site is the result of a gradual slope in the terrain (2–3°) and variation in the depth of a subsoil clay layer (Keidel et al. 2014). Soil moisture conditions are comparatively homogeneous at this particular site and the slight gradient was likely insufficient to provoke any statistically significant change in stomatal initiation. However, results indicate that an increase in SD/SI in tandem with a moderate increase in SMC may be possible in a field environment.
Prolonged exposure to elevated \([\text{CO}_2]\) resulted in no statistically significant alteration in stomatal initiation (Fig 2.4). As reasonable comparisons for this experiment are relatively few (in that other experiments of this type were either run over a much shorter time scale, looked at different growth forms or functional types and/or used different levels of carbon dioxide enrichment) it is relatively difficult to contextualise results. However, a meta-analysis of plant responses to elevated carbon dioxide under FACE conditions (Ainsworth & Long 2005) demonstrated that within an elevated range of 14-33%, experimental studies showed no effect on either SD or SI for herb or grass species although there was a definite reduction in these parameters for tree and shrub species.

Results here are largely in step with those findings in that the overall response to treatment was negligible for both C3 grasses and forbs. However, despite the lack of statistical significance, grass species response to the combination of elevated carbon dioxide and soil moisture content indicated a possible tendency to reduce stomatal numbers under dryer soil moisture conditions and increase stomatal numbers under wetter soil moisture conditions. Interaction plots (Fig 2.6) indicated that the reaction of both \(T. \text{flavescens}\) and \(A. \text{elatius}\) to enrichment could depend on how \([\text{CO}_2]\) interacts with water stress influencing stomatal initiation to produce an overall morphological response although this was not the case for either \(G. \text{pretense}\) or \(G. \text{mollugo}\).

Increases in atmospheric carbon dioxide concentrations are demonstrably effective in reducing plant stomatal density and stomatal index in some studies (Woodward & Kelley 1995; Reid 2003; Lammertsma et al. 2011) results obtained from FACE experiments are, in general terms, not in agreement (Drake et al. 1997; Long et al. 2004; Ainsworth & Long 2005). Results of this trial are in agreement with other FACE findings and indicate that either
a 20% increase in [CO₂], in much the same way as elevations of up to approx. 600 ppm, is insufficient in provoking a developmental stomatal response amongst grassland species or that additional environmental factors act in unison to subdue or mask the [CO₂] effect. In conclusion, although FACE experiments are valuable tools in assessing the effects of CO₂ enrichment on stomatal initiation, the reaction of species to moderate levels of [CO₂] enrichment is likely to depend on how this one parameter interacts with other environmental pressures (in this case soil moisture content but undoubtedly multiple others) influencing stomatal initiation to produce an overall response.
Chapter 3
Species-specific acclimation of intrinsic water-use efficiency’s in responses to moderate free-air carbon dioxide enrichment

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Abstract

Atmospheric CO₂ ([CO₂]) concentrations have risen significantly since the early twentieth century and are expected to double by the year 2100. As the primary substrate in photosynthesis, variability in [CO₂] is vitally important and a thoroughly investigated area of plant science. Although significant, the implications of contemporary [CO₂] rise (1.49 ppm/yr since 1959), in terms of provoking developmental/acclimation responses amongst modern plant taxa remains widely debated. As part of a 17 year, free-air carbon dioxide enrichment (FACE) study in a semi-natural European grassland, we examined stomatal number (stomatal density (SD) and pore geometry (theoretical maximum stomatal conductance \( g_{\text{max}} \)) as well as photosynthetic and leaf gas exchange rates for five of the most dominant species (Arrhenatherum elatus, Triestum flavescens, Geranium pretense, Sanguisorbia officinalis, and Plantago lanceolata) in response to a moderate [CO₂] enrichment regime of
20% above ambient concentrations (480 ppm). Optimal photosynthetic and \( A_{\text{sat}} \) stomatal conductance \( (g_{\text{400ppm}}) \) rates were calculated under ideal conditions (i.e. saturating light concentrations, 25°C leaf temperature and constant vapour pressure deficits) using a CIRAS-2 portable photosynthesis system and intrinsic water-use efficiencies (IWUE) were recorded as the ratio of \( A_{\text{sat}}/g_{\text{400ppm}} \). Acclimation responses were assessed by recording the \( A_{\text{sat}}, g_{\text{400ppm}} \) and IWUE values under ambient (400ppm) [CO\(_2\)] concentrations for plants grown under both ambient and elevated [CO\(_2\)] concentrations at the FACE site, as well as at imposed [CO\(_2\)] concentrations of 200, 750, 1000 and 2000 ppm. We found no consistent differences in any recorded variables for plants growing under ambient or elevated conditions nor did we find any significant acclimation response in plants when both ambient and elevated grown plants were measured under ambient [CO\(_2\)] concentrations. We did however find significant differences in species IWUE values for species that had been grown under ambient and elevated FACE conditions at values above approx. 750 ppm. Consequently, it has been the finding of this study that certain species have acclimated to prolonged exposure to moderate [CO\(_2\)] enrichment at this particular site in a manner that enhances IWUE concentrations over ambient grown counterparts at [CO\(_2\)] concentrations above circa 750 ppm. To our knowledge, this is the first study to demonstrate physiological acclimations to such a minor level of [CO\(_2\)] enrichment, a finding with potentially considerable implications for plant functional responses, ecosystem processes and community structuring under the predicted global [CO\(_2\)] regimes of the future.

Keywords: acclimation, water-use efficiency, FACE, grassland, stomata
Introduction

Variability in atmospheric carbon dioxide ([CO$_2$]) concentrations has significant implications for growth and function and provokes a wide range of physiological and morphological responses in plants (Drake et al. 1997; Long et al. 2004; Ainsworth & Long 2005). Typically these responses, or combination of responses, are unique to individual plant species and vary depending on inherent traits such as photosynthetic pathways and intrinsic growth rates resulting in alterations to nutrient uptake capability (Zerihun, A., Bassirirad 2001; Ali et al. 2013), resource use efficiencies (Dijkstra et al. 2010), allocation and partitioning (Suter et al. 2002), morphological and anatomical modifications (Haworth et al. 2011a; Haworth, Elliott-Kingston & McElwain 2013) and gaseous exchange rates (Ainsworth & Long 2005; Taylor et al. 2012).

In terms of response type, it may be considered that there are three principles. Primary plant responses are considered in this instance to be any immediate change in growth or physiological function resulting from a change in the growth environment. Acclimation responses, which are the result of long term exposure, typically result in alterations to resource allocation patterns, often with implications for leaf photosynthetic and gas exchange rates (Moore et al. 1999; Anderson et al. 2001; Buckley 2008). Adaptive responses, which require a genetic alteration and are typically characterised by permanent, hereditary changes, have rarely been demonstrated in response to contemporary [CO$_2$] rises.

The differential nature of these responses between species presents a significant challenge in assessing the likely effects of global [CO$_2$] rise not only on plant functioning generally but also in terms of alterations in community composition, underlain by changes in the population
dynamics of individual species and driven by variability in plant responses to environmental change.

As an approach to assessing the implications of CO₂ enrichment, free-air carbon dioxide enrichment experiments (FACE) may be considered a powerful tool in analysing plant responses to variable concentrations of [CO₂], alleviating or removing some of the potential limitations imposed by closed chamber experiments. Fully enclosed systems have been known to amplify the down regulation of photosynthesis in a number of cases and suppress plant responses to abiotic treatments (Sage 1994; Leakey et al. 2006, 2009; Ainsworth et al. 2008). Additionally, through an impaired capacity to comprehensively match the conditions of a natural environment, experiments may be hampered by the added complication of a chamber effect which may influence plant responses to elevated [CO₂] (Van Oijen et al. 1999), potentially leading to findings which are non-representative of those obtained from plants grown in natural systems. Consequently, it has become an important step in global change biology to reassess some of the more significant findings obtained in chamber trials in the context of more complex ecological systems under more realistic environmental conditions.

Despite the advantages in using FACE techniques to track plant responses to environmental change, there are still certain experimental issues which may potentially confound our understanding of results obtained thus far. One such factor is the propensity to impose sudden and large environmental perturbations on species and/or communities. It has been demonstrated that different rates of environmental change can affect adaptive outcomes and functional dynamics when imposed over multiple generations (Klironomos et al. 2005b) and that slower rates of environmental change result in positive adaptations acquired at a lower
cost compared with those forced to adapt to a sudden large change of equal magnitude (Collins, de Meaux & Acquisti 2006; Parton et al. 2007; Collins & de Meaux 2009).

While there are a number of long running FACE experiments (in excess of ten years), there are none that have gradually increased $[\text{CO}_2]$ concentrations in minor increments over multiple generations. Consequently, while there are a relatively large number of trials which may be sufficient to elucidate primary or acclamatory plant responses to environmental change, there are none which allow for the study of adaptive outcomes in response to minor $[\text{CO}_2]$ changes. Ultimately, the magnitude of environmental change and there effects on long term ecological processes could have serious implications for plant functioning under increased $[\text{CO}_2]$ and require extensive experimental time to evaluate (de Graaff et al. 2006).

Photosynthetic acclimation in responses to long term $[\text{CO}_2]$ enrichment has perhaps has been one of the most significant findings of FACE experiments to date. As $[\text{CO}_2]$ is currently a limiting substrate in the photosynthetic process, particularly amongst C3 species, the consequences of increasing $\text{CO}_2$ availability primarily depends on how photosynthesis may acclimate in responses to long term $[\text{CO}_2]$ enrichment. In contrast to with earlier chamber trials, where long term exposure was often seen to cause a downregulation in photosynthetic rates, FACE trials demonstrated that in the majority of cases, reduction in the enzymatic constituents of the carbon reduction cycle led to an increased nutrient and water use efficiency and, significantly, a general increase, in overall photosynthetic rates (Long et al. 2004). Despite this crucial finding however, little is known in terms of photosynthetic acclimation in response to incremental change and/or in response to minor stepped changes in $[\text{CO}_2]$. 
Evidence for an independent acclimation in operational stomatal conductance ($g_{s(opp)}$) to elevated [CO$_2$] independent of any photosynthetic acclimation is minimal and it is generally considered that stomatal sensitivity to elevated [CO$_2$] is preserved over time (Long et al. 2004). However loss of stomatal function has been observed in a number of cases, particularly in chamber trials, where it is considered that the build-up of non-structural carbohydrates in guard cells, which is typically a consequence of limitations in resource partitioning, interferes with membrane depolarisation potential, potentially resulting in impaired stomatal functioning, particularly in response to varying [CO$_2$] (Vavasseur & Raghavendra 2005; Easlon & Richards 2009) although the exact mechanism for this is currently debated. As atmospheric [CO$_2$] rates rise, CO$_2$ concentrations within the leaf also increase driving stomatal closure. However, in circumstances where stomatal functioning has become impaired an alternative approach to control gas exchange rates and improve resource use efficiencies is available via a reduction in stomatal numbers (Haworth et al. 2013).

As the number and geometry of the stomatal pores ultimately determine the maximum rate of leaf gas exchange it is expected that a reduction in overall gas exchange rates could act as a driver for the reduction in the absolute number of stomatal pores across the leaf surface. A reduction in stomatal initiation is a common finding particularly in fossil and herbarium studies which indicate a consistent inverse relationship between atmospheric carbon dioxide concentrations and stomatal densities. However, FACE studies which operate over a much shorter time frame by comparison, report very inconsistent findings in this regard with species often displaying an increase (Zhou et al. 2013), a decrease (Lammertsma et al. 2011) or no change at all (Field et al. 2015). Again it may be that restrictive time frames are
insufficient to allow for complete acclimation to new environmental conditions or that a sudden stepped increase in [CO₂] provokes an anomalous response in at least some cases.

As [CO₂] rises and photosynthetic rates increase potentially the most crucial goal for plants and communities is to maximise efficiencies as resource availability becomes increasingly limited. In reducing $g_{[\text{opp}]}$, plants loose less water and, provided there is no down regulation in photosynthetic rates in equal proportion, for an equal or greater amount of carbon assimilated. Resource use efficiencies are likely to contribute strongly to relative fitness (Godoy, Valladares & Castro-Díez 2012; Heberling & Fridley 2013) and therefore are potentially key in assessing potential biogeographic distributions in the face of global environmental change.

Here we set out to determine the effects of moderate [CO₂] enrichment in a competitive, semi-natural grassland community particularly on photosynthetic or intrinsic water-use efficiency (IWUE). Via the gradual introduction and continued maintenance of a 20% increase in [CO₂], plants at this FACE site have effectively been subjected to two different [CO₂] enrichment rates, the current global trend (represented by ambient or control plots) and an increased rate (represented by treatment or CO₂ enriched plots (Jager et al. 2003). Consequently we aim to assess physiological plant responses under FACE conditions in response to two long term enrichment rates and specifically test the hypothesis that long term exposure to moderate [CO₂] enrichment provokes acclimation responses amongst grassland community species, a relatively unique context in terms of [CO₂] enrichment experiments. Responses are assessed in terms of IWUE, $A_{\text{sat}}$, (Photosynthetic rate under optimal environmental conditions) $g_{[\text{opp}]}$, (operational stomatal conductance measured under
optimum environmental conditions) and $g_{\text{max}}$ (maximum theoretical stomatal conductance determined by specific geometrical pore traits)

**Materials and Methods**

**FACE site and CO$_2$ enrichment system**

See chapter 1 – materials and methods

**Gas exchange measurements and species selection**

Leaf gas exchange measurements were conducted using a CIRAS-2 portable photosynthesis system and PLC (6) cuvette attachment (PP-Systems, Amesbury, MA, USA). In this case a combination of cuvette head plate attachments ($4.5 \text{ cm}^2$, $2.5 \text{ cm}^2$ and $1.29 \text{ cm}^2$) were used in order to maximise the leaf area available for measurements while also reducing the amount of uncovered window space in the cuvette head. All gas exchange measurements were taken between 09:00 and 12:00 in the field and in all cases conditions in the cuvette head were set to maintain VPD (vapour pressure deficit) below 12 mb (1.2 kPa), Leaf temp at 22°C, CO$_2$ concentration at either 400 or 480 ppm and air flow through the cuvette at 200 ml min$^{-1}$.

Initially, photosynthetic irradiance curves (Pn/I) were run in order to establish the saturating light for photosynthesis. In this case, Pn (photosynthetic rate) was allowed to settle at maximum PAR (photosynthetically active radiation) of 2000 µmol (photon) m$^{-2}$ s$^{-1}$ before applying a sequence of light settings (1,600, 1,200, 1,000, 800, 600, 400, 200, 100, 50, and 0) with an imposed minimum time step of 120 s at each at each set point. Light saturated photosynthetic rate ($A_{\text{sat}}$), was then calculated using the methods of Norman, Welles &
Stomatal Responses to Biotic and Abiotic Stress

Chapter 3

McDermitt (1991). Pn/I curves were carried out on two plants for every species in every treatment (giving a total of eight light curves per species) and the maximum $A_{\text{sat}}$ value recorded for each species was then used as the set PAR value for all other gas exchange measurements. In application of the above cuvette conditions, both $A_{\text{sat}}$ and stomatal conductance ($g_{s(opp)}$) were recorded as spot measurements under optimal conditions from three plants per species in all treatments, with measurements taken from at least three leaves per plant. For each species, recordings where taken after both $A_{\text{sat}}$ and $g_{s(opp)}$ had reached steady state under cuvette conditions (approx. 30 mins).

Changes in Intrinsic water use efficiency values where measured by imposing a series of stepped increases in [CO$_2$] concentrations. Leaves clamped by the cuvette were allowed to settle until stomatal conductance stabilised at 400ppmv CO$_2$ and once measurements were recorded at this initial concentration, a series of step changes were imposed (200, 400, 750, 1000 and 2000 ppm). At each step $A_{\text{sat}}$ and $g_{s(opp)}$ were allowed to reach steady state before physiological measurements were recorded.

Intrinsic water use efficiency (IWUE) was calculated from gas exchange of CO$_2$ and H$_2$O as the ratio of light saturated CO$_2$ assimilation ($A_{\text{sat}}$) over optimal stomatal conductance ($g_{s(opp)}$).

All measurements were carried out on the youngest, fully expanded leaves of herbaceous forbs and the flag leaf of grass tillers.

Species examined in this study include *Arrhenatherum elatus*, *Triestum flavescens*, *Geranium pretense*, *Sanguisorbia officinalis*, and *Plantago lanceolata*, all of which are amongst the most dominant grass and herb species found at the Giessen FACE site.
Laboratory processing and stomatal morphological measurements

See chapter 1 – materials and methods for laboratory processing protocol.

Calculation of theoretical maximum stomatal conductance ($g_{\text{max}}$) was carried out according to the protocol of McElwain, Yiotis & Lawson (2015). For all species the following formulae was applied to stomatal data recorded on the abaxial leaf surface only except in the case of $T. flavescens$ where the adaxial surface was used;

$$ g_{\text{max}} = \frac{d \cdot v \cdot SD \cdot p a_{\text{max}}}{p d + \frac{\pi}{2} \sqrt{pa_{\text{max}}/\pi}} $$

(Eq. 3.1)

Where $d = \text{diffusivity of water vapour at } 25^\circ C$ (0.0000249 m$^2$ s$^{-1}$), $v = \text{molar volume of air} (0.0224 m^3 \text{ mol}^{-1})$, $SD = \text{stomatal density} \ (m^2)$. As it was not possible to determine the precise pore depth for the species assessed in this study, pore depth was calculated using a range of possible values (beta functions) from a fully circular pore (where the diameter is considered to be equivalent to the width of an inflated, fully turgid guard cell (Franks & Beerling 2009a; b)) to an ellipse (where diameter is equal to 20% of the pore length). In total the average diameter was calculated from five separate beta functions (100%, 80%, 60%, 50%, 40%, 20%) in the determination of the function $pa_{\text{max}}$ or maximum stomatal pore area (m$^2$).
Data Analysis

Data analysis for spot measurements of \( A_{\text{sat}}, \ g_{\text{sopp}}, \ \text{IWUE} \) and \( g_{\text{max}} \) was carried out using the same linear mixed effects models as chapter 2 (Eq 2.1).

Changes in IWUE values \( (A_{\text{sat}}/g_{\text{sopp}}) \) with stepped increases in \([\text{CO}_2]\) concentrations were carried out firstly to determine whether plants responded significantly to increasing \([\text{CO}_2]\) through application of the following linear model equation;

\[
X_{ikl} = \mu + \text{CO2R}_{ki} + \epsilon_{ikl}
\]

(Eq. 3.2)

Where \( X \) = measured variable \( (A_{\text{sat}}, \ g_{\text{sopp}}, \ \text{IWUE} \) and \( g_{\text{max}} \) recorded for each plant \( (l) \) for each step of \([\text{CO}_2]\) reference concentration \( \text{CO2R} \) \((k) \) under treatment \( (i) \), \( \mu \) is the overall mean and \( \epsilon \) represents the variability in either \( A_{\text{sat}}, \ g_{\text{sopp}}, \ \text{IWUE} \) and \( g_{\text{max}} \) for plants at the same \( \text{CO2R} \).

ANOVA testing was then carried out on linear model results to determine whether response to stepped changes differed significantly between plants grown under ambient or elevated FACE conditions.

Kruskal-Wallis tests were carried out at each individual \( \text{CO2R} \) value obtained from step change analysis (200, 400, 750 etc) to determine significant differences at each point.

As previously, all data analyses was carried out using in R (R Core Team 2012)
Results

Responses in IWUE to stepped increases in [CO₂] from 200 to 2000 ppm (Fig 3.1) revealed species specific response differences between plants grown at either ambient (400 ppm) or elevated (480 ppm) [CO₂] at the Giessen FACE site. Both A. elatius and T. flavescens (Fig 3.1, A and B) were relatively strongly affected by stepped increases in [CO₂] concentrations above ambient levels with linear models revealing a significant response to increasing [CO₂] (P<0.05) in addition to significant differences between responses of plants grown at ambient and elevated concentrations (P<0.05). A. elatius plants growing under elevated [CO₂] showed an enhanced response (i.e. a greater increase) in IWUE values compared to plants growing at ambient [CO₂]. Kruskal-Wallis tests indicated significant differences in IWUE values at 200, 400, 1000 and 2000 ppm) between elevated and ambient grown specimens of A. elatius. At imposed [CO₂] concentrations of 200 and 400 ppm ambient grown plants exhibited significantly higher IWUE values while at 1000 and 2000 ppm plants grown under elevated [CO₂] had improved IWUE’s. Results imply that prolonged exposure of A. elatius to elevated [CO₂] has led to a reduced ability to increase IWUE at ambient and sub-ambient [CO₂] concentrations, as compared with control plants, but an enhanced capacity to increase IWUE with increasingly elevated [CO₂]. T. flavescens, despite showing significant responses in IWUE values to increasing [CO₂] (P<0.05) of both ambient and elevated grown plants, displayed opposing results to those obtained for A. elatius. In this instance linear modelling revealed significant differences in response to step changes between plants grown under ambient and elevated [CO₂] concentrations (P<0.05), however, species grown under elevated concentrations showed an impaired capacity to increase IWUE values with increasingly elevated [CO₂]. Statistically, IWUE values were significantly different between
ambient and elevated grown plants at 750, 1000 and 2000 ppm while no significant differences were observed at 400 or 200 ppm indicating that plants growing under elevated [CO₂] in the field maintained IWUE responses to low [CO₂] at the expense of a lost capacity to increase IWUE with elevated [CO₂].

Increases in IWUE with increasing step changes in [CO₂] did not differ significantly at any individual level of enrichment between _G. pratense, S. officinalis, P. lanceolata_ (Fig 3.1, C, D and E) and grown at either ambient or elevated [CO₂] concentrations (P>0.05) with few exceptions. However, linear models did imply that responses between ambient and elevated grown plants may be different as significant differences were returned for all three species in this regard (P<0.05). Despite this, IWUE responses to elevated [CO₂] concentrations were less severe than observed for either of the grass species _A. elatius_ and _T. flavescens_.

Figure 3.1: Response in median IWUE values to step changes in [CO₂] between 200 and 2000ppm. Boxplots show medians, 1st and 3rd quartiles.
Spot measurements (fig 3.2 and 3.3) revealed that differences in stomatal density, $g_{\text{max}}$, $g_{\text{opp}}$, $A_{\text{sat}}$ and IWUE were not statistically significant between plants grown at ambient and elevated [CO$_2$] concentrations at the Giessen FACE site, barring minor exceptions (Fig 3.2 and 3.3). These included an increase in the photosynthetic rate of *A. elatius* when grown under elevated [CO$_2$] and a decrease in the photosynthetic rate of *P. lanceolata* (Fig 3.2 and Table 3.1). *P. lanceolata* and *G. pratense* both exhibited significantly reduced $g_{\text{opp}}$ when grown at 480 ppm however the changes in $A_{\text{sat}}$ and $g_{\text{opp}}$ significantly affected the IWUE of *A. elatius* only (Fig 3.2 and Table 3.1). In terms of morphology, only *T. flavescens* showed a significant increase in $g_{\text{max}}$ under elevated [CO$_2$] although stomatal density was not significantly altered for this, or any other species examined (Fig 3.3 and Table 3.1). In terms of % change in median values, a decrease in $A_{\text{sat}}$ and a decrease in $g_{\text{opp}}$ was observed (Fig 3.2 and Table 3.1). Exceptions to this general trend were *A. elatius*, which showed an increase in assimilation rates at 480ppm relative to 400ppm, and *S. officinalis*, which showed an increase in $g_{\text{opp}}$ at 480 ppm relative to 400 ppm (Fig 3.2). % changes in IWUE’s indicated that grasses responded to a greater extent (approx. 25% increase in IWUE at 480 ppm [CO$_2$] on average), while forbs, by comparison, showed relatively minor adjustments with an average decrease in IWUE for all three species of approx. 1% after prolonged exposure to elevated [CO$_2$] (Fig 3.2). Typically, morphological stomatal responses (stomatal density and $g_{\text{max}}$), were smaller in magnitude than physiological responses ($g_{\text{opp}}$) when all species are considered. A 6% and 5% increase was observed respectively for stomatal density and $g_{\text{max}}$ (Fig 3.3), while a 15% decrease was observed in $g_{\text{opp}}$ (Fig 3.2). Further, % changes in median stomatal density and $g_{\text{max}}$ values did not appear to relate well to changes in $g_{\text{opp}}$ with *A. elatius*, *T. flavescens*, *S. officinalis*, and *P. lanceolata* all displaying a directionally opposite response to those recorded for $g_{\text{opp}}$. *G. pratense* was the only species
to show a decreased $g_{s[opp]}$ with a corresponding decrease in $g_{\text{max}}$ (Fig 3.2 and 3.3) between species grown under ambient and elevated field conditions. Although the relationship between theoretical maximum stomatal conductance ($g_{\text{max}}$) and operational stomatal conductance ($g_{s[opp]}$) is sometimes considered well conserved in response to [CO$_2$] change (Taylor et al. 2012; McElwain et al. 2015), we find no such correlation here, where results imply that the moderate increase in [CO$_2$] may have led to a decoupling of these responses.
Figure 3.2: Median $A_{sat}$, $g_{s(opp)}$ and IWUE values for plants growing at either ambient (black) (400 ppm) or elevated (grey) (480 ppm) [CO$_2$] concentrations at the Giessen FACE site. % changes describe the change in median values at 480ppm relative to 400ppm.
Figure 3.3: Median stomatal density and $g_{\text{max}}$ values with associated standard deviations plants growing at either ambient (black) (400 ppm) or elevated (grey) (480 ppm) [CO$_2$] concentrations at the Giessen FACE site. % changes describe the change in median values at 480ppm relative to 400ppm.
Table 3.1: Median values of $A_{sat}$, $g_{s(opp)}$, IWUE and $g_{\max}$ obtained from spot measurements of plants grown under ambient (400 ppm) or elevated (480 ppm) conditions at the Giessen FACE site. Standard deviations are recorded in parenthesis alongside median values. Significant values were obtained using a linear mixed effect model where $A_{sat}$, $g_{s(opp)}$, IWUE and/or $g_{\max}$ is a function of [CO$_2$] expressed in ppm. Significant interactions between treatments are highlighted in red.

<table>
<thead>
<tr>
<th></th>
<th>$A_{sat}$</th>
<th>$g_{s(opp)}$</th>
<th>IWUE</th>
<th>$g_{\max}$</th>
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<td></td>
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<td>Elevated</td>
<td>Ambient</td>
<td>Elevated</td>
</tr>
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<td>14.6 (1.8)</td>
<td>263.5 (93.9)</td>
<td>240.0 (74.8)</td>
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<td>10.2 (4.5)</td>
<td>334.0 (72.8)</td>
<td>241.5 (47.5)</td>
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<td>17.2 (5.5)</td>
<td>814.5 (476.9)</td>
<td>626.0 (252.0)</td>
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<tr>
<td>P. lanceolata</td>
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<td>12.7 (4.4)</td>
<td>711.5 (240.6)</td>
<td>523.0 (239.6)</td>
</tr>
<tr>
<td>S. officinalis</td>
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<td>18.6 (3.2)</td>
<td>462.01 (84.2)</td>
<td>512.0 (134.1)</td>
</tr>
</tbody>
</table>
Discussion

The aim of this study has been to determine whether or not plant species have acclimated to growth under prolonged, moderate [CO$_2$] enrichment and findings in this case indicate that acclimations, in terms of intrinsic water-use efficiency, can occur even at relatively conservative levels of [CO$_2$] enrichment.

In natural, competitive ecosystems plant productivity is heavily influenced by the amount of available water and the efficiencies of plant species in using that available water supply. As global [CO$_2$] concentrations continue to rise the pressure to balance increased uptake of CO$_2$ with a reduction in water loss will be critical to success in terms of productivity and distribution and is consequently essential to our predictions of plant functioning in the context of global climate change.

In this study, exposure of individual species to increasingly elevated [CO$_2$] concentrations revealed differences in responses between grass species grown at both ambient and elevated [CO$_2$] under field conditions. _A. elatus_ evidently benefitted from growth under elevated [CO$_2$] as plants displayed an enhanced capacity to increase IWUE as [CO$_2$] was increased step-wise from 200 to 2000 ppm over plants grown at ambient [CO$_2$] (Fig 3.1). Results in this instance are typical of an acclimation in the enzymatic activity of Rubisco (Drake _et al_. 1997; Long _et al_. 2004). Often plants which display Rubisco-limited photosynthetic capacity at ambient [CO$_2$] have acclimated to become less limited by RUBP-regeneration at higher [CO$_2$] concentrations. As [CO$_2$] increases and RUBP-regeneration becomes an increasingly limiting factor in photosynthetic rates, species or functional groups which are less constrained in this regard often display an increased stimulation in both $A_{sat}$
and IWUE at the cost of lower rates under ambient or sub-ambient conditions. *T. flavescens* however, despite showing marginally higher rates of IWUE at 200 and 400 ppm over plants grown at 480 ppm, showed a decreased ability to increase IWUE at [CO₂] concentrations above ambient levels (Fig 3.1). Results in this instance indicate a possible maladaptation to growth under elevated [CO₂]. Maladaptation in this case meaning that species grown under elevated conditions showed an impaired ability to increase IWUE when exposed to stepped increases in [CO₂] above ambient levels as opposed to species which had been grown under elevated conditions at the Giessen FACE site.

None of the forb species showed significant differences in IWUE values between plants grown at either ambient or elevated [CO₂] concentrations in terms of their ability to respond to step changes in [CO₂] (Fig 3.1). There was an observed division between functional groups as grasses displayed greater sensitivity to elevated [CO₂] than forbs. This division has been recorded in FACE studies before in terms of \( g_{\text{d[opp]}} \) (Ainsworth & Long 2005; Ainsworth & Rogers 2007) and resource use efficiencies (Drake et al. 1997; Anderson et al. 2001; Lee et al. 2001). It would appear that the increased IWUE of grass species may also be linked with increased biomass under elevated [CO₂] as previous research has demonstrated an increase in yield across all grass species following [CO₂] enrichment (Kammann et al. 2005)

In the absence of any sink restrictions, step change responses should give a reasonable approximation of plant physiological behaviour, in terms of \( A_{\text{sat}} \) and \( g_{\text{d[opp]}} \), under various [CO₂] regimes. This is perhaps borne out by the fact that spot measurements are largely reflective of step change measurements in terms of IWUE. Species showing an ability to increase IWUE with stepped increases in [CO₂], i.e. *A. elatius* (Fig 3.1), tended to show an
increase in IWUE when measured under growth [CO₂] concentrations (Fig 3.2), with the exception of *T. flavescens*. Although plant responses to moderate [CO₂] enrichment may be difficult to detect in absolute terms, it is probable, based on results presented here, that acclimation and possibly adaptations are already underway enabling certain species to potentially thrive under the predicted high [CO₂] regimes of the near future (IPCC 2014).

Generally, results presented here are typical of those broadly observed in FACE studies (Ainsworth & Long 2005). In terms of percentage changes, we note that there was a slight increase of 9% in IWUE values and a 15% decrease in g₄[opp] when averaged across all species considered in this trial. However, we found that the 20% increase in [CO₂] had little or no effect on photosynthetic rate overall as there was a 0% change across all five species. Similarly, we found a very slight increase in stomatal density (6%) and g₉₅₅ (5%). Again, a lack of response or a tendency towards a slight increase in stomatal densities is a relatively common response of plants exposed to elevated [CO₂] under FACE conditions (Reid 2003; Ainsworth & Rogers 2007). The net effect of a 0% change in photosynthetic values is a relatively uncommon finding as the removal of sink restrictions imposed in pot based experiments should allow for photosynthetic stimulation in response to prolonged [CO₂] enrichment, provided no other restrictions are imposed (Garcia *et al.* 1998; Ainsworth 2003). However, we note that a decrease in assimilation rates was observed for all species bar *A. elatius* indicating that community level changes are likely to be driven by a proportion of species for which the effects of [CO₂] enrichment will be felt most strongly. Based on our findings we conclude that the nature of acclimations under elevated [CO₂] is species specific and that a comprehensive review of individual species responses are crucial to understanding fully the implications of [CO₂] rise. Further the fact that minor [CO₂] enrichment had seemingly an opposing effect on assimilation rates to those commonly observed in
FACE studies, where enrichment levels are typically in the region of 550-600 ppm, continuous, gradual increases in [CO$_2$] may affect plant acclimation responses differently to sudden, large changes (Kopp & Hermisson 2007). In that sense, ongoing, persistent environmental change may significantly influence future acclimation responses although to date there is very little evidence to support this and an incomplete understanding of the mechanisms which drive responses to chronic rather than acute environmental change. In the vast majority of cases, both short term and long term experiments are typically carried out by exposing plants or plant populations to sudden and severe disturbances after which plants are allowed to exhibit either acclamatory or adaptive responses. By contrast, few studies have assessed the effects of constantly changing environments on adaptive outcomes. However, where these outcomes have been assessed some studies have demonstrated that adaptation to slower rates of environmental change result in fitter populations relative to those adapted to sudden environmental change (Collins et al. 2006; Collins & de Meaux 2009). The implication of such studies therefore is that acclimation occurs optimally in response to relatively small alterations in environmental change and large increases, such as a near doubling of [CO$_2$], may impede adaptation/acclimation amongst plant populations.

Variability in $g_{\text{d(opp)}}$ and $A_{\text{sat}}$ responses between species led to discrepancies in IWUE values, although results indicate that grass species may be more responsive to elevated [CO$_2$] in this regard than forbs (Fig 3.1 and 3.2).

Despite the lengthy duration of [CO$_2$] enrichment at this site there was no definitive response in terms of stomatal density or $g_{\text{max}}$. *A. elatus*, *T. flavescens*, and *P. lanceolata* all showed slight increase in these values, while *G. pratense* and *S. officinalis* tended to show a slight
decrease. Overall there was an observed 6% increase in stomatal density and a 5% increase in $g_{\text{max}}$. These results fit well with other studies which report a general tendency of FACE studies to provoke increased stomatal densities (Reid 2003). Why this should be the case however is unclear as studies assessing the density responses of fossil plant leaves to variability in [CO$_2$] over geological time typically show decreasing densities with increasing [CO$_2$] (McElwain & Chaloner 1995a; Beerling, McElwain & Osborne 1998; Royer 2001) as do altitudinal studies comparing plants grown between differing [CO$_2$] partial pressures (Woodward, F. I., & Bazzaz 1988; McElwain 2004) as well as studies of herbarium samples examining anthropogenic [CO$_2$] rise (Lammertsma et al. 2011). Increasing stomatal density and decreasing stomatal size in response to moderate levels of environmental stress have been noted before and may offer increased regulatory capacity over the gas exchange process. As environmental stress becomes progressively worse however stomatal densities ultimately decrease (Xu & Zhou 2005). As [CO$_2$] enrichment is relatively mild at this particular field site this may be a possible explanation for the change in stomatal density.

Although results presented here are largely typical of results obtained from FACE studies in general, acclimation responses have never been demonstrated in response to such moderate levels of [CO$_2$] enrichment. In conclusion, through the implementation of unique and comprehensive experimental protocols we have demonstrated clear, species specific acclimations to prolonged [CO$_2$] exposure, a finding with potentially profound implications for plant functional responses, ecosystem processes and community structuring under the predicted global [CO$_2$] regimes of the future.
Chapter 4
Heritable variation in the gas exchange traits of grassland species in response to moderate free-air carbon dioxide enrichment

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Abstract

Atmospheric CO₂ ([CO₂]) concentrations significantly alter developmental plant traits with potentially far reaching consequences for ecosystem function and productivity. Over the course of Earths pre-history [CO₂] has varied widely from lows of less than 200 ppm to highs of over 3000 ppm and is often considered to be an important driver of speciation and evolutionary change. However, contemporary evolutionary responses amongst extant plant species that coincide with modern, anthropogenically driven [CO₂] rise have not, as yet, been demonstrated. Here we present findings from a long term, free-air carbon dioxide enrichment (FACE) study in a semi-natural European grassland ecosystem.

Here we use University College Dublin’s growth chamber facility in order to perform a reciprocal swap experiment in which the offspring of plants established under elevated FACE
conditions were grown at ambient [CO$_2$] concentrations while the offspring of those grown under ambient field conditions were grown under elevated [CO$_2$] concentrations. For each of five species (Arrhenatherum elatus, Triestum flavescens, Geranium pretense, Sanguisorbia officinalis, and Plantago lanceolata) we demonstrate a heritable capacity of plants grown under elevated [CO$_2$] concentrations, to adapt physiological rates of light saturated photosynthesis ($A_{sat}$), stomatal conductance ($g_{s}$) and intrinsic water-use efficiencies (IWUE) over ambient grown counterparts at.

Not all species observed in this study showed any additional physiological capacity to improve IWUE with growth under elevated [CO$_2$] however, indicating a differential capacity to adapt to increasing [CO$_2$] between species at this particular FACE site.

Keywords: contemporary evolution, heritability, water-use efficiency, FACE, grassland, stomata
Introduction

The ability to modulate gaseous exchange via the stomatal aperture has provided a means by which plants may adapt to inhabit almost any environment. Consequently, the processes that govern guard cell responses to environmental stimuli and the anatomical, morphological and physiological responses that are driven by both biotic and abiotic pressures are an important and thoroughly investigated area of plant biology. Of particular relevance is how plants will adapt gas exchange rates in response to increasing atmospheric carbon dioxide \( \text{[CO}_2 \text{]} \) concentrations. Alterations in this particular atmospheric gas have had profound effects on plant adaptation and evolution in the past (Raven, Cockell & De La Rocha 2008; Brodribb 2009; McElwain, Wagner & Hesselbo 2009; Haworth, Elliott-Kingston & McElwain 2011b; Leakey & Lau 2012) and are of significant consequence to our understanding of how extant plant species are likely to respond and adapt to the effects of future climate change.

However, despite the influence of \([\text{CO}_2]\) as a driver of plant evolution in the past, there remains little evidence that modern day plant taxa have or are developing evolutionary responses or adaptations to contemporary \([\text{CO}_2]\) increases (Leakey & Lau 2012). Yet, growth at elevated \([\text{CO}_2]\) concentrations has often been shown to provoke morphological (McElwain & Chaloner 1995b; Woodward & Kelly 1995; Royer 2001) and physiological (Drake et al. 1997; Ainsworth & Long 2005) stomatal acclimation responses which limit water loss, maximise carbon acquisition, increase photosynthetic rate and increase water-use efficiencies through increasing diffusional resistance (Ainsworth & Rogers 2007).

Acclimation responses to increased \([\text{CO}_2]\), specifically those associated with leaf gas exchange rates, have been demonstrated in numerous studies and are typically the result of
long term exposure to elevated $[\text{CO}_2]$, often resulting in alterations to resource allocation patterns which directly influence leaf photosynthetic and gas exchange rates (Anderson et al. 2001; Rogers & Ellsworth 2002; Chen 2005; Lee, Barrott & Reich 2011). Despite the large number of studies which have described acclimation responses to elevated $[\text{CO}_2]$ there are also some studies which demonstrate no such response (Herrick & Thomas 2001; Leakey et al. 2006; Usuda 2006; Bader, Siegwolf & Körner 2010; Crous et al. 2011) and consequently there may exist a differential acclimation capacity to increasing $[\text{CO}_2]$ amongst modern plant species.

Physiological acclamatory responses are widely demonstrated, however there remains ambiguity in terms of morphological plant responses, particularly stomatal responses, which set the absolute diffusive limit for gas exchange rates (Farquhar & Sharkey 1982). Significant responses to moderate levels of $[\text{CO}_2]$ enrichment, in terms of stomatal size and number, are rarely demonstrated experimentally, particularly under free-air $\text{CO}_2$ enrichment (Ainsworth & Rogers 2007), and typically are only recorded in response to large increases in $[\text{CO}_2]$, such as a doubling of current ambient levels or greater.

The ability to elevate $[\text{CO}_2]$ up to and above the typical photosynthetic saturation point for the majority of C3 species ($[\text{CO}_2]$ concentrations of 1000 ppm equating to a sub-stomatal $\text{CO}_2$ concentration of approximately 700 ppm (Wullschleger 1993)), is generally the preserve of closed environment or growth chamber studies. However, these experimental set ups are potentially limited in their capacity to mimic the climate variability inherent to most ecosystems. Opposingly, the relatively minor rises that limit the majority of free-air carbon dioxide enrichment (FACE) trials (maximum enrichment of approx. 600 ppm, with some exceptions), have thus far yielded extremely species specific responses, particularly in terms
of stomatal morphology and in certain cases physiological responses also (Bernacchi et al. 2003; Marchi et al. 2004; Blumenthal et al. 2013; Streit et al. 2014).

Results obtained from FACE trials may be considered unsurprising as the less than saturating levels of CO$_2$ enrichment in combination with additional and often conflicting environmental factors, including; light intensity, water availability, temperature and vapour pressure deficits (Hetherington & Woodward 2003; Casson & Gray 2008) are unlikely to provoke uniform responses as individual species resource requirements are typically non-uniform. In addition, the response of plant species may be further complicated by biotic factors of the natural environment in which they occur. Competitive interactions, endemic to specific plant community structures often cause conflict amongst species over resources that may be both spatially and temporarily heterogeneous (Belote et al. 2004). Potential interactions between these factors in combination with the sensitivity of stomata to a range of environmental stimuli and relatively minor levels of CO$_2$ enrichment further complicate the ability to attribute stomatal acclimation or adaptation to [CO$_2$] definitively (chapter 3).

A further confounding factor in attempting to elucidate any uniform response to increasing [CO$_2$] concentrations lies in the innate ability of plant species to alter both physiology and morphology which provides two distinct mechanistic options in the control of gas exchange and the maintenance of optimal photosynthetic rates via their stomata (Haworth et al. 2013, 2015).

Despite these difficulties, meta-analytical studies of carbon dioxide enrichment experiments demonstrate a reduction in stomatal conductance amongst modern plant species of approximately 20% with elevated [CO$_2$] concentrations of up to 600ppm (Wullschleger 1993; Drake et al. 1997; Long et al. 2004; Ainsworth & Rogers 2007). However, the lack of
Stomatal responses to biotic and abiotic stress argues for a decoupling of morphological and physiological traits over the typical range of [CO$_2$] enrichment concentrations. It has been argued that more derived plant groups, specifically angiosperm species, may be uniquely equipped to respond to [CO$_2$] enrichment, via active physiological control as they possess unique mechanisms for detecting and responding to increases in [CO$_2$] that are absent from earlier diverging lineages (Brodribb et al. 2009). Those responses, unique to angiosperm species are considered to have been a major advantage in terms of fitness and were likely a contributing factor to their radiation approx. 150 Mya (Soltis & Soltis 2004; Soltis et al. 2008; de Boer et al. 2012; McAdam & Brodribb 2012; McElwain et al. 2015). In addition, tight stomatal regulation and environmental sensitivity may confer an important advantage in terms of their resilience to the increasingly variable climactic conditions predicted in the near future (Hetherington & Woodward 2003).

Despite these apparent advantages however, the suitability of angiosperm populations to comparatively high and sustained [CO$_2$] concentrations is unclear. It may be the case, due to the observed differential capacities of individual species to increase WUE, that some species will be better suited to future environments then others as increased water-use efficiencies will alter the ecological fitness of some taxa in comparison to neighbouring competitors (Huxman & Smith 2001; Blumenthal et al. 2013; Grossman & Rice 2014).

It is currently the case that the vast majority of investigations and findings from FACE and chamber studies describe acclimation responses to elevated [CO$_2$]. Very little evidence is currently available which describe adaptive, heritable, genetic or epigenetic adaptations in plant stomatal, and/or physiological gas exchange traits in natural communities and none which describe any alterations in response to minor levels of enrichment, such as those
predicted over the next 50 years (approx. 500ppm depending on scenario (Ermenta & Nel 2014)).

In this experiment we specifically test the hypothesis that species, endemic to a semi-natural grassland community will show an enhanced capacity to improve intrinsic water-use efficiencies under elevated [CO₂] concentrations as a consequence of long term growth at a marginal CO₂ enrichment level (480 ppm). We consider that the 17 year FACE enrichment period (Jager et al. 2003) may have led to heritable adaptations in the case of some species rather than all, to the sustained carbon dioxide enrichment and that these adaptations will persist even when returned to growth under ambient [CO₂]. Here we use plant growth chambers in order to perform a reciprocal swap experiment in which the offspring of plants established under elevated FACE conditions were grown at ambient [CO₂] concentrations while the offspring of those grown under ambient field conditions could be grown under elevated [CO₂] concentrations. We therefore aim to determine the heritability of either physiological and/or morphological traits key to leaf gas exchange rates indicating the potential for genetic or epigenetic adaptations. We uniquely assess the potential of modern day plant evolutionary responses to contemporary [CO₂] rise. In order to determine possible changes in stomatal morphology we use the metric of theoretical maximum conductance (g_{max}) (McElwain et al. 2015) which incorporates stomatal number and volume of the stomatal pore, accounting for changes in overall stomatal geometry.
Materials and Methods

FACE site and CO₂ enrichment system

See chapter 1 materials and methods

Gas exchange measurements and species selection

See chapter 2 materials and methods

Laboratory processing and stomatal morphological measurements

See chapter 1 materials and methods for laboratory processing protocol and calculation of theoretical maximum stomatal conductance.

Seed collection and growth chamber conditions

Seeds for all five species were collected in August 2014. For each species, seeds were harvested at random from a minimum of five maternal plants from each of the three ambient and three elevated FACE rings that had been under continuous treatment for 18 years.

In order to capture as much inherent, within species diversity as possible, seeds were taken from widely-spaced maternal plants in order to account for the potential clustering of interrelated species. Harvested seeds for each of the five species were mixed thoroughly and stored in aluminium foil before being transferred to growth chambers for germination and experimental trials.

Seeds harvested from the University of Giessen’s FACE site were germinated and grown in two Conviron BDW-40 (Winnipeg, MB, Canada) walk-in growth chambers at University College Dublin’s Program for Experimental Atmospheres and Climate (PÉAC). In a reciprocal
swap experiment seeds collected from both ambient and elevated rings were grown at 400ppmv (ambient) and 480 (+20%) ppm [CO₂] in order to determine whether observations of plants grown under elevated [CO₂] in the field (chapter 3 results) would persist when returned to growth under ambient conditions.

Six plants per species (three each from both ambient and elevated FACE conditions) where allowed to establish in 3 litre pots containing a 3:1 potting mixture of multipurpose potting compost (Scotts Horticulture Ltd., Newbridge, Co. Kildare, Ireland) and perlite (William Sinclair Horticulture LTD, Chester, UK) and irrigated manually, as required, over the course of the experimental trial (April-Dec 2014). Plants were positioned randomly within both chambers and rotated on a weekly basis in order to minimise any chamber effects.

[CO₂] concentrations were controlled in each chamber using a WMA-4 infra-red gas analyser (PP Systems, Amesbury, MA, USA). Chamber conditions were consistently maintained for the duration of the experiment in a simulated diurnal program over a 16/8hr light–dark photoperiod (5.00–6.00 incandescent light only of 0-300 µmol m⁻² s⁻¹; 6.00–9.00 light intensity rises from 300 to 600 µmol m⁻² s⁻¹; 9.00–17.00 midday light intensity of 600 µmol m⁻² s⁻¹; 17.00–20.00 light intensity decreases 600 to 300 µmol m⁻² s⁻¹; 20.00–21.00 incandescent light only of 300-0 µmol m⁻² s⁻¹). Atmospheric O₂ concentrations were maintained using a PP-systems OP-1 O₂ sensor, and relative humidity was held constant at 70%.

Data Analysis

Spot measurements and IWUE responses to step changes in [CO₂] concentrations where carried out as in Chapter 3 materials and methods.
Results

Responses of species to stepped increases in [CO₂] from 200 to 2000ppm (Fig 4.1) revealed clear differences between the first filial generation (F₁) of plants pre-adapted to either ambient (400ppm) or elevated (480ppm) [CO₂] at the Giessen FACE site. Kruskal-Wallis tests indicated significant differences in IWUE values at each individual [CO₂] step (200, 400, 750, 1000 and 2000ppm) for both *A. elatius* and *T. flavescens* (P<0.05). Results were reinforced with linear modelling which described a positive relationship between [CO₂] and IWUE for both species (P<0.05) as well as a significant difference between the responses in the F₁ generation of plants pre-adapted to elevated [CO₂] in the field as compared to those adapted to ambient [CO₂] concentrations (P<0.05). Results in this case demonstrate that the F₁ generation of *A. elatius*, which had been grown under elevated [CO₂] in the field, showed increased IWUE values when compared with those grown at ambient [CO₂] whether or not they had been growing at 400 or 480 ppm under growth chamber treatments. Results were similar for *T. flavescens*. However, this particular species showed a decreased ability to increase IWUE when grown under elevated [CO₂] in the field (Fig 3.1 and Table 4.1) when compared with plants grown at ambient [CO₂]. Thus, IWUE responses to stepped increases in [CO₂] where the opposite of those observed in the field in this case. Both *A. elatius* and *T. flavescens* showed increasing separation in IWUE amongst the F₁ generations (Fig 4.1 and Table 4.1) of ambient and elevated grown plants as [CO₂] was increased above ambient 400 ppm stepwise.
Although differences in IWUE responses to step changes above ambient [CO₂] concentrations were statistically significant according to ANOVA procedures carried out on linear models. Kruskal-Wallis tests revealed that there were no differences in IWUE values between the F₁ generations of either *P. lanceolata*, *S. officinalis* or *G. pratense* at any individual step in [CO₂] concentration (Fig 4.1). Subsequently, the responses of the F₁ generations of these three species are deemed to be equivalent to those obtained from the parental generation at the FACE site (Fig 3.1) despite the significance of linear model testing.

As chamber treatments did not significantly alter the responses amongst the F₁ generations of plants grown at the FACE site we conclude that IWUE responses to [CO₂] step changes were heritable in this case.
Figure 4.1: Response in median IWUE values to systematic step changes in [CO$_2$] between 200 and 2000ppm. Values describe the combined responses of plants grown under chamber conditions at both 400 and 480 ppm [CO$_2$] for the offspring of plants pre-adapted to either ambient (400 ppm) [CO$_2$] (white) or elevated (480 ppm) [CO$_2$] (grey) concentrations at the Giessen FACE site. Regardless of imposed chamber conditions, plants displayed the same responses to [CO$_2$] step changes as those observed for parent plants in the field.
Table 4.1: Analysis of variance between linear models of IWUE response to step changes in [CO₂] for the parental generation (P₁), recorded under free-air CO₂ enrichment in the field, and their first filial generation (F₁) recorded in growth cabinets. Significant differences for the P₁ generation (highlighted in red) describe where the response to stepped increases in [CO₂] differ between plants grown under ambient or elevated [CO₂] in the field demonstrating an acclimation response. In the case of the F₁ generation plants derived from seed collected from ambient FACE rings are compared with plants derived from seed collected from elevated FACE rings regardless of chamber treatment ([CO₂]). Species for which there were no significant differences in IWUE values between ambient and elevated grown populations at the majority of [CO₂] step values are indicated with an asterisk (*).

<table>
<thead>
<tr>
<th>Species</th>
<th>Parental Generation [P₁]</th>
<th>First Filial Generation [F₁]</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>F-value</td>
<td>P-value</td>
</tr>
<tr>
<td>A. elatius</td>
<td>392.37</td>
<td>&lt; 2.2e-16</td>
</tr>
<tr>
<td>T. flavescens</td>
<td>155.22</td>
<td>&lt; 2.2e-16</td>
</tr>
<tr>
<td>G. pratense</td>
<td>8.2132*</td>
<td>0.4534*</td>
</tr>
<tr>
<td>P. lanceolata</td>
<td>27.739*</td>
<td>4.879e-07*</td>
</tr>
<tr>
<td>S. officinalis</td>
<td>2.2684*</td>
<td>0.1338*</td>
</tr>
</tbody>
</table>
Spot measurements carried out on the F1 generations of both ambient and elevated field grown populations at 400 and 480 ppm growth [CO2] concentrations in growth chambers demonstrated differing adaptive responses between grass and forb species.

*P. lanceolata, S. officinalis* and *G. pratense* (forb species) showed a strong adaptive response to prolonged growth under elevated [CO2] in the field. F1 generations of these species demonstrated increased IWUE’s, decreased $g_{s[opp]}$, and decreased $A_{sat}$ (although not statistically significant in the case of *P. lanceolata* (P>0.05)) when compared the F1 generations of species grown under ambient [CO2] in the field, when the two were assessed at 480 ppm in growth chambers (Fig 4.2, Table 4.3). Subsequently it was found that despite significant reductions in $g_{s[opp]}$, photosynthetic rates ($g_{s[opp]}$) were maintained relatively high resulting in an overall increase in IWUE values amongst the F1 generation of plants grown under elevated [CO2] over those grown under ambient field conditions. Conversely, neither *A. elatius* or *T. flavescens* (grass species) showed any differences between the F1 generations of elevated or ambient field grown populations in $A_{sat}$, $g_{s[opp]}$, or IWUE values when measured at 480 ppm growth [CO2] concentrations in chambers (Fig 4.2, Table 4.3).

Comparison between F1 generations when grown at 400 ppm in growth chambers where in direct opposition to those for plants grown at 480 ppm. Under these conditions the F1 generations of both *A. elatius* and *T. flavescens* grown under elevated FACE conditions showed higher IWUE, lower $A_{sat}$ and lower $g_{s[opp]}$ values when compared with the F1 generations of ambient grown plants (Fig 4.2, Table 4.2). Equally, neither *P. lanceolata, S. officinalis* or *G. pratense* showed any differences between the F1 generations of elevated or ambient field grown populations in $A_{sat}$, $g_{s[opp]}$, or IWUE values when measured at 400 ppm growth [CO2] concentrations in chambers (Fig 4.2, Table 4.2).
Overall, results demonstrate that plants at this particular FACE site display adaptive responses to prolonged growth under elevated [CO₂] concentrations. Grass species display what may be considered a typical acclimation response to elevated [CO₂] in that plants adapted to growth under elevated FACE conditions have lower photosynthetic rates, lower stomatal conductance and increased water-use efficiencies (Fig 4.2) when returned to growth under ambient [CO₂] concentrations (Grossman & Rice 2014). These differences disappear when measured under elevated [CO₂] which is indicative of a reduced investment in the manufacture of photosynthetic enzymes and the maintenance of physiological function under these atmospheric conditions. Findings obtained from spot measurements are strengthened by results obtained from exposing plants to stepped increases in [CO₂] (Fig 4.1) where reduced investment in Rubisco content leads to lower saturated photosynthetic rates, increasing build-up of sub-stomatal [CO₂] concentrations and faster rates of stomatal closure. Consequently it was seen that the F₁ generation of plants grown under elevated [CO₂] in the field demonstrated increasing water-use efficiencies in response to short term stepped increases in [CO₂] regardless of growth [CO₂] concentration (Fig 4.1, Table 4.1).

However, forb species displayed the precise opposite of this response. No differences were observed between the F₁ generations of plants grown under ambient or elevated conditions in the field when measured under ambient chamber conditions of 400 ppm [CO₂]. This result indicates a lack of an adaptive photosynthetic response, to prolonged growth under elevated [CO₂] (Fig 4.2, Table 4.2). Again results were confirmed by stepped increases in [CO₂] (Fig 4.1) where there was no clear separation in response between F₁ generations of *P. lanceolata*, *S. officinalis* or *G. pratense*. These species did show a heritable response in
that \( F_1 \) generations of plants established under elevated field conditions showed a significantly lower stomatal conductance than the \( F_1 \) generations of ambient grown plants when measured under elevated chamber conditions and significantly lower photosynthetic rates, baring the case of *P. lanceolata* for which the decrease was not significantly different.

Because photosynthetic rates between \( F_1 \) generations were equal under ambient [CO\(_2\)], but reduced in the \( F_1 \) generation of elevated grown plants in comparison with the \( F_1 \) generation of ambient grown plants, it is unlikely that the response is an adaptation to the enzymatic constituents of the photosynthetic process as is likely in the case of the grass species. Instead it may be that stomata have acclimated independently in this case, driving down photosynthetic rates under elevated [CO\(_2\)].

Despite physiological adaptations to moderate [CO\(_2\)] enrichment, chamber trials revealed no significant differences in the \( g_{\text{max}} \) values for the \( F_1 \) generations of any species when grown at either ambient or elevated [CO\(_2\)] (Fig 4.3, Table 4.2 and 4.3. Thus results may imply either a decoupling of morphological and physiological response under comparatively minor [CO\(_2\)] enrichment, or in the case that stomatal morphology and physiology are coordinated (Haworth *et al.* 2013, 2015), plant species considered in this study tend towards active physiological control as opposed to passive morphological control, in regulating gas exchange rates.
Figure 4.2: Median and corresponding standard deviation values of $A_{sat}$, $g_{e(opp)}$, and IWUE values for pants grown at both ambient (400 ppm) and elevated (480 ppm) [CO$_2$] concentrations in growth chambers. Black bars indicate the responses of the F$_1$ generations of plants grown under ambient (400 ppm) at the Giessen FACE site. Grey bars indicate the response of the F$_1$ generations of plants grown under elevated (480 ppm) at the Giessen FACE site.
Figure 4.3: Median and corresponding standard deviation values of $g_{\text{max}}$, values for plants grown at both ambient (400 ppm) and elevated (480 ppm) $[\text{CO}_2]$ concentrations in growth chambers. Black bars indicate the responses of the $F_1$ generations of plants grown under ambient (400 ppm) at the Giessen FACE site. Grey bars indicate the response of the $F_1$ generations of plants grown under elevated (480 ppm) at the Giessen FACE site.
Table 4.2: Median values of $A_{sat}$, $g_{s[opp]}$, IWUE and $g_{max}$ obtained from spot measurements for the F₁ generations of plants grown under ambient (400 ppm) or elevated (480 ppm) [CO₂] conditions at the Giessen FACE site and grown under ambient [CO₂] concentrations in growth chambers. Standard deviations are recorded in parenthesis alongside median values. Significant values were obtained using linear mixed effect models where $A_{sat}$, $g_{s[opp]}$, IWUE and/or $g_{max}$ is a function of [CO₂] expressed in ppm. Significant interactions between treatments are highlighted in red.

<table>
<thead>
<tr>
<th>F₁ Ambient x F₁ Elevated [400 ppm]</th>
<th>$A_{sat}$</th>
<th>$g_{s[opp]}$</th>
<th>IWUE</th>
<th>$g_{max}$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Ambient</td>
<td>Elevated</td>
<td>Ambient</td>
<td>Elevated</td>
</tr>
<tr>
<td>A. elatius</td>
<td>13.85(0.53)</td>
<td>10.15(1.30)</td>
<td>254(40.36)</td>
<td>94(36.81)</td>
</tr>
<tr>
<td>T. flavescens</td>
<td>12.3(3.91)</td>
<td>8.2(1.56)</td>
<td>255(10.82)</td>
<td>68.5(22.19)</td>
</tr>
<tr>
<td>G. pratense</td>
<td>17.6(10.69)</td>
<td>17.95(5.17)</td>
<td>319.5(219.68)</td>
<td>420.5(261.73)</td>
</tr>
<tr>
<td>P. lanceolata</td>
<td>22.1(1.84)</td>
<td>12.4(0.59)</td>
<td>481(152.39)</td>
<td>448.5(31.99)</td>
</tr>
<tr>
<td>S. officinalis</td>
<td>16.95(3.95)</td>
<td>11.8(4.72)</td>
<td>295(88.22)</td>
<td>274.5(152.10)</td>
</tr>
</tbody>
</table>
Table 4.3: Median values of $A_{\text{sat}}$, $g_{s(opp)}$, IWUE and $g_{\text{max}}$ obtained from spot measurements for the F$_1$ generations of plants grown under ambient (400 ppm) or elevated (480 ppm) [CO$_2$] conditions at the Giessen FACE site and grown under elevated [CO$_2$] concentrations in growth chambers. Standard deviations are recorded in parenthesis alongside median values. Significant values were obtained using linear mixed effect models where $A_{\text{sat}}$, $g_{s(opp)}$, IWUE and/or $g_{\text{max}}$ is a function of [CO$_2$] expressed in ppm. Significant interactions between treatments are highlighted in red.

<table>
<thead>
<tr>
<th>F$_1$ Ambient x F$_1$ Elevated [480 ppm]</th>
<th>$A_{\text{sat}}$</th>
<th>$g_{s(opp)}$</th>
<th>IWUE</th>
<th>$g_{\text{max}}$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Ambient</td>
<td>Elevated</td>
<td>Ambient</td>
<td>Elevated</td>
</tr>
<tr>
<td>A. elatius</td>
<td>13.5 (2.06)</td>
<td>12.55 (1.40)</td>
<td>187 (238.48)</td>
<td>195.5 (291.56)</td>
</tr>
<tr>
<td>T. flavescens</td>
<td>12.4 (3.40)</td>
<td>14.15 (4.54)</td>
<td>177 (89.83)</td>
<td>236 (35.58)</td>
</tr>
<tr>
<td>G. pratense</td>
<td>23.9 (1.78)</td>
<td>17.3 (4.00)</td>
<td>386 (72.94)</td>
<td>161 (113.70)</td>
</tr>
<tr>
<td>P. lanceolata</td>
<td>12.8 (1.68)</td>
<td>11.7 (1.67)</td>
<td>739 (13.89)</td>
<td>238 (22.76)</td>
</tr>
<tr>
<td>S. officinalis</td>
<td>23.9 (0.06)</td>
<td>17.75 (1.67)</td>
<td>379 (1.00)</td>
<td>261.5 (11.58)</td>
</tr>
</tbody>
</table>
Discussion

The aim of this study has been to determine whether or not any long term, adaptive responses to [CO$_2$] enrichment have occurred under FACE conditions since enrichment began in 1997. Of the five species included in this study both _A. elatius_ and _T. flavescens_ showed a significantly enhanced capacity to increase IWUE (P<0.05) as [CO$_2$] increased to levels above ambient (Fig 4.1). This was a response unique to plants that had been grown under 480 ppm [CO$_2$] at the Giessen FACE site and persisted amongst the F$_1$ generations of those species even when returned to growth at 400ppm [CO$_2$] in growth chambers. As these results match those observed under FACE conditions in the field (Fig 4.1 and Fig 3.1) we conclude that this particular response was heritable, in that it was observable in the F$_1$ generation, and irreversible despite the imposition of altered growth [CO$_2$] concentrations, as imposed by growth chambers, in a reciprocal swap trial. Despite the significance of understanding plant responses to elevated [CO$_2$] there are relatively few studies which have addressed the possibility of long term adaptations to this particular environmental condition and even fewer where adaptations have been demonstrated.

Physiological responses of _A. elatius_ and _T. flavescens_ recorded in this study are typical of acclimation responses recorded in many other studies (Anderson _et al._ 2001; Rogers & Ellsworth 2002; Chen 2005; Lee _et al._ 2011; Grossman & Rice 2014) although the differential capacity to adapt to moderate [CO$_2$] enrichment amongst species is a unique finding.

Of those studies that have demonstrated heritable trait changes, similar results to those presented here have been described. In an assessment of stomatal conductance and reproductive fitness (Grossman & Rice 2014) for an invasive desert grass species (_Bromus madritensis_), growth chambers were used to assess the responses in the offspring of
ambient and elevated field grown populations in a trial methodologically similar to our own. Analogous to results obtained here, offspring of this particular grass species were shown to exhibit lower stomatal conductance amongst the offspring of plants pre-adapted to elevated [CO$_2$] (560 ppm) when grown under ambient conditions as compared with the offspring of plants pre-adapted to growth at ambient [CO$_2$] (360 ppm). In this case the evolution of lower conductance was accompanied by reproductive fitness as the maintenance of relative fitness under elevated [CO$_2$] resulted in reduced fitness under ambient [CO$_2$] by comparison with plants pre-adapted to [CO$_2$] concentrations of 360 ppm. A limitation of this study however was that only a single species was considered and uncertainty remains as to whether or not heritable alterations of this nature would occur universally across all species endemic to this particular FACE site.

Körner & Diemer (1994) who examined the differences in growth responses of alpine plant species from both high altitude (low CO$_2$ partial pressure) and lowland (ambient CO$_2$ partial pressure) populations, described higher percentage change in photosynthetic rates of plants established under ambient partial pressures when grown alongside plants pre-established under high altitude partial pressures. By contrast, species established under low CO$_2$ partial pressure showed greater stimulation in photosynthetic rates when grown under elevated [CO$_2$] then lowland populations. The muted ability of lowland populations to respond to elevated [CO$_2$] was considered to be the consequence of excess accumulated non-structural carbohydrate and dilution of leaf nitrogen when grown under relatively elevated CO$_2$ partial pressures. Similar to findings presented here, these results were highly species specific as all species considered in this experiment did not necessarily follow the same trend. However, the finding that differences in responses to experimental treatments were the result of long
term exposure to unique growth conditions and were apparently heritable, in that they were non-reversible in at least a single generation, is a finding that matches those presented here.

In terms of this particular FACE study, and uniquely from those carried out to date, we obtained what may be considered atypical responses for the forb species *P. lanceolata, S. officinalis* and *G. pratense*, in that apparent alterations where inhibitory to growth under elevated [CO$_2$] as demonstrated by their poor performance, in comparison to the F$_1$ generations of ambient grown plants, when measured under elevated chamber conditions (Fig 4.2). The hereditary nature of this response was at least counter intuitive in that the progeny of plants grown under elevated [CO$_2$] at the FACE site did not perform as well as the progeny of plants adapted to growth under ambient [CO$_2$].

The significance of canalisation, or the relative robust ability of species to buffer phenotypes against environmental pressures, has gained significant recent interest due to its importance in the preservation of the genotype to phenotype translation. A review of plant responses to free air [CO$_2$] enrichment indicates significant capacity for acclimation amongst modern plant taxa and, in some cases, the capacity for those acclimations to be inherited amongst offspring (Nakamura *et al.* 2011; Grossman & Rice 2014). The lack of any response in this instance may therefore be due a comparatively weak canalisation (consistent ability to reproduce a particular phenotype (Wagner, Booth & Bagheri-Chaichian 1997; Lempe *et al.* 2013)) capacity of forb species. However, the detection of heritable, multi-generational responses to [CO$_2$] amongst particular plant taxa in this experiment could also be attributable to a number of unconsidered factors.

Although the performance of offspring depends on the prevailing environmental conditions under which they are assessed as well as their genotypic characteristics, maternal effects
(the influence of the maternal genotype or phenotype on the offspring phenotype) may also play a role in the observed patterns in species IWUE responses to [CO$_2$] enrichment. Previous investigations incorporating the use of seed-propagated annuals have demonstrated the impacts of environmental conditions on seed characteristics amongst the maternal generations of plant species, having the knock-on effect of significantly impacting the performance of the offspring through changes in seed germination, seedling survival and growth (Bezemer, Thompson & Jones 1998; Steinger, Gall & Schmid 2000). Similarly, results from our own experiment imply that plastic responses of the maternal generation to environmental conditions may extend to those individual’s offspring, influencing offspring trait expression. However, although the possibility of maternal influences on the offspring phenotype is a possibility the mechanism for this in this instance is unclear.

Over extended time periods and multiple generations, the possibility for genetic changes amongst species populations is also a possibility (Studer & Edwards 2002). However, as this experiment made no attempt to quantify genotypic variation within species populations it is not possible to comment on the relative success of genotypes. In addition, the proximity of treatment rings at the FACE site may facilitate gene flow between ambient and elevated populations making a genetic response difficult to determine under these circumstances.

In addition to genetic differentiation resulting from natural selection, plants exposed to elevated [CO$_2$] concentrations may also display heritable changes in gene expression and function (Maloof et al. 2001). As these effects are not driven by changes in DNA sequence but by molecular processes that regulate the expression of particular genes (Bossdorf, Richards & Pigliucci 2008) apparent heritable responses may also have an epigenetic root.
As results of this experiment have demonstrated the potential for heritable responses amongst certain species, further research to determine the driver for these responses is needed at this site.

In terms of morphological traits, we found no significant changes in the $g_{\text{max}}$ values for any of the species considered here. As alterations in stomatal morphology can be in terms of size and number (Franks & Beerling 2009b), and because parental FACE populations demonstrated no significant differences in stomatal density (chapters two and three) the $g_{\text{max}}$ metric was utilised to describe all potential morphological alterations. The utilisation of complex morphology, phenology and growth traits in the assessment of evolutionary responses to elevated [CO$_2$] have proven problematic, particularly amongst modern day plant species (Lau et al. 2007; Leakey & Lau 2012).

It is possible that these traits are more conserved as they are potentially complexly interlinked with other functional traits. In the case of [CO$_2$], responses in terms of stomatal initiation may link with other downstream processes, such as drought induced hormone signalling (ABA), which in themselves are intrinsically linked [CO$_2$] (Chater et al. 2015). By contrast, traits related to stomatal conductance and/or water-use efficiencies may be controlled by relatively few genes and consequently respond more rapidly to selection processes (Panio et al. 2013)

The fact that maximum leaf diffusive conductance, stomatal density and stomatal pore length have all been shown to link strongly to [CO$_2$] across several geological time scales (McElwain & Chaloner 1995a; Royer 2001; Beerling & Royer 2002; McElwain et al. 2002; Franks & Beerling 2009a), suggests a close coupling between morphological stomatal traits and leaf gas exchange capacity, however the fact that none were observed in this study, and
that FACE studies in general (Ainsworth & Rogers 2007) show highly inconsistent responses in this regard may imply that any significant and consistent response in terms of $g_{\text{max}}$ may be dependent on time of exposure and level of [CO$_2$] enrichment.

Based on these findings we conclude that a differential evolutionary capacity exists amongst modern grassland species to adapt to elevated [CO$_2$] concentrations. Contemporary evolutionary processes that favour adaptive species over non adaptive species will potentially have major implications for ecosystem structure and functioning over longer timescales.
Chapter 5
Stomatal mediated gas exchange reflects resource partitioning amongst grassland species at differing soil moisture deficits

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Abstract

It is often the case that co-occurring plant species in naturally competitive communities adopt differing strategies which allow for the spatial and temporal partitioning of limiting resources. These strategies are often reflected by alterations to key functional traits of roots, stems and leaves which may provide the means by which co-occurring plant species differentiate to fill particular ecological niche space.

Leaf stomatal conductance is a key eco-physiological attribute of plants and one which is particularly sensitive to environmental change. Alterations to stomatal conductance are essential for acclimation of a plant to environmental stimuli, particularly soil moisture availability, increasing relative productivity rates through enhanced water-use efficiencies. Consequently, effective stomatal functioning is an
important determinant of plant survival, particularly under drought stress, although is a characteristic that is perhaps underutilized in studies of niche segregation.

Here we investigated stomatal conductance's of four typical and agronomically important grassland species grown at two distinct levels of soil moisture concentration (25% for control treatments and between 5 and 8% for drought treatments) and species richness (monoculture and four species mixes). Niche space was defined using non-parametric kernel density functions of stomatal conductance and niche overlaps (NO\textsubscript{K}) were calculated as the area under the smaller of the two population density functions.

Increasing competition led to increased convergence in conductance trait values while increasing drought stress in competitive assemblages resulted in increasing divergence in trait values.

Understanding the effects of water availability and competitive plant interactions on community structuring is critical to predictions of how these communities might alter with future forecast global change.

Keywords: Convergence, divergence, water-use efficiency stomatal conductance, coexistence, drought, competition.
Introduction

Mechanisms of coexistence in diverse plant communities are of particular significance as they facilitate population dynamics, influence ecosystem function and, crucially, provide a methodology through which community structure might be assessed with forecast environmental change (Chesson 2000; Silvertown 2004; Kearney et al. 2010; Wilson 2011; Silvertown, Araya & Gowing 2014). To that extent, a number of theories which attempt to explain the stable coexistence between competing species within particular habitats have been developed (Silvertown 2004; Wilson 2011) with the classical principle being that, to achieve successful partitioning of resources, individual species must occupy unique niche space along environmental axes (Grinnell 1917; Elton 1927; Hutchinson 1957).

Despite an abundance of theory however, precisely how species respond to various biotic and abiotic stresses facilitating robustness and coexistence in diverse assemblages has persistently lacked resolution. Predictions have in fact been so contentious that the null hypothesis or neutral theory of biodiversity (Hubbell 2001), in which characteristic species traits are considered to be irrelevant in terms of their success or ability to withstand environmental change within a particular habitat, has challenged niche-based community theories as methods of predicting both the diversity and relative abundances of species within ecosystems (Alonso, Etienne & McKane 2006).

More recently however, the reliability of the neutral method has been closely scrutinised and found, in some cases, to be insufficient (Silvertown 2004; Levine & HilleRisLambers 2009; Adler, Ellner & Levine 2010). In particular, the inadequate consideration of the ecologically dominant species and their significance to ecosystem function has been cited as a potential flaw in the methodology (Purves & Turnbull 2010; Connolly et al. 2014). In general terms
dominant species may be said to exhibit unique, physiological or morphological characteristics which provide for their success within a community. Consequently they deliver a significant proportion of ecosystem services and play a fundamental role in habitat functioning. Therefore it is the species with characteristics most appropriate to the occupation of vacated niche space and the maintenance of key ecosystem services which are more likely to display greater abundance as opposed to the random ascension of any co-occurring species following environmental disturbance. In that sense the neutral theory may not be capable of a comprehensive explanation regarding the relative abundances of common and rare species within a specific habitat.

As recent findings suggest (Silvertown 2004; Levine & HilleRisLambers 2009), dependable methods of analysing large numbers of species in order to assess probable changes to habitat structure with changing environmental conditions remain elusive in population ecology. However, the consideration that key traits of an organism, physiological traits in particular, are the most relevant indicators of ecological success within a particular environment has remained popular (Kearney & Porter 2009; Pérez et al. 2014; Higgins & Richardson 2014) and has often been shown to correlate with relative success of species within an ecosystem (Kattge et al. 2011; Woodin et al. 2013; Violle et al. 2014). In addition, the occurrence of species displaying physiological variability directly related to environmental gradients of resource availability or environmental stress have also been demonstrated (Araya et al. 2011; Moreno-Gutiérrez et al. 2012; Woodin et al. 2013; Jaime et al. 2014) and indicates the need for these traits to be considered in any predictive modelling of ecosystem change.

In the case of plant communities, soil moisture availability is perhaps the most vitally accessible resource in that it is of distinct importance to plant growth and function. However,
It is also a highly variable ecosystem property and has previously been used to demonstrate hydrological niche segregation amongst species based on their particular requirements (Araya et al. 2011; Moreno-Gutiérrez et al. 2012; Jaime et al. 2014). Natural heterogeneity, both spatially and temporally, as well as variability in individual species demand makes soil water availability particularly suitable to investigations of trait divergence and/or niche segregation providing an obvious axis of ecological differentiation that can facilitate species co-habitation. However, although soil moisture availability has previously been explored, the mechanisms through which individual species control the rate of uptake of this resource and the potential of utilising these mechanisms as a bio-physical basis for hydrological niche segregation have not been thoroughly investigated.

In order to investigate the possibility of niche segregation by means of trait differentiation within a community we apply a key tenet of ecological theory (limiting similarity principle (Macarthur et al. 2014)) which posits that resource competition and/or environmental stress should produce patterns where co-occurring species segregate into distinct niche space or demonstrate increased inter-specific variability in key traits specifically associated with the capture and usage of limiting resources. Variability in these characteristics can potentially be recorded as alterations in physiological or morphological traits which may subsequently reflect niche segregation as a mechanism of species coexistence.

The objective of this study has been to assess whether the effect of interspecific competition provokes differences in stomatal control of gas exchange rates and whether or not, in response to the imposition of severe drought, species grown in competitive assemblages will demonstrate significantly different alterations in their resource use profiles (in terms of their absolute range of stomatal conductance values) than they would in monocultures. Regulation of the stomatal aperture in order to control water loss often results in an increase in the
intrinsic water-use efficiency value of photosynthesis (IWUE) or the ratio of light saturated photosynthetic rate ($A_{sat}$) to maximum operational stomatal conductance ($g_{s,opp}$). It has previously been asserted that this ratio might be used as a basis for hydrological niche segregation, particularly at lower soil moisture concentrations, as the extent to which species control IWUE is demonstrably unique (Silvertown et al. 2014). Here, we consider the recorded range of stomatal conductance values as indicative of species soil water use strategies for a given set of environmental conditions. In this context we assess how species adapt water use strategies in response to drought at different levels of interspecific competition.
Materials and Methods

Establishment of the experiment began in June 2012 at Johnstown Castle environmental research centre. Forty large pots (approx. 65 L volume) were sown with: *Lolium perenne* (perennial ryegrass), *Cichorium intybus* (common chichory), *Trifolium repens* (red clover) and *Trifolium pratense* (white clover). Sixty seeds were sown in each pot using a hexagonal grid allowing for all four species to be sown in either monoculture or equal abundance four-species communities. In the case of four species mixtures, grid templates were used to sow the plants in order that individual seeds of all four species were randomly distributed throughout the pots. All pots were filled with a five cm layer of coarse gravel (for drainage) followed by a homogeneous soil-sand mixture which consisted of: 75% agricultural top-soil and 25% fine sand (sandy-loam texture: 8.3% clay, 22.4% silt and 69.3% sand). Pots were watered using a drip irrigation system (Irritec Technology Ltd.) incorporating two separate watering lines allowing separate watering regimes to be implemented. Scheduled watering took place on an automated basis once per day at 7:00am using a Gardena C1060 plus (Gardena International GmbH.) water computer and to minimise preferential water flow three drippers were placed equidistant from each-other in pots. To calculate the relative mm rainfall being applied to pots and to check for any inconsistencies between watering lines the system was calibrated after installation and again in August 2013. Soil moisture content (SMC) was measured formally at least once per week over the course of the experiment and measurements were taken between two and five pm, using a ML2 Theta-probe (Delta-T devices Ltd.). Due to high variability across the pot surfaces, three random SMC measurements were taken in each pot (always at least 5 cm from the edge) and a pot average used to calculate the line average. Outside of the experimental drought period all
lines received the same amount of water which was altered as necessary to maintain the line average pot SMC at approximately 25%.

The target range of the drought treatment was between 5% and 8% SMC while the control treatment was 25% SMC. Treatments were subsequently considered “wet” or “dry” dependent on the target range of soil moisture concentration. Irrigation schedules were altered to maintain the line average SMC across all plant communities within the target range, and were changed according to fluctuations in glasshouse temperature and humidity (see supplementary data).

Gas exchange measurements

Leaf gas exchange measurements were carried out in June 2014 using a CIRAS-2 portable photosynthesis system and PLC (6) cuvette attachment (PP-Systems, Amesbury, MA, USA). In this case a combination of head plate attachments (4.5 cm², 2.5 cm² and 1.29 cm²) were used in order to maximise the leaf area available for measurements while also reducing the amount of uncovered window space in the cuvette head. All gas exchange measurements were taken between 09:00 and 12:00 and in all cases conditions in the cuvette head were set to maintain VPD (vapour pressure deficit) below 12 mb (1.2 kPa), Leaf temp at 22 °C, CO₂ concentration at 400 ppm and air flow through the cuvette at 200 ml min⁻¹.

Initially, photosynthetic light curves (Pn/I) were run in order to establish the saturating light for photosynthesis. In this case, Pn (photosynthetic rate) was allowed to settle at maximum PAR (photosynthetically active radiation) of 2000 µmol (photon) m⁻² s⁻¹ before applying a sequence of light settings (1,600, 1,200, 1,000, 800, 600, 400, 200, 100, 50, and 0) with an imposed minimum time step of 120 s at each set point. Light saturated photosynthetic rate (A_s), was then calculated using the methods of Norman, Welles & McDermitt (1991). Pn/I
curves were carried out on two plants for every species in every treatment (giving a total of 8 light curves per species) and the maximum \( A_{sat} \) value recorded for each species was then used as the set PAR value for all other gas exchange measurements. In application of the above cuvette conditions, both \( A_{sat} \) and \( g_{s(opp)} \) were recorded from between 5 and 6 plants per species in all treatments with measurements taken from at least two leaves per plant. For each species, recordings where taken after both \( A_{sat} \) and \( g_{s(opp)} \) had reached steady state under cuvette conditions.

Calculating resource-use-overlap

In this case the absolute range of stomatal conductance values for each species is taken as the resource use profiles (RUP) of four agriculturally significant grassland species. Individual RUP’s are taken to represent the realised niche space of those species for a given set of environmental factors (light, temperature, humidity, soil structure, competition, etc). Through the imposition of an imposed soil moisture deficit on species grown in both monoculture and four species mixtures we then examined if, or how, resource use profiles were altered. Variability in the extent of RUP overlap represents increasing convergence or divergence in stomatal conductance and is consequently a novel way of assessing these community assembly processes in response to the imposed biotic and abiotic disturbances of this particular trial.

For each treatment, resource-use-overlap between species was calculated in the application of the kernel function method described by Mouillot et al (2005) to the obtained stomatal conductance values for each species in each treatment. This provides a nonparametric method of calculating the density distributions for a particular functional trait, in this case optimal stomatal conductance \( (g_{s(opp)}) \), which is then used as an axis of differentiation.
Once the kernel density distributions for the measured $g_s$ values where generated, the kernel density derived resource-use-overlap ($RUOK$) between each pair of species ($I$ and $J$) using stomatal conductance values as axis $T$, was calculated as the area of overlap between the population density distributions of each species using the following expression:

$$RUOK_{I,J,T} = 1 - \frac{1}{2} \int |F_{IT}(X) - F_{JT}(X)| \, dx$$

(Eq. 4.1)

This method implies minimal assumptions in the density distribution of measured $g_s$ values for a given species, and is consequently less sensitive to departures from normality in the recorded data, especially when analysing skewed distributions (Mason et al. 2011).

Kernel density distributions where derived using R (R Core Team 2014). Analysis of resource-use-overlap was also run using the R statistical package using the script supplied by Geange et al. (2011).
Results

Fig 5.1 displays the kernel density distributions of stomatal conductance for all species across all treatments. The x-axes in this case are the log transformations of conductance values and the y-axes the output of the probability functions (as described by Mouillot et al. (2005) for each transformed conductance data point. Table 5.1 describes the total overlap in resource use profiles between species (as derived from Eq 4.1) in dry and wet four species mixtures as well as in dry and wet monocultures. In this case pairwise comparisons of overlap in resource use profiles are compared between species, with a value of one indicating complete overlap while values of zero are indicative of no overlap. Results demonstrate that, under well-watered conditions (25% SMC), resource use profiles of plants grown in monocultures showed greater separation. However, the effect of increased interspecific competition (as highlighted by values taken from species grown in well-watered mixtures) showed an increased overlap in resource use profiles (Fig 5.1 and Table 5.1). In this case, the effect of growth in competitive mixtures had the effect of increasing the resource use overlap of the constituent species regardless of SMC.

All species responded to increased interspecific competition with increased IWUE (P < 0.05) except Lolium perenne which showed lower IWUE in wet mixtures then wet monocultures (P < 0.05) and Trifolium pratense which showed lower IWUE in dry mixtures then dry monocultures (P < 0.05). Four species mixtures always showed enhanced IWUE at the overall community level regardless of SMC (Fig 5.2).
Figure 5.1: Kernel density estimates of $g_s$ values obtained for all species across all treatments. The estimated, non-parametric population densities ($f(x)$) are the sum of normal kernel distributions for each log transformed $g_s$ data point recorded from gas exchange measurements. Density distribution curves represent the functional range of stomatal conductance or resource use profiles for each species which fluctuate according to experimental treatment. A = Kernel density distributions for all species in wet monocultures. B = Wet mixture. C = Dry monocultures. D = Dry mixture.
Figure 5.2: Mean IWUE values of all species for each of the experimental treatments. Fig A describes the community level IWUE as the stacked mean values of individual species IWUE’s for each experimental treatment. Fig B describes the differences in individual species responses to experimental treatments as facets of Fig A.
Table 5.1: Estimated overlap in stomatal conductance profiles for each species pair for each of the experimental treatments. Overlap is derived from the kernel-based index of stomatal conductance values (equation 1). A = Pairwise overlaps, reflecting similarity in resource use rates, between species in wet monocultures; B = Wet mixtures; C = Dry monocultures and D = Dry mixtures

<table>
<thead>
<tr>
<th></th>
<th>C. intybus</th>
<th>L. perenne</th>
<th>T. pratense</th>
<th>T. repens</th>
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<tr>
<td>A</td>
<td></td>
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<tr>
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Table 5.2: IWUE – Kruskal-Wallis Test: Dry Monoculture V’s Dry Mixture

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<tr>
<th>Species</th>
<th>chi squared</th>
<th>df</th>
<th>p-value</th>
<th>Significant</th>
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</thead>
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<tr>
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<td>7.54</td>
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<td>P &gt; 0.05</td>
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<tr>
<td><em>Lolium perenne</em></td>
<td>9.6731</td>
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<td>P &lt; 0.05</td>
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Table 5.3: IWUE - Kruskal-Wallis Test: Wet Monoculture V’s Wet Mixture

<table>
<thead>
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<th>df</th>
<th>p-value</th>
<th>Significant</th>
</tr>
</thead>
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<td><em>Trifolium pratense</em></td>
<td>13.8087</td>
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<td>6.1906</td>
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<td><em>Cichorium intybus</em></td>
<td>31.1221</td>
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<td>21.677</td>
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<td>P &lt; 0.05</td>
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Table 5.4: IWUE - Kruskal-Wallis Test: Wet Mixture V’s Dry Mixture

<table>
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<th>p-value</th>
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<td>13.3813</td>
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<td><em>Trifolium repens</em></td>
<td>2.6734</td>
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<td><em>Lolium perenne</em></td>
<td>68.7771</td>
<td>1</td>
<td>P &lt; 0.05</td>
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</tbody>
</table>
The effect of a severe soil moisture deficit (5-8% SMC) on four species mixtures reduced the overlap in resource use profiles compared with those observed in the well irrigated treatment. Where RUP's where relatively tightly grouped in the well-watered mixtures the imposition of a soil moisture deficit caused increasing differentiation in resource use profiles (Fig 5.1 and Table 5.1).

Although there was a general trend to increase IWUE with decreasing SMC in four species mixtures *Trifolium pratense* displayed the opposite tendency. However, alterations in the IWUE values for *Trifolium pratense* were not significant in this case (P < 0.05) (Table 5.4).

Decreasing soil moisture concentration had the effect of decreasing stomatal conductance values, as demonstrated by a negative shift in the log $g_s$ values in the dry monocultures and mixtures, as compared with their better irrigated comparisons, the wet monocultures and mixtures (Fig 5.1). Decreased soil moisture concentration also had the effect of increasing the variability in stomatal conductance values as demonstrated by a decrease in probability ($\int f(x)$) values (Fig 5.1).

Fig 5.1 displays the resource use profiles of individual species highlighting the shifts in profiles with the imposition of a drought in both monocultures and four species mixtures. Here it was seen that both *Cichorium intybus* and *Lolium perenne* displayed increased differentiation in resource use profiles between wet and dry mixtures but decreases in overlap between wet and dry monocultures. *Trifolium pratense* and *Trifolium repens* on the other hand showed an increase in overlap between wet and dry mixtures but decreases in overlap between wet and dry monocultures.
Discussion

Plants grown in competitive mixtures tended to show greater similarity in resource use/consumption rates than those same species grown in monoculture (Fig 5.1, Table 5.1). Findings in this case suggest that species grown in four species mixtures developed consumption rates that were more highly optimised when resources were freely available as highlighted by the increase in species IWUE’s (Fig 5.2, Table 5.2 and 5.3).

Our results broadly agree with this hypothesis in that species grown in competitive mixtures showed greater similarity in resource use rates than they did in equivalent monocultures. As well as increased overlap in resource use profiles, plants grown in well-watered, four species mixtures also displayed increased water use efficiencies. This was true for all species except *Lolium perenne* which showed a slightly lower IWUE in wet mixtures compared to wet monocultures and *Trifolium pratense* which showed a slightly lower IWUE in dry mixtures as compared with dry monocultures. Despite the species specific responses to growth in competitive assemblages however, four species mixtures always showed enhanced IWUE at the overall community level when compared with the combined IWUE of their equivalent monocultures (Fig 5.2).

Although empirical evidence of trait convergence under increasing interspecific competition is scarce there is some theoretical precedent for the process. Fox et al (2008) described character convergence under competition for essential resources based on the earlier work of Abrams (1987) and Leon and Tumpson (1975). In this case it was demonstrated that species may adaptively alter their resource use rates in response to the presence of a strong competitor as competition reduces resource availability. In order to persist in situations where resources are non-substitutable, species must have similar consumption rates in order to
gain equal share or successful partitioning of that resource which leads to increasing similarity in consumption rates as well as improved resource use efficiencies.

Although there was an observed decrease in resource-use overlaps in this case, water-use efficiencies where again increased under drought stress, as had been the case under increasing interspecific competition.

This study is one of very few that has considered the direct effects of interspecific competition on plant IWUE independent of any additional environmental stress. One other study (De Boeck et al. 2008) however, which examined the mixed effects of increased warming and competition on experimental grassland communities, demonstrated that plants in multi-species communities typically showed increased WUE compared to monocultures regardless of any induced warming. These results, in combination with those presented here suggest that interspecific competition may drive convergence in consumption rates between species typically increasing water use efficiency’s. In addition, the significant number of studies which describe increasing productivity with increasing species richness (Hector et al. 1999; van Ruijven & Berendse 2005; Tilman, Reich & Knops 2006; Reich et al. 2012; Liang et al. 2015) may further infer that the processes of convergence in competitive mixtures contribute to the increased productivity of those communities over monocultures.

Plants adapted to growth in competitive mixtures tended to be more dissimilar in resource use profiles under drought stress then they were under non-stressed conditions but more similar then their equivalent monocultures (Fig 5.1, Table 5.1).

The combined pressure of increasing interspecific competition and increasing drought stress are in direct opposition in this case as competition between species drives increasing convergence while drought stress promotes divergence.
As drought pressure acts to reduce consumption rates to minimum values, which may not be identical for all species, the result has been increasing dissimilarity in stomatal conductance profiles. However, as growth in mixed assemblages encourages optimal consumption rates and increasing similarity based on the presence of competitors the result has been a convergent/divergent trade-off with diverse communities being more water use efficient than their equivalent monocultures.

Trait divergence and niche complementarity are comparatively well documented and a number of examples across a range of ecosystems, have previously been described (Verheyen et al. 2008; Wacker et al. 2008; Medlyn et al. 2011; De Bello et al. 2013; Laliberté, Norton & Scott 2013). One particularly relevant study describing niche differentiation in a Mediterranean open woodland; Moreno-Gutiérrez et al. (2012) demonstrated niche segregation along a functional continuum based on leaf level isotope discrimination. In this case δ¹³C and δ¹⁸O where strongly correlated with species distance from source water and governed by tight stomatal control over the leaf physiology regulating carbon and oxygen isotopic ratios at the leaf level. Lower proportions of δ¹³C and δ¹⁸O were linked with higher stomatal conductance and lower WUEᵢ (instantaneous water use efficiency. See table 1 – Introduction) which were indicative of a more profligate water use strategy. By contrast, higher proportions of δ¹³C and δ¹⁸O were linked with lower stomatal conductance and higher WUEᵢ and characteristic of a more conservative water use strategy. The finding that coexisting species displayed a range of water use strategies which were conserved over time suggests eco-physiological niche segregation amongst dryland plant species.

Our results concur with these previous findings in that communities are likely to demonstrate trait divergence in response to stress, particularly drought stress, as they show increased separation in terms of stomatal conductance values (Fig 5.1, Table 5.1). However, here we
extend that observation to demonstrate specifically the moderating effect of diversity on individual species responses. In this case, species responses to drought stress were not uniform but dependent on community composition. For example, water use efficiencies between mixtures and monocultures were not significantly different for either *Trifolium pratense* or *Cichorium intybus* in response to drought although they were for *Trifolium repens* and *Lolium perenne* (Fig 2). Despite the species specific alterations in IWUE at the species level however, community level IWUE increased with both increasing interspecific competition and drought stress (Fig 2).

In terms of this experiment, all four grassland species are considered to be in competition for a non-substitutable, limiting resource at the same time and in the same confined space. Under these conditions where no adequate alternative resource exists character or trait divergence is not an option, and models predict that the four competing species will tend to converge in their resource uptake rates (Fox & Vasseur 2008). We did not find this to be the case in this instance as results demonstrate greater similarity in resource use rates in well supplied mixtures by comparison with to those observed under reduced soil moisture availability (Fig 5.1, Table 5.1).

Alterations in stomatal conductance ranges amongst species resulted in variation in intrinsic water use efficiencies between species with species becoming more or less profligate in their resource use rates in accordance with environmental conditions. Alterations in water use efficiencies via trait plasticity and dynamic stomatal functioning are considered indicative of effective resource partitioning and the adoption of either a more conservative or profligate water use strategy of individual species, as such, trait plasticity providing for divergence in response to short term environmental stress may be critical to successful cohabitation under variable environmental conditions.
Here we exhibit how stomatal conductance profiles may be used to demonstrate the processes of convergence and divergence within plant communities as well as how those processes are driven. In this case we demonstrate how increasing species richness always increases convergence whereas disturbance pressures, in this instance drought stress, typically result in divergence of trait values.

Conclusion

Despite the wealth of knowledge surrounding plant physiological responses to environmental variables, comparatively little is understood in terms of how those responses might be mediated through inter/intra-specific competition in dynamic, species rich assemblages. As resources become limiting plants often display phenotypic responses to neighbouring species in order to maximize resource acquisition, although the direct implications of this on plant physiological process is not often considered. Results in this instance have demonstrated that species adapted to growth in competitive communities may alter resource acquisition rates and improve water use efficiencies in response to drought where those same species grown in the absence of interspecific competition may not.

With renewed interest in trait based methods in the analysis of community assembly processes there has been an increasingly concerted effort to determine the significant mechanisms involved in maintaining community diversity and equilibrium. Here, we set out to determine how specific plant physiological process where affected by inter/intra-specific competition and how growth in mixed communities, as opposed to monocultures, affected responses to an imposed soil moisture deficit. We conclude that interspecific variability in species stomatal responses to environmental stimuli, which is well documented in terms of stomatal conductance (Abril & Hanano 1998; Fay et al. 2002; Zweifel, Rigling & Dobbentin
2009; Miranda-Apodaca et al. 2015), offer a unique method of monitoring community assembly process and further endorse the benefits of species diversity in productivity and drought resistance.
Chapter 6
Synthesis

Biochemical models of plant leaf physiology predict that increasing atmospheric carbon dioxide concentrations ([CO$_2$]) should enhance rates of photosynthetic uptake as well as improving water use efficiency (Farquhar & Sharkey 1982; Von Caemmerer 2000). However, under ‘real world’ conditions species responses to elevated [CO$_2$] are demonstrably complex and inconsistent leading to disparity in predicting the actual implications for species and ecosystems in the face of unprecedented anthropogenic climate change.

Variability in the abiotic factors which are fundamental to the biological and chemical processes of plants, such as [CO$_2$] and soil moisture concentrations, will impact the structure, function and community composition of terrestrial plant communities due to the typically unique way in which individual species sense and/or respond to changes in these environmental components (Loreau et al. 2001; Angert et al. 2009; de Mazancourt et al. 2013). Individuality of response is therefore a crucial consideration to the way in which we examine and interpret the implications of environmental change and highlights the significance in assessing responses across a broad range of species at the community level. A more complete understanding of species specific reactions when grown in natural ecosystems, will allow us to more accurately assess the implications for a range of important patterns and functions, including; biological diversity (Tilman, Wedin & Knops 1996; Loreau et al. 2001; de Mazancourt et al. 2013), water cycling (Gebauer, Horna & Leuschner 2012; Kunert et al. 2012) and carbon sequestration (Steinbeiss et al. 2008; De Deyn et al. 2011). In terms of this thesis, research has demonstrated that a suite of experimental approaches, including long-term field trials (chapters two, and three), growth chamber experiments (chapter four) and competition experiments (chapter five) are required to advance a fuller
understanding of how future grassland ecosystems will respond to anthropogenic global change. Key findings of this thesis based on these methodologies have demonstrated that;

- Species responses to moderate [CO$_2$] enrichment in a semi-natural European grassland system are likely to be physiological rather than morphological in nature, although responses at this level of enrichment are species specific (chapters two and three).
- Physiological acclimations do occur, even at moderate levels of enrichment, although again there is a differential capacity between species to acclimatise to minor changes in the growth environment (chapter three).
- Acclimation responses are hereditary amongst [CO$_2$] sensitive species demonstrating the possibility of an evolutionary response to moderate [CO$_2$] enrichment (chapter four).
- Responses of plants to changes in their environment are modified by growth in competitive communities (chapter five).

In focusing on the morphological and physiological diversity of stomatal patterning and function it is possible to gain an insight into how these traits might be linked with the functional diversity (the elements of biodiversity that influence how ecosystems function) within an ecosystem and what the environmental context of this might be.

**Phenotypic plasticity in stomatal development**

Variability of stomatal traits, within and across plant species, and under a variety of conditions, is typically strongly correlated with assimilation rates and tends to conserve the relative gradient for CO$_2$ diffusion into the leaf (Buckley 2008; Franks et al. 2013). This
suggests that stomatal development and function are closely coordinated with the biochemical capacity for photosynthesis across developmental and even evolutionary timescales. The strength of this coupling is often demonstrated in crop breeding, where selection for higher productivity is usually accompanied by higher stomatal conductance and lower WUE (French & Schulz, 1984; Fischer et al., 1998; Condon et al., 2004). Disruption of this correlation has been shown in transgenic plants, which maintain normal stomatal conductance despite an impaired photosynthetic mechanism (Quick et al., 1991; von Caemmerer et al. 2004). In these cases, impaired photosynthesis results in reduced WUE, but breaking the stomatal/photosynthesis connection in this way provided early indications that stomata could be similarly targeted for manipulation, independently of photosynthetic capacity, to change WUE.

Perhaps the strongest indication of an alteration in overall conductance in response to environmental factors may be obtained from an analysis of changes in stomatal morphology. As stomatal density and pore size combined set the absolute limits for gas exchange (Franks & Farquhar 2007; Franks et al. 2012; Taylor et al. 2012; Franks & Casson 2014; McElwain et al. 2015) they are potentially a more reliable indication of changes in conductance/transpiration rates, particularly when exposed to environmental stresses which drive increases or decreases in conductance over extended time periods. By contrast physiological measurements indicate the actual conductance at a given time and are strongly influenced by the conditions of the immediate environment (light, humidity, temp etc). Photosynthetic rates, conductance and by extension water-use efficiencies are further dependent on the time of day, year, or the growth stage at which measurements are taken (Konrad et al. 2008) and consequently there is often observed little agreement between physiological stomatal conductance and the absolute stomatal conductance as determined
by stomatal limitations. In reality stomata set the limits for physiological conductance and thus provide a level of plasticity which enables plants to optimise physiological performance dependent on a host of abiotic and biotic stresses which may act on plants independently or in unison at any particular time.

For the reasons outlined above, this PhD thesis included an initial assessment of plant stomatal densities and plant stomatal indices in response to elevated [CO$_2$] concentrations at the Giessen FACE site in order to determine the potential for plants to shift not only physiological stomatal conductance but also their stomatal conductance envelope as set by stomatal limitations.

Findings from this initial assessment (Chapter two) revealed that stomatal initiation was not influenced by a 20% above ambient increase in [CO$_2$] inferring that overall plasticity was unaffected and conductance/transpiration rates, if affected at all, could be adjusted within the limits set by morphological stomatal traits.

**Physiological acclimations under elevated [CO$_2$]**

Based on the available literature covering modern day stomatal initiation rates from FACE studies (Long *et al*. 2004; Ainsworth & Long 2005; Ainsworth & Rogers 2007) it is possible that only a relatively large alteration in [CO$_2$], (such as may be imposed above the capacity of modern FACE trials), will be sufficient to provoke consistent changes in stomatal morphological traits and implies that there remains large scope for the physiological adjustment without the necessity for morphological change for any increase in [CO$_2$] of at least up to double the current ambient concentrations and possibly more.
A study by carried out on the effects of \([\text{CO}_2]\) enrichment (572 ppmv) on the stomatal conductance of sun and shade leaves of over story sweetgum (\textit{Liquidambar styraciflua}) grown at the Duke FACE experiment (Herrick, Maherali & Thomas 2004) demonstrated that gas exchange measurements taken in June and September, every year for a four year period, showed a reduction of 26% in both sun and shade leaves despite no significant alteration in stomatal densities. Another study (Lauber & Körner 1997) focusing on the stomatal responses of calcareous grassland community species growing under full season \([\text{CO}_2]\) enrichment (600 ppm) at low altitude in the Swiss Jura mountains showed variability in response at the species level with some species responding to experimental treatment and some showing no alterations in stomatal conductance. Again none of the species studied showed changes in stomatal density or stomatal index between treatments. It has even been demonstrated that in response to long term \([\text{CO}_2]\) enrichment, where daily concentrations are regularly in excess of 1000 ppm (van Gardingen \textit{et al.} 1995) and where species have been exposed over multiple generations, that despite persistent reductions in stomatal conductance, associated changes in stomatal density and index were not observed (Bettarini, Vaccari & Miglietta 1998).

It is important to note however, that studies of plant material over the last 100 to 200 years often show a strong and consistent decrease in stomatal density and/or \(g_{\text{max}}\) in response to \([\text{CO}_2]\) rises from approx. 300 to 400 ppm (Wagner \textit{et al.} 1996; Lammertsma \textit{et al.} 2011) which directly opposes results obtained from FACE studies.

Results obtained from the Giessen FACE site (chapter two) in addition to the fact that elevated \([\text{CO}_2]\) has driven an increase in aboveground biomass by 20 – 25% (Kammann \textit{et al.} 2005), provoked further investigation into leaf level gas exchange processes (chapters three and four). In absolute terms however, we were unable to determine any consistent
differences in stomatal conductance, photosynthetic rate or intrinsic water-use efficiencies, between ambient and elevated grown plants for any of the species examined (chapter two). Aside from the influence of environmental factors, it is considered that the timing of measurements may have had a significant bearing on physiological results (chapter two). In a recent study of this particular experimental FACE site carried out on spring growth, Haworth et al (in press) observed significant differences in photosynthetic rates in plants measured under optimal cuvette conditions (photosynthetically saturating light intensity, 20°C leaf temp, and constant vapour pressure deficit). Under these conditions there was a stimulation in photosynthetic rates of approximately 8 and 43% relative to ambient grown plants and a significant reduction in stomatal conductance for both *A. elatius* and *G. pratense*. The influence of [CO$_2$] enrichment on photosynthetic rate was not apparent under ambient cuvette conditions where it was considered that the heterogeneity of local environmental conditions and possible biotic pressures has impeded the recording of any significant change on plant gas exchange Haworth et al. in press). Similar to findings of this thesis (chapter three), there were no observable changes in stomatal morphology or initiation rates at this level of enrichment.

Although statistically significant evidence for photosynthetic acclimation was not universally observed in this study there were indications of partial acclimations for several species, including; *A. elatius*, *G. pratense*, and *P. lanceolata*, all of which showed slight increases in the carbon to nitrogen ratio of between 3.1 and 5.8%. Alterations in the carbon to nitrogen ratio of leaves is a factor that has been frequently linked with photosynthetic acclimation in many studies (Drake et al. 1997; Long et al. 2004) as it is indicative of a down regulation of Rubisco content at elevated [CO$_2$], where less Rubisco is required as photorespiration becomes increasingly inhibited (Rogers & Humphries 2000; Long et al. 2004), and may also
occur in conjunction with a build-up of non-structural carbohydrate within the leaf. In addition there was also seen a decrease in both photo respiration ($R_d$) and dark respiration rates ($R_n$), potentially due to prolonged exposure to $[\text{CO}_2]$ and a subtle, but chronic reduction in nitrogen availability occurring at the elevated rings.

These findings are corroborated by our own in which exposure of plants to instantaneous step changes in $[\text{CO}_2]$ revealed that despite no significant differences in intrinsic water-use efficiencies between ambient and elevated treatments, differences were apparent at high concentrations of $[\text{CO}_2]$, typically 750 ppm and above (chapter three). These findings indicate that acclimations may be occurring at relatively minor concentrations of $[\text{CO}_2]$ enrichment. However, as these alterations are relatively subtle, they are only detectable at very high levels of $[\text{CO}_2]$.

**Heritability of eco-physiological traits**

Increased genetic variation in response to elevated $[\text{CO}_2]$ has been detected in many plant taxa, indicating the potential for adaptive responses (Wieneke et al. 2004; Steinger, Stephan & Schmid 2007; Bishop et al. 2014) Despite this however, heritable trait responses have not been widely demonstrated amongst grassland species in response to moderate $[\text{CO}_2]$ enrichment. Chapter four addresses this current gap in understanding by investigating if species specific acclimations to $[\text{CO}_2]$ observed at the Giessen FACE site (chapter three) are heritable, thus advancing the idea elevated $[\text{CO}_2]$, even at a moderate level, may provoke genotypic response of modern plant species. If resource partitioning or alterations to C/N ratios, as described by Haworth et al. (in press) are heritable traits of plant species It may translate into heritable changes in physiological stomatal and/or photosynthetic function and
consequently be apparent amongst the offspring of trial species despite further alterations to environmental conditions.

In terms of precedent, there are some studies have demonstrated heritability of leaf traits which may in turn influence stomatal function (Geber & Dawson 1997; Case, Curtis & Snow 1998; Rae et al. 2006; Johnson et al. 2009). Heritable responses to elevated [CO₂] were demonstrated in a study carried out by Nakamura et al. (2011) where it was shown that plants transferred from habitats with higher [CO₂] had higher relative growth rates, greater leaf to root ratios, lower photosynthetic rates, and lower stomatal conductance, traits which were shared by the offspring of the particular species under consideration (*Plantago asiatica*). Other studies, such as that carried out by Collins & Bell (2004) have further described differences in the derivation of traits over multiple generations. In this particular study the multi-generational responses between separate genetic lines of green algae to [CO₂] enrichment evolved differing rates of photosynthesis and respiration, differing chlorophyll content and inter-specific variability in cell size thus highlighting the significant variability associated with the development of responses and the difficulties that may be linked with predicting species distributions under these conditions based on grouped plant assessments such as by functional type.

Building on observations from field studies we examined the possibility of a hereditary response by collecting seeds from plants exposed to both ambient and elevated [CO₂] concentrations at the Giessen field site, reversing those conditions and measuring IWUE responses to stepped changes in [CO₂] as had been done in the field. In this way the offspring of plants grown under elevated [CO₂] in the field could be grown at ambient [CO₂] concentrations in the chambers and vice versa. Results demonstrated that for the majority of species (*Trisetum flavescens* being the exception) physiological responses to treatment were
preserved amongst off-spring. In so doing we believe that this is the first study to demonstrate heritable trait responses of grassland species to moderate [CO₂] enrichment.

**Increasing species diversity and implications for leaf gas exchange rates**

Conscious of the fact that plants grown in competitive communities may not respond in precisely the same way as those grown as individuals or in monocultures an additional aim of this thesis was to decouple the inherent responses of species to environmental stress from the responses of plant–plant interactions.

In the conducting of chamber trials plants are very often grown as individuals, removed from any competitive pressures potentially imposed by the multitude of coexisting species typical of species rich grassland communities. In order to ascertain whether or not growth in increasingly species rich communities may alter physiological plant responses to environmental stimuli we conducted a glasshouse trial combining varying levels of species richness, or inter-specific competition, and drought stress via irrigation control of soil moisture concentrations.

This study carried out in chapter five describes the influence of plant community diversity on individual species responses to environmental stress (in this case drought treatment) and based on Investigations which have shown that species diversity can affect community stability/structure through influencing the responses of individual species to changes in their environment (Loreau *et al.* 2001; Tilman *et al.* 2006)). Here, plant assemblages with different levels of species richness (1 or 4 species mixes) were exposed to severe drought in order to determine any effect on plant stomatal behaviour, particularly in the role of preserving or
modifying plant Intrinsic water use efficiencies. Uniquely, we assessed plant stomatal conductance responses to drought conditions in species rich (mixed communities of four species) and species poor (monocultures) communities incorporating stomatal conductance as an axis of niche differentiation. For each species, the realised niche was considered to be the functional range of observed stomatal conductance values for a given set of environmental conditions. Principally findings demonstrate how competition for limiting resources increases convergence of trait values, implying that the majority of species present within a given environment possess similar nutrient/resource requirements and consequently similar optima in terms of use and acquisition of those resources, while drought stress provoked increasing divergence, or dissimilarity in resource use, highlighting the significance of niche segregation under periods of environmental stress. A further advantage of this approach to measuring physiological rates is that it incorporates the natural variability of responses which is perhaps a short coming of typical mean/median assessments (Violle et al. 2012). Based on these findings, we consider the incorporation of interspecific trait variability potentially key to improving our understanding of the effects of environmental changes on key plant traits which may, by extension, improve predictions in terms of how those changes will influence both biodiversity and ecosystem functioning.

In summary, we provide evidence for heritable responses and contemporary evolutionary responses to moderate levels of [CO₂] enrichment at a physiological level in a semi-natural grassland system. Significantly these responses are not uniform across all species, a finding which extensive ecological significance. Adaptation in growth and developmental traits has often been considered an ecological strategy providing a mechanistic basis for persistence in response to environmental change (Anderson, Panetta & Mitchell-Olds 2012). However, additional ecological strategies (distributional/range shifts and phenotypic plasticity) provide
additional means by which species may cope with environmental disturbance which potentially require no evolutionary modifications (Lau et al. 2007; Maestre et al. 2007; Lenoir et al. 2008). Currently little is known in terms of how ecological and evolutionary processes/responses may interact and in terms of future study, an assessment of relative abundance and growth rates of species at the Giessen FACE site may give a clearer insight into weather adaptive responses alone will translate into ecological success in response to elevated [CO₂]. Further, it is the assertion of this thesis that neither FACE nor Chamber experiments offer a comprehensive solution to assessing plant responses to environmental change. However, complementary use of both protocols allows for the isolation of significant environmental effects and an assessment of their true ecological implications by incorporating often unaccounted for biotic (chapter five) and abiotic factors present in a natural field environment (chapters two and three). Consequently we recommend a combined approach (chapter four) in the assessment of potential environmental disturbances on plant communities.
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Additional Published Work
Fossil plant stomata indicate decreasing atmospheric CO$_2$ prior to the Eocene-Oligocene boundary


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Abstract

The Cenozoic geological record is an ideal test lab to study the drivers, feedbacks and consequences of climate change. Concentrations of greenhouse gases, in particular carbon dioxide (pCO$_2$), are believed to play a pivotal role in climate change, but these can not be estimated accurately beyond ice core measurements. Recently, proxy-based estimates of pCO$_2$ and mass balance climate models have largely converged regarding the long-term evolution of pCO$_2$, but more needs to be done to determine the exact role of pCO$_2$ in climate change. The role of – and crucially the sensitivity of climate to – pCO$_2$ is a critical research question, still unsolved for the Cenozoic, as well as for present and future climate change.
Here we present a terrestrial stomatal proxy-based pCO$_2$ record from Saxony, Germany, derived from the extinct fossil plant species *Eotrigonobalanus furcinervis*, covering the latest Eocene, the important climate change boundary between the Eocene and Oligocene, as well as the latest Oligocene and the Oligocene-Miocene boundary. The stomatal proxy utilizes an inverse relationship between the density of stomata and pCO$_2$, to reconstruct paleo-pCO$_2$ using stomatal densities of fossil plants. This inverse relationship is well documented, and because it is species-specific, the most reliable stomatal proxy records are reconstructed using single species. Here we show that pCO$_2$ decreased continuously throughout the latest Eocene, in congruence with marine proxy temperature records, but does not change markedly at the Eocene-Oligocene boundary when marine isotope proxies register the most precipitous cooling of the Cenozoic. pCO$_2$ was similar at the Eocene-Oligocene boundary as it was in the latest Oligocene and at the Oligocene-Miocene boundary. Whereas the stomatal density record demonstrates a large relative decrease in pCO$_2$, calibrated values coinciding with the timing of Antarctic ice sheet formation are surprisingly low (<550 ppm) and likely require further independent validation and/or calibration. The new results presented here suggest that a decrease in pCO$_2$ may have significantly preceded the large decrease in temperatures that characterizes the Eocene-Oligocene transition, perhaps related to the “hysteresis effect” previously proposed – where a certain threshold of pCO$_2$ change was crossed before the cumulative climate change effects caused a rapid decline in temperatures and thus climate mode.
Introduction

The role of pCO₂ in Cenozoic climate

The Cenozoic era is characterized by large climatic variations, including the fundamentally important transition from an ice-free ‘greenhouse’ planet to the modern ‘icehouse’ planet with polar glaciations. The overall trend displays decreasing temperatures, punctuated by rapid temperature shifts in both directions, generally thought to have been driven primarily by changes in greenhouse gases, most importantly pCO₂ (DeConto and Pollard, 2003; Zachos et al., 2008; Hansen et al., 2013; Inglis et al., 2015). The full extent of the role of pCO₂ in Cenozoic climate change transitions remains however unresolved. To date, the most detailed Cenozoic temperature and pCO₂ proxy records are derived from marine isotope proxy methods (e.g. Foster et al., 2012; Pagani et al., 2011; Pearson et al., 2009; Zachos et al., 2001, 2008). Isotope records, however, may be influenced by a variety of taphonomic and diagenic biases (see Coxall and Pearson, 2007 for review; and Pagani et al., 2011), that obscure the climatic signal. Proxy-based pCO₂ reconstructions – including those based on fossil plant stomata, paleosols and carbon isotopes – have until recently differed up to an order of magnitude, in particular for the early part of the Cenozoic – with estimates ranging from hundreds to thousands ppm pCO₂ (see Royer et al., 2001). More recently however, these have converged, documenting an emerging coherent pCO₂ pattern with an obvious relationship with the global temperature records (Beerling and Royer, 2011). New results based on the marine Tex₈₆ proxy indicate that pCO₂ played a dominant role in Eocene cooling and the “descent into the icehouse”, with only a minor role ascribed to ocean gateway reorganization or paleogeographic change (Inglis et al., 2015).
The climate of the Eocene still constitutes one of the greatest unsolved problems in paleoclimate research. Temperatures were globally much higher than today, leading to a weakened equator-to-pole temperature gradient and muted seasonal cycle compared to today; the so-called ‘Eocene equable climate problem’ (Sloan and Barron, 1992). Climate modelling has been able to reconstruct this climatic pattern with excessively high pCO₂ levels (~4500 ppm: Huber and Caballero, 2011), but such elevated pCO₂ atmospheres do not agree with most proxy records. It has therefore been speculated that Eocene climate sensitivity was elevated compared to today and/or other forcing in addition to high pCO₂ was involved (Caballero and Huber, 2013; Hansen et al., 2013). In order to solve this enigma, reliable proxy records of pCO₂ are of paramount importance.

The fundamental climatic reorganization that occurred close to the Eocene-Oligocene boundary (~34 Ma), often referred to as the Eocene-Oligocene transition (EOT), had drastic consequences characterized by both terrestrial and marine faunal and floral extinctions accompanied by evolutionary turnover (Prothero, 1994; Coxall and Pearson, 2007; Sheldon et al., 2009: Kunzmann, 2012; Kvaček et al., 2014), although vegetation changes in the European terrestrial record appear to be less dramatic and more gradual (Kvaček et al., 2014; Kunzmann et al., submitted). This event marks a fundamental shift in climate mode when Earth descended from a globally warm ‘greenhouse’ climate with minimal polar ice into the ‘icehouse’ characterized by polar glaciations that prevail today. Deep-sea marine proxy records reveal a pattern of global cooling in the late Eocene, culminating in a rapid growth (< 200,000 years) of the first continental-scale ice sheet on Antarctica in the basal Oligocene (Coxall et al., 2005; Miller and Devernal, 1992; Zachos et al., 1996; 2001). General circulation models of Paleogene climate have shown that continuously declining pCO₂, amplified by Milankovitch forcing and ice-albedo feedbacks, could cause significant
temperature reduction resulting in a permanent continental Antarctic ice-sheet once a critical pCO₂ threshold is crossed, e.g. (DeConto and Pollard, 2003; Pollard and DeConto, 2005; Zachos and Kump, 2005; Hansen et al., 2013). Proposed mechanisms for the sudden EOT climatic shift thus include changes in global heat transport due to the opening of Southern Ocean gateways (Kennett, 1977), greenhouse gas drawdown as a consequence of mountain uplift and associated weathering (Molnar and England, 1990) or reduced volcanic outputs, and orbital forcing (Coxall et al., 2005; DeConto and Pollard, 2003). Modelling studies also indicate that lowering of atmospheric pCO₂ may have been the primary mechanism forcing this cooling transition, with a likely threshold for initiating Antarctic glaciation being pCO₂ of < 700 ppm (DeConto et al., 2008; Pagani et al., 2011). Recently, the timing of the EOT cooling has also been re-examined, with new results indicating that rather than a sudden cooling episode close to the EOT, pCO₂ decrease and subsequent substantial cooling took place gradually during the mid-late Eocene (Inglis et al., 2015). Reconstructing Eocene and EOT pCO₂ is thus a key requirement for constraining the links between climate and radiative forcing during the transition.

Four proxies have been identified as being particularly useful for Cenozoic reconstructions by the Intergovernmental Panel on Climate Change (IPCC report 2007); the terrestrial proxies based on stomatal densities of fossil plants and on carbon isotope composition of paleosols, as well as the marine proxies based on carbon isotope composition of fossil phytoplankton and boron isotope composition of fossil foraminifera. Each proxy is continually being fine-tuned to correct for inherent uncertainties and errors have been greatly reduced. For example, estimates based on paleosols tended, until recently, to overestimates pCO₂ (Cerling, 1991; Ekart et al., 1999; Breecker et al., 2010; Montanez, 2013), whereas stomatal proxy- pCO₂ datasets tend to underestimate Cenozoic pCO₂ by 150-250 ppm, requiring the
assignment of a “correction factor” and upwards revision (Kürschner et al., 2008; Beerling et al., 2009). Previous studies using the stomatal proxy method of pCO$_2$ reconstructions for the time intervals surrounding the EOT have been inconclusive. For example, results based on datasets of the gymnosperms Ginkgo biloba and Metasequoia glyptostroboides suggest that pCO$_2$ was more or less stable at between 300 and 450 parts per million by volume during the Eocene, Oligocene and Miocene (Royer, 2001; Royer et al., 2001) - however, the stomatal proxy-based pCO$_2$ are of low resolution and lack necessary detail: data are from the early Eocene and middle Miocene only, with the EOT pCO$_2$ estimated by interpolation. A later study based on data gathered from published images of G. biloba suggests a decrease in pCO$_2$ at the EOT (Retallack, 2001), but still with a large data gap across the EOT. More recent studies also suggest higher, and possibly rapidly decreasing, pCO$_2$ (ranging ca. 1000-500 ppm) in the late middle Eocene using M. glyptostroboides (Doria et al., 2011) and angiosperm species (Grein et al., 2011). Stomatal data based on fossil leaves from three angiosperm taxa suggest that pCO$_2$ were significantly higher at the EOT than during the Early Oligocene, however there is no data for the EOT proper and the stratigraphic resolution again is very low (Roth-Nebelsick et al., 2004). Subsequent studies using several angiosperm species in a leaf gas exchange model reconstruct early Oligocene to early Miocene pCO$_2$ to ca. 400 ppm throughout (Grein et al., 2013; Roth-Nebelsick et al., 2014). Nonetheless, most stomatal proxy-based pCO$_2$ records do seem to fall into the same general range of values for the same time periods and a consistent pattern is emerging for the Cenozoic (see Fig. 4, left panel).

Resolving the role of pCO$_2$ for Cenozoic climate requires improved proxy records of atmospheric pCO$_2$ at sufficient resolution to resolve leads and lags, revealing the primary forcing mechanism behind the climate change observed. All pCO$_2$ proxy tools carry inherent
weaknesses as well as strengths, therefore all available methods are required to provide information on paleo-pCO\(_2\) and thus substantiate consensus. Here we contribute to this effort by presenting a new stomatal proxy-based record spanning the late Eocene, EOT, late Oligocene and Oligocene-Miocene boundary. The record contributes towards covering the prevalent data gap in the stomatal proxy record near the EOT, is of substantial resolution when compared to other stomatal proxy records, and is based on a large dataset of plant leaf stomatal densities of a single fossil species throughout - considered ideal when employing this proxy method. The mechanisms behind and specific approaches involved in the stomatal proxy method are introduced in more detail below.

The stomatal proxy method of paleo-pCO\(_2\) reconstruction

Stomata are pores on plant leaf surfaces through which gas exchange takes place; i.e. carbon is attained for photosynthesis from CO\(_2\) and at the same time water vapour and oxygen are lost by diffusion. An inverse relationship exists between the frequency of stomata and pCO\(_2\), due to optimization of gas exchange by plants, which results in plants preserving water by reducing the number of stomata when CO\(_2\) is readily available. Woodward (1987) established this from observations of herbarium material, showing that modern tree species have responded to the anthropogenic rise in pCO\(_2\) by reducing their stomatal frequency significantly. The inverse relationship between stomatal frequency (recorded as “stomatal density”: the number of stomata per mm\(^2\) (SD) or the percentage of stomata relative to epidermal cells: the “stomatal index” (SI)) and pCO\(_2\) has been repeatedly demonstrated for a wide variety of plant taxa from disparate geological and ecological settings from the Palaeozoic until today and is thus established as a strong proxy for paleo-pCO\(_2\) (e.g. Beerling et al., 1998; McElwain, 1998; Retallack, 2001; Royer et al., 2001; Kürschner et al., 2008; Steinthorsdottir et al., 2011b; 2013: Steinthorsdottir and Vajda, 2015). The increasingly
A close match between stomatal proxy pCO$_2$ results and independent proxy records, climate modelling and actual pCO$_2$ measurements (e.g. Finsinger and Wagner-Cremer, 2009; Foster et al., 2012; Kürschner et al., 2008; Retallack, 2001; Rundgren and Björck, 2003; Steinthorsdottir and Vajda, 2015) instils growing confidence in this proxy for recording past pCO$_2$ and therefore for providing independent constraints on pCO$_2$ dynamics. Strongly supporting the validity of the stomatal proxy is also the recent identification of the mechanism by which plants control their stomatal densities based on atmospheric pCO$_2$: All plants use the enzyme carbonic anhydrase to detect pCO$_2$ around their leaves (Frommer, 2010; Hu et al., 2009); mature leaves (early shoots) then control stomatal development of younger leaves through long-distance signalling (Lake et al., 2002), involving the HIC gene signalling pathway (Brownlee, 2001; Gray et al., 2000).

In order to transform stomatal frequency data derived from fossil plants into paleo-pCO$_2$ estimates it is usually necessary to compare stomatal data from present day plants that are either relatives or in other ways equivalent to the fossil plants. Nearest living relatives (NLR) should be used when possible, but when they are not available nearest living equivalents (NLE= present day species that are of comparable ecological setting and/or structural similarity to their fossil counterpart) should be used instead (McElwain and Chaloner, 1995).

The stomatal response of plants is species (but in some cases broadly genus) specific (Kelly and Beerling, 1995; Kürschner et al., 1996; Kürschner et al., 1997; Steinthorsdottir et al., 2011a; Steinthorsdottir et al., 2011b) and it is therefore ideal to study fossil material with close modern analogues – most ideally same-species. However, the fossil records does not usually offer same-species fossil plant material further back than the Quaternary, so when reconstructing older material, assumptions about the most appropriate NLE must be made. Modern-day stomatal frequencies, in particular SI, do not show great intra-species variation,
but when studying fossil material, databases including abundant leaf material for each time interval studied must be amassed as far as possible, to minimize intra- and inter-species variation.

There are three stomatal paleo-pCO$_2$ calibration methods in use – the stomatal ratio method (McElwain and Chaloner, 1995; McElwain, 1998), which relies on a ratio between stomatal frequencies of fossil plants and their NLE to semi-quantify pCO$_2$; the transfer function methods, which rely on herbarium material and/or experimental datasets for NLR/NLE responses to calculate pCO$_2$ curves (e.g. Beerling and Royer, 2002); and more recently developed taxon-independent mechanistic gas exchange models (e.g. Franks et al., 2014; Grein et al., 2013; Roth-Nebelsick et al., 2014), which at their core use measurements of stomatal density and pore size to estimate maximum theoretical gas exchange rates from which palaeo-assimilation rate and pCO$_2$ are simultaneously estimated by iteration. Whereas the transfer function and gas exchange modelling approaches aim towards reconstructing the accurate absolute (quantitative) past pCO$_2$ using NLR and/or plant physiological measurements, the stomatal ratio estimates relative (qualitative) pCO$_2$ – in particular changes in past pCO$_2$ - using NLE for older fossil material, or NLR for younger material. The stomatal ratio method has however been shown to closely match results produced with transfer function methods (Beerling and Royer, 2002; Barclay et al., 2010; Steinthorsdottir et al., 2011b) and is seen as a good alternative where detailed estimates of other photosynthetic parameters, which are required to initialize mechanistic models, are not readily available. In addition, the stomatal ratio method is simple and can be applied to material that is less well preserved than required for the alternative methods, or does not have a well-defined NLR or NLE. The stomatal ratio method of calibrating paleo-pCO$_2$ relies on two so-called standardizations: the “modern” standardization that assumes that the ratio
between past and modern pCO₂ (taken as pre-industrial, 300 ppm) is 1 (RCO₂ = 1) and is applied to young material, typically from the Quaternary, as well as the “Carboniferous” standardization that sets the ratio between past and modern pCO₂ at two times preindustrial levels of 300 ppm (RCO₂ = 2 = 600) (McElwain and Chaloner, 1995). Given the semi-quantitative nature of the stomatal ratio method, both standardizations are usually applied to fossil leaf material of Mesozoic and Cenozoic age, to provide minimum and maximum pCO₂ estimates.

In this study we use the stomatal ratio method with SD and both standardizations. Many of the database leaves were preserved in such a way that it was not possible to quantify epidermal density (and thus SI), nor conduct accurate measurements on stomatal dimensions. Furthermore, obvious NLR do not exist for *E. trigonobalanus*, therefore a NLE had to be chosen based on taxonomy and structure of the fossil material, as well as the availability of herbarium and living leaf samples to determine SD at recent pCO₂ levels.
Material and methods

Fossil leaf database

_Eotrigonobalanus furcinervis_ (Rossm. 1840) Walther et Kaček in Kvaček and Walther 1989, an extinct evergreen Fagaceae, occurred from the middle Eocene to the Oligocene-Miocene boundary and was geographically widely distributed, i.e. from central Europe to Russia, as well as to the Mediterranean area (Mai and Walther, 2000). It is considered as a thermophilous species in evergreen broadleaved forests as well as in mixed mesophytic forests adapted to humid and warm-temperate to subtropical climate (Mai and Walther, 2000). _E. furcinervis_ tolerated a wide range of water table conditions and soil characteristics. It was therefore present in fossil taphocoenoses derived from riparian forests, back swamps, peat bogs and zonal vegetation. Whereas in Eocene it often predominated zonal Fagaceae-Lauraceae forests (Mai and Walther 2000), in the Oligocene mixed mesophytic forest this species was ecologically sub-dominant. Based on the record of cupules, seeds and leaves including cuticles it is commonly accepted that the fossils represents a single long-living but rather variable fossil species, although minor changes in leaf anatomy have led to the distinction of two subspecies, ssp. _furcinervis_ (mainly Eocene, rare in Oligocene) and ssp. _haselbachenses_ (only Oligocene; Kvaček and Walther 1989). The latter one is distinguished by the absence of pubescences (trichome clusters) on the abaxial leaf epidermis. Furthermore, a variety of leaf morphotypes are distinguished which can be interpreted as ecological variants (ecotypes) based on interdependencies between leaf morphology and environment.

The database analysed here consists of 233 _E. furcinervis_ leaf cuticle fragments on as many slides, representing 151 separate individual leaf specimens. All specimens are hosted at the
Senckenberg Natural History Collections Dresden, Germany (Fig. 1 and Table 1). Specimens were all collected from the central German Weiβelster Basin, a coastal alluvial plain at the southern margin of the North German–Polish ‘Tertiary’ Basin (Standke, 2008). This basin is well-known for its extensive record of middle Eocene to early Miocene megafloral assemblages that are mainly derived from azonal vegetation, i.e. riparian and swamp forests (e. g. Kunzmann, 2012). Here, we investigate a succession of cuticle rich megafossil assemblages or “taphocoenoses” that contain E. furcinervis, covering the late middle Eocene to the Oligocene-Miocene boundary (Fig. 2). Respective assemblages were reassessed according to the most recent lithostratigraphic concepts for central and East Germany (Standke, 2008; Standke et al., 2011). Their phytosтратigraphic ages were evaluated based on the regional palynomorph-based scheme of Krutzsch (2011). Accurate stratigraphic chronology for terrestrial lignite-bearing sedimentary successions is complicated by the lack of common index fossils caused by dissolution of any hard parts of animals by humic acids, hindering the level of stratigraphic resolution attainable for marine deposits (Rothe-Nebelsick et al., 2014), and all estimated ages must thus be regarded as approximate (Fig. 3). In the manuscript, age estimates have been assigned an error of ±1-2 million years, depending on the quality of age constraints. In particular, material representing a total of eight separate fossil plant chronological levels of the Weiβelster Basin is investigated (Table 1). In contrast to the mainly azonal vegetation from the coastal plains of the Weiβelster Basin, a single late Oligocene locality, Kleinsaubernitz (KS, LA/KS; fig. 2), is volcanic in origin and lies within the Lausitz basin, in particular at its southern margin or even in the hinterland (Standke, 2008). Leaf specimens derive from a sediment-filled maar, preserving a parautochtonous assemblage mainly representing zonal vegetation (Walther, 1999).
Stomatal density quantification

Pre-prepared cuticle samples provided by Senckenberg Natural History Collections Dresden were examined microscopically by an adaptation of the methodology set out by Poole and Kürschner (1999) in order to determine SD and SI. The fragmented nature of the fossil material did not allow establishing where individual cuticle samples were located on the original leaf surface, and consequently whether material was obtained from mid lamina or less preferable marginal regions of the leaf surface (see Fig. 1). In addition, individual epidermal cells were not easily discernible in the majority of the *E. furcinervis* material, making SI determination impossible, and thus SD only was recorded. SD was obtained using a Nikon SK Polarized Light Microscope at x200 magnification with a graticule (grid) providing a counting field of 0.042 mm$^2$. The graticule was centred over areas where stomata occurred in greatest numbers (i.e. probably away from veins and margins) and between up to five counts were recorded for each slide, resulting in 659 SD counts for the database of 151 individual leaf specimens (Table 1). Data was stored in Microsoft Excel 2010 before being statistically manipulated using MINITAB (version 16.1.1 for Windows).

Paleo-pCO$_2$ calibration

*Eotrigonobalanus furcinervis* belongs to the Fagaceae, but its position is not well phylogenetically defined. Based on cupule morphology, genus *Eotrigonobalanus* belongs to a basal clade of the family, exhibiting intermediate characters between modern *Trigonobalanus* and *Castanopsis* (Mai, 1995), but comparison of leaf venation and leaf cuticle micromorphology place *Eotrigonobalanus* with *Trigonobalanus* and *Lithocarpus*, away from *Castanopsis* (Kvaček and Walther, 1989) and this affiliation has recently been confirmed (Denk et al., 2012). However, since the phylogeny of Fagaceae has changed
considerably (Manos et al., 2001; Manos et al., 2008), an improved systematic framework is required to confirm the evolutionary position of *Eotrigonobalanus* within the family. Because its exact relationship to crown group genera is unknown and, therefore, a nearest living relative (NLR) could not be obtained for stomatal proxy-based pCO$_2$ reconstruction the the nearest living equivalent (NLE) approach was selected.

In this study, *Trigonobalanus doichangensis* was chosen as NLE, due to it being a basal species within the Fagaceae family and having leaf macro-morphological and cuticle micro-morphological affinities with *E. furcinervis*, including cyclocytic stomata and similar structured trichomes (Kvaček and Walther, 1989; see also Denk et al., 2012). Leaf cuticle specimens collected in 1988 were sampled at the Kew Herbarium (Royal Botanical Gardens, Kew, Richmond, Surrey, UK) and analysed in the laboratory. SD was determined to be 546.11/mm$^2$ at pCO$_2$ of 351 ppm (collection year levels according to NOAA ESRL data).

Using the stomatal ratio method with *T. doichangensis* NLE for *E. furcinervis*, we calibrated paleo-pCO$_2$ using the equations below to derive minimum and maximum paleo-pCO$_2$ ("Modern" and "Carboniferous" Standardization of McElwain and Chaloner, 1995), respectively:

$$\text{Paleo-pCO}_2\min\ (\text{ppm}) = \left( \frac{\text{SD}_\text{NLE} = 546.11}{\text{SD}_\text{fossil}} \right) * 351 \text{ ppm}$$

$$\text{Paleo-pCO}_2\max\ (\text{ppm}) = \left( \frac{\text{SD}_\text{NLE} = 546.11}{\text{SD}_\text{fossil}} \right) * 600 \text{ ppm}$$
Results

Stomatal density

SD of *E. furcinervis* are unusually high, ranging between ca. 425 and 740 stomata/mm². The lowest SD values (signifying highest pCO₂) are found in the oldest deposits, mid to late Eocene in age (ca. 39 Ma) and the highest values (signifying lowest pCO₂) are found in late Eocene (ca. 35.5 Ma), representing the most significant SD change during the time period covered by the dataset: SD increases by >300 stomata/mm² or by ca. 75%, a very significant change indicating a sizeable decrease in pCO₂ in ca. 3.5 million years (Table 1). Stomatal densities then decrease slightly again and remain around 640/mm² in the latest Eocene and earliest Oligocene (representing the Eocene-Oligocene transition at 34 and 33.8 Ma respectively), as well as in the late Oligocene. At the Oligocene-Miocene boundary, SD decreases again to ca. 570/mm². The overall pattern is therefore a ca. 75% decrease in SD from the Mid-Late Eocene to the late Late Eocene prior to the Eocene-Oligocene transition, and relatively stable SD values from the latest Late Eocene to the Oligocene-Miocene boundary.

Paleo-pCO₂ estimates

Paleo-pCO₂ calibrated using the stomatal densities of *E. furcinervis* will be discussed mainly as average values and evaluated in terms of relative change, as introduced above. The largest change in palaeo-pCO₂ is the decrease from the mid-late Eocene (ca. 39 Ma) to Late Eocene (ca. 35.5 Ma) of >250 ppm, from ca. 630 ppm to ca. 365 ppm – a decrease in pCO₂ of ca. 40% (Fig. 4; Table 1). Concentrations of CO₂ then increase again by ca. 45 ppm to ca. 410 ppm throughout the latest Eocene and earliest Oligocene. Further increases then take
place, to ca. 430 ppm by the Late Oligocene and ca. 475 ppm at the Oligocene-Miocene boundary.

Overall, the pCO$_2$ pattern detected includes a significant decrease in pCO$_2$ taking place during the late Eocene; pCO$_2$ of ca. 410 ppm surrounding the Eocene-Oligocene boundary; as well as the subsequent Late Oligocene warming culminating at the Oligocene-Miocene boundary (Fig. 4).
Discussion

Fidelity of the Saxony stomatal pCO\textsubscript{2} record

The Saxony fossil leaf database and the derived pCO\textsubscript{2} record are unique in two important ways: Firstly, this relatively large database derives from a well-constrained stratigraphic succession and consists of a single species throughout – *E. furcinervis* – which is, as discussed above, the most ideal situation when using fossil leaf material to reconstruct paleo-pCO\textsubscript{2}, since inter-species variability is eliminated and stomatal responses to pCO\textsubscript{2} is likely to be consistent through time. The procurement of a single-species dataset from multiple stratigraphic levels across several million years is not common, in particular when the stratigraphy represents time intervals of significant climate and/or environmental change, as is the case here. Secondly, the derived pCO\textsubscript{2} record is the first terrestrial stomatal proxy pCO\textsubscript{2} record that so closely envelopes the EOT, an important climate change interval, previously marred by a stomatal proxy data gap.

The principal challenge concerning the Saxony stomatal density record was translating the stomatal signal into reliable levels of pCO\textsubscript{2}. One of the main limitations associated with the use of paleo-proxies is the preservational state of fossil material and in this case the preservation of fossil leaves did not allow paleo-pCO\textsubscript{2} reconstruction using gas exchange models, but only using stomatal densities with the stomatal ratio method, identified as the least quantitative of the three stomatal proxy-based methods for pCO\textsubscript{2} reconstruction. Additionally, there is a lack of available transfer functions for potential NLEs of *E. furcinervis*, so it was not possible to obtain independent pCO\textsubscript{2} reconstructions for comparison and consensus. Secondly, the absence of an obvious NLE for *E. furcinervis* – an extinct species
of uncertain phylogenetic affinity – further introduces errors in pCO$_2$ calibration. Although we consider *T. doichangensis* the best available NLE, there is no guarantee that its stomatal density and degree of response to pCO$_2$ closely mirrors that of its distant fossil relative. The pCO$_2$ levels calibrated here are indeed low – probably artificially low – compared to most previously published pCO$_2$ datasets, although broadly comparable to stomatal pCO$_2$ records (Fig. 4, left panel). When testing three additional potentially suitable NLE species for constructing separate pCO$_2$ records; *Trigonobalanus verticillata, Castanopsis cuspidata* and *Lithocarpus henryi*, the resulting paleo-pCO$_2$ values were extremely low – considerably lower than when using the chosen NLE *T. doichangensis* – in many cases being incompatible with photosynthesis. This indicates that, for some reason, the stomatal proxy-derived pCO$_2$ estimates presented here are in all likelihood artificially low and should be adjusted upwards until this methodological problem is solved. This problem is by no means limited to this study, as will be discussed below. To summarize, the single-species stomatal density record is of high fidelity, likely to reflect stomatal responses to changes in pCO$_2$ which can be used to semi-quantify paleo-pCO$_2$, in particular relative change over time. However, we consider the pCO$_2$ numerical values presented here to be an approximation to the actual pCO$_2$ levels present in the late Eocene to the Oligocene-Miocene boundary.

Comparison with other pCO$_2$ records

Previously published stomatal proxy-based Cenozoic pCO$_2$ records do not always agree, but report highly elevated (McElwain, 1998; Doria et al., 2011; Grein et al., 2011; Smith et al., 2010), intermediate (Retallack, 2009) or similar to modern (Royer et al., 2001;) pCO$_2$ for the Eocene. Similarly high variability in estimated pCO$_2$ levels exists for the Oligocene as well as the Miocene (Grein et al., 2013; Kürschner et al., 2008; Roth-Nebelsick et al., 2014; Royer et al., 2001). In a series of papers studying stomatal parameters of plants from the Late Eocene
to Early Miocene of Saxony, some including analysis of *E. furcinervis*, an overall trend emerged of higher pCO$_2$ in the Late Eocene, lower pCO$_2$ in the Early Oligocene and intermediate, stable levels of pCO$_2$, showing no statistically significant variation, in the Late Oligocene, at the Oligocene-Miocene boundary and in the Early Miocene (Grein et al., 2011, 2013; Roth-Nebelsick et al., 2004, 2014). These trends are independently supported by flora-based climate proxies and partially reflect the global marine temperature data, in particular the cooling at the Eocene-Oligocene transition (Roth-Nebelsick et al., 2004; 2012; Zachos et al., 2001, 2008). When pCO$_2$ levels are quantified based on stomatal parameters in a mechanistic model (Konrad et al., 2008), concentrations ranging from 400-700 ppm, but most consistently around 400 ppm, are found throughout the studied intervals, notably despite changes in stomatal parameters (Grein et al., 2013; Roth-Nebelsick et al., 2014).

Using a rigorous generalized statistical framework, Beerling et al. (2009) revised previously published pCO$_2$ estimates based on *Ginkgo* and *Metasequoia* from the early Eocene and middle Miocene upwards by150-250 ppm. Based on this revision, average stomatal proxy-based pCO$_2$ is 450-700 ppm in the Paleogene and 500-600 ppm in the Neogene (Beerling et al., 2009). Interestingly, the younger set of pCO$_2$ estimates was fully compatible to marine proxy data and modelling results (e.g. Pagani et al., 2005; Hansen et al., 2008), whereas the older set of estimates seemed to underestimate pCO$_2$ compared to the other approaches, even after the upwards revision of stomatal pCO$_2$ values (see Fig. 4 in Beerling et al., 2009). The seemingly more pronounced underestimation for pCO$_2$ values based on older material is also found in the present study, where mid Eocene to earliest Oligocene pCO$_2$ values are at the very low end or lower than previously published stomatal estimates, whereas values from the Oligocene-Miocene boundary and early Miocene are in broad agreement with previous estimates (see Fig. 4, left panel). However, Kürschner et al. (2008) indicated that an upwards
correction factor of 150-200 ppm was necessary also when reconstructing Miocene paleo-pCO$_2$ with two species from the Lauraceae family. In general therefore, there is a tendency for Cenozoic stomatal proxy-based pCO$_2$ values, reconstructed using the available methods, to report consistently lower pCO$_2$ values than alkonone- or boron-based proxies as well as those from mass balance modelling. More work needs to be done in order to refine these methods and obtain more accurate quantitative pCO$_2$ values based on stomatal densities, but stomatal proxy records are still highly valuable in showing relative changes as well as baseline pCO$_2$ values for the past.

Pearson et al. (2009) reconstructed pCO$_2$ for the first time across the EOT using the planktonic foraminifera boron isotope pH proxy and found that the main reduction in pCO$_2$ took place before the main phase of ice growth (ca. 33.6 Ma: DeConto et al., 2008), followed by a sharp recovery to pre-transition levels and then a more gradual decline. Their results thus confirm the central role of declining pCO$_2$ in Antarctic ice sheet initiation and development and agree broadly with carbon cycle modelling (e.g. Merico et al., 2008). The quantitative estimates of pCO$_2$ varied greatly however, according to which d$^{11}$B value was used to derive pH, with geochemical models of the boron cycle suggesting a range of 37-39 ‰ for sea water (sw) d$^{11}$B during this time (Simon et al., 2006). Pearson et al. (2009) use the central estimates for d$^{11}$B with sw = 38 ‰ and find that pCO$_2$ was in the range of 900-1100 ppm before the main phase of ice growth (ca. 34.6-34 Ma), decreasing to ca. 750 ppm at 34.59 Ma, before recovering briefly to ca. 1100 ppm (33.47-33.33 Ma) and then decreasing again to ca. 700 ppm. Comparing these results with estimates of pCO$_2$ with d$^{11}$B with sw = 37 ‰ produce concentrations which are considerable lower and very similar to the estimates reported here, ranging from ca. 550 ppm before the main phase of ice growth, decreasing by 100 ppm to ca. 450 ppm at 33.59 Ma, increasing briefly to ca. 620 ppm and then declining to
ca. 400 ppm. Estimates of pCO$_2$ with $d^{11}$B with sw = 39 ‰ produce results which are much higher – ca. 2000 ppm before main ice growth, ca. 1500 at 33.59 Ma and first increasing thereafter to >2000 ppm, before declining again.

Recently published alkenone-based pCO$_2$ records also found significantly declining pCO$_2$ before and during the Antarctic glaciation (EOT and earliest Oligocene), supporting the pCO$_2$ pattern of Pearson et al. (2009) and the role of pCO$_2$ as the primary forcing agent of Antarctic glaciation, consistent with model derived thresholds (Pagani et al. 2011; Zhang et al., 2013). The alkenone-derived dataset values are overall higher, with pre-EOT values of ca. 1000 ppm, minimum value of ca. 670 at 33.57 Ma and then gradual decline to ca. 350 ppm at the Oligocene-Miocene boundary.

Comparison with vegetation change and proxy continental climate records

Paleoclimate reconstructions based on Central European megafloras reveal a sharp decline in continental cold month mean temperature (Mossbrugger et al., 2005) and mean annual temperature (Moraweck et al., 2015; Kvaček et al., 2014) prior to the EOB which is consistent with the timing of the CO$_2$ decline that we report here (Fig 4, mid panel). Furthermore, palaeo-vegetation analysis of the Weiβelster and North Bohemian basins reveal that gradual restructuring of dominantly evergreen forests by immigration of deciduous species such as *Platanus neptuni*, *Trigonobalanopsis rhamnoides* and *Taxodium dubium* (Kunzmann et al. subm.) took place in the late Bartonian to early Priabonian interval at ca. 38 Ma (Kvaček, 2010; Teodoridis and Kvaček, in press), also prior to the EOT. The temporal coincidence of pCO$_2$ decline and major vegetation transition – from angiosperm-dominated notophyllous evergreen forests to mixed mesophytic forests – suggest a potential causal role
of pCO₂ decline in the changing ecological composition of forests. It may have been in part triggered by differential responses of evergreen and deciduous taxa to declining pCO₂ (Fig 4). The functional trait of deciduousness is an adaptation to episodic cooling (Zanne et al., 2014), however it has also been demonstrated experimentally (McElwain et al., 2015) and on theoretical grounds (Niinemets et al., 2011) that taxa with low leaf mass per area (i.e. those that are deciduous or herbaceous) and high stomatal conductance have faster photosynthetic rates that evergreens at lower atmospheric pCO₂. In contrast evergreens have higher responsiveness in terms of photosynthetic rates at elevated pCO₂ (Niinemets et al., 2011). Further experimental investigation is now required to tease apart the relative importance of ‘CO₂ starvation’ and increased seasonality on the late Bartonian to early Priabonian vegetation transition.

The issue of stomatal proxy-based pCO₂ reconstructions almost exclusively showing considerably lower pCO₂ than other pCO₂ proxies is of yet unresolved. An important advance was made when it was demonstrated that Cenozoic pCO₂ estimates based on stomata should be adjusted upwards by 150-250 ppm to closely match the estimates based on additional proxies (Kürschner et al., 2008; Beerling et al., 2009). However, the fact remains that by now numerous Cenozoic pCO₂ records based on stomatal parameters of a range of woody plant species all indicate considerably lower pCO₂ than those reconstructed using other proxies. Stomatal proxy-based pCO₂ records that are independently calibrated using different species/genera and families usually agree with one another and show Eocene-Miocene pCO₂ in the range of 300-800 ppm (Fig. 4). Although the stomatal proxy clearly needs to be improved before significant reevaluation of the role of pCO₂ in Cenozoic climate change and thus climate sensitivity during this time is warranted, it should not be a priori
rejected that stomatal proxy records may accurately indicate much lower pCO₂ levels during the Cenozoic than previously assumed.
Conclusions

A new terrestrial stomatal proxy-based pCO$_2$ record, derived from fossil leaves of species *Eotrigonobalanus furcinervis* from Saxony, Germany, covers the late Eocene to the Eocene-Oligocene transition at high resolution, as well as the late Oligocene and the Oligocene-Miocene boundary. The record indicates that pCO$_2$ decreased continuously and gradually during the late Eocene, from ca. 600 ppm in the earliest late Eocene to ca. 410 ppm at the Eocene-Oligocene boundary, but did not experience a precipitous fall at the Eocene-Oligocene boundary in contrast with marine isotope temperature records. Late Oligocene and Oligocene-Miocene boundary pCO$_2$ is stable at around 450 ppm. The late Eocene gradual but significant decrease in pCO$_2$ reported here is independently supported by terrestrial records of vegetational change and reconstructions of coldest month mean temperatures. Although the pCO$_2$ values reported here may be artificially low, due to errors introduced in pCO$_2$ calibration, they nonetheless broadly agree with previously published stomatal proxy records where there is overlap, indicating that Cenozoic pCO$_2$ may have been considerably lower than previously thought. The Saxony record further strongly indicates that decrease in pCO$_2$ took place much more gradually than the recorded decrease in global sea surface temperatures, indicating that a tipping point was reached in the latest Eocene, plunging the Earth System into icehouse conditions, supporting the hysteresis hypothesis for this important climate change event.
Stomatal Responses to Biotic and Abiotic Stress

Additional Published Work

Tables

Table 1. Lithostratigraphic and phytosratigraphic positions of the *Eotrigonobalanus furcinervis*-containing fossil taphocoenoses in the Weißeelster Basin (central Germany); lithostratigraphy after Standke et al. 2010, spore-pollen zonation after Krutzsch (2011).

<table>
<thead>
<tr>
<th>Assemblage / site</th>
<th>Reference for fossil flora</th>
<th>Formation</th>
<th>Member</th>
<th>Horizon</th>
<th>Epoch</th>
<th>Spore-pollen zone</th>
</tr>
</thead>
<tbody>
<tr>
<td>Witznitz</td>
<td>Mai and Walther 1991</td>
<td>Cottbus</td>
<td>Thierbach</td>
<td>Witznitz</td>
<td>end of Oligocene</td>
<td>II</td>
</tr>
<tr>
<td>Espenhain-Störmthal</td>
<td>Mai and Walther 1991</td>
<td>Cottbus</td>
<td>Thierbach</td>
<td>Witznitz</td>
<td>end of Oligocene</td>
<td>II</td>
</tr>
<tr>
<td>Haselbach 2</td>
<td>Mai and Walther 1978</td>
<td>Böhlen</td>
<td>Gröbers</td>
<td>Haselbach</td>
<td>earliest Oligocene?</td>
<td>20A/B</td>
</tr>
<tr>
<td>Schleenhain 4</td>
<td>Kunzmann and Walther 2012</td>
<td>Böhlen</td>
<td>Gröbers</td>
<td>Haselbach</td>
<td>earliest Oligocene?</td>
<td>20A/B</td>
</tr>
<tr>
<td>Schleenhain 3</td>
<td>Kunzmann and Walther 2002</td>
<td>Borna</td>
<td>Domsen</td>
<td>overlying bed of lignite seam 23o</td>
<td>latest Eocene</td>
<td>19 (?)</td>
</tr>
<tr>
<td>Schleenhain 2</td>
<td>Ferdani 2014, Mai and Walther 2000</td>
<td>Borna</td>
<td>Bruckdorf</td>
<td>underlying bed of lignite seam 23o and leaf measure in lignite seam 23o</td>
<td>late Eocene</td>
<td>18o</td>
</tr>
<tr>
<td>Haselbach 1</td>
<td>Mai and Walther 2000</td>
<td>Borna</td>
<td>Bruckdorf</td>
<td>intercalated bed between lignite seam 23u and 23o</td>
<td>late Eocene</td>
<td>18uo</td>
</tr>
<tr>
<td>Schleenhain 1</td>
<td>Hennig and Kunzmann 2013</td>
<td>Borna</td>
<td>Bruckdorf</td>
<td>overlying bed of lignite seam 23u</td>
<td>late Eocene</td>
<td>18u</td>
</tr>
<tr>
<td>Knau</td>
<td>Mai and Walther 2000</td>
<td>Borna</td>
<td>uncertain</td>
<td>fluvial deposit</td>
<td>late Eocene</td>
<td>17/18</td>
</tr>
<tr>
<td>Profen-Süd</td>
<td>Fischer in Mai and Walther 2000</td>
<td>Profen</td>
<td>Wallendorf</td>
<td>underlying bed of lignite seam 1</td>
<td>late middle Eocene</td>
<td>17</td>
</tr>
</tbody>
</table>
Table 2. The Saxony *Eotrigonobalanus furcinervis* database, including spore-pollen zones (Krutzsch 2011) and epoch inferred from them, stomatal density counts and pCO₂ calibration results, all shown with standard deviation, average pCO₂ in bold. Comparison to previously published stomatal proxy-based pCO₂ results from central Germany and nearby regions listed in the far right column.

<table>
<thead>
<tr>
<th>Sites</th>
<th>Epoch</th>
<th>Spore/pollen zone</th>
<th>SD (stomata/mm²)</th>
<th>pCO₂ Min (ppm)</th>
<th>pCO₂ Max (ppm)</th>
<th>pCO₂ Average (ppm)</th>
<th>No. of leaves</th>
<th>Other studies CO₂ ppm</th>
</tr>
</thead>
<tbody>
<tr>
<td>Witznitz, Espenhain-Störmthal</td>
<td>latest Oligocene</td>
<td>II</td>
<td>569.02 ± 108.40</td>
<td>351.6 ± 79.12</td>
<td>600.02 ± 135.03</td>
<td>475.81 ± 107.08</td>
<td>45</td>
<td>~420 to ~530 ppm¹</td>
</tr>
<tr>
<td>Kleinsaubernitz</td>
<td>late Oligocene</td>
<td>20G</td>
<td>623.29 ± 97.82</td>
<td>316.8 ± 58.41</td>
<td>540.71 ± 99.7</td>
<td>428.76 ± 79.05</td>
<td>25</td>
<td>~400 ppm¹</td>
</tr>
<tr>
<td>Schleenhain 4, Haselbach 2</td>
<td>earliest Oligocene</td>
<td>20 A/B</td>
<td>657.13 ± 118.98</td>
<td>302.5 ± 59.31</td>
<td>516.29 ± 101.23</td>
<td>409.40 ± 80.27</td>
<td>21</td>
<td>n/a</td>
</tr>
<tr>
<td>Schleenhain 3</td>
<td>latest Eocene</td>
<td>19</td>
<td>642.88 ± 84.05</td>
<td>303.1 ± 35.54</td>
<td>517.24 ± 60.66</td>
<td>410.17 ± 48.10</td>
<td>11</td>
<td>n/a</td>
</tr>
<tr>
<td>Schleenhain 2</td>
<td>late Eocene</td>
<td>18 o</td>
<td>740.65 ± 148.90</td>
<td>269.56 ± 53.01</td>
<td>460.05 ± 90.48</td>
<td>364.80 ± 71.74</td>
<td>39</td>
<td>n/a</td>
</tr>
<tr>
<td>Haselbach 1</td>
<td>late Eocene</td>
<td>18 uo</td>
<td>505.88 ± 47.06</td>
<td>373.50 ± 35.99</td>
<td>637.45 ± 61.43</td>
<td>505.48 ± 48.72</td>
<td>2</td>
<td>~470 (ave.)² ~270 (min)² ~710 (max)²</td>
</tr>
<tr>
<td>Schleenhain 1</td>
<td>late Eocene</td>
<td>18 u</td>
<td>661.18 ± 90.93</td>
<td>296.15 ± 44.65</td>
<td>505.43 ± 76.206</td>
<td>400.79 ± 60.429</td>
<td>4</td>
<td>n/a</td>
</tr>
<tr>
<td>Knau</td>
<td>late Eocene</td>
<td>17/18</td>
<td>495.50 ± 77.80</td>
<td>397.33 ± 68.7</td>
<td>678.12 ± 117.25</td>
<td>537.73 ± 92.98</td>
<td>4</td>
<td>n/a</td>
</tr>
<tr>
<td>Profen-Süd</td>
<td>late middle Eocene</td>
<td>17</td>
<td>426.14 ± 83.56</td>
<td>467.87 ± 101.78</td>
<td>798.51 ± 173.71</td>
<td>633.19 ± 137.74</td>
<td>1</td>
<td>~470 (ave.)² ~270 (min)² ~710 (max)²</td>
</tr>
</tbody>
</table>
Applying Konrad et al. (2008) stomatal optimization model in a multispecies consensus approach (Grein et al., 2013)

Applying Konrad et al. (2008) stomatal optimization model to stratigraphically lumped *Eotrigonobalanus furcinervis* samples from Profen and Haselbach (Roth-Nebelsick et al., 2012). n/a = Individual site CO\(_2\) data not reported so direct comparison not possible.

**Figures**

Figure 1. *Eotrigonobalanus furcinervis* (Rossm. 1840) Walther et Kvaček in Kvaček and Walther 1989, A: mass occurrence of leaves in lignite, Schleenhain opencast mine, Saxony, Germany, site Schleenhain 2, Borna Formation, Bruckdorf Member, late Eocene (Priabonian), SPP zone 18o, MMG PB SchleOE 535; B: abaxial leaf cuticle with stomata and trichome bases, Schleenhain opencast mine, Saxony, Germany, site Schleenhain 4, Böhlen Formation, Gröbers Member, earliest Oligocene (Rupelian), SPP zone 20A/B, slide MMG PB SchleMO 11/05 from leaf SchleMO 556/2.
Figure 2: Sites (asterisks) of *Eotrigonobalanus furcinervis*-containing fossil taphocoenoses in central and east Germany considered in the present investigations, note: the Schleenhain and Haselbach opencast mines revealed taphocoenoses in four and two distinct lithostratigraphic positions respectively (see also stratigraphic chart in fig. 3). Map legend: D = Germany, CZ = Czech Republic, PL = Poland, FR = France, NL = the Netherlands.
Figure 3. Stratigraphic position of the assemblages with *Eotrigonobalanus fucinervis*, regional lithostratigraphy and Krutzsch’s (2011) correlation to the spore-pollen zones including his proposed correlation of spore-pollen zones to global scale (see text section 2.2 for explanation and comments on dating uncertainty); black vertical bars next to assemblage names are the temporal uncertainty (based on a combination of lithostratigraphic information of the respective unit and spore-pollen zonation); bars of Schleenhain 1 and 2 are not to scale because gaps in the sediment deposition of the respective units are not equivalent to the duration of spore-pollen zones; gaps between the Eocene spore-pollen (sub-)zones illustrate gaps in the terrestrial sediment record, i.e. erosion. For horizon information see table 1.
Figure 4. CO₂, vegetation and climate trends through the Cenozoic. The most significant changes in pCO₂, forest ecosystem composition (A) and continental climate as tracked by terrestrial plants (B) take place in the late Eocene, whereas the most significant change in global temperatures as tracked by marine isotopes (C) takes place at the Eocene-Oligocene boundary, indicating that the significant climate transition at the Eocene-Oligocene boundary was preceded by a gradual decrease in pCO₂ during the late Eocene. (A): pCO₂ estimates from fossil stomata (this study pink with black error bars) in the context of existing stomatal proxy estimates (in grey from Beerling and Royer, 2011) in a chronostratigraphic framework. Vertical bar shows the gradual late Eocene vegetational restructuring of the dominantly evergreen forests of the Weißenster and North Bohemian basins studied here (dark green to light green), suggesting a potential causal role of pCO₂ decline in the changing ecological forest composition (*Kunzmann and Walther, 2012; ** Kvaček et al., 2014; Kunzmann et al., submitted). Note that the assigned ages for CO₂ values from this study are estimated based on the biostratigraphic controls presented in Fig 3. Absolute ages were not available for any of the nine fossil study sites (Table 2) although clear superposition information is available throughout allowing good estimates of the temporal sequence of CO₂ estimates (see Fig 3). (B): Continental temperature curve: Record of continental cold month mean temperature for Central Europe during the last 45 My, redrawn from Mosbrugger et al (2005). Horizontal bars represent coexistence intervals. Orange curve shows data from the Weißenster and Lausitz Basins, northeast Germany; blue curve shows data from the Lower Rhine Basin, northwest Germany (see Mosbrugger et al., 2005).
for details). (C): Global climate (temperature) curve derived from stacked records of deep-sea benthic foraminiferal oxygen-isotopes: a proxy for relative changes in marine temperature in the late Eocene prior to ice build up, based on updated records from Deep Sea Drilling Project and Ocean Drilling Program sites. Raw data is smoothed by using 15-point running mean, to minimize biases introduced by uneven temporal and spatial distribution of records (data from Zachos et al., 2001; 2008; and references therein).
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