Long-term ecophysiological responses to climate change

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Publication date:
2013

Document Version
Publisher's PDF, also known as Version of record

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PhD thesis

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May 2013

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PhD thesis

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“ The road

to wisdom:

Well, it’s plain
and simple to express.
Err and err and err again,
but less and less and less. ***”***

- Piet Hein

Preface

This thesis is the result of a three year PhD in the CLIMAITE project. The work was financed by the Villum Kann Rasmussen Foundation and carried out at the ECO center, Department of Chemical and Biochemical Engineering (KT), Technical University of Denmark - but more than 200 days was used at the field-site of CLIMAITE, Brandbjerg.

Numerous people have been involved through the process of this thesis; to whom I am deeply grateful. First, thanks to my supervisors; Teis N. Mikkelsen, Andreas Ibrom and Helge Ro-Poulsen always for being ready for discussions and supporting when needed. In line with my supervisors, I would like to send a special thanks to Kristian R. Albert for all the fruitful discussions and the extraordinary support throughout the 3 years. Moreover, thanks to Nina W. Thomsen for helping with all the field work and for keeping the spirit high also in rain and doing cold times.

The multi-disciplinary scientific environment in the CLIMAITE project has opened new perspectives in my individual work and for that I am grateful. Thanks to all the scientists involved in CLIMAITE for all the good time and discussions. Special thanks to the great technicians; Preben Jørgensen, Poul T. Sørensen, Nina W. Thomsen, Andreas Fernqvist and Svend Danbæk for keeping the CLIMAITE project running. Further a special thanks to the all the PhD students in CLIMAITE for the useful discussions on graphical and statistical issues – and also for all the fun and laughter.

Thanks to all the people in the ECO center at KT, for increasing my knowledge in new scientific fields and for making the daily life more fun. Thanks to all for the great time, cake and parties. Obviously also thanks to all the PhD students in the ECO-group for sharing ups and downs. Special thanks to Sabine Reinsch, Cathrine Heinz Ingvordsen and Eike Marie Thaysen for patient listening to my frustration and support during the final stage of this thesis.

More, I would thanks friends and family for believing in me and be understanding and patient in the absent of my company. Final a special thanks to Lars for always to be their when I need it - without you it would not have been possible!

Kristine Stove Boesgaard
May 2013

Abstract

Plant physiology is affected by climate change. Acclimations of photosynthetic processes are induced by short-term changes in climatic conditions. Further acclimation can be caused by long-term adjustments to climate change due to ecosystem-feedbacks. The aim of this PhD was to investigate plant physiological responses to climate change in a seasonal and long-term perspective.

The effects of elevated CO_2, passive night time warming and periodic summer drought as single factor and in combination, on plant physiology were investigated in the long-term multi-factorial field experiment CLIMAITE in a Danish heathland (www.climaite.dk). The responses of plant physiological parameters, such as photosynthetic capacity, stomatal conductance and respiration were measured after six years of treatments.

A small leaf adaptor frame was developed to conduct high precision leaf gas exchange measurement in the field (Paper I). The leaf adaptor frame increased the precision of the commonly used leaf gas exchange method. It was used to conduct all physiological measurements on the two dominated heathland species at the CLIMAITE-site, the grass *Deschampsia flexuosa* and the shrub *Calluna vulgaris*.

In *Calluna*, differences in magnitude of physiological responses to the climate change treatment were found between warm and cold season. In the warm season no down-regulation of the photosynthetic capacity under elevated CO_2 was found. Opposite significantly down-regulated photosynthetic capacity was observed during the cold season. However, in both seasons the stimulation of photosynthesis was maintained in elevated CO_2. No effect of warming was found in either of the seasons, but drought was found to counterbalance the CO_2-induced stimulation of photosynthesis during warm season (Paper II).

Besides the study of seasonality, long term responses of plant physiology to the climate change factors were investigated. In the CLIMAITE-experiment it has been shown that 2 years of treatment altered physiological responses in *Deschampsia* and *Calluna*. In the work of this PhD similar responses were observed after 6 years of treatment. The magnitudes of physiological responses were related to differences in soil water content in the respective years. Elevated CO_2 was the main driver for physiological changes in the two species with different growth strategies. The growth strategies of *Deschampsia* and *Calluna* defined the physiological responses to elevated CO_2 and only severe drought was observed to change the magnitude of responses (Paper III).

In conclusion, the leaf adaptor frame greatly improved the measurement precision of leaf gas exchange. High precision photosynthetic measurements showed that leaf level responses to climate change factors are stable upon a wide range of seasonal and inter-annual variation. Long-term ecosystem adjustments after 6 years of treatments did not cause further physiological acclimation in either *Deschampsia* or *Calluna*. The study indicates robustness of the Danish heathland ecosystem to moderate climate change.

Sammendrag

Planters fysiologi påvirkes af ændringer i klimaet. Planters fysiologi tilpasses hurtigt til ændringer i det miljø de vokser i og tilpasninger over lang tid kan ses som langsomme feedbacks-mekanismer. Formålet med dette Phd-arbejde har været at undersøge planters fysiologiske tilpasninger til klimaforandringer, både over sæson-variation og på lang sigt.

Plantefysiologiske responser blev undersøgt i fler-faktor eksperimentet CLIMAITE (www.climaite.dk) beliggende i et dansk hede-økosystem efter seks års behandlinger med forhøjet atmosfærisk CO_2, passiv nat-opvarmning og periodisk sommertørke som enkel og fler-faktor. Klimafaktorenes indflydelse plantefysiologiske parametre; som f.eks. fotosyntesekapaciteten, stomatal konduktans og respiration, blev undersøgt på græssen bølget-bunke (*Deschampsia flexuosa* L.) og dværgbusken, hedelyng (*Calluna vulgaris* L.).

For at kunne udføre høj-præcisions målinger på bladenes gas-udveksling i felten udvikledes en blad-klemme. Blad-klemmen øgede præcisionen af den eksisterende blad gas-udvekslings metode (Artikel I). Den udviklede metode blev benyttet til samtlige af de fotosyntetiske-målinger i dette Phd-arbejde.

Det blev observeret at hedelyng tilpassede sig klimabehandlingerne forskelligt under den varme og den kolde sæson. I den varme sæson blev ingen ned-regulering fundet i fotosyntesekapaciteten under forhøjet CO_2 og på trods af en ned-reguleret fotosyntesekapacitet i den kolde sæson, var fotosyntesen øget i begge sæsoner. Der blev heller ikke fundet nogen effekt af nat-opvarmningen i den kolde sæson. Tørke udlignede den forhøjede fotosyntese i forhøjet CO_2 (Manuskript II)

Udover sæsoneffekten af klimabehandlingerne blev lang-tidstilpasninger til behandlingerne også undersøgt. Et tidligere studie fandt at de to undersøgte arter havde tilpasset sig de nye klimatiske forhold efter 2 års klimabehandlinger i CLIMAITE-projektet. I dette studie blev de samme tilpasninger observeret efter 6 år med klimabehandlinger. Forskelle imellem de to år menes forklaret ud fra forskellen i jordvandsindholdet de pågældende år. Forhøjet CO_2 var den klimafaktor der påvirkede planternes fysiologi mest. Tilpasningen til forhøjet CO_2 i de to arter, hedelyng og bølge-bunke, var forskellige og bestem at deres forskellige vækst strategier. Udover effekter af forhøjet CO_2 var det kun tørke der påvirkede planterne fysiologi (Manuskript III).

Det kan konkluderes at blad-klemmerne i høj grad ødede præcisionen af gas-udveksling målingerne. De meget præcise fysiologiske målinger viste at planternes tilpasninger til klimabehandlingerne forblev det samme på trods af forskelligheder i både årstider og år. Økosystem-tilpasninger til 6 års klimabehandlinger ændrede ikke tilpasningsmønstre i enten hedelyng eller bølget-bunke. Det udførte studie viser at det danske hede-økosystem er robust overfor moderate klimaforandringer.

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List of Papers

I. Boesgaard KS, Mikkelsen TN, Ro-Poulsen H, Ibrom A (2013) Reduction of molecular gas diffusion through gaskets in leaf gas exchange cuvettes by leaf-mediated pores. *Plant, Cell and Environment*, DOI: 10.1111/pce.12064

II. Boesgaard KS, Albert KR, Ro-Poulsen H, Ibrom A, Larsen KS, Mikkelsen TN (2013) Photosynthetic stimulation by long term climate change manipulations in *Calluna vulgaris* is maintained on top of seasonal acclimation in cold season. *Submitted to Journal of Experimental Botany*.

III. Boesgaard KS, Ro-Poulsen H, Mikkelsen TN (2013) Leaf level ecophysiological responses to climate change are consistent over six years of *in-situ* manipulations. *In preparation*.

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1. Ecophysiology in a world of climate change

1.1 Climate change

During the last century increased atmospheric concentrations of greenhouse gasses has been observed, primarily caused by human activities. Since the industrial revolution in the middle of the 19th century the atmospheric concentration of carbon dioxide (CO_2) has increased from ca. 270 ppm to over 400 ppm in 2013 (http://co2now.org), which has mainly been related to the burning of fossil fuel and changes in land use. Atmospheric CO_2 concentration is expected to increase even further depending on the magnitude of future human CO_2 emission and land use changes.

Models have been used to predict the consequences of the increased greenhouse gas emissions on the global climate (e.g. IPCC, 2007). Climate change models provide different outputs, but a general prediction is that the increasing atmospheric CO_2 concentration will lead to a global temperature increase of about 1.4-5.8 °C over the next 100 years, depending on the magnitude of the CO_2 increase used in the model scenarios. Increased global temperature influences precipitation patterns, increasing the frequency of drought periods and more heavy rainfall episodes (IPCC, 2007).

In Denmark, a temperature increase of 2-3 °C is expected for the yearly mean temperature around 2100 compared to 1990. The increased temperature is predicted to be higher during nighttime and winter compared to day time and summer, respectively (Danish Metrological Institute, http://dmi.dk ; Easterling et al., 1997). The higher temperature increase in winter is expected to be followed by more precipitation (20-40%). In summer, on the other hand, the precipitation is expected to decrease by 10-15%. Higher frequency of heavy rainfalls in combination with less precipitation is expected to result in longer drought periods in summer time (Danish Metrological Institute, http://dmi.dk).

1.2 Ecosystem feedback processes

Terrestrial ecosystems are affected by climate changes at all levels from single leaf physiology to ecosystem species composition (e.g Reich et al., 2006; Walther, 2003). Complicated feedback mechanisms are involved in rebalancing the ecosystem after environmental changes (Körner, 2006). Figure 1 shows the ecosystem feedback to elevated CO_2 concentration. Increased atmospheric CO_2 is known to stimulate ecosystem production via increased photosynthetic assimilation, resulting in an increased aboveground and belowground biomass (de Graaff et al., 2006).

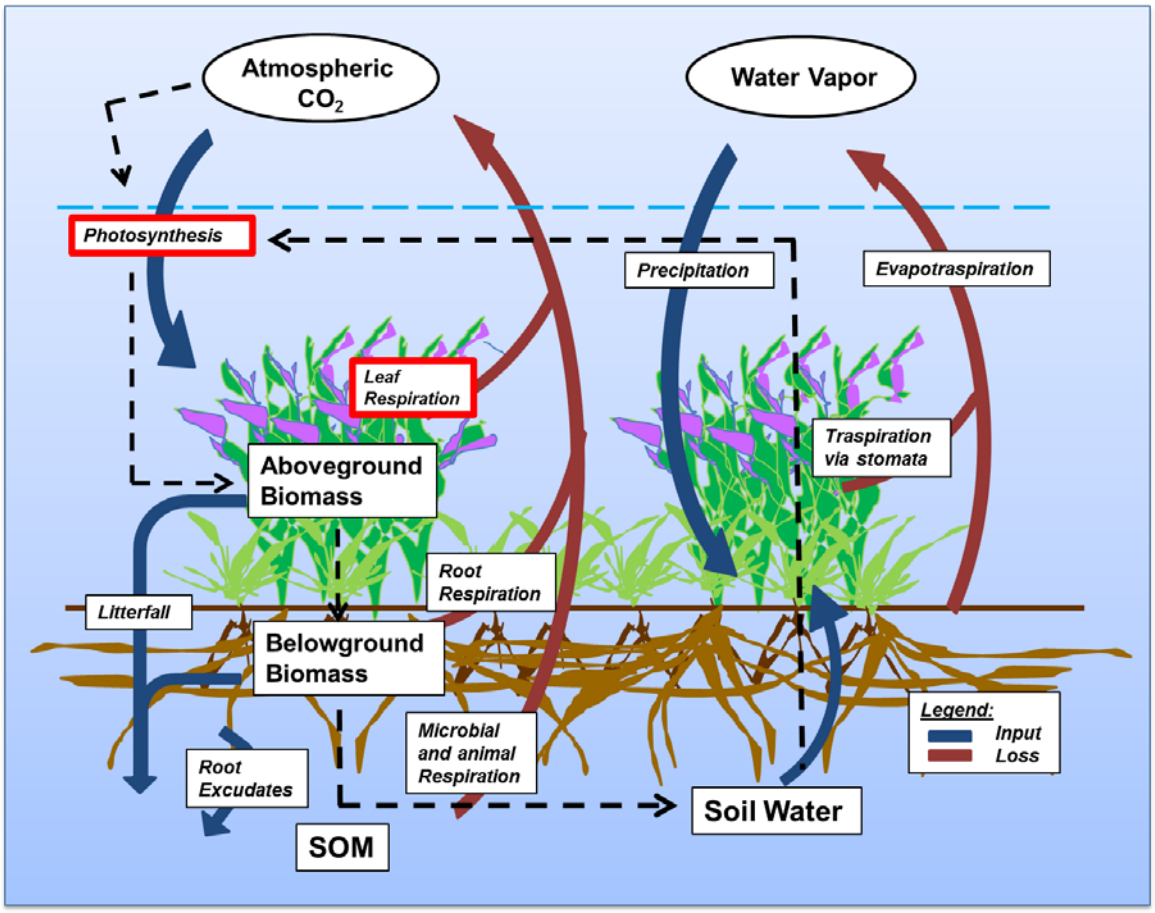

Figure 1. Ecosystem carbon and water fluxes. Blue arrows are indicating carbon and water input to the system and brown losses from the system. Points of interest in the present thesis are highlighted with red boxes and the dotted-line arrows indicate an example of an ecosystem feedback-system. Feedback response direction is not described on the figure.

Increased plant biomass potentially increases the overall plant water use and will thus decrease soil water availability. Nutrient and water limitations have been shown to mitigate the CO_2-induced ecosystem production, resulting in more limited ecophysiological responses in natural ecosystems than in human impacted systems like agricultural fields (Leakey et al., 2009). Ecophysiological responses are rapid (second-minutes), and within a short time (day-month) plants can acclimate to new environmental conditions. Direct environmental impact on ecophysiology is thus expected to be fast, and long term responses are the result of slower ecosystem feedbacks.

1.3 What can we learn from leaf level processes?

Leaf level physiology can respond rapidly, and changes in CO_2, nutrient availability, water supply and temperature can affect the responses in contrasting directions. Photosynthetic carbon assimilation is the only source of carbon for the terrestrial ecosystem, why responses at this level

are of highest interest in understanding the direction of ecosystem responses. Single factor experiments have been conducted for decades (e.g. reviewed in Ainsworth & Long 2005; Newsham & Robinson, 2009) However, the predicted climate changes do not only influence single environmental factors, as many different factors are simultaneously influenced by the climate (e.g. IPCC, 2007). Ecosystem scale and global models predict the impact of multiple factors based on the knowledge from single factor experiments. Models are useful as long as interactions between climate change factors are additive, but experiments including more than one factor have provided insight in how complex the interactions between different single factors can be (e.g. Albert et al., 2011a/b/c; Larsen et al., 2011; Crous et al., 2011; Leuzinger et al., 2011). A recent study by Leuzinger et al. (2011) explains how multifactor experiments and upscaling reduce single factor responses. Models can easily overestimate ecosystem responses which emphasizes the importance of multifactor experiments.

1.4 Ecophysiological responses to climate change factors

1.4.1 Elevated atmospheric carbon dioxide

Large amounts of experiments have been conducted, focusing on elevated atmospheric CO_2 responses on plant physiological processes (e.g. reviewed in Leakey et al., 2009; Ainsworth & Rogers, 2007; Ainsworth & Long, 2005; Long et al., 2004). For example, higher partial pressure of atmospheric CO_2 immediately increases the intercellular concentration of CO_2 (c_i) and increased photosynthesis (A) as a direct effect of an increased ratio of CO_2/O_2 at the active site of the enzyme ribulose-1.5-bisphosphate carboxylase/oxygenase, Rubisco (Von Caemmerer, 2000). Thus Long et al. (2004) argued that most physiological responses to elevated CO_2 in plants, and thereby ecosystems, can be divided into effects on photosynthesis and stomatal conductance (g_s). Growth under elevated CO_2 predominately results in stimulated light-saturated net photosynthesis, decreased stomatal conductance and increased water use efficiency (Leakey et al., 2009; Ainsworth & Rogers, 2007; Ainsworth & Long, 2005; Medlyn et al., 2001). Acclimation to elevated CO_2 often results in decreased photosynthetic capacity via down regulation of the maximum Rubisco carboxylation rate (V_{cmax}) and to some degree the rate of electron transport (J_{max}) (e.g. Leakey et al., 2009; Ainsworth & Rogers, 2007; Ainsworth & Long, 2005). At the current level of CO_2, photosynthesis is often limited by the Rubisco carboxylation rate, but as the atmospheric CO_2 concentration increases, photosynthesis becomes more frequently limited by electron transport (Rogers & Humphries, 2000; Long & Ainsworth, 2006). A shift to electron transport limited light saturated photosynthesis in higher CO_2 reduces the need for carboxylation capacity, making it possible for the plant to redistribute the nitrogen invested in Rubisco (Drake et al., 1997). Thus a common finding from elevated CO_2 experiments is a reduction of nitrogen in

leaves and an increased carbon to nitrogen ratio (e.g. Ainsworth & Rogers 2007; Long et al., 2004). The magnitude of down regulation of the photosynthetic capacity has been related to the availably of nitrogen, with highest responses in ecosystems with low nitrogen availability (Ainsworth & Roger, 2007; Nowak et al., 2004). As mentioned above (Leakey et al. 2009), the magnitudes of responses are typically smaller in natural ecosystems, where not only nutrients but also water can be limiting.

When the CO_2/O_2 ratio in leaf tissue increases under elevated CO_2, the losses of energy due to photorespiration are decreasing, resulting in an increased efficiency of photosynthesis (Lambers et al., 1998). Mitochondrial respiration (dark respiration) has been found to be directly down regulated by elevated CO_2 in some studies but not in others. Tjoelker et al. (2001) found little evidence that a doubling in CO_2 concentration had any direct effect on dark respiration. Hamilton et al. (2001) found no effect of long-term growth in elevated CO_2 on maintenance respiration rates or the response to changes in temperature (e.g. Q_{10}) and concluded that the influence of elevated CO_2 on plant respiratory carbon fluxes is primarily related to increased biomass.

Respiration and photosynthesis are closely connected, and photosynthesis is strongly dependent on stomatal opening. Thus, environmental change induced effects on stomatal conductance cannot be neglected in an ecophysiological perspective (Long et al., 2004). Reduced stomatal conductance is a common response to elevated CO_2 and can affect water status of plants and increase water use efficiency (Ainsworth & Rogers, 2007). Studies have indicated that elevated CO_2 reduced the water consumption, which results in reduced soil water depletion and induces so called “water saving” (Leuzinger & Körner, 2007; Robredo et al., 2007). However, studies have also indicated that water saving as a result of reduced stomatal conductivity probably only occurs during severe drought periods and is strongly dependent of the species growth strategies (e.g. Robredo et al., 2007; Albert et al., 2011a & 2012).

1.4.2 Nighttime warming

Increased temperature, as predicted for the future, can strongly affect photosynthesis (e.g. Berry and Björkmann 1980, Medlyn et al 2002). Photosynthesis involves a lot of biochemical reactions and their overall temperature response can be understood in the scene of the temperature dependency of photosynthesis and its interactions with other processes (Farquhar et al. 1980, Kirschbaum and Faquhar 1984, Medlyn et al. 2001). According to the temperature impact on biochemical reaction rates (the Arrhenius relationship), the activation of the photosynthesis bioprocess is increased with increased temperature at low to moderate temperatures. At higher temperatures, photosynthesis is decreased due to conformational changes of key enzymes and thus reduce the CO_2 fixation. Photosynthesis has a temperature optimum and plants are able to

acclimate considerably to different growth temperature, changing their CO_2-fixation optimum (Sage & Kubien, 2007)**.**

Direct effects on photosynthesis and leaf respiration resulting from increased temperature is well documented (e.g. Atkin et al., 2005a/b; Campbell et al., 2007). However, in the context of climate change, it is argued that temperature effects on the plant physiology is more related to effects at other levels in the ecosystem (Nowak *et al.*, 2004). In Europe, warming during the last decades resulted in an overall earlier onset of the spring/summer season of 2.5days/°C (Menzel *et al.*, 2006), and warming has been documented to increase soil mineralization and ecosystem evaporation (e.g. Rustad et al., 2001; Schmidt et al., 2004). Changed seasonality as well as nutrient and water availability, can be of great importance for ecosystem carbon sink capacity mediated by photosynthetic CO_2 fixation (e.g. Llorens & Penuelas, 2005; Peñuelas et al., 2007). Warming can stimulate photosynthesis; however, increased daytime respiration or changes in the temperature acclimation of respiration can occur and can lead to an overall reduction of carbon uptake (e.g. Campbell et al., 2007). Effects of warming are expected to be most pronounced during nighttime (Easterling *et al.*, 1997), and studies with increased night temperatures have shown that warming increased nighttime respiration, positively influencing the carbon sink strength, and then stimulated daytime photosynthesis the following day (e.g. Griffin et al., 2002; Turnbull et al. 2002 & 2004). Positive effects of e.g. temperature mediated increased mineralization rate can potentially increase the nutrient availability for plants, increasing the photosynthetic capacity (Kattge et al., 2009).

1.4.3 Extended spring/summer drought

Drought is a strong environmental stress factor for most plants (e.g. Schmidt et al., 2004; Pérez-Ramos et al., 2010). Precipitation patterns are expected to change (IPCC, 2007), and in temperate ecosystems it is expected that longer periods of drought and episodic heavy rainfall will become more frequent. Drought has an intensive negative effect when prolonged, but during rewetting, photosynthetic physiological processes can be restored (e.g. Albert et al., 2011a). Physiological processes are not only affected during dry periods but also in the post-drought period. For example, in a study of evergreen oak and broad leaved *Phillyrea* (Ogaya &Peñuelas, 2003) clear carry-over effects of summer-drought were found during the following colder season, decreasing photosynthetic capacity in plants previously exposed to drought. Slow growing species as trees and shrubs are competitive in their responses to drought, where fast growing opportunistic species have been found to show leaf-die back, maintaining high photosynthetic performance in the remaining leaves (e.g. Albert et al,. 2012; Versules et al., 2006).

Water shortage limits plant growth, mainly as a result of a changed plant carbon balance, increasing the rate of respiration more than the reduction in photosynthesis (e.g Flexas et al., 2006). Plants exposed to drought show negative responses in most physiological parameters such as A_{sat}, g_s, V_{cmax} and electron transport (J_{max}). Additionally, drought is also known to delay phenological processes related to flowering and germination (e.g. Signarbieux & Feller, 2011; Albert et al. 2011 a/b; Jentsch et al., 2009; Prieto et al., 2008; Llorens & Penuelas, 2005). Single factor climate change experiments as described in the above sections have been conducted in a large number (e.g. reviewed in Ainsworth & Long, 2005; Ainsworth & Rogers, 2007; Leakey et al., 2009; Nowak et al., 2004; Newsham & Robinson, 2009). However, the predicted climate change is not only involving single environmental factors and much fewer studies have been combining several experimental factors (e.g. Blooret al., 2010; Ellsworth et al., 2012; Nogués & Baker, 2000; Schmidt et al., 2004). Therefore there is a continuous interest and need for long-term multi factorial experiments (Leuzinger et al., 2011; IPCC, 2007).

1.5 Objective of the thesis

The main focus of the thesis has been to explore long-term ecophysiological responses to climate change in a temperate heath/grassland. Moreover, the aim has been to provide useful model-validation data for up-scaling leaf level gas exchange measurements to ecosystem scale. Photosynthetic and, particularly, respiration fluxes at leaf level are small, why an additional aim has been to improve the current technique of measuring photosynthesis to increase the precision of small scale gas exchange measurements. The following major questions were addressed during the 3 years of work covered in this thesis:

- Is it possible to correct leaf gas exchange measurements to increase the precision of small CO_2 and H_2O fluxes at the leaf level? (Paper I)
- How does climate change affect *Calluna vulgaris* and are the effects the same in the cold and warm seasons? (Paper II)
- How does long-term climate change affect heathland-ecosystem feedbacks and influence *Deschampsia flexuosa* and *Calluna vulgaris* physiology? (Paper III)

2. Methodological considerations and descriptions

2.1 Climate change manipulation – field studies

Climate change experiments have been conducted over a broad range of ecosystems using all kinds of different techniques. Changed temperatures, nutrient availability and soil water content are relatively easy to manipulate *in-situ* using shelters, tents, open top chambers or irrigation whereas elevated CO_2 concentrations are more complicated to manipulate. The development of the Free-Air-CO_2-Enrichment (FACE) technique has provided a tool for *in-situ* manipulation of elevated CO_2 and similar techniques are used for manipulating ozone and humidity (e.g. AspenFACE and FAHM). The FACE technique has been used in all kinds of ecosystems from grassland to forest and agricultural fields, and some experiments have been maintained for more than ten years. FACE experiments have been employed in combination with other factors such as ozone, nutrient additions, warming or manipulation of precipitation. In few cases, FACE experiments have been combined with more than one climate change factor; e.g. OzFACE, combining three levels of CO_2 with defoliation and nutrient addition, and the CLIMAITE experiment, which combines FACE with passive nighttime warming and periodic spring/summer drought.

2.2 The CLIMAITE experiment

The long-term ecophysiological responses to climate change manipulations presented in paper I-III were carried out within the climate change experiment CLIMAITE. In 2005, CLIMAITE was establishing to investigate long term climate change effects on biological processes in terrestrial ecosystems. The experiment is situated in a temperate heathland close to Jægerspris, North Zealand, Denmark (55°53'N, 11°58'E). The vegetation is co-dominated by the evergreen dwarf shrub *Calluna vulgaris L.* (~ 30%) and the grass *Deschampsia flexuosa L.* (~ 70%), and a minor occurrence of other grasses, herbs, mosses and lichens (Kongstad *et al.*, 2012). The soil is nutrient poor and sandy with a pH_{CaCl_2} of ~ 4.5 in the top 5 cm of soil. Mean annual temperature is ~10 °C and the annual mean precipitation is ~700 mm (Mikkelsen et al., 2008)

The CLIMAITE experiment includes the following treatments: Untreated control (A), elevated CO_2 (CO2), passive nighttime warming (T), periodic summer drought (D) and all combinations (TD, TCO2, DCO2, TDCO2), replicated in six blocks within a complete split-plot design. Each block includes two octagons of 6.8 m diameter, divided in four plots (Figure 2). The FACE technique is used to expose one octagon in each block to 510 ppm CO_2 during daylight hours. The passive nighttime warming is performed by automated infrared reflective curtains covering one half of each octagon.

Nighttime warming results in an increased air temperature of 1.4 °C in 20 cm height, on average (Mikkelsen et al., 2008). Every year of the experiment, experimental drought has been established during two to five weeks in the spring or summer by automated rain-activated curtains covering one half of the octagons each. To avoid total die-out, the drought treatment was stopped when the soil water content in the top 20 cm was reduced to less than 5 %. The experimental treatments with CO_2 and warming were initiated in October 2005, and the first drought was imposed in June 2006. In each experimental plot the soil water content over two depths (0-20 cm and 0-60 cm) was continuously recorded using time domain reflectometry (TDR). Simultaneously, air temperature has been measured in 20 cm height, and soil temperature has been monitored in 0 cm and 5 cm depth. Two climate-stations are located in the experimental area, where temperature, radiation within the photosynthetic spectrum (PAR) and the precipitation have been measured in 2 m height. To follow intensively the seasonality in the photosynthetic performance, two additional "High Temporal Resolution" plots (HTR plots) outside the treatments were established in January 2011. Within this PhD project, field work was conducted throughout the years 2011 and 2012. In the period from May 2011 to May 2012, the HTR plots were measured on every second week and monthly campaigns were conducted in from April 2011 to February 2012 (Figure 3).

Figure 2. Outline of the experimental treatments at the CLIMAITE experimental site. A) One experimental block, containing two separated octagons, the upper panel shows the control ring with ambient CO_2 concentration and the lower panel the ring with elevated CO_2 concentration at 510 ppm (FACE-ring). Each octagon is equipped with drought and warming curtains. Each block includes all eight treatment combinations of elevated CO_2 (CO2), passive nighttime warming (T), extended summer drought (D) and non-treated control (A). B) Aerial photo of the CLIMAITE experimental site, where all curtains are out for demonstration purpose (Photo: Kim Pilgaard).

2.3 Plant ecophysiological performance

All applied methods are described in details in each manuscript, but in the following section the use of methods are discussed.

Leaf physiological processes can be divided into energy production and energy consumption processes. Energy production includes light harvesting and processes through the photosynthetic apparatus resulting in available energy units (ATP and NADPH). Consumption of energy is related to the energy demanding physiological processes, mainly CO_2-assimilation in the Calvin-cycle. Assimilated carbon is stored as energy rich molecules, such as sugars, which are allocated from leaves to other parts of the plant and used for growth and maintenance. The assimilation of CO_2 is closely connected to the plant water balance and the CO_2 and H_2O fluxes between leaves and atmosphere have been the background for the development of many different gas exchange methods to determine photosynthesis and respiration. Since photosynthetic and respiratory fluxes are always related to leaf area, biomass or nutrient concentration, gas exchange measurements cannot stand alone, but need to be combined with leaf trait characteristics.

2.3.1 Leaf gas exchange measurements (Paper I)

Leaf photosynthetic and respiratory fluxes of CO_2 and H_2O are small, and high precision is required to conduct reliable measurements (e.g. Bruhn et al. 2002; Pons et al. 2009). In the laboratory under controlled environmental conditions, high precision equipment provides the opportunity to measure even the smallest fluxes. However, this type of equipment is often large and immobile, and thus not suitable for field work. Smaller portable gas exchange systems have been developed using small leaf chambers, where the leaf (or twigs) are sealed between to gaskets. Unfortunately, diffusion through the gasket material are unavoidable and has been demonstrated and described previous (e.g. Long & Bernacchi, 2003, Rodeghiero et al. 2007, Flexas et al. 2007). Most leaf gas exchange manufactures provide methods for correction, and methods to minimize the advective leakage through gaps between plant and gasket material have been suggested (e.g. Rodeghiero et al. 2007, Flexas et al. 2007). In short, the two common approaches are either to seal the leaf chamber in an additional gas tight container (Flexas et al., 2007) or correct measurements with dead leaf data (Rodeghiero et al., 2007). However, the suggested methods are difficult, if not impossible, to apply under extensive field work, why I decided to develop a new approach to increase precision of field conducted leaf gas exchange measurements.

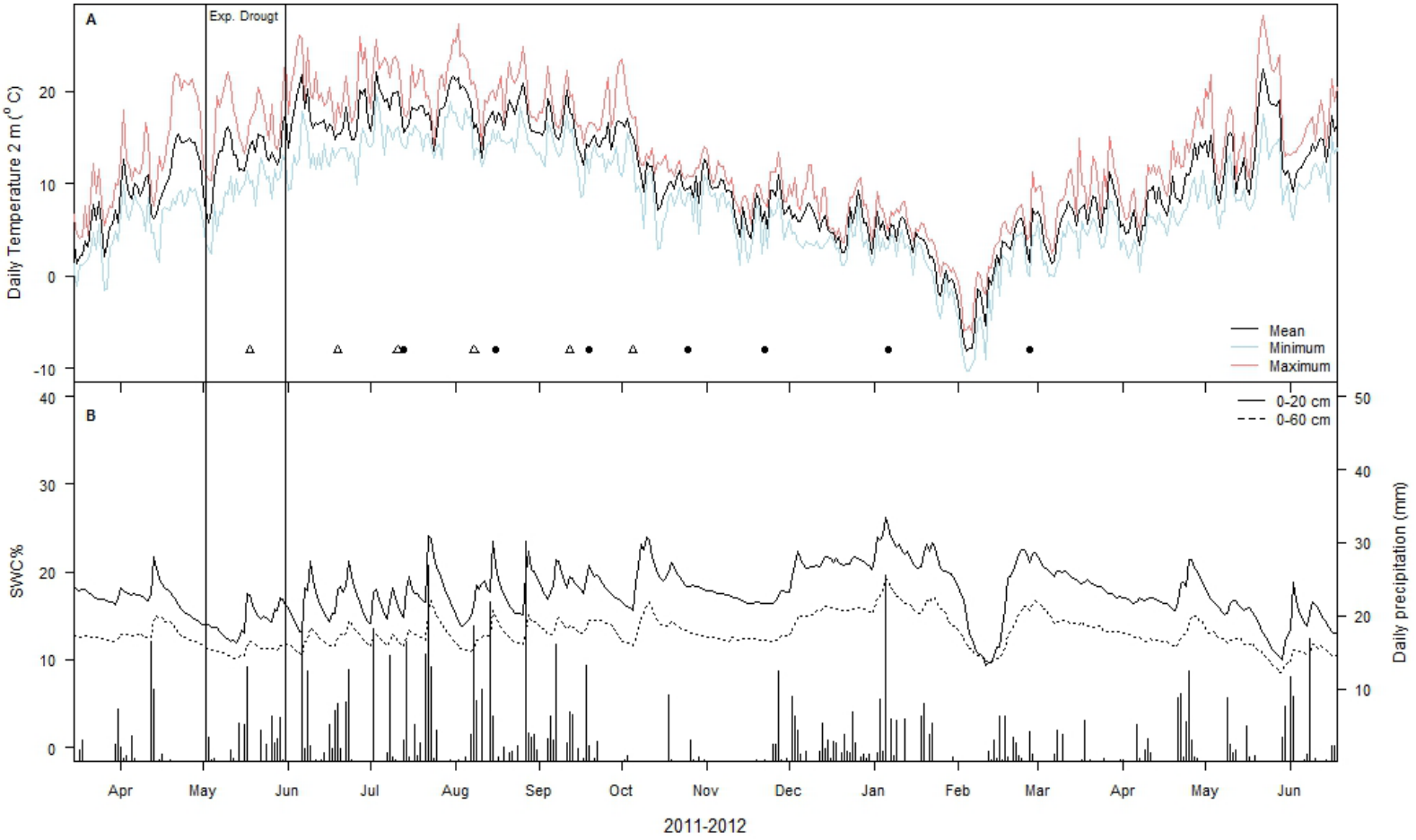

Figure 3. climatic condition at the CLIMAITE experimental site during the mearsurements of the thesis work. A) Daily mean (black), minimum (blue) and maximum (red) temperature in 2 m height (n=2). Date of campaign start of leaf gas exchange measurements on *Deschampsia flexuosa* (Δ) and *Calluna vulgaris* (●). B) Daily mean soil water content (SWC%) in ambient plots (n=6) in 0-20 and 0-60 cm depth and daily mean precipitation in mm (n=2).

To minimize the leakage between plant material and the gasket, we developed a gas tight leaf adaptor frame, LAF (figure 4), believing that this would increase the precision of the measurement. However, early pilot-studies showed that the LAF did not minimize the diffusion leakage. The arising question to answer was now: '*why does exclusion of leaf mediated leakage not decrease the diffusion leakage*?' After many experiments including turbulent wind conditions and different gasket materials we finally found the answer and concluded that avoiding leaks enables precise correction and highly increases the reliability of the measurement (Paper I).

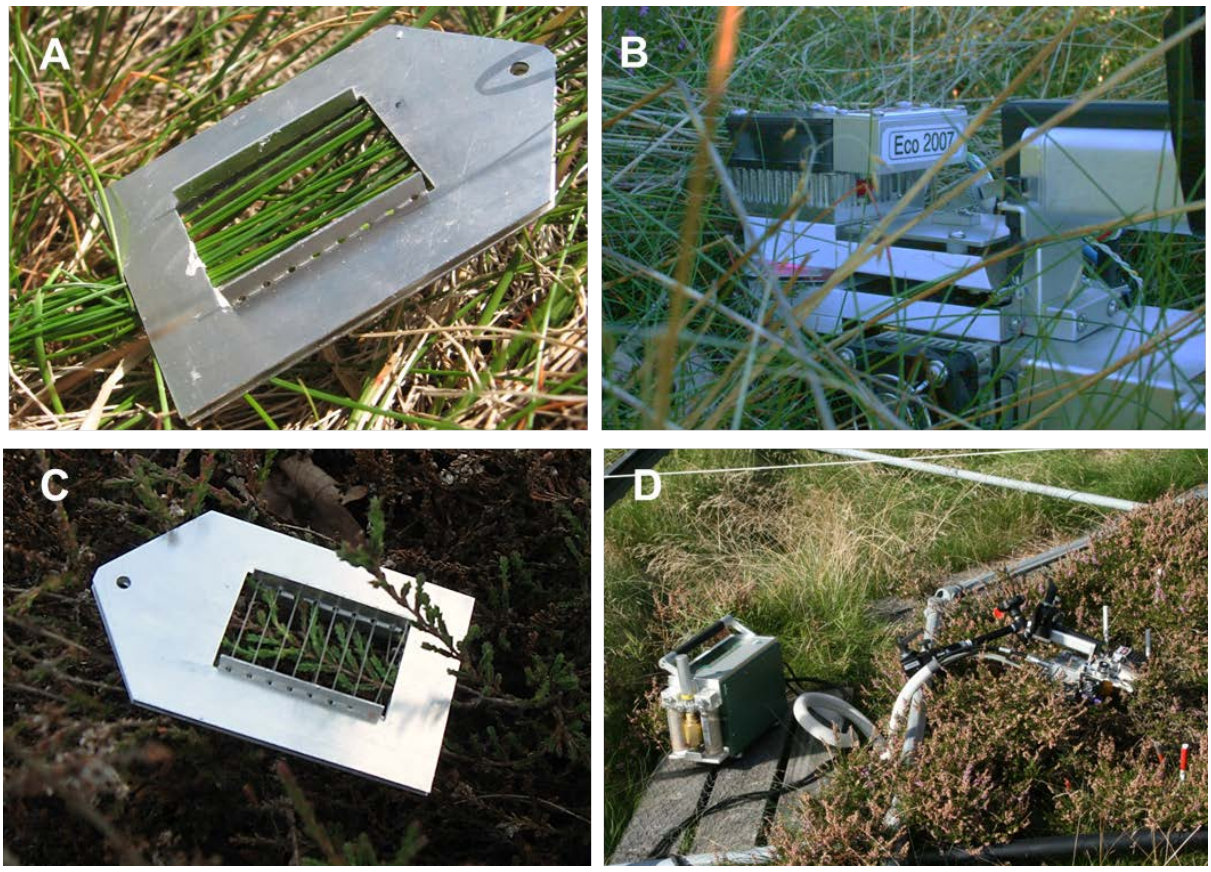

Figure 4. Leaf adaptor frame (LAF) used under field conditions, detailed description can be found in Boesgaard et al., (2013), Paper I. A) + C) LAF attached to a sample of *Deschampsia flexuosa* and *Calluna vulgaris*, respectively. B) + D) Licor 6400 attached to samples of *Deschampsia* and *Calluna* within LAF, respectively. All photos by Kristine Boesgaard.

2.3.3 Measurements on leaf trait characteristics

As explained above, measurements of photosynthesis and respiration should always be related to leaf area, biomass or nutrient concentration. Portable gas exchange systems normally have small chambers with an inside area < 6 cm^2, however it would be difficult to fill out an area of that size with one or even with more leaves of the plants species investigated in this thesis, *Deschampsia* and *Calluna* (figure 4). Again, LAF was found to be a great tool for precise area estimates and

later for dry weight and nutrient content determinations. After a measurement of a leaf sample within the LAF, leaves were cut out and brought to the laboratory. In the laboratory, estimation of leaf area were done using photographs and scanned images to present the area as projected leaf area, as described in Smith et al. (1991).

Plants are able to re-allocate nutrients such as nitrogen to different part of the plants, thus one leaf can have a different nitrogen content than the one next to it. To be able to connect carbon and nitrogen concentration directly to the measured photosynthesis or respiration, the same sample was used for elemental analysis after dry weight determination.

2.3.4 Photosynthesis parameters – models and fitting procedure

Evaluation of the impact of climate change factors on ecophysiological processes was conducted using the relation between the rate of photosynthetic assimilation (A) and either the intercellular CO_2 concentration (c_i) or the incident light intensity (I). A more simple approach was used to evaluate stomatal conductance (g_s) and leaf dark respiration (R_D). Details on estimation of photosynthetic parameters from the A vs. I relation (light-response), g_s and R_D can be found in paper I-III.

The relation between A and c_i was evaluated using the C3-photosynthesis model, the Farquhar-von Caemmerer-Berry (FvCB) model, described in 1980 and later modified (Farquhar et al., 1980; Harley & Sharkey, 1991; Bernacchi et al., 2001). We adapoted the model-fitting approach from Dubois et al. (2007) for data processing A-c_i data in SAS (SAS Intsitute Inc.) and converted the algorithm to be used in the free-software R (R Develpment Core Team, 2010). The FvCB model considers biochemical reactions of photosynthesis to be in one of three steady states (Long & Bernacchi, 2003). At low c_i, the rate of photosynthesis can be predicted by the properties of ribulose 1,5-bisphosphate carboxylase/oxygenase (Rubisco) assuming a saturating supply of substrate, Ribulose-1,5-bisphosphate (RuBP). Photosynthesis is here limited by the carboxylation rate of Rubisco (V_{cmax}) and is normally referred to as the Rubisco-limited state. As c_i increases, the photosynthetic rate becomes limited by the regeneration-rate of the substrate RuBP and, assuming that RuBP is used at a constant rate, the electron transport rate (J) is limiting the photosynthesis, referred to as the RuBP-limited photosynthesis. The rate of triose-phosphate utilization (TPU) can become the limiting step for photosynthesis as c_i increases. However, this will only occur under both high light and highCO_2 concentrations, which is rarely the case for plants under outdoor conditions, and therefore the model improvement provided by Harley and Sharkey (1991) is not used with in this thesis. The approach from Dubois et al (2007) is based on a model-fitting, where the lowest value of one of the three described photosynthetic limitations defines the rate of photosynthesis at a given c_i. To improve the model the temperature correction

by Bernacchi et al. (2001) was used to normalize data that were not measured at the standard temperature of 25 °C.

Model fitting is validated by the amount of measurement points. The more points on the curve the more unknown variability can be evaluated. Using the minimum detecting approach, fitting of photosynthesis to one of the two limitation stages of photosynthesis (Rubisco-limited or RuBP-regeneration-limited) was not always an option for our data series. Thus, when no minimum could be identified for the photosynthetic limitation, V_{cmax} was fitted using data with c_i<500 ppm and J was fitted using data with c_i > 550 ppm (example in figure 6).

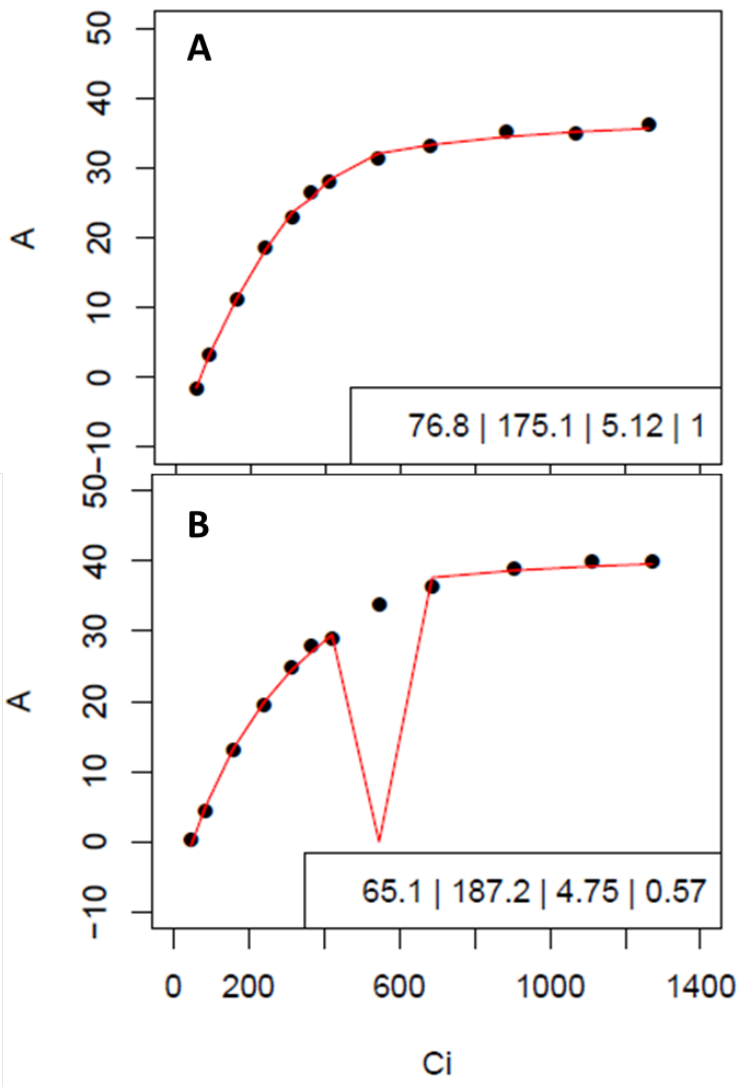

Figure 5. Example of an A- c_i curve fitting using the minimum-seeking model A) and the fixed model B). The values in the bottom right corner of each of the panels are fitted values for V_{cmax}, J, respiration estimated from the A- c_i -fitting (R_d) and the last number is the R^2-value for the fitted red line. The red line connects the two fitted functions. The drop seen in B) appears due to no overlap between the fitted functions.

3. Photosynthetic performance to climate change

In the following ecophysiology chapter, responses of plant ecophysiology to long-term climate change manipulations on the heathland species *Calluna vulgaris* and *Deschampsia flexuosa* is summarized and discussed. A detailed discussion of the results is presented in papers II and III.

3.1 Seasonality and growth strategy

Plant physiology is rapidly influenced by environmental changes, i.e. light, temperature and water availability. The plants are facing diurnal, seasonal and annual variations in environmental conditions. In temperate climate, where environmental conditions is variable throughout the year, an evaluation of ecosystem and ecophysiological responses to climate change, and particularly the seasonality in the responses, are of high interest. Seasonal variability within single months is of great magnitude, thus high temporal resolution in data is needed.

Ecosystem carbon exchange can be evaluated with high temporal resolution (seconds – hours) using techniques as eddy-covariance or automatic ecosystem gas exchange chambers. These kinds of techniques are particularly useful to evaluate ecosystem performance under ambient conditions, but cannot be used to evaluate the ecosystem carbon source or sink potential. Leaf level C3- photosynthesis and light-relation models (Farquhar et al., 1980; Lambers et al. 1998) provide information about the plant photosynthetic potential and are important tools for up-scaling procedures and in evaluating ecosystem carbon source/sink potentials in a climatically changed future. Leaf level gas exchange measurements are suitable for these kinds of evaluations but are highly time-consuming and difficult to perform under field conditions.

Covering seasonal variation with a higher temporal resolution than a monthly scale within eight treatments replicated six times, as in the CLIMAITE experiment, is costly, time and money consuming. Therefore, seasonality investigations with two-week intervals in HTR plots outside the treatments were performed from May 2011 to May 2012. Assuming that the seasonality under ambient conditions roughly follows the same pattern as in the different treatments, data from HTR plots can be used to define the seasonality within treatments and provide an important tool for up-scaling carbon and water fluxes to an annual ecosystem scale. In paper II, seasonality of the maximum carboxylation rate (V_{cmax}) at ambient temperatures was used to remove the seasonal variability to compare treatment effects in different time a year. In combination with the improved gas exchange approach presented in section 2.3 and paper I, seasonal variation in leaf dark respiration was also investigated. Leaf respiration is one of the most difficult parameter to measure, particularly under field conditions, because the leaf-level fluxes are extremely small and high precision equipment is needed. However, information about

leaf respiration is valuable for the up-scaling of ecosystem responses to climatic changes (see more in Chapter 4).

The two co-occurring species at the CLIMAITE experimental site, the grass *Deschampsia* and the dwarf shrub *Calluna*, have different life strategies (Albert *et al.*, 2012). Therefore, it is expected that their seasonal pattern in light-saturated net photosynthesis and leaf dark respiration is different. Across all time, high temporal data showed that *Deschampsia* had a higher photosynthetic rate and lower respiration rates than *Calluna*. This finding was in line with earlier observations from monthly campaigns at the CLIMAITE experimental site (Albert et al. 2012).

The two species are also different in their way to deal with seasonal changes in temperatures (Paper II). Where *Calluna* re-translocates leaf nitrogen in cold seasons, *Deschampsia* does not. This is in line with findings from other studies on evergreen species (e.g. Bryant et al., 1983). The opportunistic growth strategy of *Deschampsia*, such as leaf die-back during unfavorable periods with little water and low temperature, maintains higher productivity in remaining leaves (Albert et al. 2012). *Calluna*, on the other hand, is more vulnerable to drought and temperature changes by maintaining leaf biomass and surviving unfavorable conditions by acclimatizing the photosynthetic apparatus to new conditions (Albert et al. 2012.). *Calluna* not only down regulates V_{cmax} and leaf nitrogen content, but it also closes stomates in dry periods (Albert et al. 2012).

Unfortunately, the season evaluated in the present thesis cannot be considered as normal. An extremely dry winter and daytime temperatures below 0°C without protecting snow cover lead to a major dieback of the standing *Calluna* biomass at the experimental site. In April 2011, only around 5-10% of all *Calluna* stands had functioning green shoots. New shoots regrew from the dried stands during May-July 2011, with an approximately 75% regrowth. The dieback was not observed to be different between treatments. The delayed growing season was seen as a slow start-up of A_{sat} of *Calluna* in the early regrowth phase (May – June), compared to *Deschampsia* which showed a high A_{sat} already during the same period (figure 6).

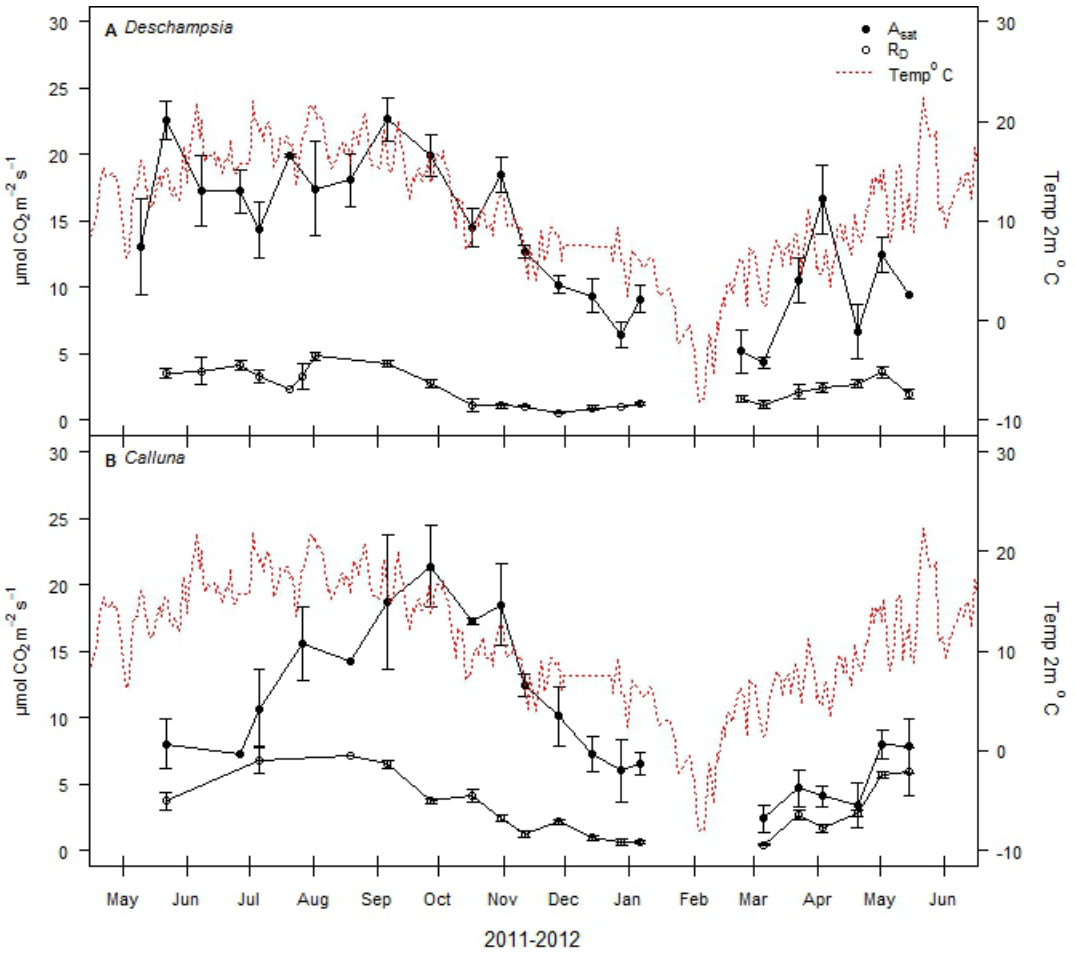

Figure 6. Seasonality in light-saturated net photosynthesis (A_{sat}, ●) and dark respiration (R_D, ○) in HTR plots in combination with daily mean air temperature in 2 m height (°C, ····). A) *Deschampsia flexuosa* and B) *Calluna vulgaris*.

3.2 Seasonal responses to climate change in *Calluna vulgaris* (Paper II)

Considering the seasonal variation in ecophysiological processes led to a major question: '*Will species respond to climatic changes the same way at the different times of the year?*' To address this question the warm season was defined to be between May and October, as the main growing season, and the cold season was defined to start in the beginning of October, according to Larsen et al. (2007). Most recent studies of impacts of climate change on ecosystems have been carried out in the growing season (e.g. Ainsworth et al., 2004; Day et al., 2008; DeLucia & Thomas, 2000; Llorens & Penuelas, 2005; Robredo et al., 2007). The seasonal data provided knowledge about how seasonal changes in particular temperature affect the rates of photosynthesis and respiration. In *Calluna* A_{sat} was reduced by more than 50 % in the cold season compared to the warm season, whereas *Deschampsia* reduced A_{sat} by ca. 40%. As discussed in section 3.1, *Deschampsia* induced leaf dieback under unfavorable conditions, leaving remaining leaves with

high fitness and photosynthetic potential. This makes it difficult to distinguish between physiological responses and growth-strategy-induced responses. Thus, investigations of ecophysiological responses in the cold season were conducted on *Calluna* only. Studies of evergreen scrubs and trees in the temperate climate zone have shown a potential for continued carbon uptake during the cold season (e.g. Larsen et al., 2007; Campbell et al., 2007). At the CLIMAITE experimental site, Andresen & Michelsen (2005) showed that *Calluna* sustained uptake of nitrogen in December, indicating that photosynthesis is potentially maintained throughout the cold season. Larsen et al. (2007) estimated that 22% of the annual ecosystem photosynthesis and 30% of the annual ecosystem respiration could be assigned to the cold season, between October-Marts, in a Danish heathland ecosystem.

The FvCB model of C3 photosynthesis is based on leaf gas exchange measurements conducted at standard temperature (see section 2.3.4). In controlled environments, such as greenhouses or growth chambers, temperature is easy to control. However, under outdoor environmental conditions, temperature stabilization and control is highly difficult (e.g. Li-Cor Inc., 2008). To improve the FvCB model, Bernacchi et al. (2001) included a temperature correction to the model to take temperature fluctuations during measurements into account. Normalization of ecophysiological measurements, such as V_{cmax} are needed for the evaluation of seasonal differences between experimental treatments. Therefore, ambient high temporal gas exchange measurements were used as normalization background.

The effects of the experimental treatments were investigated within the earlier defined warm and cold season (paper II). Elevated CO_2 stimulated A_{sat} ,which is in line with other findings (e.g. reviewed in Drake et al., 1997; Nowak, et al., 2004; Ainsworth & Long, 2005; Leakey et al., 2009). However, *Calluna* did not show any down-regulation of the photosynthetic capacity during the warm season, which is in contrast to other observations on trees and shrubs (e.g. Nowak et al., 2004). CO_2-induced stimulation of A_{sat} was found to be related to an increased intercellular CO_2 concentration via an unchanged stomatal conductance (Paper II). Earlier findings from the CLIMAITE experimental site in the second year of treatment indicated a photosynthetic down-regulation under elevated CO_2 concentration (Albert et al., 2011a). However, in the 2nd year, soil water content was ca. 50 % lower across the growing season than in the presented year, which resulted in a lower stomatal conductance Thus, c_i/c_a in either ambient or elevated CO_2 was not maintained as high as in the present study. Lower stomatal conductance has been related to photosynthetic down-regulation and the absence of response can be related to this (more on this in section 3.3 and Paper III).

The temperature normalized V_{cmax}^{25} revealed that there was no photosynthetic down-regulation during the warm season, but *Calluna* significantly down-regulated V_{cmax}^{25} during the cold season (Paper II). Despite the down-regulation of V_{cmax}^{25}, A_{sat} was maintained at higher levels

in the elevated CO_2 treatment, resulting in a 5 % lower reduction of A_{sat} in the cold season compared to the warm season. A smaller difference between warm and cold season in combination with a continuous higher A_{sat} indicates the presence of a higher annual carbon uptake under elevated CO_2. In line with this, a measured higher soil respiration under elevated CO_2 at the CLIMAITE site within the first 3 years of treatment was hypothesized to be induced by a higher photosynthetic carbon uptake (Selsted *et al.*, 2012). Furthermore, leaf nitrogen was strongly reduced in our study in the cold season, which is in line with the growth strategy of *Calluna* (Aerts et al., 1990; Gimingham, 1960; Jackson et al., 1999) and was observed to be equal among all treatments. The CO_2-induced reduction in photosynthetic capacity (V_{cmax}^{25}) found in the cold season is argued to be induced by a natural re-translocation of nitrogen in colder seasons to maintain a constant c_i/c_a. In a meta-analysis by Ainsworth and Roger (2007) it was shown that CO_2-induced photosynthetic down-regulation was largest when plants were grown with low nitrogen supply. The CLIMAITE site has not been found to be particularly nitrogen limited (Larsen *et al.*, 2011), and the increase in magnitude of the V_{cmax}^{25}down regulation in the cold season is argued to be induced by a natural re-translocation of nitrogen from the leaves to other plant parts.

Neither nighttime warming nor extended drought was observed to influence ecophysiological processes in *Calluna* in the cold season, and responses in the warm season were similar to earlier findings (Albert et al., 2011a/c). The absence of a warming effect on photosynthetic parameters in the cold season was related to the fact that measurements of full ACi-curves were stopped before days with strong nighttime frost, due to the limited time of daylight. In Albert et al. (2013, paper IV) the effect of nighttime frost events on the photosystem II performance was significantly improved in warmed plots before and after frost events. This finding indicates that the passive nighttime warming treatment effectively extends the growth period. Warm season responses are discussed in details in the next section and Paper III. Summarizing ecophysiological responses in the cold season: A smaller reduction of A_{sat} under elevated CO_2 was found to increase the potential annual carbon uptake, as in the full-factorial treatment (TDCO2). Furthermore, I can conclude that the magnitudes of ecophysiological responses to climate change are highly dependent on seasonal and inter-annual variation.

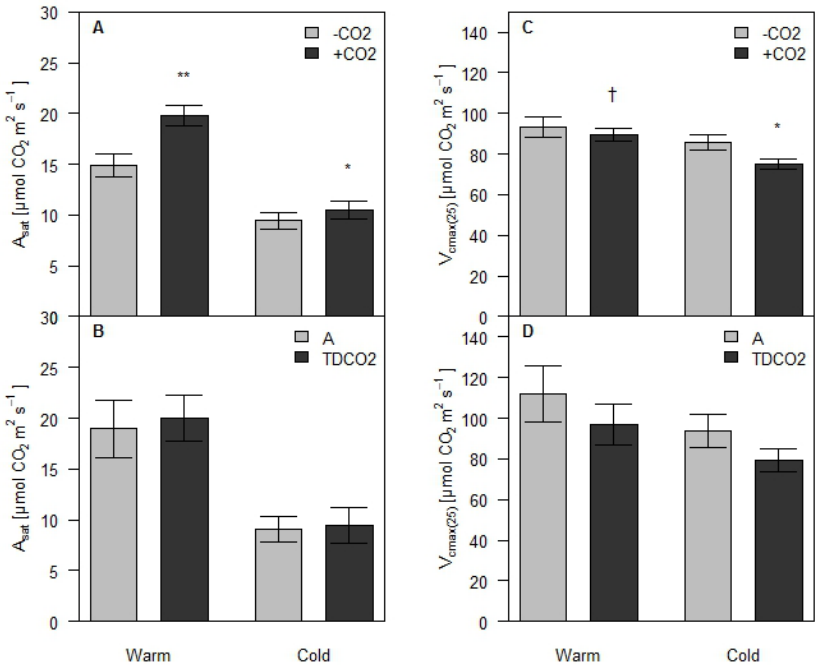

Figure 7. Photosynthetic response during warm and cold season. A) + B) Mean ± SE of light saturated net photosynthesis at ambient temperature, A_{sat} (µmol CO_2 m^{-2} s^{-1}). C) + D) the maximum carboxylation rate normalized to 25 °C, V_{cmax}^{25}(µmol CO_2 m^{-2} s^{-1}). Seasonal treatment effects are indicated as * $p<0.05$, ** $p<0.01$, in the respective season. Tends at $p<0.1$ are noted with †. A) + C) Only responses to ambient CO_2 versus elevated CO_2 (-CO2 and +CO2, n=24) and B) + D) the un-manipulated control A versus the full combination of warming, drought and elevated CO_2, TDCO2 (n=6).The figure is from paper (II).

3.3 Ecophysiological responses to long term climate change (Paper III)

Long term ecophysiological responses to climate changes are indirect results of ecosystem feedbacks originating from short-term plant induced responses (section 1.2.3). The long-term exposure of plants to elevated CO_2, warming and drought can induce different responses in the ecosystem, and since feedback processes can be slow it is difficult to establish how long time is needed for the system to reach a new steady state. Within the second year of experimental treatments in the CLIMAITE-project, photosynthetic performance was evaluated during the warm season (Albert et al., 2011 a/b/c). After this short time (1-2 years) *Deschampsia* and *Calluna* responded differently to the experimental treatments. To evaluate the potential long-term feedback of the climate change manipulations on photosynthetic performance, a similar investigation was conducted during this PhD (Paper III). The two investigated years (2006/2011) differed in precipitation and temperatures. Additionally, in the last year, a dry and cold winter resulted in a major dieback of *Calluna* biomass, as described in section 3.2. The ecophysiological responses that were observed after two years of manipulation were often found in the presented long-term study too (Paper III). Differences in the magnitude of responses and directions were mainly related to differences in water availability and to the die-back of *Calluna* biomass, rather than to treatment effects.

Table 1. Ecophysiological responses to long-term climate change treatments in *Deschampsia flexuosa* and *Calluna vulgaris.*

Variable	CO2	T	D	TxDxCO2
Light-saturated photosynthesis (A_{sat})				
Deschampsia	~	~	~	~
Calluna	↑	~	↓	‡‡
Dark respiration (R_D)				
Deschampsia	~	~	~	‡‡
Calluna	~	~	~	~
Maximum Rubisco carboxylation rate (V_{cmax})				
Deschampsia	↓	~	~	‡‡
Calluna	~	~	↓	‡‡
Maximum electron transport rate (J_{max})				
Deschampsia	~	~	~	~
Calluna	~	~	~	~
Light- & CO_2-saturated photosynthesis (A_{max})				
Deschampsia	↓	~	~	‡‡
Calluna	↑	~	~	‡‡
Stomatal conductance (g_s)				
Deschampsia	~	~	~	~
Calluna	~	~	~	↑↓
Water use efficentcy (*WUE*)				
Deschampsia	↑	~	~	
Calluna	~	~	~	↑↓
Intercellular CO_2 concentration (c_i)				
Deschampsia	~	~	~	~
Calluna	↑	~	~	‡‡
Specific leaf area (*SLA*)				
Deschampsia	~	~	~	↑↓
Calluna	↓	~	~	‡‡
Nitrogen per. leaf area ($mg\ N\ cm^{-2}$)				
Deschampsia	~	~	~	~
Calluna	~	~	~	~
Carbon and nitrogen ratio (*C/N*)				
Deschampsia	↑	~	~	‡‡
Calluna	↑	~	~	‡‡

Note: Responses are compared to non-treated ambient conditions across the growing season after 6 years of treatments. *Symbols*: No effect (~); significant increase (↑); significant decrease (↓); additive response (not significant) of single factor treatments (‡‡); significant antagonistic interaction between single factor treatments (↑↓). Responses are considered significant on a level of $p < 0.05$.

Grasses are known to be more affected by elevated CO_2 concentration than slow growing shrubs and trees. Grasses reduce their photosynthetic capacity and maintain similar rate of photosynthesis as under ambient CO_2 concentration (Ainsworth & Long, 2005; Nowak et al., 2004). In the 6th year of treatment *Deschampsia* met the hypothesis that growth under elevated CO_2 concentration led to a down-regulated photosynthetic capacity that was observed as a reduction in the maximum carboxylation rate (V_{cmax}) and a decline in the maximum light and CO_2 saturated photosynthesis (A_{max}). This finding was in line with the observed short-term response of *Deschampsia* (Albert et al., 2011b). However, photosynthetic stimulation was no longer significant under elevated CO_2, and only tended to be increased after 6 years. The opposite response was found for *Calluna*. In the second year, photosynthesis in *Calluna* was found to be significantly stimulated by elevated CO_2, despite a down-regulation in photosynthetic capacity (Albert et al., 2011a/c). In contrast no down regulation was observed in the present study and photosynthetic stimulation under elevated CO_2 concentration is argued to be the result of high stomatal conductance and high c_i/c_a (Paper III).

Despite the different growth strategies of the two species, leaf nitrogen content was found to be significantly reduced under elevated CO_2 concentration in both species (e.g. Larsen et al., 2011, Ellsworth et al., 2012). However, the reduction was caused by different mechanisms: In line with the opportunistic growth form of *Deschampsia*, it was argued that the grass reduces the amount of nitrogen via reallocation (Andresen & Michelsen, 2005; Andresen et al. 2009; Nielsen et al, 2009). On the other hand, *Calluna* increased the carbon content to produce thicker leaves, which resulted in a reduction of leaf nitrogen content (%) as a result of tissue dilution, which is in line with other studies (e.g. Kongstad et al., 2012; Andresen et al. 2005; Kattge et al., 2009). Nitrogen reducing mechanisms were strongly related to whether or not the photosynthetic capacity within the two species was down-regulated.

Significantly higher biomass of *Deschampsia* in the spring within the 3th year of treatment (April-May 2007) and an increased photosynthetic capacity within warmed plots support the hypothesis that the warming treatment prolonged the growing season (Kongstad et al., 2012 and Albert et al. unpublished). Nevertheless, the earlier start of the growing season did not increase the total biomass later in the season, and the effects on the photosynthetic capacity also disappeared (Kongstad et al., 2012; Albert et al., 2011c). On the other hand, *Calluna* was not stimulated to initiate early growth in the warmed plots and in general (Kongstad *et al.*, 2012). In the present study, measurements were conducted from May to October 2011 and no stimulation of the photosynthetic capacity was found during the warm season. Thus, it can be argued that the onset of the growing season of *Calluna* has been earlier than May, or that an early onset did not take place at all due to the dry spring conditions observed in February to May in 2011.

The extended drought treatment significantly decreased the photosynthetic capacity via stomatal closure, resulting in a lower photosynthetic performance in *Deschampsia*, in the second year of treatments (Albert et al., 2011b/c). The observed drought effect on the photosynthetic performance of Deschampsia was absent after 6 years of treatment. Albert et al. (2012) points out that under mild to moderate drought, *Deschampsia* is able to maintain both stomatal conductance and photosynthetic capacity by reducing the aboveground leaf biomass. As water availability decreases within a dry period, osmotic adjustments take place in the remaining leaves, and this results in a strongly improved water use efficiency (WUE) and enables the plant to maintain photosynthetic performance (Verslues et al., 2006). In line with this, WUE was increased during the experimental drought and in the following lag phase, where soil water was still lower than in the non-drought plots (May-June). *Calluna* is a drought tolerant species and the growth strategy does not induce leaf dieback in unfavorable periods (Grime et al., 1988). Thus, it was expected that in dry periods photosynthesis will be down-regulated, either due to reduced stomatal conductance or V_{cmax}. In both of the evaluated years, a lower photosynthesis in the drought plot was observed within the experimental drought and lag phase where SWC was still lower than in non-drought plots. However, in the 6th year of treatment, the reduced A_{sat} could not be explained by a reduced stomatal conductance, as it was found in the second year of treatment (Albert et al., 2011a). The experimental drought in 2011was initiated on top of an already natural dry period, which resulted in a giant dieback of *Calluna*. The dieback did, as mentioned earlier, not differ in magnitude between treatments and thus, a slightly decreased photosynthetic capacity was observed in combination with a decreased photosynthetic activity, indicating a later onset of growth after the dieback in July.

After 6 years of treatment, my leaf physiology data showed the same responses as seen in the beginning of the experiment (within 2 years of treatment) for both species at the site. Overall, no long-term ecosystem response was observed to influence leaf level physiology.

4. Outlook – Ecosystem climate change feedbacks in the perspective of leaf level responses.

Long-term responses on the leaf level are caused by ecosystem feedbacks and a major question to answer is: '*Has 6 years of experimental treatments of a Danish heathland ecosystem led to ecosystem changes that are detectable as altered leaf level physiology?*' This question has been dealt with in this thesis, particularly in paper III. However, to detect effects on different spatial scales, up-scaling from leaf level responses to ecosystem feedbacks are needed.

4.1 Upscaling from leaf to ecosystem scale.

In addition to papers I-III, it is planned to make a final paper using the mechanistic ecosystem model MAESTRA (http://bio.mq.edu.au/maestra) to up-scale leaf level photosynthetic carbon input to the carbon balance on ecosystem scale. Some reflections and considerations about up-scaling processes from the leaf level to ecosystem carbon exchange evolved during this PhD, and are presented in the following section.

The MAESTRA model combines leaf level photosynthetic parameters, such as photosynthetic capacity and leaf water relations, with environmental factors and aboveground biomass. It is used to scale up carbon, nitrogen and water fluxes in the ecosystem on an annual scale. Models like MAESTRA are limited by the quality of the input data, and thus data validation is necessary and high quality defined relations between photosynthetic parameters and environmental factors (water, temperature, CO_2) for each plant species are highly desirable. One goal of this PhD was to provide high quality leaf respiration measurements on *Deschampsia* and *Calluna*. High temporal resolution measurements (every 2 weeks) in ambient plots (figure 2) will provide the backbone for the up-scaling of leaf respiration to the ecosystem scale. Additionally, the relationship between leaf dark respiration (R_D) and mitochondrial respiration (R_{light}) estimated from the FvCB-model will be used to improve the MAESTRA model. The default relation between R_D and R_{light} in the MAESTRA model is 0.4 and is used to define R_{light}. Estimating R_{light} as $0.02*V_{cmax}$ (after von Caemmerer, 2000) and relating this relationship to the measured values of R_D , indicated that the relation of R_D/R_{light} = 0.4 is not a valid assumption for either of the two species. The relationship was ca. 0.2 for *Calluna* and ca. 0.6 for *Deschampsia* (figure 8). Over- or underestimation of R_{light} can strongly influence the modeled carbon balance, and thus a correct estimation of R_D/R_{light} is of high importance to improve up-scaling from leaf to ecosystem level.

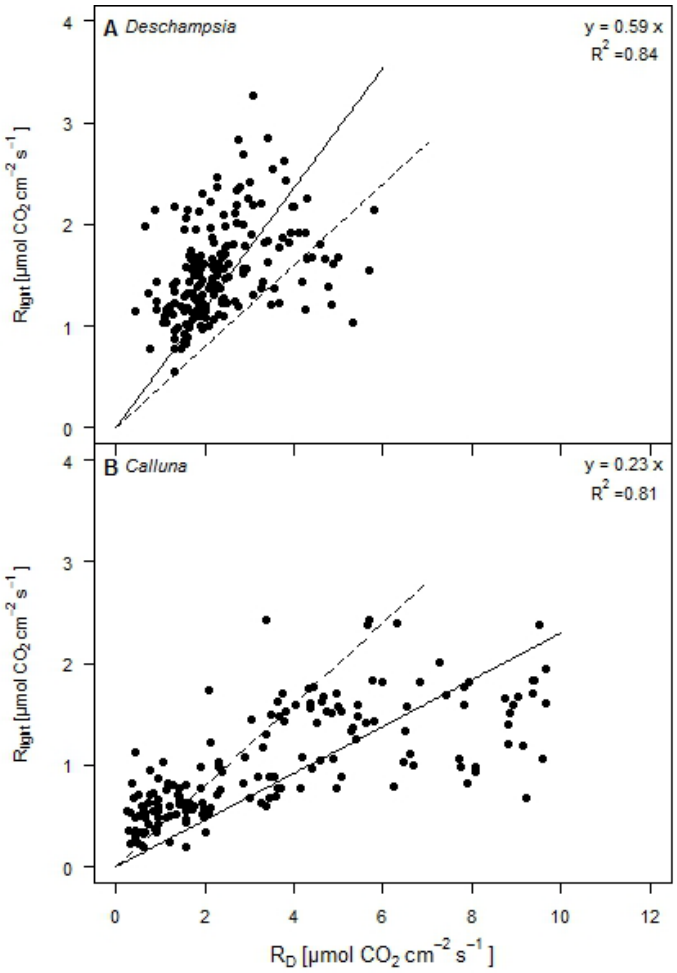

Figure 8. Relationship between leaf dark respiration (R_D) and respiration in light (R_{light}) per leaf area. Solid line is the fitted relationship and the stippled line is the MAESTRA default relation of 0.4. A) *Deschampsia flexuosa* and B*) Calluna vulgaris.*

Stomatal conductance is decreasing when atmospheric CO_2 is increased. However, the response is slow and therefor associated with technical limitations. Enclosure of leaves in small leaf cuvettes for long time (> hours) can lead to undesirable edge-effects, why it is difficult, if not impossible, to define 'true' stomatal responses to increased CO_2 using these techniques. Therefore, a part of this PhD was the development and evaluation of a technique to investigate the effect of increased atmospheric CO_2 on stomatal conductance in *Deschampsia* and *Calluna*. Using mesocosms, the master-student Andreas Brændholt, in collaboration with me and the supervisors Andreas Ibrom and Teis Mikkelsen, developed a gas exchanges system (figure 9) to investigate the effect of CO_2 concentration on stomatal conductance under controlled environmental conditions. He concluded that the stomatal response to CO_2 concentration was related to the different growth strategies and he observed that none of the species showed linear responses. The developed gas exchange system and the first measurements of the CO_2 effect on stomatal conductance can be used in the validation process of the MAESTRA model.

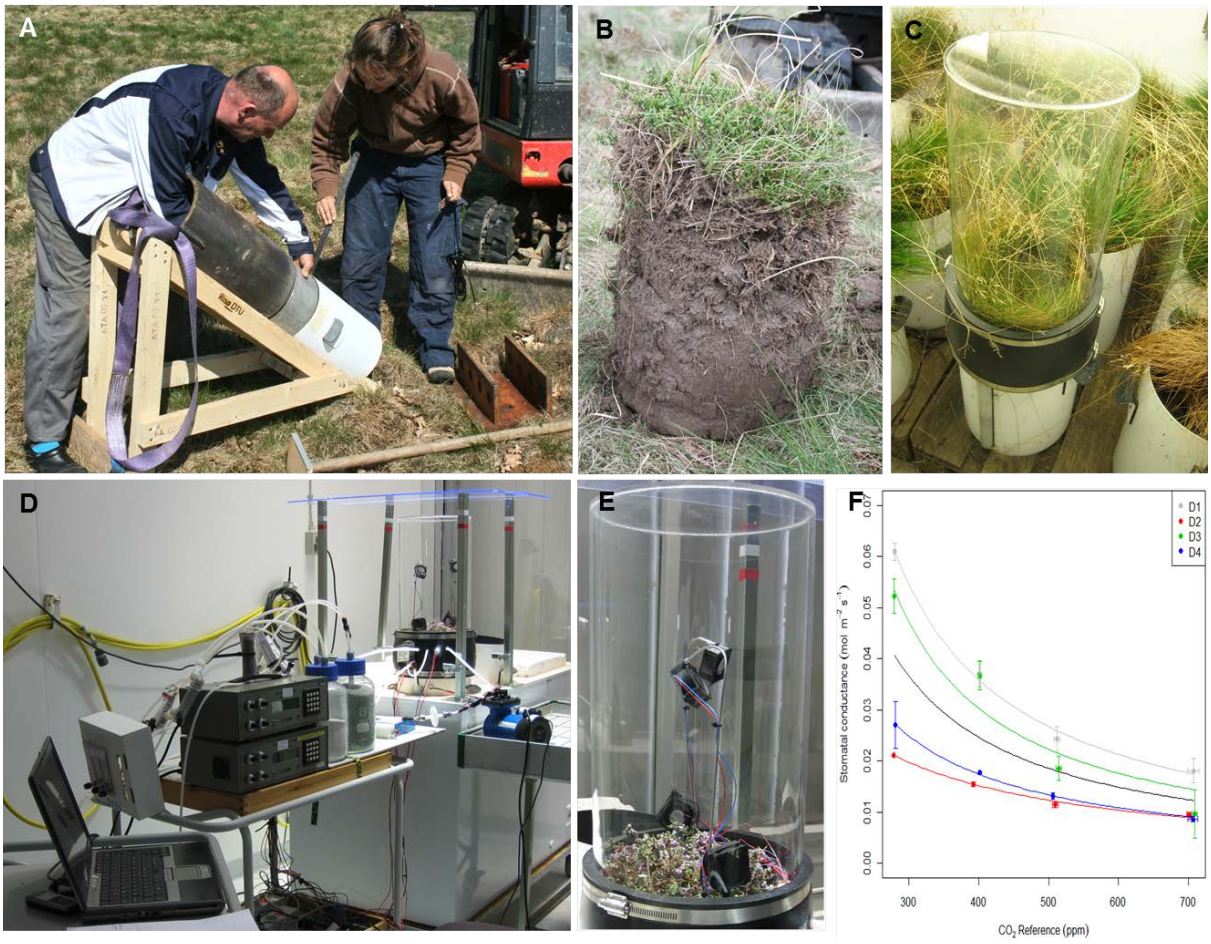

Figure 9. Basic overview of the mesocosm experiment and sampling results. A) Sampling device. B) Sampled mesocosm of *Calluna vulgaris*. C) Final mesocosm of *Deschampsia flexuosa* with a gas exchange chamber on top. D+E) Final gas-exchange system during measurements of CO_2 and H_2O exchange. F) CO_2 response of stomatal conductance in four different mesocosms containing *Deschampsia* (x-axis: ambient CO_2 reference, ppm, y-axis: stomatal conductance, mol m^{-2} s^{-1}) Photos: Andreas Brændholt and Kristine Boesgaard.

4.2 Conclusions and Perspectives

High precision leaf gas exchange measurements have frequently been limited to controlled environments, such as laboratory or indoor growth-facilities. The improvement of the leaf gas exchange measurements using leaf adaptor frames (LAF) showed that it is possible to conduct high precision measurements under field conditions. Furthermore, LAFs can be used for plant species with small and three-dimensional round leaves like leaves from *Deschampsia* and *Calluna*, demonstrating that LAFs can be used for high precision leaf level measurements. Additionally, LAFs was used to conduct *in-situ* gas exchange measurements and leaf trait analyses on the same leaf sample, improving the relationship between leaf parameters. LAFs were used to investigate the effects of climate factor treatments on leaf gas exchange parameters in *Deschampsia* and *Calluna* under field conditions.

Leaf level responses of *Calluna vulgaris* to the experimental treatments were different between the cold and the warm season. Despite a 50 % reduction of the photosynthetic potential (A_{sat}) in the cold season, the overall effect of elevated CO_2 was an increase of the annual carbon uptake with up to 5%. This was caused by a more pronounced difference in photosynthesis between the warm and cold season in ambient CO_2 plots compared to elevated CO_2. In the cold season, the reduction in photosynthetic capacity under elevated CO_2 seems to be related to a translocation of nitrogen. Conversely, nitrogen re-translocation can explain the only marginal increase in A_{sat} found during the warm season. Warm season processes differ from the cold season and elevated CO_2 concentration increases the potential annual carbon uptake in *Calluna*.

The leaf level physiological responses to the treatments were the same after 6 as after 2 years. Different ecophysiological responses, for both *Deschampsia* and *Calluna*, could be explained by different soil water contents during the two years. In the drier second year, photosynthesis in *Deschampsia* was significantly stimulated by elevated CO_2 concentration, and this effect combined with either warming or drought was additive (Albert et al. 2011b/c). In the wetter sixth year, the CO_2-induced stimulation of photosynthesis in *Deschampsia* was only minimal and not significant. *Calluna* showed strong CO_2 stimulated photosynthesis in both the dry and the wet year, and responses to multifactor-treatments were additive or antagonistic. In conclusion, 6 years of treatment did not change leaf level parameters in the investigated Danish heathland plants dramatically. Physiological acclimations to climate change manipulations seem to have taken place within the first year of treatment, and the responses were consistent over longer time (6 years). However, to evaluate ecosystem feedbacks at a leaf level scale in such a highly resilient ecosystem, longer time is needed. The consistency of the leaf level responses during a more than 6 years treatment period would be very useful to investigate to improve the knowledge of ecophysiological responses to climate change.

It was shown that measurements of leaf level parameters are highly important to understand ecosystem feedbacks and carbon fluxes, as the main input of carbon to terrestrial ecosystems is through photosynthesis. However, effects of climate change induced photosynthetic responses on an ecosystem or global scale can be extremely difficult to predict, why models are good tools. Unfortunately, models are limited by the quality of input data and require that data are precise and relevant. Data from field experiments like CLIMAITE can, to some extent, be used to validate ecosystem models and can be used to point out which factors in a climate changed future that might determine the ecosystem sink or source capacity. To be able to evaluate treatment effects on a seasonal scale, the gas exchange measurements in this PhD work were conducted to mimic field temperature conditions and then normalized to a common temperature. Ecosystem models like MAESTRA are trying to estimate ecosystem carbon balances based on relationships between photosynthetic parameters, leaf traits and environmental factors. The presented work in

paper II-III illustrates that there are certain relationships between photosynthetic parameters, leaf traits and environmental factors that could be of high importance in an up-scaling process. However, these kinds of relationships would need to be investigated under more controlled conditions to be validated for model-approaches. Thus, more specific experiments focusing on different ecophysiology-related relationships might be of higher importance in future research.

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Appendix

The appendix present additionally results collected within this PhD. The presented data are not included in either the synopsis or paper I-III, but would be used for upscaling purpose

Table A1+A2) Physiological parameters collected in HTR- plots in *Deschampsia* and *Calluna*, respectively.

Table A3 +A4) Leaf trait from leaf samples in HTR- plots in *Deschampsia* and *Calluna*, respectively.

Table A1. Means ± SE (n=6) of photosynthetic parameters in *Deschampsia flexuosa* from HTR-plots. Parameters are light-saturated photosynthesis (A_{sat}), maximal rate of Rubisco carboxylation (V_{cmax}),), maximal rate of RuBP regeneration (J_{max}), light- and CO_2-saturated photosynthesis (A_{max}), dark respiration (R_D), mitochondrial respiration rate (R_{light}), intercellular CO_2 concentration (c_i), stomatal conductance (g_s), water use efficiency (WUE).

Date	A_{sat}	V_{cmax}	J_{max}	A_{max}	R_D	R_{light}	c_i	g_s	WUE
22-05-2011	22.59 ± 1.45	88.52 ± 4.54	199.37 ± 5.55	-	3.54 ± 0.31	1.77 ± 0.09	226.52 ± 6.84	0.26 ± 0.03	5.93 ± 0.19
08-06-2011	17.28 ± 2.64	131.99 ± 22.8	163.12 ± 27.1	47.39 ± 1.15	3.70 ± 0.99	2.64 ± 0.45	235.23 ± 6.31	0.20 ± 0.03	4.14 ± 0.67
27-06-2011	17.23 ± 1.64	132.90 ± 25.8	160.42 ± 27.6	39.90 ± 7.65	4.17 ± 0.34	2.66 ± 0.52	230.44 ± 6.25	0.21 ± 0.02	3.44 ± 0.54
06-07-2011	14.36 ± 2.10	128.10 ± 8.60	186.22 ± 24.6	47.64 ± 6.36	3.28 ± 0.45	2.56 ± 0.17	217.99 ± 16.4	0.16 ± 0.04	3.55 ± 0.12
21-07-2011	19.91 ± 0.10	77.39 ± 2.55	141.07 ± 27.7	42.26 ± 10.6	2.32 ± 0.00	1.55 ± 0.05	259.81 ± 23.4	0.33 ± 0.08	5.51 ± 0.49
02-08-2011	17.45 ± 3.60	159.23 ± 21.0	205.96 ± 31.4	48.10 ± 8.13	4.85 ± 0.29	3.18 ± 0.42	231.16 ± 6.10	0.22 ± 0.05	3.19 ± 0.27
19-08-2011	18.11 ± 1.99	59.99 ± 2.30	157.89 ± 5.26	39.62 ± 2.50	-	1.20 ± 0.05	277.33 ± 14.7	0.35 ± 0.08	5.34 ± 0.61
06-09-2011	22.64 ± 1.60	127.78 ± 12.3	240.42 ± 14.0	60.18 ± 4.24	4.27 ± 0.29	2.56 ± 0.25	236.90 ± 16.1	0.29 ± 0.05	7.03 ± 1.15
27-09-2011	19.88 ± 1.57	73.13 ± 10.9	180.71 ± 21.2	48.47 ± 3.25	2.77 ± 0.28	1.46 ± 0.22	228.60 ± 23.5	0.23 ± 0.06	9.84 ± 0.00
17-10-2011	14.47 ± 1.44	39.55 ± 3.07	127.02 ± 9.61	38.46 ± 3.04	1.13 ± 0.43	0.79 ± 0.06	310.29 ± 4.80	0.37 ± 0.07	6.11 ± 0.89
31-10-2011	18.50 ± 1.36	63.00 ± 17.9	202.13 ± 42.2	45.79 ± 2.72	1.10 ± 0.15	1.26 ± 0.36	309.37 ± 6.49	0.50 ± 0.09	6.49 ± 0.33
11-11-2011	12.66 ± 0.50	27.01 ± 1.51	88.63 ± 4.39	-	1.06 ± 0.11	0.54 ± 0.03	284.59 ± 8.44	0.23 ± 0.02	-
28-11-2011	10.22 ± 0.70	18.20 ± 1.08	72.98 ± 3.54	22.68 ± 1.16	0.57 ± 0.08	0.36 ± 0.02	305.44 ± 3.91	0.23 ± 0.02	9.10 ± 0.00
14-12-2011	9.37 ± 1.22	21.40 ± 2.45	82.23 ± 8.13	21.90 ± 1.75	0.94 ± 0.17	0.43 ± 0.05	265.58 ± 12.2	0.15 ± 0.04	-
28-12-2011	6.44 ± 0.94	15.61 ± 1.80	60.84 ± 5.67	17.98 ± 2.22	1.04 ± 0.03	0.31 ± 0.04	308.41 ± 6.74	0.14 ± 0.01	9.39 ± 0.44
06-01-2012	9.12 ± 1.05	17.82 ± 1.53	69.54 ± 5.09	18.80 ± 1.24	1.26 ± 0.13	0.36 ± 0.03	305.95 ± 11.7	0.22 ± 0.05	-
24-02-2012	5.21 ± 1.63	14.45 ± 3.97	56.64 ± 15.2	11.86 ± 2.71	1.64 ± 0.25	0.29 ± 0.08	331.56 ± 15.6	0.19 ± 0.05	6.34 ± 1.03
06-03-2012	4.33 ± 0.44	15.59 ± 7.33	41.69 ± 8.94	8.70 ± 2.12	1.15 ± 0.30	0.31 ± 0.15	275.92 ± 33.0	0.07 ± 0.02	9.93 ± 0.00
23-03-2012	10.54 ± 1.65	34.20 ± 5.30	107.74 ± 13.6	33.42 ± 7.01	2.14 ± 0.56	0.68 ± 0.11	273.38 ± 29.0	0.23 ± 0.08	7.12 ± 0.78
03-04-2012	16.65 ± 2.62	33.42 ± 3.92	103.05 ± 12.4	35.98 ± 5.07	2.43 ± 0.35	0.67 ± 0.08	242.45 ± 11.8	0.22 ± 0.06	8.90 ± 0.84
20-04-2012	6.69 ± 2.05	54.35 ± 19.0	122.35 ± 16.2	37.06 ± 3.94	2.75 ± 0.33	1.09 ± 0.38	267.29 ± 10.6	0.11 ± 0.05	6.93 ± 0.05
02-05-2012	12.44 ± 1.33	68.85 ± 10.7	182.58 ± 38.4	49.95 ± 6.97	3.60 ± 0.45	1.38 ± 0.21	281.43 ± 29.9	0.23 ± 0.05	5.90 ± 1.91
15-05-2012	9.38 ± 0.00	54.20 ± 7.01	139.14 ± 12.4	34.25 ± 0.95	1.96 ± 0.37	1.08 ± 0.14	246.04 ± 0.00	0.12 ± 0.00	5.75 ± 0.00

Table A2. Means ± SE (n=6) of photosynthetic parameters in *Calluna vulgaris* from HTR-plots. Parameters are light-saturated photosynthesis (A_{sat}), maximal rate of Rubisco carboxylation (V_{cmax}),), maximal rate of RuBP regeneration (J_{max}), light- and CO_2-saturated photosynthesis (A_{max}), dark respiration (R_D), mitochondrial respiration rate (R_{light}), intercellular CO_2 concentration (c_i), stomatal conductance (g_s), water use efficiency (WUE).

Date	A_{sat}	V_{cmax}	J_{max}	A_{max}	R_D	R_{light}	c_i	g_s	WUE
22-05-2011	8.02 ± 1.85	33.83 ± 5.50	74.25 ± 8.67	11.33 ± 2.02	3.73 ± 0.65	0.68 ± 0.11	328.47 ± 12.7	0.29 ± 0.03	2.75 ± 0.31
06-07-2011	10.66 ± 2.99	66.53 ± 9.19	120.02 ± 15.3	37.16 ± 4.00	6.83 ± 1.03	1.33 ± 0.18	324.83 ± 5.56	0.41 ± 0.16	1.67 ± 0.63
27-07-2011	15.54 ± 2.78	82.11 ± 0.00	130.59 ± 0.00	49.81 ± 6.77	-	1.64 ± 0.00	327.01 ± 0.25	0.54 ± 0.11	1.50 ± 0.00
19-08-2011	14.20 + 0.00	56.54 + 8.21	150.72 + 28.0	45.52 + 3.92	7.10 + 0.00	1.13 + 0.16	351.48 + 0.00	0.86 + 0.00	2.04 + 0.00
06-09-2011	18.70 ± 5.05	73.59 ± 18.0	154.64 ± 18.5	57.44 ± 3.81	6.50 ± 0.30	1.47 ± 0.36	332.05 ± 10.6	0.68 ± 0.04	1.73 ± 0.54
27-09-2011	21.40 ± 3.08	62.63 ± 5.63	148.27 ± 14.22	47.40 ± 3.28	3.71 ± 0.16	1.25 ± 0.11	309.87 ± 18.3	0.56 ± 0.05	4.01 ± 0.10
17-10-2011	17.25 ± 0.26	49.88 ± 3.88	131.57 ± 12.2	36.25 ± 2.87	4.15 ± 0.50	1.00 ± 0.08	291.33 ± 5.94	0.33 ± 0.03	4.50 ± 0.22
31-10-2011	18.50 ± 3.07	50.31 ± 4.57	142.38 ± 14.7	40.04 ± 3.22	2.44 ± 0.27	1.01 ± 0.09	321.91 ± 9.13	0.62 ± 0.18	4.72 ± 0.36
11-11-2011	12.42 ± 0.89	28.04 ± 1.93	96.35 ± 5.01	-	1.25 ± 0.21	0.56 ± 0.04	333.12 ± 11.9	0.47 ± 0.07	5.42 ± 0.31
28-11-2011	10.10 ± 2.21	21.64 ± 4.37	76.08 ± 10.7	20.97 ± 2.94	2.13 ± 0.15	0.43 ± 0.09	305.64 ± 13.3	0.25 ± 0.06	7.20 ± 2.52
14-12-2011	7.22 + 1.31	14.51 + 1.77	54.54 + 4.61	15.50 + 1.82	0.93 + 0.18	0.29 + 0.04	291.50 + 10.1	0.13 + 0.03	8.36 + 1.11
28-12-2011	6.01 + 2.38	12.67 + 4.60	54.87 + 17.1	17.92 + 5.13	0.65 + 0.23	0.25 + 0.09	359.84 + 7.28	0.48 + 0.30	4.15 + 0.13
06-01-2012	6.55 ± 0.88	10.44 ± 1.05	44.23 ± 3.31	14.72 ± 0.84	0.63 ± 0.11	0.21 ± 0.02	320.99 ± 7.41	0.18 ± 0.04	6.76 ± 2.13
06-03-2012	2.38 ± 1.06	4.99 ± 3.24	21.82 ± 12.8	7.23 ± 3.91	0.43 ± 0.04	0.10 ± 0.06	317.06 ± 33.0	0.06 ± 0.00	4.38 ± 2.64
23-03-2012	4.69 ± 1.38	11.10 ± 3.02	37.40 ± 9.86	21.47 ± 2.37	2.70 ± 0.37	0.22 ± 0.06	331.34 ± 3.22	0.15 ± 0.04	4.76 ± 0.74
03-04-2012	4.08 ± 0.79	9.40 ± 1.84	32.69 ± 6.99	11.30 ± 2.14	1.70 ± 0.30	0.19 ± 0.04	313.28 ± 5.44	0.09 ± 0.02	3.85 ± 0.58
20-04-2012	3.43 ± 1.69	13.24 ± 2.07	48.66 ± 5.89	17.53 ± 1.61	2.79 ± 0.24	0.26 ± 0.04	343.45 ± 19.6	0.14 ± 0.01	3.60 ± 2.12
02-05-2012	7.99 + 1.07	44.99 + 5.06	134.03 + 11.2	43.36 + 4.67	5.69 + 0.20	0.90 + 0.10	339.25 + 3.63	0.35 + 0.06	2.18 + 0.13
15-05-2012	7.86 ± 2.03	35.18 ± 9.79	107.51 ± 29.1	32.31 ± 8.51	5.91 ± 1.83	0.70 ± 0.20	351.83 ± 2.52	0.51 ± 0.14	1.51 ± 0.09

Table A3. Means ± SE (n=6) of leaf traits in *Deschampsia flexuosa* from HTR-plots. Traits are leaf nitrogen concentration (*N %*), leaf nitrogen per leaf area (*mg N cm^{-2}*), leaf carbon to nitrogen concentration (*C/N*) , specific leaf weight (*SLW*) and natural aboundance of 13C ($\delta^{13}C$)

Date	*N%*	*mg N cm^{-2}*	*C/N*	*SLW*	$\delta^{13}C$
19-01-2011	2.50 ± 0.12	0.33 ± 0.03	19.73 ± 0.97	0.13 ± 0.01	-27.95 ± 0.39
09-02-2011	2.24 ± 0.10	0.32 ± 0.02	21.75 ± 0.97	0.14 ± 0.01	-27.80 ± 0.32
24-03-2011	2.41 ± 0.18	0.33 ± 0.04	19.76 ± 1.43	0.14 ± 0.01	-28.58 ± 0.42
07-04-2011	3.04 ± 0.21	0.40 ± 0.03	15.80 ± 1.14	0.14 ± 0.01	-28.10 ± 0.19
10-05-2011	2.35 ± 0.16	0.40 ± 0.02	20.32 ± 1.47	0.17 ± 0.01	-27.42 ± 0.27
22-05-2011	2.23 ± 0.15	0.36 ± 0.04	21.52 ± 1.44	0.16 ± 0.01	-27.50 ± 0.28
08-06-2011	1.82 ± 0.19	0.27 ± 0.07	27.72 ± 2.84	0.14 ± 0.02	-27.01 ± 0.24
27-06-2011	1.67 ± 0.11	0.33 ± 0.03	29.15 ± 2.02	0.20 ± 0.01	-27.27 ± 0.33
06-07-2011	1.96 ± 0.17	0.34 ± 0.05	25.40 ± 1.96	0.17 ± 0.02	-26.58 ± 0.45
21-07-2011	2.06 ± 0.19	0.35 ± 0.08	24.36 ± 2.02	0.17 ± 0.03	-26.84 ± 0.37
27-07-2011	3.00 ± 0.11	0.37 ± 0.03	15.57 ± 0.58	0.13 ± 0.01	-28.84 ± 0.24
02-08-2011	2.17 ± 0.12	0.34 ± 0.02	22.77 ± 1.20	0.16 ± 0.01	-27.04 ± 0.56
19-08-2011	2.03 ± 0.03	0.32 ± 0.01	23.92 ± 0.38	0.16 ± 0.00	-27.00 ± 0.22
06-09-2011	1.94 ± 0.07	0.39 ± 0.03	25.00 ± 0.89	0.20 ± 0.01	-27.10 ± 0.25
16-09-2011	1.78 ± 0.08	-	26.95 ± 1.19	-	-27.42 ± 0.38
27-09-2011	2.20 ± 0.09	0.31 ± 0.01	21.82 ± 0.84	0.14 ± 0.01	-26.85 ± 0.43
17-10-2011	1.92 ± 0.08	0.32 ± 0.02	24.67 ± 0.87	0.17 ± 0.01	-26.96 ± 0.39
31-10-2011	2.31 ± 0.09	0.31 ± 0.02	20.83 ± 0.84	0.13 ± 0.01	-28.30 ± 0.69
11-11-2011	2.22 ± 0.05	0.27 ± 0.02	21.19 ± 0.51	0.12 ± 0.01	-28.47 ± 0.49
28-11-2011	2.30 ± 0.09	0.31 ± 0.01	20.49 ± 0.81	0.13 ± 0.01	-28.67 ± 0.21
14-12-2011	2.33 ± 0.04	0.33 ± 0.02	20.14 ± 0.32	0.14 ± 0.01	-28.38 ± 0.37
28-12-2011	2.18 ± 0.21	0.24 ± 0.03	21.69 ± 2.21	0.11 ± 0.00	-28.42 ± 0.46
06-01-2012	2.36 ± 0.08	0.32 ± 0.02	19.78 ± 0.59	0.14 ± 0.01	-27.43 ± 0.29
25-01-2012	2.42 ± 0.10	-	18.95 ± 0.77	-	-27.53 ± 0.22
09-02-2012	2.46 ± 0.12	-	18.92 ± 0.83	-	-27.40 ± 0.41
24-02-2012	2.42 ± 0.13	0.35 ± 0.04	19.59 ± 1.05	0.15 ± 0.01	-28.48 ± 0.50
06-03-2012	2.38 ± 0.07	0.35 ± 0.02	19.80 ± 0.57	0.15 ± 0.01	-27.42 ± 0.37
23-03-2012	2.73 ± 0.32	0.40 ± 0.04	18.49 ± 1.63	0.15 ± 0.01	-27.93 ± 0.42
03-04-2012	2.79 ± 0.19	0.44 ± 0.04	17.08 ± 1.13	0.16 ± 0.01	-28.37 ± 0.20
20-04-2012	2.56 ± 0.13	0.38 ± 0.04	18.22 ± 1.01	0.15 ± 0.02	-28.36 ± 0.15
02-05-2012	2.57 ± 0.24	0.45 ± 0.02	19.15 ± 1.96	0.19 ± 0.02	-28.14 ± 0.22
15-05-2012	2.23 ± 0.17	0.33 ± 0.02	21.19 ± 1.56	0.15 ± 0.01	-27.97 ± 0.13

Table A4. Means ± SE (n=6) of leaf traits in *Calluna vulgaris* from HTR-plots. Traits are leaf nitrogen concentration (*N %*), leaf nitrogen per leaf area (*mg N cm^{-2}*), leaf carbon to nitrogen concentration (*C/N*) , specific leaf weight (*SLW*) and natural aboundance of 13C ($\delta^{13}C$)

Date	*N%*	*mg N cm^{-2}*	*C/N*	*SLW*	$\delta^{13}C$
22-12-2010	1.37 ± 0.09	-	39.55 ± 2.61	-	-29.27 ± 0.35
19-01-2011	1.57 ± 0.05	0.27 ± 0.03	32.58 ± 1.02	0.17 ± 0.02	-29.60 ± 0.15
09-02-2011	1.40 ± 0.03	0.23 ± 0.03	35.56 ± 0.98	0.16 ± 0.02	-29.67 ± 0.29
02-03-2011	1.49 ± 0.05	-	33.81 ± 1.27	-	-29.46 ± 0.32
24-03-2011	1.40 ± 0.04	0.27 ± 0.02	36.14 ± 1.18	0.19 ± 0.01	-29.58 ± 0.29
18-04-2011	1.84 ± 0.10	0.31 ± 0.01	27.95 ± 1.56	0.17 ± 0.01	-29.71 ± 0.35
22-05-2011	1.96 ± 0.06	0.34 ± 0.03	25.19 ± 0.80	0.17 ± 0.02	-28.79 ± 0.31
08-06-2011	1.96 ± 0.25	0.27 ± 0.03	26.86 ± 2.81	0.14 ± 0.01	-28.27 ± 0.48
27-06-2011	1.76 ± 0.12	0.32 ± 0.02	28.08 ± 2.47	0.19 ± 0.01	-28.80 ± 0.24
06-07-2011	1.89 ± 0.13	0.34 ± 0.03	26.92 ± 1.93	0.18 ± 0.02	-29.41 ± 0.25
21-07-2011	1.82 ± 0.11	0.26 ± 0.03	28.48 ± 1.86	0.14 ± 0.01	-29.66 ± 0.53
27-07-2011	2.04 ± 0.16	0.33 ± 0.05	25.62 ± 2.41	0.16 ± 0.02	-29.49 ± 0.05
02-08-2011	2.19 ± 0.13	0.43 ± 0.03	23.83 ± 1.73	0.20 ± 0.02	-29.16 ± 0.37
19-08-2011	2.37 ± 0.22	0.37 ± 0.03	23.09 ± 2.98	0.16 ± 0.01	-28.54 ± 0.35
06-09-2011	2.14 ± 0.09	0.34 ± 0.02	23.93 ± 1.20	0.16 ± 0.01	-28.86 ± 0.29
16-09-2011	1.76 ± 0.18	0.36 ± 0.07	29.95 ± 3.89	0.21 ± 0.04	-28.99 ± 0.33
27-09-2011	1.98 ± 0.05	0.38 ± 0.02	25.35 ± 0.60	0.19 ± 0.01	-29.21 ± 0.34
17-10-2011	1.70 ± 0.06	0.39 ± 0.01	29.22 ± 0.98	0.23 ± 0.01	-28.98 ± 0.23
31-10-2011	1.75 ± 0.10	0.43 ± 0.03	28.69 ± 1.63	0.25 ± 0.01	-29.47 ± 0.25
11-11-2011	1.69 ± 0.08	0.40 ± 0.02	29.27 ± 1.55	0.24 ± 0.01	-29.64 ± 0.32
28-11-2011	1.59 ± 0.03	0.41 ± 0.02	30.98 ± 0.71	0.26 ± 0.01	-29.35 ± 0.45
14-12-2011	1.69 ± 0.04	0.45 ± 0.02	29.15 ± 0.83	0.27 ± 0.01	-28.87 ± 0.54
28-12-2011	1.67 ± 0.01	0.45 ± 0.01	29.42 ± 0.13	0.27 ± 0.01	-29.85 ± 0.18
06-01-2012	1.77 ± 0.05	0.47 ± 0.02	28.09 ± 0.72	0.27 ± 0.01	-28.79 ± 0.58
25-01-2012	1.71 ± 0.05	-	28.53 ± 0.93	-	-29.49 ± 0.31
09-02-2012	1.59 ± 0.06	-	30.92 ± 1.33	-	-29.01 ± 0.40
24-02-2012	1.56 ± 0.04	0.40 ± 0.01	32.00 ± 1.00	0.27 ± 0.01	-29.16 ± 0.22
06-03-2012	1.74 ± 0.07	0.45 ± 0.02	28.94 ± 1.09	0.26 ± 0.01	-29.08 ± 0.17
23-03-2012	1.87 ± 0.10	0.40 ± 0.04	26.84 ± 1.58	0.22 ± 0.02	-28.54 ± 0.78
03-04-2012	1.94 ± 0.04	0.44 ± 0.05	25.52 ± 0.57	0.23 ± 0.02	-29.45 ± 0.45
20-04-2012	2.09 ± 0.05	0.58 ± 0.04	23.78 ± 0.51	0.28 ± 0.02	-28.58 ± 0.67
02-05-2012	2.36 ± 0.09	0.61 ± 0.04	21.46 ± 0.80	0.26 ± 0.01	-27.88 ± 0.60
15-05-2012	2.09 ± 0.09	0.56 ± 0.02	24.17 ± 1.08	0.27 ± 0.01	-28.16 ± 0.04

PAPER I

Plant, Cell & Environment

Plant, Cell and Environment (2013) doi: 10.1111/pce.12064

Reduction of molecular gas diffusion through gaskets in leaf gas exchange cuvettes by leaf-mediated pores

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ABSTRACT

There is an ongoing debate on how to correct leaf gas exchange measurements for the unavoidable diffusion leakage that occurs when measurements are done in non-ambient CO_2 concentrations. In this study, we present a theory on how the CO_2 diffusion gradient over the gasket is affected by leaf-mediated pores (LMP) and how LMP reduce diffusive exchange across the gaskets. Recent discussions have so far neglected the processes in the quasi-laminar boundary layer around the gasket. Counter intuitively, LMP reduce the leakage through gaskets, which can be explained by assuming that the boundary layer at the exterior of the cuvette is enriched with air from the inside of the cuvette. The effect can thus be reduced by reducing the boundary layer thickness. The theory clarifies conflicting results from earlier studies. We developed leaf adaptor frames that eliminate LMP during measurements on delicate plant material such as grass leaves with circular cross section, and the effectiveness is shown with respiration measurements on a harp of *Deschampsia flexuosa* leaves. We conclude that the best solution for measurements with portable photosynthesis systems is to avoid LMP rather than trying to correct for the effects.

Key-words: CLIMAITE project; CO_2 leakage; diffusion leakage; gasket density; leaf adaptor frame (LAF); leaf respiration; portable gas exchange system.

INTRODUCTION

Small leaf chambers are widely used for measurements of leaf gas exchange. Measurements of small gas fluxes such as leaf respiration strongly depend on accuracy, and even small artificial changes of the CO_2 flux can be of significant magnitude relative to the correct rate of leaf gas exchange (e.g. Bruhn, Mikkelsen & Atkin 2002; Pons *et al*. 2009). Unfortunately, diffusion through the gasket material in modern commercial portable leaf gas exchange systems is unavoidable and has been demonstrated and described previously (e.g. Long & Bernacchi 2003; Flexas *et al*. 2007; Rodeghiero, Niinemets & Cescatti 2007). Most manufacturers provide methods to correct for the diffusion, and different methods to avoid or minimize the advective leakage through gaps between plant and gasket material, that is, leaf-mediated pores (LMP), and have been suggested. Rodeghiero *et al*. (2007) suggested enclosing the leaf chamber in a bag and let the gas concentration inside the bag approach that inside the leaf chamber. Flexas *et al*. (2007) observed that using dead or inactive broadleaf material as reference for correction resulted in more reliable flux estimates than using the manufacturer's correction method alone. However, these methods are difficult, if not impossible, to apply under extensive field work, and correction with specific dead leaf material is not useful concerning small leaf structures such as grasses, where the number of leaves and thereby LMP between the gaskets cannot be kept constant. Whereas Flexas *et al*. (2007) only describe the effects of using dead leaf material, this study aims at suggesting a theory that can be used to understand and correct the unintended effects of LMP in gas exchange equipment. We tested the theory using the portable photosynthesis system LI6400 (Li-Cor Inc., Lincoln, NE, USA). A newly developed leaf adaptor frame (LAF) for measurements of small but thick leaves, that are of much smaller width than the cuvette opening area, is tested for minimizing the effects of LMP. The use of LAF aims to measure reliable gas exchange rates under field conditions, even at small fluxes such as leaf dark respiration, and to conduct repeatable and reproducible *in situ* measurements on exactly the same plant material in a sequence of measurements.

Further, we investigate the effects of different diffusion correction methods applied to field measurements on Wavy hair-grass (*Deschampsia flexuosa*). Finally, we aim at recommending a procedure that minimizes errors in leaf respiration measurements.

THEORY

The development of LAF aimed at minimizing possible leakage effects through LMP. However, pilot studies of the relation between using LAF and the effects of artificial LMP showed that the opposite was the case: Diffusive losses in gas exchange measurements were lower in presence of LMP than without. The following theory describes the influence of LMP on the accuracy and precision of leaf gas exchange measurements, and is able to answer the obvious question: 'How can LMP reduce leakage through the gaskets of gas exchange cuvettes?'

Direct leakage, caused by leaf structures creating small pores between the gaskets, will result in a mass flow of air between the chamber and the surrounding air. To avoid an inflow of air into the chamber, a small overpressure inside the

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chamber is maintained. In the situation of a completely sealed chamber, that is, no pores that allow advection of air between the inside and the outside of the chamber, gasses will diffuse through the gaskets according to the concentration gradient across the gasket and the diffusion coefficient of the sealing material. This situation is the basis for the manufacturers' correction (Li-Cor Inc. 2008).

Gas diffusion through a material can ideally be described by Fick's first law,

$$F_D = -\frac{K_D(\rho_o - \rho_i)}{S_g l}, \quad (1)$$

where F_D is the flux across the material due to diffusion out of the cuvette (mol m^{-2} s^{-1}), ρ_o–ρ_i is the difference in molar density between the inside (index *i*) and the outer surface (index *o*) of the material (mol m^{-3}), S_g an l are the area and length of the path through the compressed gasket (m), respectively, and K_D is the diffusion coefficient of the compressed gasket material (m^2 s^{-1}) for the gas of interest. The term $\frac{\rho_o - \rho_i}{l}$ can be referred to as the concentration gradient.

In a completely closed chamber, that is, without any open pore between the gaskets, the total loss or gain of CO_2 according to the measurement can be described with Eqn 1, where the diffusion leakage only varies with the CO_2 concentration gradient.

In a situation where leaves or other plant organs create small pores as a result of their structure, the overpressure inside the chamber results in a continuous loss of air from the inside of the cuvette caused by mass flow through the pores. Such advective loss of air (and thereby a given gas mass transport) from the interior of the chamber can be described as,

$$F_{air} = vC_i, \quad (2)$$

where v is the volumetric flow of air (m^3 s^{-1}) and C_i is the concentration of CO_2 (mol m^{-3}) inside the chamber (Fig. 1). Because of the small overpressure in the chamber, F_{air} is outward directed. Therefore, it is assumed that F_{air} does not affect C_i and thus the flux measurements. It will, however, be shown that this is an oversimplification.

The total flux of CO_2 across the gasket in the presence of LMP, that is, $F_t = F_D + F_{air}$, is depending on the direction and the magnitude of the CO_2 concentration gradient across the gasket (Fig. 1). Here, it is important to consider the CO_2 concentration in the boundary layer surrounding the gasket and the wind conditions surrounding the leaf cuvette. In Figure 2, two possible scenarios are shown, where the CO_2 concentration inside the chamber are higher than the ambient CO_2 concentration outside the cuvette. With increased thickness of the boundary layer, the CO_2 concentration in the quasi-laminar boundary layer (C_{bl1}) will be charged with gas from the inside as a result of the advective mass flow, and thus, C_{bl1} approaches C_i, a situation that normally would occur under indoor conditions (Fig. 2a). As a consequence, the concentration gradient decreases, and F_t decreases as a result of decreased F_D through the gasket

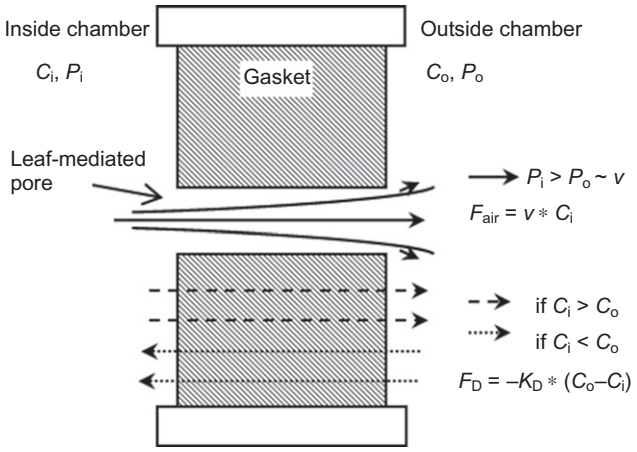

Figure 1. Schematic presentation of the mass flow (black arrow) and diffusion (dotted, dashed arrow) in different concentration situations where leaf-mediated pores are present. Sketch dimensions are not real to improve the illustration of the theory. Fair is the rate of advective CO_2 mass flow out of the chamber depending on the volumetric flow of air (v) and the CO_2 concentration inside the chamber (C_i). P_i and P_o are the pressure inside and outside of the chamber, respectively. F_D is the rate of CO_2 loss/increase as a result of diffusion depending on the diffusion coefficient K_D and the difference in CO_2 concentration outside and inside the chamber ($C_o - C_i$).

material. The thickness of the boundary layer decreases with increasing wind velocity at the surface (e.g. Nobel 1991). Thus, under windy conditions that are typical for outdoor measurements, a higher CO_2 concentration gradient will be kept across the gasket, thus increasing F_D (Fig. 2b). In non-windy conditions, the effect of LMP will thus, counter-intuitively, lead to a smaller diffusive flux across the gasket as compared to a gasket without plant-mediated pores. The effect of LMP on the diffusion through the gasket will be as variable as are the wind conditions in the field.

With no LMP present, the windy conditions around the leaf cuvette should, according to the theory, reduce the concentration gradient, but because of the lack of additional mass flow of air from the inside of the cuvette, this effect would be much smaller if not insignificant. To support the theory, we tested the following three hypotheses: (H1) the leakage flux is a problem of diffusion through the gasket, (H2) LMP reduce the diffusive flux in non-windy conditions and (H3) in windy conditions, the reducing effect of LMP on the diffusion leak is approaching the diffusion without LMP. H1 was tested by sealing the cuvette completely with a gas-tight material and investigate the leakage at a strong CO_2 concentration gradient. If replacing the gasket by gas-tight material removes the leakage, the leak observed when using gaskets must be caused by molecular diffusion through the gasket material. H2 was tested by comparison of empty cuvette measurements with gaskets and measurements with LMP caused by leaf artefacts. H3 was tested with the same approach as for H2 at differing wind speeds outside the gasket.

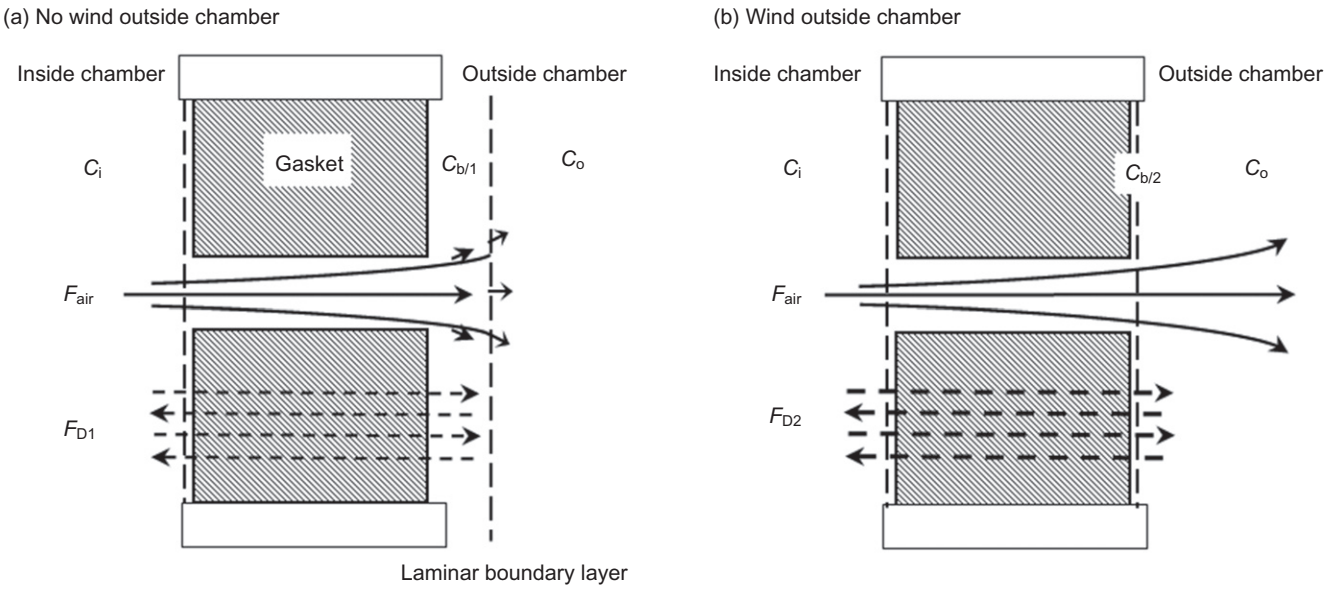

Figure 2. Schematic presentation of the diffusion theory under two turbulent regimes on the outer side of the chamber gaskets. Fair is the rate of advective mass flow of CO_2 depending on the velocity of the mass flow of air (v) and the CO_2 concentration inside the chamber (C_i). F_{D1} and F_{D2} are the rates of CO_2 transport as a result of diffusion depending on the diffusion coefficient K_D and the CO_2 concentration gradient across the gaskets, in the two cases (a and b). C_{bl1} and C_{bl2} are the concentrations of CO_2 in the boundary layer directly at the outer surface of the gasket in the two cases (a and b) and C_o is the concentration in the surrounding air outside the chamber.

MATERIALS AND METHODS

Plant material and locations

Wavy hair-grass (*Deschampsia flexuosa* (L.) Trin.) was used for the experiments with living plant material. We used this plant because accurate gas exchange field measurements on *D. flexuosa* and *Calluna vulgaris* were needed for the multifactorial climate manipulation experiment, CLIMAITE, Brandbjerg, North-Zealand, Denmark (see Mikkelsen *et al*. 2008). With respect to gas exchange measurements, the plant species has the disadvantage of having small and thick leaves. For gas exchange measurements, a bundle of 10–15 parallel leaves were fixed carefully inside an aluminium frame (LAF, see below), avoiding overlapping leaves, that is, a similar procedure as in Albert *et al*. (2011a).

Regular leakage tests with empty cuvettes were conducted about every second week during 2011 in the experimental area of CLIMAITE. Outdoor measurements were all done under the specific environmental conditions at ambient CO_2 concentrations. Indoor measurements were conducted at two different places with differing background CO_2 concentrations. Analyses with gaskets were performed in a well-ventilated room at ambient CO_2 concentrations around 400 ppm. All other tests were conducted under controlled CO_2 concentrations in a fume cupboard with continuous ventilation. The CO_2 concentration in the cupboard was continually measured and logged with a LI-7550 infrared gas analyser (Li-Cor) connected to a laptop computer. The background CO_2 concentration in the cupboard was 454.5 ± 0.3 ppm (n = 12 346) during all measurements.

LAF description

The LAF consisted of two small aluminium frames 40 × 60 mm (1 mm sheet). Each frame had an opening matching exactly the dimensions of the cuvette opening of 20 × 30 mm. The LAF was infolded with a 4 mm fold (Fig. 3a,b). The two frames and the plant material were sealed with blue tack (Lyreco, Marly, France), that is, establishing similar surface contact conditions to the gaskets as large flat leaves. The blue tack was proven to be gas tight and thereby suitable for sealing the LAF (presented under 'Diffusion tests with and without LAFs'). In each of the folds, there were eight holes (Ø 1 mm) to establish a harp of 0.3 mm nylon strings, in order to support *C. vulgaris* shoots inside LAF and guarantee minimal shoot overlap. The usefulness of this feature is not tested here.

Gas exchange measurements

The present study is performed using the LI-6400 open Portable Photosynthesis System from Li-Cor Biosciences, a type of system widely used for leaf level gas exchange measurements. The LI-6400 was connected to a standard 20 × 30 mm chamber with a LED light source (6400-02B) and a CO_2-mixing device controlling the level of reference CO_2. Other manufactures provide similar systems, and the theory applies for all portable photosynthesis systems that provide a slight overpressure inside the chamber and are sealed with non-gas-tight foam material.

We used the following protocols for CO_2 response curves and light response curves in the field. Leaves were

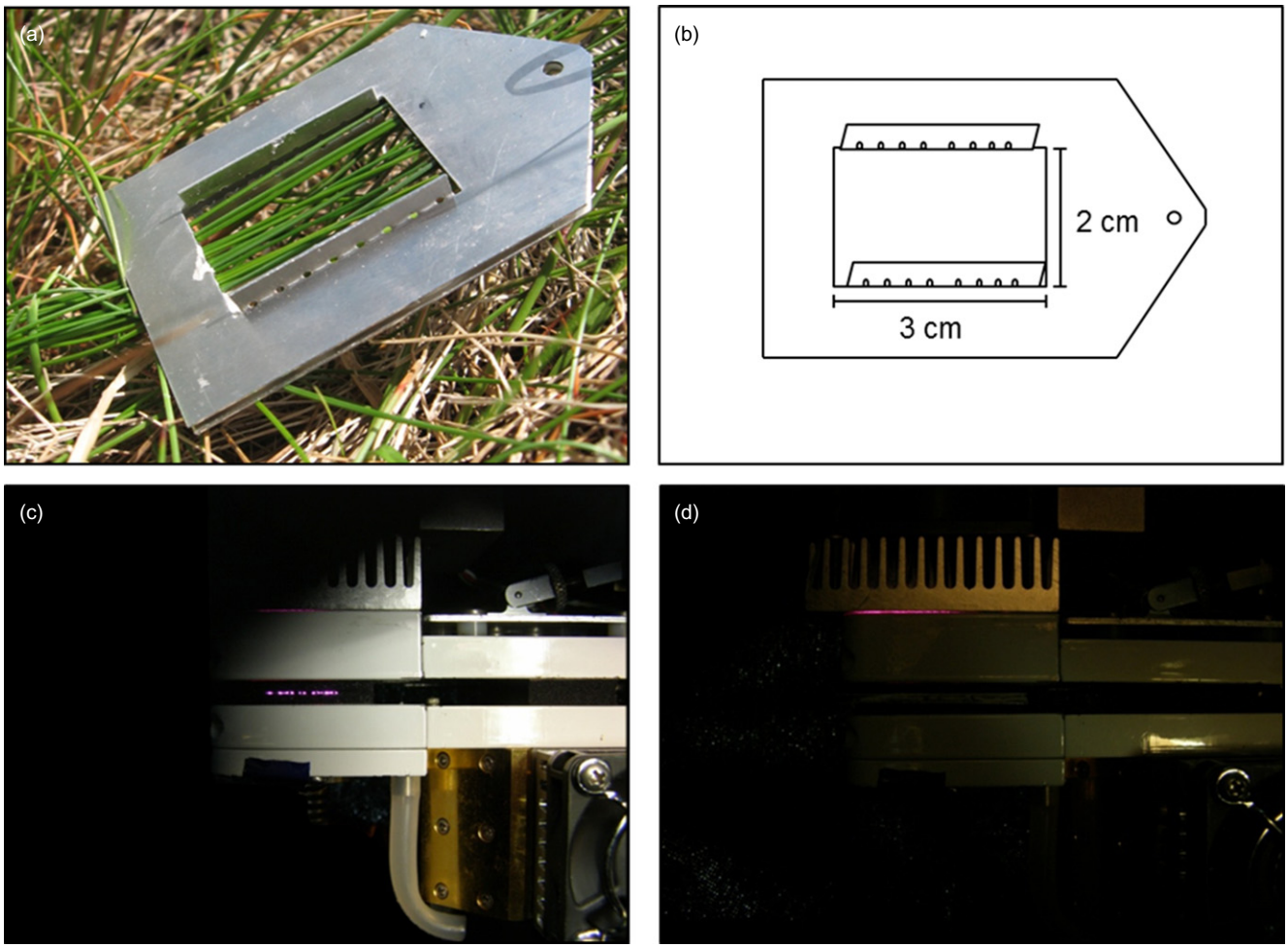

Figure 3. (a) The use of leaf adaptor frames (LAF) at the field site. *Deschampsia flexuosa* leaves are attached in LAF and ready for measurements. (b) Schematic sketch of LAF, in correct scale. (c) Visual illustration of the occurrence of artificial leaves (AL). The purple light comes from the RBG (red-blue-green) light source of LI6400. No light can be seen when the AL are sealed with blue tack in the LAF. (d) The same as (c) but here, the AL are kept inside LAF.

acclimated to the chamber condition for 6 min at 390 ppm, until net photosynthesis and stomatal conductance were stabilized [coefficient of variation (CV) < 1%]. A CO_2 response curve was measured stepping down the CO_2 concentration from 390 to 50 ppm CO_2 and then re-establishing it to the 390 ppm level again, for at least 3 min. Then, the concentration of CO_2 was stepped up to complete saturation at 1400 ppm CO_2. Measurements were performed at a light saturating level of 1500 μmol photosynthetically active photons m^{-2} s^{-1}, using the Li-6400 auto-program 'ACi-curves' with these settings: time between measurements min 45 and max 55 s, reference CO_2 (C_r) [mol mol^{-1}] and intracellular CO_2 concentration (c_i) stable in 10 s with CV < 1%. Matching was performed between every step. Block temperature was set to 25 °C. Relative humidity was adjusted to 45–60% during measurements. Non-photochemical respiration (R_{light}), maximum carboxylation (V_{cmax}) and electron transport (J_{max}) rates were calculated from curve fitting to the Farquhar–von Caemmerer–Berry (FvCB) model equations (Bernacchi *et al*. 2001; Dubois *et al*. 2007). Immediately after running the ACi-curve protocol, the light response curve was measured. The auto-program 'Light curves' on the Li-6400 was used by stepping down the light from 2000 μmol photosynthetic photons m^{-2} s^{-1} [photosynthetic active radiation (PAR)] in nine steps to zero. The photosynthesis saturating reference CO_2 concentration was set to 1400 μmol m^{-2} s^{-1}. From the light response curve the maximum dark respiration (R_{dark}) and maximum light-saturated rate of photosynthesis (A_{max}) was calculated using a non-rectangular hyperbola as regression model (Lambers, Chapin & Pons 1998). In a last step, leaf dark respiration (R_D) was measured directly in the dark at 390 ppm, that is, ambient CO_2 concentration, and estimated from 6 min of flux data at 2 s resolution.

All data were recalculated for correct leaf area and corrected for leakage with three different methods (see further details in the paragraph 'Data corrections').

The three different estimates for leaf respiration (R_{light}, R_{dark}, R_D) are compared.

Diffusion tests with and without LAFs

Tests were conducted to evaluate the influence of the use of a chamber gasket for sealing (H1). Firstly, the leaf chamber was completely sealed with blue tack not using gaskets at all. The differences of the fluxes in a completely sealed versus a gasket-sealed empty cuvette are thus caused by diffusion through the gasket. We assumed that the fluxes measured with a completely sealed empty cuvette are zero μmol CO_2 s^{-1}.

The second test was done by comparing two different chamber gasket materials, white gaskets (spare part no. 6400-30 and black gaskets (spare part no. 6400-33). Three different combinations were obtained using either only white or black or a combination of the two (upper and lower).

Thirdly, the use of an empty LAF was compared to an empty chamber, in both cases sealed with gaskets.

In a fourth test, the effects of pores across the gasket, established by using a bundle of seven tin solder wires (Ø 0.8 mm) that mimic the dimensions of grass leaves (henceforth artificial leaves, AL), was investigated either with or without using LAF (H2).

Effects of ALs on the pressure difference between inside and outside the cuvette

To test whether or not there is a pressure difference across the gaskets at different regimes, a needle attached to a pressure sensor (Model 278, Setra System Inc., Boxbourgh, MA, USA) was inserted through the gasket.

Effects of wind on gas exchange measurements

To evaluate the effect of turbulence around the leaf cuvette (H3), tests with AL with or without LAF in otherwise empty chambers were conducted under two conditions: with a small fan (model: embpapst 412 (Embpapst, Brøndby, Denmark) with an approximate wind speed of 0.35 m s^{-1} close to the fan) in front of the chamber, creating turbulence in the air. This was compared to the same measurements in still air, also taking care that the Peltier cooler would not generate a wind field under those measurements.

Area estimations

After the measurements were performed, the LAF containing the leaves were cut off. The LAF with leaves were placed in a flatbed scanner with the light-exposed side downward and scanned. To avoid damaging the scanner, LAFs were placed inside a gasket attached to a transparency sheet. The non-light-exposed side was filled with gasket material to avoid shading effects. Area estimations were quantified using the image processing program (ImageJ, National Institute of Health, Bethesda, MA, USA). The inside length of the LAF (3 cm) was in all cases the reference length, and area was determined from 8-bit colour pictures with the threshold approach. The scanned leaf areas are given as projected leaf areas, after Smith, Schoettle & Cui (1991).

Data correction

The correction for even small leaks is important for the correct estimation of leaf respiration, because it is itself a relatively small flux. Two different methods to correct for diffusion through the gasket are used and compared.

Firstly, the manufacturer provides a flow-dependent normalized diffusion rate $k = 0.46$ (mol s^{-1}). The CO_2 gas exchange rate can be corrected using the following equation:

$$A_K = \frac{u(C_r - C_s)}{100S} - C_s E + \frac{k}{100S}(C_a - C_s), \quad (3)$$

Where A_K is the corrected assimilation rate, u is the flow rate through the chamber, E is the calculated transpiration rate to account for the CO_2 dilution through water vapour flux from the leaf, S is the leaf area inside the chamber (cm^2). Note that C_r, C_s and C_a are the mole fractions (mol mol^{-1}) in the reference cell, sample cell and in the surroundings, respectively, and not the molar densities as in Eqn 1). The first term of the Eqn 4) represents the assimilation rate without any diffusion correction and the last term is the correction term, $\frac{k}{100S}(C_a - C_s)$. The k-value provided by the manufacture is estimated for the use of one white and one black gasket (Li-Cor Inc. 2008). Note the differing sign conventions: Li-Cor provides assimilation rates, which are positive when the leaf takes up CO_2 via photosynthesis. In a physical gas exchange perspective, positive fluxes are a result of CO_2 addition to the chamber, which in biology refers to the situation of leaf respiration.

The relationship between k, u, C_r and C_s can be expressed as

$$\frac{C_s - C_r}{C_r} = -\frac{k}{u}. \quad (4)$$

This relationship is used for estimation of k for the use of two black gaskets and the use of LAF with AL. These different k-values will later be used for correction of photosynthesis rates.

An alternative empirical method to correct the data is using an empty chamber approach, described by, for example, Bernacchi *et al.* (2001). All C_s (sample cell) values on the ACi curves are corrected with the corresponding $\Delta C_E = C_{s,E} - C_{r,E}$, from a LI6400 machine-dependent mean of empty chamber measurements for each of the concentration levels. The mean ΔC_E used in this study is based on a minimum of 35 machine-dependent, empty chamber measurements done in the field across the season of 2011. After adding the correct area, the corrected assimilation rate (A_E) is calculated as:

$$A_E = \frac{u(C_r - (C_r - \Delta C_E))}{100 * S} - (C_s - \Delta C_E) * E, \quad (5)$$

where A_E is the empty-apparent assimilation rate measured in an empty cuvette and u is the flow rate through the chamber. This approach assumes that the atmospheric concentration is invariant, which is justified because the measurements were taken at daytime when the atmosphere is well

mixed. The seasonal variation of the ambient daytime CO_2 concentrations is small compared to the range of C_r and the ACi and light response protocol.

Statistical analysis

Statistical analyses are done using the R software (R Development Core Team 2010). The linear dependency of F_D to the concentration gradient across the gasket was tested with linear regression. We represented F_D by the measured concentration difference ($C_s - C_r$), which is proportional to the flux because the flow rate of air through the cuvette under the experiment was held constant. The concentration gradient was represented by the CO_2 concentrations in the cuvette, noting that the ambient concentration was held constant under the experiment. The reason for doing so is firstly, that these values are given as data pairs from the analyser, and further, as the results will show, that the relevant C_a, that is, the concentration directly at the outer surface of the gaskets was not possible to measure, anyway. The advantage is that the results can directly be compared (all concentration units) and interpreted. The slopes of ($C_s - C_i$) versus C_i were tested to be different from zero. If the slope differed from zero, this was a result of diffusion. Differences between slopes of different experiments were tested using pairwise *t*-test and Tukey's grouping test. The differences between different correction methods and the three different respiration estimates were also tested for significance using pairwise *t*-test and Tukey's grouping test.

RESULTS

Effects of LAFs on diffusive leakage

Measurements with completely blue tack-sealed cuvettes did not show any linear relationship between the flux, represented by the concentration difference in the air before entering and after leaving the cuvette ($C_s - C_r$), and internal CO_2 concentration inside the cuvette (C_i; Fig. 4). C_i in this case represents the concentration difference across the blue tack material because the outside concentration was held constant running the CO_2 response protocol ($P = 0.88$, $R^2 = 0.012$). The same result was found when testing the effect of eventually remaining artificial pores introduced in the blue tack sealing ($P = 0.37$, $R^2 = 0.00$). The effective sealing of the LMP was also demonstrated visually comparing Fig. 3c and d (see below). The non-linear patterns that both treatments showed in Fig. 4 are not significant. They can be seen in all test measurements and are machine dependent (see, e.g. Fig. 4). These interesting patterns are not the subject of this study but we note that there is a small, systematic under or overestimation of the flux beyond the leakage through the gasket in this particular cuvette design depending on the choice of C_i.

Sealing the leaf chamber with gaskets showed a linear relation of the artificial CO_2 flux with the CO_2 concentration inside the chamber ($P < 0.001$, $R^2 > 0.5$; Fig. 5). There was a significant difference between the different gasket materials

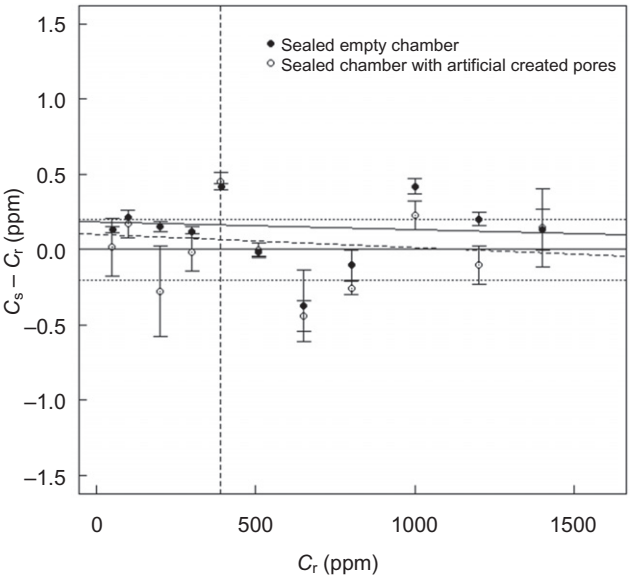

Figure 4. CO_2 concentration differences between the sample (C_s) and the reference cell (C_r) and the CO_2 concentrations inside the leaf chamber (C_i) for measurements done with blue tack-sealed chamber (●) and blue tack-sealed chamber with artificial created pores (○). Solid and dashed lines are the linear regression lines for sealed and sealed with artificial created pores, respectively. The two horizontal dotted lines represent the detection limits for the Li6400 (Li-Cor Inc. 2008) and the vertical dotted line is the CO_2 concentration in the surroundings during measurements.

($P = 0.04$). If two white gaskets were used, the regression lines were significantly different from the regression using two black gaskets ($P = 0.018$). Figure 5 shows that the diffusion was highest using the white gaskets. The combination of a black and white gasket resulted, as expected, in intermediary diffusion rates, with significantly different slopes compared to the use of white gaskets ($P = 0.018$). Table 1 shows the estimated *k*-values for all cases. The *k*-value from white/black gasket accurately confirmed the Li-Cor *k*-value of 0.46 mol s^{-1}.

Testing the effect of an empty LAF compared to an empty cuvette did not show any significant difference between the slopes ($P = 0.24$). In both cases, there was a clear linear relationship with increasing CO_2 concentration inside the leaf chamber; both slopes where significantly different from zero ($P < 0.001$, $R^2 > 0.35$).

We used a qualitative test to show the existence of LMP, by illuminating the cuvette from the inside with the instrument internal red light source. In Fig. 3c, LMP caused by AL are visualized by the light shining between them towards the outside of the cuvette. When sealing the AL with blue tack inside the LAF, no light could be seen from outside (Fig. 3d). Comparison of the linear relationship between the fluxes and the CO_2 gradient using AL kept between the two black gaskets and AL kept in the LAF, showed a significant difference between slopes ($P < 0.001$). The slope was smaller for AL alone than AL fitted inside LAF; however, both were different from zero ($P < 0.001$, $R^2 > 0.6$). The *k*-value

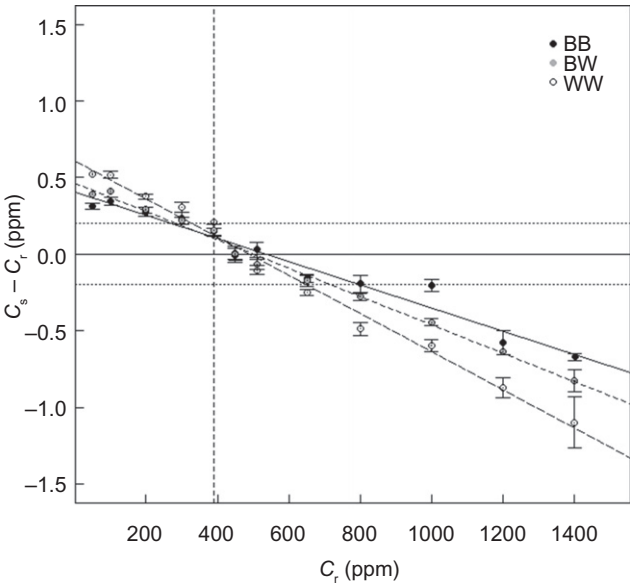

Figure 5. Influence of sealing an empty chamber with different gasket combinations, black/black (●), white/black (grey ●) and white/white (○), on the CO_2 concentration difference between the sample (C_s) and reference air in relation to the inlet CO_2 concentration (C_r). Solid, dashed and dotted lines are the linear regression lines for measurements with black/black, white/black and white/white gaskets, respectively. The two horizontal dotted lines represent the detection limits for the Li6400 (Li-Cor Inc. 2008) and the vertical dotted line is the CO_2 concentration in the surrounding air during measurements (approximate 400 ppm CO_2).

using two black gaskets were $k_{BB} = 0.38 \pm 0.02$ mol s^{-1} and $k_{LAF} = 0.38 \pm 0.025$ mol s^{-1} when AL were kept inside LAF (Table 1).

There was a detectable but very small increase of the pressure inside of the AL chamber compared to outside (103.57 kPa outside to 103.59 kPa inside). Artificial pores created with needles (Ø = 0.8 mm, i.e. much larger than the LMP caused by AL) did not change this pressure difference.

Disturbance of the boundary layer surrounding the gasket with a fan reduced the concentration gradient. However, it was not significant when AL were kept inside LAF ($P = 0.86$). Wind had a significant effect when AL were kept between gaskets ($P < 0.01$), that is, LMP were established between the gasket and AL.

Effect of the correction method on the respiration estimates

Photosynthetic model parameters based on CO_{2-} and light response data from four individual plants of *D. flexuosa* were corrected using two different methods, correction with the Li-Cor provided correction term using two different diffusion rates, $k = 0.46$ and $k_{BB} = k_{LAF} = 0.38$, and using the mean of 35 empty chamber measurements collected throughout the year 2011 under many different kinds of environmental conditions (wind: c. 0–15 m s^{-1}, temperature: c. 0–30 °C and humidity: c. 50–99%; $P < 0.001$, $R^2 = 0.35$).

The only parameters of the FvCB model (A/C_i) that were significantly affected by the correction method were the respiration parameters (Table 2). Both correction methods, that is, the *k*-value approach or subtraction of empty cuvette measurements from the measurements, resulted in a significantly lower respiration rate compared to the non-corrected respiration rate ($P < 0.008$). In addition, the result of the light response fitting only showed a significant influence of the correction method in the parameterization of respiration (Table 2). No difference was found between the two correction methods. Dark respiration measurements were not affected by any correction, which was expected as they were performed under ambient concentrations and serve as a reference.

The three different respiration estimates (R_{light}, R_{dark} and R_D) were significantly different from each other when no correction of the data was done ($P = 0.001$). In contrast, no difference was found when data were corrected by either of the *k*-values ($P > 0.35$) or ΔC_E ($P = 048$). The only parameter where the correction method influenced the result was R_{light}. R_{light} corrected with $k_{BB} = 0.38$ was significantly different from the ΔC_E-corrected R_{light} ($P = 0.015$). When R_{light} was corrected by *k*, it tended to be different from the ΔC_E-corrected R_{light}. No difference was seen between *k* and k_{BB} corrected parameters.

DISCUSSION

The influence of LMP on gas exchange measurements

Above all, to note is that the CO_2 leakage from or into the leaf chamber is a result of diffusion determined by the gasket material. Sealing the leaf chamber with gas-tight material stopped any diffusion, as proven by the measurements, that is, the absence of any relationship between the measured flux in the empty chamber and the CO_2 concentration inside the leaf chamber. Even when LMP were artificially introduced through the gas-tight sealing material, no effects of the concentration gradient were seen (Fig. 4). This supports the initial hypothesis of the manufacturer that a

Table 1. Estimates of diffusion coefficients (*k*) with different gaskets combinations, with or without AL or inside LAF

Treatment	Intercept	Slope	R^2	*k*-value
WB	0.47	–0.00092	0.98	0.46
WW	0.61	–0.00124	0.96	0.62
BB	0.41	–0.00076	0.93	0.38
LAF + AL	0.40	–0.00077	0.89	0.38
AL	0.33	–0.00064	0.82	0.32

All *k*-values are calculated using a flow rate (*u*) at 500 mol s^{-1} under indoor conditions in a well-ventilated room.
WB, one white and one black gasket; WW, two white gaskets; BB, two black gaskets; LAF + AL, artificial leaves attached between two black gaskets using LAF; AL, artificial leaves attached between two black gaskets without using LAF; R^2, adjusted *R*-squared.

Table 2. Comparison of different correction methods for the estimation of physiological parameters from raw gas exchange data

Parameter	No correction	$k = 0.46$	$k_{BB} = 0.38$	ΔC_E
FvBC model				
V_{cmax}	183.83 ± 32.0	167.6 ± 29.6	170.1 ± 29.6	166.4 ± 31.1
J_{max}	212.9 ± 27.2	194.6 ± 27.4	197.8 ± 27.4	189.6 ± 27.8
R_{light}	7.8 ± 0.2	5.7 ± 0.1***	6.0 ± 0.1***	5.0 ± 0.3***
Light response model				
A_{max}	63.3 ± 11.3	63.2 ± 11.3	63.2 ± 11.3	63.1 ± 11.3
R_{dark}	2.9 ± 0.3	5.9 ± 0.7*	5.4 ± 0.7†	5.7 ± 0.7*
Dark respiration				
R_D	5.1 ± 0.1	5.1 ± 0.1	5.1 ± 0.1	5.0 ± 0.1

All data are collected using LAF. Data are corrected by the Li-Cor diffusion coefficient $k = 0.46$, with the estimated $k_{BB} = 0.37$ for black gasket or by ΔC_E mean of outdoor empty chamber measurements ($n > 35$). R_{light} is the respiration in light extracted from the FvBC model from A/Ci curves, R_{dark} is the dark respiration extracted from the light response model and R_D is the actual measured rate of respiration under ambient conditions. V_{cmax} is the maximum carboxylation rate, J_{max} the maximum capacity of electron transport and A_{max} is the maximum light and CO_2 saturated photosynthesis. Significance levels are given as $^{\dagger}P < 0.10$, $*P < 0.05$, $**P > 0.01$, $***P < 0.001$. In no cases, the three corrected values were significantly different from each other.

small overpressure in the cuvette will offset any effects of LMP on the cuvette internal concentrations. The small slope seen in Fig. 4 with LMP present is due to a mass loss of CO_2 across the cuvette gaskets, but it is not significant. In Fig. 5, the results of testing different gasket materials and their combination show significant linear relationships between the flux and the CO_2 concentration inside the leaf chamber, representing the concentration gradient across the gaskets as the external concentration was held constant. These slopes differenced significantly using different gasket material. In the light of the presented theory on diffusion and the fact that mass flow is only outward directed from the leaf chamber because of the small overpressure in the chamber (Li-Cor Inc. 2008), the observed leakage is purely the result of molecular diffusion through the gasket material.

The CO_2 leakage from a closed, empty chamber has been found to be constant, independently of the surrounding environmental conditions (empty chamber measurements conducted across the season 2011). There were no significant differences between measurements with an empty LAF and a LAF with test leaves (AL); consequently, the CO_2 leakage using LAF is only depending on the concentration gradient and not on wind speed around the cuvette or number and size of AF. The diffusion rates estimated from indoor measurements of an empty chamber sealed with two black gaskets and with AL inside the LAF did not change (0.38 mol s^{-1}, in both cases). This supports our first hypothesis (H1) that turbulence around the cuvette does not affect the gas exchange measurements in the absence of any LMP.

Measurements with AL without LAF demonstrated a different phenomenon, which can be explained with the theory described above. Correction of the CO_2 concentration inside the chamber with an empty chamber reference has been suggested by most manufacturers and described by, for example, Bernacchi *et al*. (2001). Attaching leaves inside the chamber will create small pores or air channels (LMP) between the gaskets and the sides of leaf veins or grass leaves, as seen in Fig. 3c. Clearly, the number and sizes of the pores will vary between all individual leaves and thus the mass flow of air out of the chamber will vary from sample to sample and maybe even depend on the pressure applied when pressing the gaskets against each other (Flexas *et al*. 2007). The theory including the boundary layer around the gaskets explains why LMP lead to a decreased CO_2 leakage. It is the consequence of the reduced CO_2 concentration gradient across the gasket, which results from the dilution of the CO_2 concentration in the boundary layer (C_{bl}) on the outer side of the gasket with air from inside the chamber (C_i). The effectiveness of the reduction of the CO_2 concentration gradient depends on three parameters: (1) the concentration difference $C_a - C_i$; (2) the size and amount of LMP; and (3) the development and size of the boundary layer around the gasket (Fig. 2a). In Fig. 6, this phenomenon can be seen as a less steep slope when LMP are present. The fact that none of the two lines in Fig. 6 (AL inside and without LAF) intercept the *x*-axis (zero flux), but are higher, at ambient CO_2 concentrations, where no CO_2 concentration gradient should be present, supports the theory further. The concentrations directly at the outer surface of the gasket and the thickness of the boundary layer at the outside of the gasket determine the gradient. The consequence of the lower diffusion coefficient due to LMP is that the manufacturer's correction will overcorrect the diffusion rate across the gasket because it neglects the change in the CO_2 gradient across the gaskets. The estimated *k*-value from measurements with LMP are significantly lower than those given by the manufacturers (0.32 ± 0.02 compared to 0.46 mol s^{-1}) and even though we used black gaskets as a reference, the diffusion rate *k* is still lower when LMP are present (0.32 ± 0.02 to 0.38 ± 0.02 mol s^{-1}).

Long & Bernacchi (2003) found that the CO_2 leakage varied depending on the type of leaf and suggested to use dead leaf material of the investigated species as reference for correction. In contrast, Flexas *et al*. (2007) showed in laboratory experiments that the rate of CO_2 leakage decreased

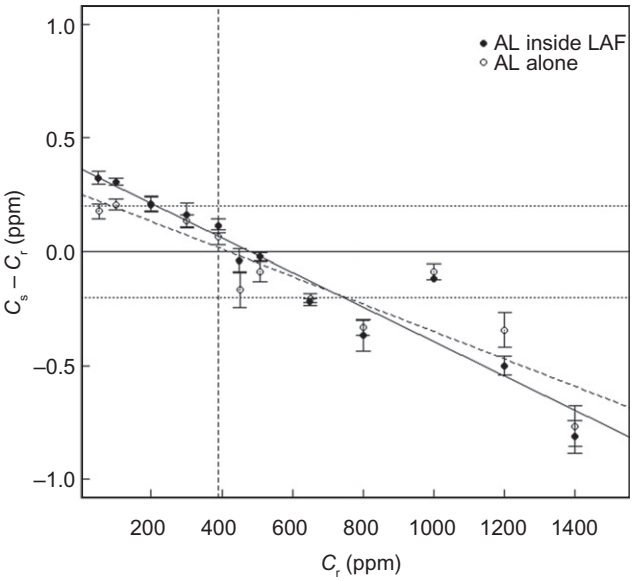

Figure 6. Effects of chamber reference CO_2 concentrations (C_r) on $C_s - C_r$, the difference between the sample and reference air CO_2 concentration, using artificial leaves (AL) either inside a leaf adaptor frame (LAF) (●) or without (○).The solid and the dashed lines are the linear regressions in the case where AL are attached inside a LAF or not, respectively. The two horizontal dotted lines represent the detection limits for the Li6400 (Li-Cor Inc. 2008) and the vertical dotted line is the CO_2 concentration in the surroundings during measurements.

when attaching a dead boiled leaf inside the chamber, explaining that most of the leakage from the chamber takes place in the interface between the gaskets because of different structures in leaf surface and not through the gaskets. Rodeghiero *et al*. (2007) concluded that the CO_2-diffusive molar flow rate (μmol CO_2 s^{-1}) increased with LMP and thereby increased the CO_2 diffusion across the gasket, which supports Long & Bernacchi (2003) but disagrees with the interpretations of Flexas *et al*. (2007). Individually, these studies (Long & Bernacchi 2003; Flexas *et al*. 2007; Rodeghiero *et al*. 2007) support different parts of the theory of the present study.

The studies by Flexas *et al*. (2007) and Rodeghiero *et al*. (2007) conclude that a dead leaves correction strongly influenced the parameterization of the FvCB model and also agreed in their conclusion that correction of data using a constant diffusion coefficient are not useful. The two papers are both dealing with the hypothesis that minimizing the CO_2 concentration gradient can improve the accuracy of measurements and following photosynthetic parameterization. Testing the influence of enclosure of the leaf chamber in plastic bags both studies resulted in the reduction of CO_2 diffusion across the gaskets (Flexas *et al*. 2007) or the CO_2-diffusive molar mass flow (Rodeghiero *et al*. 2007). However, the studies do only conclude that a decrease in CO_2 concentration gradient is resulting in an improved parameterization, but no explanations of how this is related with LMP have been suggested.

All mentioned studies of the CO_2 leakage problem have been performed in laboratory environments where wind did not disturb the air surrounding the leaf chamber. According to our theory, laboratory experiments like the ones described above, can lead to misleading conclusions about the diffusion leakage.

Our result did not show any difference between empty LAF or LAF + AL measurements with and in windy or still air conditions at the outer gasket. However, in the absence of LAF, we found a significant influence of AL both under windy and not windy conditions. Under calm conditions, the LMP lead to a decrease in the rate of CO_2 leakage, which supports the findings of Flexas *et al*. (2007) and can be explained by the diluted outer surface CO_2 concentration and thus reduced gradient between C_i and C_{bl}. Results from bag experiments show a similar reduction of CO_2 leakage and can be explained by the same theory as described above. When Rodeghiero *et al*. (2007) found a reduction in the CO_2 leakage from LMP after enclosure of the chamber with a bag, it is due to a drop in the CO_2 concentration gradient between inside of the bag and C_i.

Under windy conditions, the boundary layer thickness is reduced, and therefore, the CO_2 gradient that controls the CO_2 diffusion will approach the difference between the CO_2 concentration inside and outside of the chamber. Thus, as suggested by Long & Bernacchi (2003), there will be an increased diffusion leakage. This is supported by our results.

Field conditions imply a varying disturbance of the boundary layer, and thus, a correction must take the thickness of the layer as depending on wind speed into account. This is virtually impossible because the wind speed close to the gasket is unknown and variable. We therefore advocate avoiding LMP and propose to seal irregular shoot and leaf structures with LAFs fitted to the actual leaf structure, such as the LAF developed in this study. This will lead to reproducible results that can be corrected with the methods proposed by the manufacturers. Our results support the correctness of these methods in the absence of LMP as will be discussed in the next section.

The use of LAF and the influence on respiration estimations

Gas exchange measurements on small leaves like grasses are challenging because of the small CO_2 fluxes that enhance the demand of accuracy, especially concerning respiration measurements. Our study has proven that LAF seal LMP in a way that only the diffusion across the gasket needs to be considered as a source of error. Li-Cor provides a flow-dependent diffusion rate k of 0.46 (Li-Cor Inc. 2008). Licor's k-value was found to be reproducible in our study, which has also been the case in earlier studies (e.g. Flexas *et al*. 2007; Rodeghiero *et al*. 2007). From this, the correction term provided by Li-Cor seems to be a good approach for correction of gas exchange data. Using LAF necessitates the use of two black gaskets since the white material is too sensitive to the shape folds of the LAF. We found different diffusion coefficients (k) using different gaskets or using LAF; which shows the importance

to choose an appropriate *k*-value for correction. We did not find any difference in *k*-values using LAF or only an empty chamber with two black gaskets. This is why we only presented the difference in correction with the LiCor provided *k* and our k_{BB} value.

The earlier mentioned correction using an empty chamber reference of a given CO_2 concentration inside the chamber has been suggested and used several times (e.g. Bernacchi *et al*. 2001; Albert *et al*. 2011b). Since no LMP are present when using LAF, this correction seems reasonable as long as the empty chamber measurements are performed under the same CO_2 regime as the measurements. Contrary to the study by, for example, Flexas *et al*. (2007), our parameterization of parameter estimates from the FvCB model only showed effects of the correction on the respiration value (R_{light}). Like the photosynthesis estimates from the FvCB model, results from the light response data only showed effects of the correction on the respiration parameter (R_{dark}), too. However, measured dark respiration (R_D) taken under ambient CO_2 concentrations was not changed by any of the correction methods. We conclude that such R_D measurements can serve as a true reference.

Unexpectedly, there was no difference between the influences of the two *k*-values (k and k_{BB}). The only difference between the correction methods on the parameterized estimates was between the R_{light} corrected with k_{BB} or ΔC_E ($P = 0.015$). A trend was also seen comparing R_{light} corrected with k and ΔC_E ($P = 0.15$). The R_{light} was estimated from the ACi curves where the C_r changed. Other parameters were obtained at stable C_r (1400 or 390 ppm), where the small changes in ΔC_E over C_r caused by the machine have a larger influence (clearly shown in, e.g. Fig. 5).

No significant differences between the three respiration estimates (R_{light}, R_{dark}, R_D) were found, when data was corrected with either of the *k*-values ($P > 0.35$) or ΔC_E ($P = 0.48$), proving that avoidance of LMP clearly improves leaf gas exchange measurements.

Beyond the problem of direct CO_2 leakage through LMP, a study by Pons & Welschen (2002) challenged the assumption that photosynthesis and respiration measured in the chamber are only related to leaf area between the inner boundaries of the gaskets, that is, the cuvette opening. Pons & Welschen (2002) argued that the leaf tissue between the gaskets is continuously contributing with a respiratory CO_2 flux transported through the gasket to the inside of the chamber. In addition here, LAF can be argued to improve the accuracy of measurement. Leaves inside LAF are sealed with blue tack, which eliminates the space around the leaf surface and the sealing material since the blue tack has been shown to be strongly gas tight. The only path for a small flux from the respiring leaf area will then be through the leaf tissue during measurements. The effect of lateral CO_2 diffusion inside leaves on the rate of photosynthesis has been shown to be very small and only over less than 0.3 mm (Morison & Lawson 2005). Thus, it can be neglected using LAF.

Several studies showed that leaf respiration rates are not sensitive to elevated CO_2 concentrations (e.g. Jahnke 2001; Bruhn *et al*. 2002; Jahnke & Krewitt 2002). Any CO_2 response analysis requires proper correction of the CO_2-diffusive leackage because this does also depend on the cuvette internal concentration via the concentration gradient compared to the ambient air concentration. If the correction overcorrects the diffusion, the corrected values might in fact falsely indicate even an increase of leaf respiration with increasing CO_2 concentration instead of a possibly expected but apparently non-existing product inhibition. The LAF technique can provide new insight by eliminating a major error regarding the accuray of leaf gas exchange measurements of small fluxes in small leaf chambers. The advantage of the empty chamber correction is, however, that it also corrects for the so far unexplained machine-dependent systematic deviations that have been shown in Fig. 4 and can also be seen as systematic patterns of residuals in the other experiments (Figs 5 & 6). The origin of these sytematic errors still remains to be investiaged.

CONCLUSIONS

Certain leaf structures cause small holes or LMP across the contact zone of the upper and lower gaskets of gas exchange leaf cuvettes. Including the effects of such pores on the concentration in the boundary layer outside the cuvette and thereby reducing the concentration difference across the gasket, we were able to explain, at first glance, the counterintuitive reduction of CO_2 diffusion rates through the presence of LMP. The involvement of the boundary layer makes the effects of LMP on diffusion across the gasket wind speed dependent. Because the wind speed in field gas exchange measurements cannot be controlled, LMP need to be avoided. We showed that this can successfully be done with LAF, which we developed for this purpose. When avoiding LMP, the usual correction methods that describe diffusion through the gasket can be applied with large confidence. However, if possible, correction by means of empty chamber measurements done at same environmental conditions is the best correction resulting in most reliable results because it also corrects for measuring system-dependent biases that are unrelated to diffusion through the gaskets.

ACKNOWLEDGMENTS

The work was carried out within the CLIMAITE project, financially supported by the Villum Kann Rasmussen Foundation. Support has also been received from Air Liquide and DONG energy. Special thanks to Eike Marie Thaysen for patiently listening to Kristine's ongoing frustration from the first remarkably counterintuitive results to the final conclusive theory.

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Received 24 September 2012; received in revised form 21 December 2012; accepted for publication 28 December 2012

PAPER II

Title:

Photosynthetic stimulation by long term climate change manipulations in *Calluna vulgaris* is maintained on top of seasonal acclimation in cold season.

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Keywords:

V_{cmax}, CLIMAITE, elevated CO_2, warming, drought.

List of abbreviations:

CO_2, experimental elevated CO_2; FACE, free air carbon enrichment; D, experimental drought; C, leaf carbon concentration; N, leaf nitrogen concentration; C/N-ratio, leaf carbon to nitrogen ratio; and J_{max} , maximal velocity of RuBP regeneration; J_{max}^{25}, maximal velocity of RuBP regeneration normalized to 25°C; A_{sat}, light saturated net photosynthesis at ambient CO_2; A_{max}, light- and CO_2 saturated net photosynthesis; c_i, calculated intercellular CO_2 concentration; E, leaf transpiration; WUE, instaneus water use efficiency = A_{sat}/E; HTR, high temporal resolution plots; T, passive nighttime warming; V_{cmax}.maximal carboxylation rate of Rubisco at ambient temperature; V_{cmax}^{25}, maximal carboxylation rate of Rubisco normalized to 25°C; *PI*, performance index based on chlorophyll-a fluorescence induction curve analysis; F_v/F_m, maximal quantum yield of PSII in dark.

Submitted to Journal of Experimental Botany

Abstract:

Photosynthetic responses to elevated CO_2 (Free-Air-CO_2-Enrichment, CO2), passive night time warming (Infrared reflective curtains, T) and prolonged summer drought (rain excluding curtains, D) and all combinations were investigated in the temperate heathland after six years of manipulation. In elevated CO_2 the net photosynthesis of the evergreen shrub, *Calluna vulgaris*, was stimulated across the warm and the cold seasons via increased intercellular CO_2 concentration despite a down-regulated photosynthetic capacity in the cold season. Elevated CO_2 induced leaf nitrogen dilution and together with the autumn re-translocation of leaf nitrogen, these combined responses were found to explain the strong down-regulation of the photosynthetic capacity in cold season.

The responses in the full-factorial combination (TDCO2) were mainly explained by additive and some antagonistic dampening effects. In the TDCO2 treatment, *Calluna* grew thicker leaves and had less nitrogen, similar to single factor treatment with elevated CO_2. In warm season the drought counterbalanced photosynthetic stimulation of elevated CO_2 and the photosynthetic capacity was unchanged in the TDCO2.

In conclusion, the responses to the combination of climate change factors seem to be dominated by the responses of elevated CO_2 in both the warm and the cold season. However, a changed precipitation pattern with more intense periods of drought can strongly reduce the stimulation of carbon uptake. Considering up-scaling carbon budgets to an ecosystem scale, N re-translocation and thereby seasonal changes in photosynthetic responses need to be taken into account and maintained carbon uptake during cold season cannot be excluded.

Introduction

Climate is changing and factors such as increased atmospheric CO_2 concentration, temperature and precipitation are primary drivers of biological processes and influence all levels of ecosystems (e.g. Walther, 2003; Kirschbaum, 2004). Experiments have shown strong impacts of the single climate factors on eco-physiological processes; however studies of single climate factors conclude that the responses to different climate factors are contradicting (e.g, Luo et al., 2008; Crous et al., 2011). Thus, multifactor-experiments with two or more factors are of great importance (e.g. reviewed in Nowak et al., 2004; Leuzinger et al., 2011).

Climate change impact studies on plant eco-physiology have to our knowledge mainly been conducted during the warm season (growing season), while only few studies have been conducted across all seasons (e.g. Crous et al., 2011, Gorsuch et al., 2010). Evergreen plants have evolved strategy including processes maintaining rate of photosynthesis at a sustainable level, even within

periods of low temperature and low light intensity (Oquist and Huner, 2003) . However Miller (1979) argued that the temperate evergreen heathland species *Calluna vulgaris* is dormant from October to February. However, Andresen and Michelsen (2005) showed that *Calluna* sustains uptake of nitrogen in December indicating photosynthesis potential throughout the winter time. Larsen et al. (2007) estimated that 22% of the annual photosynthesis and 30% of annual ecosystem respiration could be assigned to the cold season, between October-Marts in a Danish heathland ecosystem. The relatively high contribution of the cold season to the annual C-budget indicates that climate change effects on photosynthetic processes can have profound effects during cold season.

Plants respond to the strong seasonal temperature variation in temperate climates by regulating the temperature sensitivity of physiological processes according to the seasonal temperature level. Photosynthesis are strongly affected by temperature, and the overall temperature response can be understood as the combined temperature dependency of its component processes and their interactions (Farquhar et al, 1980; Medlyn, et al., 2002a /b; Kattge and Knorr, 2007; Bernacchi et al., 2009). Following an Arrhenius type relationship, the activation of the carboxylation process in controlled by the enzyme ribulose 1.5-bisphosphate carboxylase/oxygenase (Rubisco) is increased with increasing leaf temperature at low to moderate temperatures. At higher temperatures photosynthesis is decreased due to conformational changes in e.g. key enzymes (e.g. Sage, 2002).

Down-regulation of photosynthetic capacity during cold-season in response to the lower temperatures are reducing rates of photosynthesis (Vogg et al. 1998; Bigras and Bertrand, 2006). Therefore it can be difficult to separate responses to particular climate factors within the cold season. In the warm season, elevated CO_2 concentrations have been shown to stimulate light-saturated net photosynthesis (A_{sat}) as a result of increased intercellular CO_2 concentration, despite reduced stomatal conductance (g_s) and down-regulation of the carboxylation velocity (V_{cmax}) and dilution of leaf nitrogen concentration in the leaves (e.g. Long et al., 2004; Ainsworth and Long , 2005; Leakey et al., 2009). At longer time scales, stimulation of A_{sat} by elevated CO_2 can be diminished. For example can an increase plant carbon uptake lead to increased biomass accumulation and high leaf area index, moving the ecosystem to a new steady state, where limitation of the essential elements as nitrogen can induce progressive nitrogen limitation or other limitations as water shortage (PNL, Luo et al., 2004; Reich et al., 2006; Körner 2006; Morgen et al., 2004).

To our knowledge no studies have investigated leaf level responses to growth in elevated CO_2 during colder seasons, within a multifactorial climate manipulation experiment. Low growth

temperatures will as mentioned above reduce the photosynthetic capacity; also V_{cmax}; thus an additive response of both the elevated CO_2 and the seasonal induced down-regulation of V_{cmax} can be expected.

Increased air temperatures are one of the consequences of elevated atmospheric CO_2 concentrations and growth in a warmer environment has e.g. been found to induce earlier onset of the growing season (e.g. Menzel et al., 2006; Hovenden et al., 2008; Prieto et al., 2009). Menzel et al. (2006) reported due to increased temperatures in the last decades, an overall advancement of leafing and flowering of 2.5days/°C, on average throughout Europe. Increased seasonal temperature is closely coupled to the reduction of the freezing-period in most mid- to high-latitude regions (G. Walther et al., 2002) which can have consequences for the acclimation of overwintering plants in these regions (e.g. Greer et al., 2000). Additionally higher temperature is expected to be coupled not only to increasing the minimum temperature on an annual scale, but also on daily scale, especially at nighttime (Walther, 2003). Night time warming increases the minimum temperatures; e.g. reducing the days of frost events in the outer growing seasons, which can influence the processes related to the seasonal acclimation (Oquist and Huner, 2003). Therefore night-time warming may prolong photosynthetic capacity in late growing season. A recent study on *Calluna* showed that night time warming increased photosynthetic performance measures as *PI* and F_v/F_m before and after frost events in early winter (Albert et al., 2013), thus it is reasonable to a higher photosynthetic performance in the early state of the cold season.

Drought is a strong environmental stress factor for most plants (e.g. Schmidt et al., 2004; Pérez-Ramos et al., 2010). Precipitation patterns are expected to change and in temperate ecosystems it is expected that longer periods of drought and episodic heavy rain will become more frequent (Boberg et al., 2010; Christensen et al., 2010). Drought has an intensive negative effect when prolonged, but during rewetting, photosynthetic physiological processes can be expected to be restored (e.g. Albert et al., 2011a). However, studies on evergreen oak and broad leaved *Phillyrea* (Ogaya andPeñuelas, 2003) have shown a clear carry-over effect of summer drought during the cold season, decreasing photosynthetic capacity in plants previously exposed to drought. *Calluna* had also been shown to have a lower physiological performance (*PI*) in late season beyond the drought and rewetting periods, indicating a carry-over effect (Albert et al., 2011a). Plants exposed to drought shows negative responses in the case of most physiological parameters; reducing A_{sat}, g_s, V_{cmax} and electron transport (J_{max}) and to delay phenological process related to flowering and germination (e.g. Signarbieux and Feller, 2011; Albert et al. 2011 a/b; Jentsch et al., 2009; Prieto et al., 2008; Llorens and Penuelas, 2005).

Effects of elevated CO_2 concentrations, temperature and prolonged summer drought on the seasonality of plant eco-physiological parameters, were investigated in a long-term multi-factorial experiment, designed according to a scenario for climate conditions in Denmark 2075 (Mikkelsen et al., 2008). Cold season effects of climate change on the dominating evergreen shrub, *Calluna vulgaris* were investigated. Additional to treatment responses, the seasonal acclimatization were investigated, in order to clarify if cold season responses to the treatments were still be present despite the strong physiological seasonal acclimatization. We here report the impact on plant eco-physiology and leaf characteristics. To simplify the potentially complex interactive responses (i.e. additive, antagonistic or synergistic effects) related to the multifactorial design have concentrated on the following hypotheses.

I. Elevated CO_2 will increase plant carbon uptake both in the warm and cold seasons, despite seasonal difference in down-regulation of V_{cmax}.
II. Nighttime warming will increase plant carbon uptake in the cold seasons (spring and autumn) by increasing V_{cmax}.
III. Drought will reduce plant carbon uptake during and just after the drought-period via lower stomatal conductance and down-regulated V_{cmax}.
IV. Combined single factor treatments will lead to different responses; either additive or interactive, i.e. antagonistic or synergistic.

Method and Material

Site Description

The study was performed in the experimental site of the long-term climate change experiment CLIMAITE situated in a dry heathland in North Zealand, Denmark (55°53'N, 11°58'E). The vegetation is dominated by the evergreen dwarf scrub Heather (*Calluna vulgaris L.*) and Wavy hairgrass (*Deschampsia flexuosa L.*). The soil is nutrient poor and sandy with a pH of 4.5 in the topsoil. Mean annual temperature is 9.8 °C and the annual mean precipitation is 697 mm (Mikkelsen et al., 2008).

The experiment included the following treatments: Un-manipulated control (A), elevated CO_2 (CO2), passive nighttime warming (T), periodic summer drought (D) and all combinations (TD, TCO2, DCO2, TDCO2), replicated in six blocks in a complete split-plot design (n=6). Each block includes two octagons of 6.8 m diameter, divided in four plots. With the Free-Air-CO_2-Enrichement (FACE) technique one octagon in each block are exposed to 510 ppm CO_2 during daylight hours (Miglietta et al., 2001). The passive nighttime warming is performed by automated infrared reflective

curtains covering one half of each octagon. Nighttime warming results in an increased air temperature (20 cm) by 1.4 °C on average (Mikkelsen et al., 2008). Experimental drought is established, during a period of between two and five weeks in the spring or summer in each year of the experiments, by automated rain-excluding curtains covering each one half of the octagons. In order not to kill the plants drought treatment was stopped when the soil water in the top 20 cm was reduced to 5 %. Together with the nighttime warming curtains the rain-excluding curtains create the split-plot design. The experimental treatment of CO_2 and warming was started in October 2005. In each experimental plot the soil water content over two depths (0-20 cm and 0-60 cm) was continuously recorded using time domain reflectometry (TDR). Simultaneously, air temperature was measured in 20 cm height, and soil temperature in 0 cm and 5 cm depth. Two climate-stations are located in the experimental area, where temperature, radiation within the photosynthetic spectrum (PAR) and the precipitation were measured in 2 m height. For further technical detail of the setup, see Mikkelsen et al. (2008).

To measure the seasonality in photosynthetic performance of *Calluna*, with high temporal resolution, two additional high temporal resolution plots (HTR plots) outside the treatments were established in January 2011 and sampled every second week from mid-May 2011 to mid-May 2012.

Leaf Gas exchange

Leaf level CO_2 and H_2O fluxes were measured *in situ* using open, portable leaf gas exchange analysis systems (LI-6400, LI-COR Biosciences, Lincoln, Nebraska, USA)). Three LI-6400s with 2*3cm chamber and LED light sources (6400-02B) were used stomatal conductance (g_s), light-saturated photosynthesis (A_{sat}) and light and CO_2-satuarated photosynthesis (A_{max}) (full campaign) were measured within the experimental treatments during 13-21 July, 16-18 August, 19-22 September, 25-27 October and 22-23 November 2011. Short campaigns measuring g_s, A_{sat} and A_{max} were carried out 10-11 January and 27-28 February 2012.

Healthy top-shoots of *Calluna vulgaris*, from the upper part of the canopy, were selected for every measurement. To minimize gas diffusion and leaking problems using leaf cuvettes, shoots were placed in leaf adaptor frames (LAF) as described in Boesgaard et al. (2013).

We used two different protocols, a long (full campaign) and a short one (snapshot campaign). In the long protocol both a CO_2-response-curve and a light-response-curve were measured. For both protocols, leaves were acclimated to the chamber condition for 6 min at 390 ppm CO_2 (510 ppm in FACE plots), until net photosynthesis and stomatal conductance were stabilized (CV < 1%). In the

snapshot protocol a small CO_2 response curve containing 4 steps at 4 CO_2 levels were preformed (390, 510 1200 1400 ppm, starting at the level of CO_2 in the measured treatment, 390/ 510 ppm). In the long protocol a CO_2 response-curve was measured stepping down the CO_2 concentration from 390 ppm (510 ppm) to 50 ppm CO_2 and then brought back to the 390/510 ppm level again, for at least 3 min. After the reestablishment of the CO_2 level the concentration of CO_2 was stepped up to complete saturation at 1400 ppm CO_2. All measurements (both protocols) in the period of April through October were performed at a light saturating level of 1500 µmol photosynthetically active photons m^{-2} s^{-1} (PAR) using the Li-6400 auto-program "A/C_i-curves" (operating system Open 6.2). From October to April the light level was set to 800 ppm (saturating). Settings: time between measurements-log min 45 and max 55 seconds, reference CO_2 [mol mol^{-1}] and intercellular CO_2 concentration stable in 10 seconds with CV<1%. Matching was performed between every step of CO_2. To reflect the true ambient temperature of the day, block temperature was set to the expected mean temperature of the measuring day (with ± 5 °C fluctuations around the target value, due to limitation of the equipment). Relative humidity was manually adjusted to 45-55% during measurements. Maximum carboxylation (V_{cmax}) and electron transport (J_{max}) rates were calculated from curve-fitting to the Farquhar-von Caemmerer-Berry (FvCB) model equations (Dubois et al. 2007 and Bernacchi et al., 2001) When no minimum between the Rubisco-limited and RuBP-regeneration-limited phases could be found by the fitting procedure, V_{cmax} was fitted using data with C_i<500 ppm and J_{max} was fitted using data with C_i > 550 ppm. . Non-photochemical respiration (R_{light}) was calculated as 0.02*V_{cmax} (after von Caemmerer, 2000). Within full campaign protocol (described above) the A/C_i-curve protocol was followed by a light-response curve.

For comparison of the fitted V_{cmax} and J_{max} value they were normalized to 25°C. Data normalization of fitted values of V_{cmax}^{25} and J_{max}^{25} to 25°C was done in relation to leaf temperature, as explained under 'Estimation of photosynthetic capacity and temperature normalization.

The light-saturated net photosynthesis (A_{sat}), intercellular CO_2 concentration (C_i) and transpiration (E) were extracted from the first point in the CO_2 response-curves from both protocols at CO_2 reference = 390 ppm CO_2 in non-FACE plots and at 510 ppm in FACE plots. The water use efficiency (WUE) was calculated as $WUE = A_{sat}/E$. All data were corrected for diffusion leakage following the protocol by Boesgaard et al. (2013).

Leaf characteristics, area, weight, nitrogen, carbon and $\delta^{13}C$

After each measurement the leaf adaptor frame (LAF) containing the leaves was cut off the plant and the plant material inside the frame was brought to the laboratory. Leaves and stems were detached from each other and placed on a paper together with a square of the fixed dimension of 3x3 cm, and a photo was taken placing the paper with leaves and stems on top of a light table. Area estimations were quantified using the image processing program ImageJ (National Institute of Health, USA). The side of the square (3 cm) was in all cases the reference length, and area was determined from 8-bit colour pictures with the threshold approach. The photographed leaf area are given as projected leaf area, as described in Smith et al. (1991).

The dry weight of the plant material was measured after drying 48 hours at 60°C. The specific leaf area, SLA (cm^2/g) and specific leaf weight, SLW (g/cm^2) were determined from the dry weight and the projected leaf area. The dried leaf material was analyzed for carbon and nitrogen concentrations and for ^{13}C natural abundance ($\delta^{13}C$) with an elemental analyzer (CE 1110, Thermo Electron, Milan, Italy) coupled to a Finnigan MAT Delta PLUS isotope ratio mass spectrometer (Thermo Scientific, Bremen, Germany). The $^{13}C/^{12}C$ isotope ratios are given as delta notation ($\delta^{13}C$) i.e. relative measurement against the $^{13}C/^{12}C$ ratio in an international standard (Vienna Pee Dee Belemnite): $\delta^{13}C = \frac{R_{sample} - R_{standard}}{R_{standard}} * 1000‰$.

Estimation of photosynthetic capacity and temperature normalization

In the C3-photosynthesis model, first presented by Farquhar, von Caemmerer and Berry (1980) (FvCB-model), photosynthesis is at all conditions limited by one out of three processes: 1) the maximum rate of Rubisco-catalyzed carboxylation (Rubisco-limited), 2) the regeneration of RuBP controlled by the electron transport rate (RuBP-limited) or 3) triose-phosphate utilization controlled regeneration of RuBP (TPU-limited). The original model was parameterized at a standardized leaf temperature of 25 °C, but the uncertainty of the model increased when the temperatures differed from 25°C. Under ambient CO_2 concentration Rubisco-limited photosynthesis is common (Rogers and Humphries, 2000). Thus, light saturated photosynthesis can be expressed by (Farquhar et al., 1980):

$$A_c = \frac{V_{cmax(25)}(C_i - \Gamma^*)}{C_i + K_c(1 + \frac{O}{K_o})} - R_{light} , \qquad (1)$$

where A_c is the light-saturated Rubisco-limited photosynthesis, V_{cmax}^{25} the maximum Rubisco-catalyzed carboxylation rate at 25°C, C_i the intercellular CO_2 concentration, Γ^* the CO_2 compensation point in the absence of mitochondrial respiration (R_{light}), and K_c and K_o are Michaelis-Menten constants for Rubisco activity for CO_2 and O_2, respectively. According to Bernacchi et al. (2001), the constants for Γ^*, K_c and K_o can be assumed to be 42.8 µmol mol^{-1}, 405 µmol mol^{-1} and 278 mmol mol^{-1} at 25°C, respectively.

Hikosaka et al. (2006) explain that light saturated net photosynthesis at ambient CO_2 concentrations (A_{sat}) at any given temperature is mainly limited by the carboxylation rate for Rubiso, irrespectively of growth temperature. Therefor A_{sat} can be expressed as A_c (Eq. 1). Under ambient outdoor temperature it is difficult, if not technically impossible (Li-Cor Inc., 2008), to collect A/C_i-curve-data *in situ* at a strictly standardized temperature of 25°C. Because V_{cmax} is strongly sensitive to temperature (e.g. Bernacchi et al., 2001) normalization to temperature is necessary for a comparison of data collected at ambient temperature in the field, when seasonal changes in temperature highly affect V_{cmax}.

Plants response respond to environmental changes (e.g. temperature and light) at several time scales, from daily to seasonal. Acclimation to changed temperature is known especially to influence enzymatic driven processes and with low temperatures many photosynthesis related processes have been found to decrease (Kattge and Knorr, 2007; Leuning, 2002).To evaluate the treatment effects in the present study photosynthetic capacity, V_{cmax} and J_{max} were measured across the whole period at ambient temperatures. The data from the ambient and the HTR plots were used to fit the seasonal temperature response of the photosynthetic capacity. The seasonal temperature response combines the instantaneous temperature responses and that part of their seasonal variation that is related to the seasonal course of the temperatures.

The V_{cmax} and leaf temperature values were fitted to the model of the instantaneous temperature response (de Pury and Farquhar, 1997):

$$V_{cmax} = V_{cmax}^{25}\, exp^{\left(\frac{\left(T_{leaf}-25°C\right) Eac}{\left(R*\left(T_{leaf}+273K\right)\left(25+273K\right)\right)}\right)} \qquad (2)$$

where R is the molar gas constant, T_{leaf} is the leaf temperature and E_{ac} is the activation energy of the Rubisco carboxylation reaction. Assuming that the temperature response of V_{cmax} is constant, equation (2) can be used to calculate V_{cmax}^{25} from any measured V_{cmax} value at given ambient temperature. The same approach and the same assumptions were used for normalize J_{max} to 25°C (J_{max}^{25}).

The temperature normalization makes it easier to compare photosynthetic capacity that is measured in different seasons and at different temperatures. The differences in normalized photosynthetic capacity between differently treated plots describe here the treatment effects and their variability in time.

Statistical Analysis

Treatment effects were analyzed with a linear mixed effect model using the lmer function in the lme4-package of the free statistical software R (R Develpment Core Team, 2010). The lmer function provides the option to incorporate both fixed-effect parameters (main factors) and random effects (random factors). Main effect factors of the model were elevated CO_2 (CO2), warming (T), drought (D) and their interactions (T×CO2, T×D, D×CO2, T×D×CO2), while block, including the group of two adjacent octagons, was set as a random factor to exclude systematic site heterogeneity from the analysis of treatment effects. Testing the seasonal patterns across warm (April-September) and cold (October-Marts) seasons, Time was used as a random factor, too. F-values and p-values were extracted with the MixMod-package using the SAS-algorithm. The data was tested for normal distribution with Levene's test and the data was either log(x) or 1/x transformed, if necessary. Homogeneity of variance was visually inspected from residual plots.

The significance level was set to p-values < 0.05 and trends were noted if p-values < 0.1. An additive effect is when effects of two or three factors are simply added to each other. Statistically, an additive effect will not be shown as significant due to the lack of single factor significance. Looking at interaction plots produced by the MixMod-package, significant interactions were categorized as synergistic if the combined effects of single factors have a stronger impact than the expected additive effect, or as antagonistic if the combined response is lower than the additive response.

Results

Climate and experimental control measurements

During winter 2010-2011 the site experienced a long period with snow cover from November 2010 to mid-January 2011, followed by 1-2 weeks of warm sunny weather with daytime air temperature between 2-8 °C. From end of January to end of February 2011 there was no snow, sun and repeatedly hard frost events (below zero during day-time). This caused a major die-back of the standing population of *Calluna vulgaris*. In April 2011 we estimated that only around 5-10% of all *Calluna*

stands had functioning green shoots. New shoots regrew from the dried stands during May-July 2011, with an approximate 75% regrowth of *Calluna* (personal observation, Kristine S. Boesgaard and Nina W. Thomsen). The die-back was not observed to differ between treatments (personal observation, Johannes Ransjin). Due to slow regrowth in spring, homogenous canopy structures across the entire experimental area were first established in early July (personal observation, Kristine S. Boesgaard and Nina W. Thomsen). Measurements at the HTR plots started already in mid-May.

The late winter and early spring 2010-11 was drier compared to the Danish average climate during the period from 1960 to 1999 (http://www.dmi.dk) and was combined with higher than average temperatures in the early to the late spring (March-end May). The summer of 2011 was generally warmer and wetter than the 40 year average ensemble values. The autumn was still warmer but with very little precipitation. The experimental drought was performed during one month starting on May 2nd and excluded 57.8 mm of rain, corresponding to 8.3% of the annual precipitation in 2011.

The soil water contents (SWC%) in 0-20cm in the experimental plots did not change much during the evaluated period (July to February). In May and June 2011 SWC was significantly lower in all drought treated plots (May $p=0.017$, June $p= 0.007$). In the non-drought treated plots SWC varied between 13.6 – 16.5% as compared to the drought treated plots with only 9.9-15.2%,. The two highest SWC values were found in the treatments combined with elevated CO_2. This effect was, however not significant. SWC values in 0-60 showed the same effects of treatments that 0-20 cm and were in general c. 4% lower than in 0-20cm.

Soil temperature in 5 cm depth was significantly higher in treatments with nighttime warming across almost the whole measurement period of January 2011 – June 2012, except for the periods where the treatments have stopped due to snow and frost, damaging the technical system (generally in periods between December-February). Daily mean temperature in 2 m height reached the maximum during summer, and in winter 2010-2011 it reached a minimum of -8.7 °C (22 December 2010), here in combination with complete snow cover. In a snow free period in February 2011 daily mean temperature in 2 m height reached a minimum of – 5.5°C (20 February 2011). Night time minimum temperatures in vegetation height (20 cm) were significantly higher in warmed plots during all time (except for the period indicated on figure 1 with dotted lines, where warming-treatment, due to technical problems with snow, were turned off), with a mean increase of 1.00 ± 0.05 °C. Highest impact of warming was found in springtime in both of the years 2011 and 2012 with mean temperature increases above + 4^{o}C.

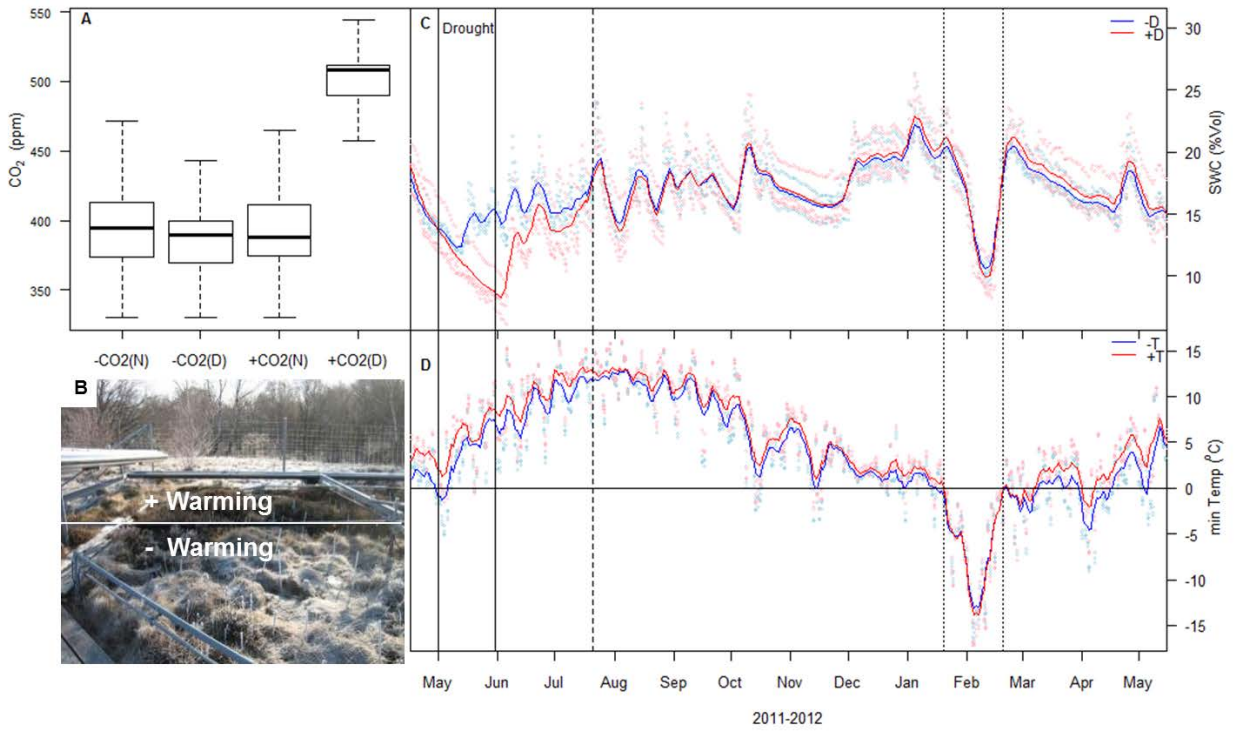

Figure 1. Climatic data and treatments effects: A) Median of CO_2 concentration (ppm) in none-FACE plots (-CO2) and in FACE plot (+CO2) across the whole experimental period and during daytime (D) and nighttime (N). Boxes show 25-75% of values, vertical lines indicate the median, whiskers length are 1.5×IQR. B) Photograph showing the effect of nighttime warming after a night with rime frost (the white material on the none-warmed part of the octagon is rime). C) 7-days running means in soil water content in 0-20 cm (SWC %) in all non- drought and all drought plots (n=24). The drought period is marked with a line on the graph and the treatment resulted in an exclusion of 57.8 mm of rain. D) 7-days running means of the observed minimum temperature in 20 cm height in all non-warmed (-T) and all warmed plots (+T) (n=24).Solid line in panels C) and D) marks the experimental drought period, split line indicates the lack period where SWC is still influenced of experimental drought. Small dotted line indicates the period where treatments were not functional due to snow cover.

Leaf gas-exchange

The photosynthetic parameters varied substantially across the year. Parameters referring to photosynthesis process (ex. A_{sat}, g_s, V_{cmax}, V_{cmax}^{25}, J_{max} and J_{max}^{25}) all followed the same general seasonal pattern (Figure 2) with high rates during summer and lowest through winter. Fitted values of the maximum carboxylation rate ($V_{cmax,}$ V_{cmax}^{25}) and the maximum rate of electron transport (J_{max}, J_{max}^{25}) were all highest from June- September 2011, with the absolute highest rates in September. V_{cmax} ranged from 65.0±7.9 to 50.1±3.0 µmol m^2 s^{-1} in the period June- September 2011, where the temperature normalized V_{cmax}^{25} was overall ca. 30 % higher during the whole year. In the same time-period J_{max} ranged from 118.4±11.2 to 138.3±9.4 µmol m^2 s^{-1}; the J_{max}^{25} values were ca. 25% higher than this level. The A_{sat} and A_{max}, however, had highest rates in the period from August to October

2011(A_{sat}: 18.0±1.7 to 18.7±1.5 µmol CO_2 m^2 s^{-1}, A_{max}: 31.1±7.8 to 31.1±7.8 µmol CO_2 m^2 s^{-1}) (data in supplementary material). Stomatal conductance (g_s) was high during the period from July-October, highest during measurements in August (0.84±0.02 mmol H_2O m^2 s^{-1}).

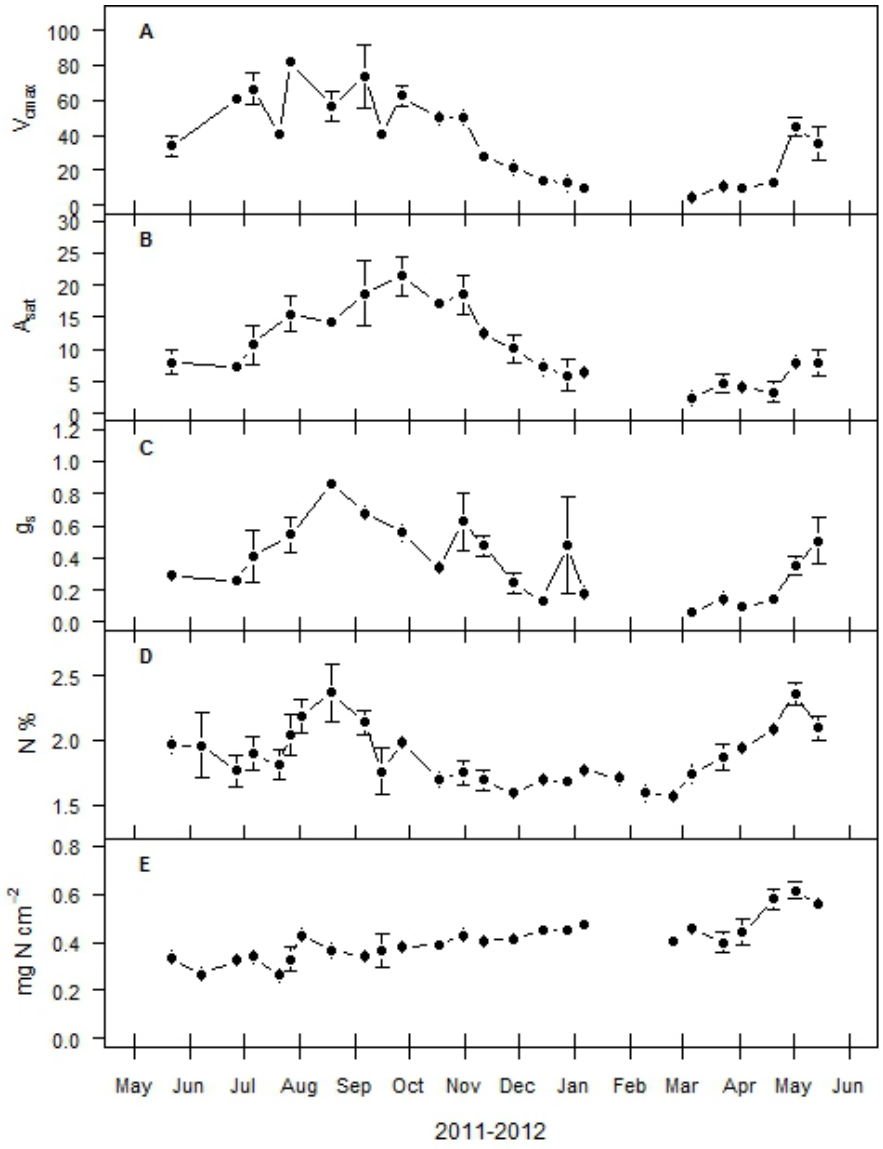

Figure 2. Seasonality of different photosynthetic parameters and leaf traits. A) The maximum carboxylation rate at ambient temperature, V_{cmax} (µmol CO_2 m^{-2} s^{-1}) at ambient temperature across the year 2011-2012. B) Light saturated photosynthesis at ambient temperature, A_{sat} (µmol CO_2 m^{-2} s^{-1}). C) Stomatal conductance at ambient temperature under light saturated conditions and ambient temperature, g_s (mmol H_2O m^{-2} s^{-1}). D and E) leaf nitrogen expressed per weight and area, N% and mg N cm^{-2}, respectively.

Within treatments, monthly campaigns were performed during the warm (July-September) and the cold (October-February) season. Means across the seasons are presented in Table 1. Light-saturated photosynthesis (A_{sat}) was significantly higher in all CO_2 plot across both the warm and the cold season ($p < 0.05$) and showed the same seasonal pattern as observed in the HTR plots (not shown). The highest rates were found in September and the lowest were seen after snowmelt in end February (not shown). In the warm season the experimental drought was found to reduce A_{sat} significantly across all drought-treated plots ($p<0.05$). Although the drought treatment was already completed at the end of May 2011, the soil water contents were still lower in the drought treated plot until August and affected the photosynthesis parameters. No carry over effect was seen during the cold season. No other than additive responses were found in either of the seasons (Table 2).

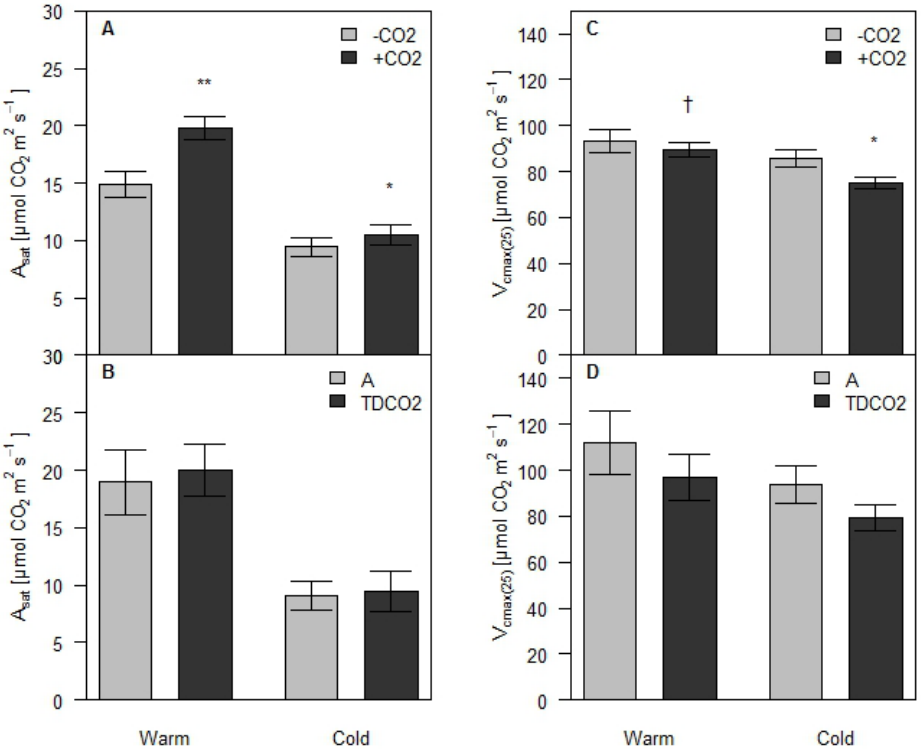

Figure 3. Photosynthetic response during warm and cold season. A) + B) Mean ± SE of light saturated net photosynthesis at ambient temperature, A_{sat} (µmol CO_2 m^{-2} s^{-1}). C) + D) the maximum carboxylation rate normalized to 25 °C, V_{cmax}^{25}(µmol CO_2 m^{-2} s^{-1}). Seasonal treatment effects are indicated as * $p<0.05$, ** $p< 0.01$, in the respective season. Tends at $p<0.1$ are noted with †. A) + C) Only responses to ambient CO_2 versus elevated CO_2 (-CO2 and +CO2, n=24) and B) + D) the un-manipulated control A versus the full combination of warming, drought and elevated CO_2, TDCO2 (n=6).

The patterns seen in the HRT plots were also found for light- and CO_2-saturated photosynthesis (A_{max}) (not shown). However, no significant treatment effects were found in either of the two seasons. Drought tended to influence A_{max} negatively but the effect was smaller in combination with elevated CO_2, and night time warming ($p<0.1$).

Across all seasons the intercellular CO_2 concentration (c_i) was found to be significantly higher ($p<0.001$) across all CO_2-treatments (CO2). c_i at ambient CO_2 conditions ranged between 308.2±4.3 to 376.5±6.3 ppm for A and 400.7±9.3 to 488.6±5.0 ppm for CO_2. The relation between C_i and the ambient CO_2 concentration (c_i/c_a) was increased in all CO_2-treatment across all time ($p<0.01$).

Antagonistic interactions between elevated CO_2 and drought (CO2×D) were found for stomatal conductance (g_s) across the warm season ($p<0.05$), compensating for the negative tendency of the drought treatment. Additional antagonistic interactions between all three treatments (T×D×CO2) were found across the warm season ($p<0.05$), even though no single factor effect was found (Table 2). No effect was found on g_s during the cold season.

The water use efficiency (*WUE*) varied between 1.0-8.8 µmol CO_2 $mmol^{-1}$ H_2O, with lowest seasonal values in July and August 2011, high values in November 2011 and low values again in February 2012. Across seasons *WUE* was not significantly affected by single factors, however drought tended to decrease WUE in the warm season ($p=0.06$). Additionally a significant antagonistic interaction between all factors (T×D×CO2) was observed in warm season. No effect on WUE was seen in cold season.

Table 1. Seasonal means ± SE (n=18-24) of photosynthetic parameters and leaf traits across warm and cold season. Treatments are un-manipulated (A), elevated CO_2 (CO2), passive nighttime warming (T), periodic spring/summer drought (D) and all combination of single factors (DCO2, TCO2, TD, TDCO2). Parameters are light-saturated photosynthesis (A_{sat}), light- and CO_2-saturated photosynthesis (A_{max}), maximal rate of Rubisco carboxylation at ambient temperature and normalized to 25 °C (V_{cmax} and V_{cmax}^{25}), maximal rate of RuBP regeneration at ambient temperature and normalized to 25 °C (J_{max} and J_{max}^{25}), mitochondrial respiration rate (R_{light}), intercellular CO_2 concentration (c_i), ratio between intercellular and atmospheric CO_2 concentration (c_i/c_a), stomatal conductance (g_s), water use efficiency (*WUE*), leaf nitrogen concentration (*N %*), leaf carbon to nitrogen concentration (*C/N*) and specific leaf weight (*SLW*) (n=6)

Parameter	Season	A	CO2	D	T	DCO2	TCO2	TD	TDCO2
A_{sat}	*warm*	18.95±2.84	18.23±2.06	13.98±1.98	15.22±2.17	18.60±2.50	21.64±1.23	12.11±2.51	20.05±2.25
	cold	9.26±1.70	9.83±1.75	9.13±1.37	10.36±1.72	1.23±1.56	11.17±1.64	9.27±1.50	10.80±2.22
A_{max}	*warm*	33.72±3.33	28.83±2.52	25.57±2.38	29.14±2.83	29.46±2.57	32.71±2.03	28.54±2.80	32.37±2.23
	cold	16.64±2.61	14.91±2.47	17.98±2.41	18.20±2.87	15.15±2.09	16.22±2.06	16.41±2.31	15.03±2.80
V_{cmax}	*warm*	88.93±8.50	69.45±5.65	68.64±6.30	71.93±6.41	74.87±6.69	78.68±7.24	66.68±5.64	72.48±7.20
	cold	35.50±4.72	27.32±2.73	27.89±2.93	30.95±2.99	26.41±2.12	26.21±1.82	29.62±2.91	26.54±3.60
V_{cmax}^{25}	*warm*	112.05±13.7	84.43±7.17	89.39±8.20	90.45±9.05	88.50±7.13	91.51±4.52	87.75±10.3	93.75±8.04
	cold	97.05±10.06	74.42±6.09	76.32±7.23	88.25±8.05	74.84±5.51	72.81±3.47	81.74±4.57	79.24±5.64
J_{max}	*warm*	193.44±17.2	157.93±10.1	158.84±11.7	163.48±13.1	183.30±12.1	180.28±11.7	159.47±12.8	174.82±12.7
	cold	107.37±11.19	94.89±7.98	98.50±9.17	102.90±10.61	92.23±6.02	94.06±3.89	101.72±7.09	101.26±9.58
J_{max}^{25}	*warm*	141.2±5.69	143.1±5.99	136.76±4.62	141.16±5.08	144.63±7.04	143.52±6.09	137.79±4.55	139.55±5.75
	cold	83.98±4.07	84.76±4.16	85.4±3.43	83.04±3.98	83.75±3.93	84.27±3.74	83.71±3.99	83.81±4.39
R_{light}	*warm*	1.78±0.17	1.39±0.11	1.37±0.13	1.44±0.13	1.50±0.13	1.57±0.14	1.33±0.11	1.45±0.14
	cold	0.71±0.09	0.55±0.05	0.56±0.06	0.62±0.06	0.53±0.04	0.52±0.04	0.59±0.06	0.59±0.07
c_i	*warm*	329.20±7.38	417.78±8.73	318.19±18.0	331.81±6.45	440.05±5.37	421.15±5.84	339.01±11.2	427.95±9.02
	cold	337.70±8.42	444.75±11.9	319.66±8.34	314.39±17.91	451.04±7.80	447.82±9.87	337±52±6.73	451.01±9.67
c_i/c_a	*warm*	0.75±0.05	0.94±0.04	0.70±0.05	0.76±0.03	1.03±0.04	0.96±0.03	0.76±0.04	0.97±0.04
	cold	0.76±0.03	1.00±0.04	0.74±0.04	0.69±0.04	1.01±0.04	1.01±0.04	0.76±0.03	1.01±0.04
g_s	*warm*	0.73±0.07	0.48±0.06	0.51±0.05	0.60±0.05	0.62±0.06	0.56±0.04	0.64±0.07	0.58±0.05
	cold	0.37±0.05	0.35±0.06	0.29±0.05	0.33±0.06	0.35±0.05	0.40±0.06	0.36±0.33	0.33±0.06
WUE	*warm*	3.36±0.62	4.75±0.46	3.80±0.72	3.27±0.40	3.12±0.38	3.86±0.30	2.87±0.56	3.91±0.41
	cold	5.88±0.72	5.47±0.85	6.85±0.76	6.74±0.76	5.87±0.59	5.84±0.62	5.35±0.69	5.29±0.73
N%	*warm*	1.97±0.08	1.81±0.09	2.05±0.08	1.92±0.10	1.79±0.08	1.87±0.11	1.96±0.09	1.72±0.12
	cold	1.62±0.03	1.49±0.03	1.64±0.03	1.64±0.04	1.44±0.02	1.51±0.03	1.67±0.03	1.45±0.03
C/N	*warm*	26.52±1.11	29.09±1.45	25.31±0.99	27.70±1.41	29.08±1.22	28.71±1.97	26.94±1.21	32.66±2.79
	cold	30.97±0.72	34.00±0.67	30.58±0.49	30.76±0.75	34.97±0.60	33.40±0.62	30.29±0.53	34.99±0.64
SLW	*warm*	0.17±0.01	0.18±0.01	0.16±0.01	0.17±0.01	0.18±0.01	0.19±0.01	0.16±0.01	0.19±0.01
	cold	0.22±0.01	0.25±0.01	0.22±0.01	0.23±0.01	0.24±0.01	0.24±0.01	0.23±0.01	0.24±0.01

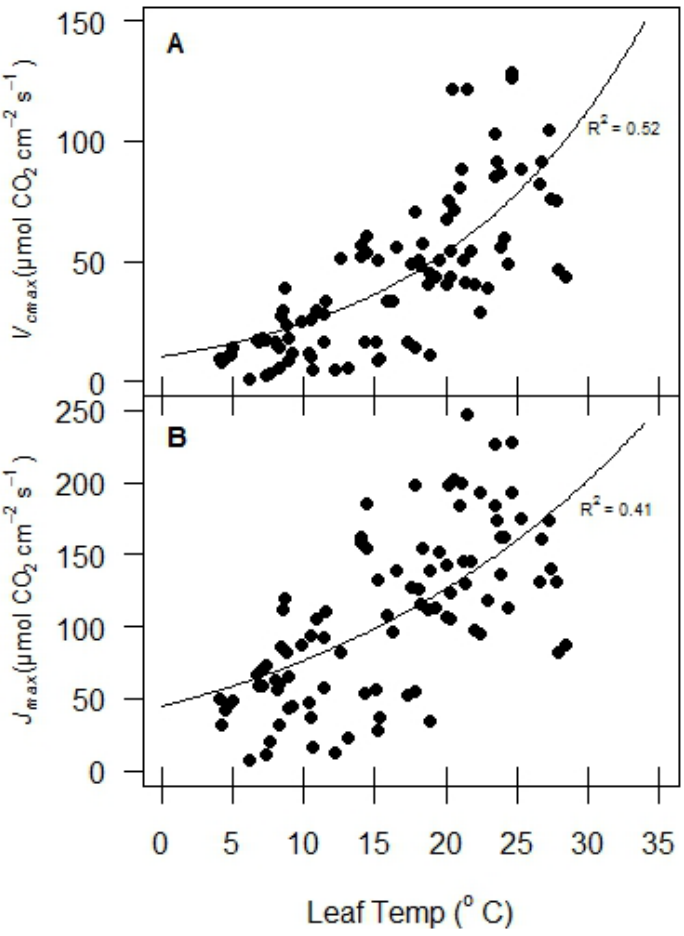

Figure 4. Relations between V_{cmax} and J_{max} to ambient leaf temperature from all ambient plots collected in monthly campagins and from the HTR plots,in the period from July 2011 to May 2012. These relations are the basis for the temperature normalization. Data from May to July 2011 was excluded to avoid problems with different physiological performace in the regrow phase after die-back (furter discribed in 'Climate and experimental control measurements'). The relationship was fitted using equation 2. A) V_{cmax}^{25} = 78.1±4.4 µmol m^{-2} s^{-1}, E_{ac} = 54.6±6.6 KJ mol^{-1}, p<0.01. B) J_{max}^{25} = 159.8±8.5 µmol m^{-2} s^{-1}, E_{ac} = 34.4±4.8 KJ mol^{-1}, p<0.01.

Normalization to 25°C was done using the relationship between seasonal measurements in none-treated plants of V_{cmax} and J_{max} to the actual leaf temperature. HTR plots were followed from May 2011to May 2012, but as a result of the big die-back with regrow phase of *Calluna* from May to July 2011 data from this period was exclude from the fitting. Thus leaves in early developmental phase have a different physiological performance than fully develop leaves (e.g Reich et al., 1991). Figure 4 shows the fittings and residual analysis for both V_{cmax} and J_{max} showed no pattern of concern. The relationship between J_{max} and V_{cmax} was found to be: J_{max}=49.5+1.66* V_{cmax} with a significant

correlation-factor R^2=0.88. Similar response was found for temperature normalized values J_{max}^{25} = 36.2+1.83*V_{cmax}^{25}.

In cold season V_{cmax}^{25} was found to be significantly lower in elevated CO_2 where no effect was seen in warm season (p<0.05). Drought tended to decrease V_{cmax}^{25} in the warm season and in both cold and warm season there was found a trend of an antagonistic interaction between drought and elevated CO2. No effect was found on temperature normalized values for J_{max}^{25}.

Relationships between N% and V_{cmax} was found significant (R^2=0.23, p<0.001) for non-temperature normalized data while no relation was found between N% and V_{cmax}^{25}. However N per leaf area (mg cm^2) was significantly correlated to V_{cmax}^{25} (R^2=0.11, p<0.001). There was no relation between leaf nitrogen and J_{max} or J_{max}^{25} (not shown).

Leaf chemistry and structure

From May- July, across all treatments, *Calluna* shoots changed from being small, thin and light green to thicker and dark-green. Fully grown shoots showed an increased specific leaf weight (SLW) during the whole year May 2011-May 2012 (from 0.17±0.02 g cm^{-2} in May 2011 to 0.26±0.01 g cm^{-2} in May 2012) in untreated seasonality-plots. Within treatments the same was observed (A: 0.18±0.01 g cm^{-2} in July 2011 to 0.25±0.01 g cm^{-2} in February 2012). Across both, cold and warm seasons SLW was significantly increased by elevated CO_2 (p<0.01). SLW did on average increase ca. 4% more in all elevated CO_2-plots compared to ambient CO_2-plots. The experimental drought or night time warming influenced SLW individually and no interactions were found.

The C/N ratio in the leaves increased significantly from May 2011 until July 2011, followed by a significant decrease in August 2011. In August 2011 the low C/N ratio was a result of a significantly lower N% in the leaves. From August the ratio increased to an even higher value than before until decreasing to the same level in May 2012 as in August 2011. Within treatments a significantly higher C/N ratio was found across all CO_2 treated plots (p<0.01) in both, warm and cold seasons (Table 2). Across the cold season an overall D×CO2 interaction was found (p<0.05). The CO_2 treatment did in general deplete the leaf $\delta^{13}C$ due to the lower $\delta^{13}C$ signature of the added CO_2 gas (p<0.0001). The leaf $\delta^{13}C$ was similar in new grown shoots compared to two samples from September and November 2010 (data not shown) and did not change across cold or warm season.

Table 2. F-values, level of significance and response directions. Single factor effects of elevated CO_2 (CO2), night time warming (T) and drought (D), and their interactions on light-saturated photosynthesis (A_{sat}), light- and CO_2-saturated photosynthesis (A_{max}), maximal rate of Rubisco carboxylation at ambient temperature and normalized to 25 °C (V_{cmax} and V_{cmax}^{25}), maximal rate of RuBP regeneration at ambient temperature and normalized to 25 °C (J_{max} and J_{max}^{25}), mitochondrial respiration rate (R_{light}), intercellular CO_2 concentration (c_i), ratio between intercellular and atmospheric CO_2 concentration (c_i/c_a), stomatal conductance (g_s), water use efficiency (*WUE*), leaf nitrogen concentration (*N %*), leaf carbon to nitrogen concentration (*C/N*) and specific leaf weight (*SLW*). Analysis of variance included the repeated measures approach and the significance levels are * p<0.05, ** p<0.01 and *** p<0.001, tendency are denoted as p<0.1 with increased (↑), decrease (↑) and antagonistic interactions as (↑↓).The time effect testing differences between warm and cold season was significant for all parameters.

Parameter	Season	CO2		D		T	D×CO2		T×CO2		T×D		T×D×CO2	
A_{sat}	*warm*	11.00	**↑	6.41	*↓	0.20	2.20		2.85		0.50		1.40	
	cold	3.95	*↑	0.35		0.04	0.06		0.03		0.14		0.54	
A_{max}	*warm*	1.55		3.20	†↓	0.04	1.35		1.16		1.78		2.79	†↓↑
	cold	0.63		0.04		0.14	0.02		0.19		0.40		0.18	
V_{cmax}	*warm*	0.00		3.07	†↓	1.19	2.15		2.46		0.08		2.47	
	cold	3.07	†↓	1.29		0.15	2.30		0.54		2.09		0.08	
V_{cmax}^{25}	*warm*	0.17		2.01		1.25	1.91		3.08	†↓↑	0.99		1.72	
	cold	5.29	*↓	1.24		0.00	3.48	†↓↑	0.11		1.22		0.20	
J_{max}	*warm*	0.30		0.73		0.75	2.28		1.83		0.00		3.78	†↓↑
	cold	0.95		0.10		0.00	0.34		0.48		0.90		0.00	
J_{max}^{25}	*warm*	0.51		0.09		0.29	1.78		1.32		0.14		2.15	
	cold	1.03		0.03		0.13	0.48		0.16		0.43		0.00	
R_{light}	*warm*	0.00		3.07	†↓	1.19	2.15		2.46		0.08		2.47	
	cold	3.07	†↓	1.29		0.15	2.30		0.54		2.09		0.08	
c_i	*warm*	205.06	***↑	0.67		0.28	1.73		0.89		0.00		1.20	
	cold	261.36	***↑	0.03		0.54	0.18		0.00		1.87		2.53	
c_i/c_a	*warm*	225.43	***↑	1.11		0.12	2.38		0.64		0.02		0.99	
	cold	179.72	***↑	0.09		0.39	0.23		0.00		1.76		2.60	
g_s	*warm*	1.77		0.18		0.10	5.06	*↓↑	0.00		2.63		8.80	**↓↑
	cold	0.19		1.72		0.04	0.35		0.35		0.12		3.75	†↓↑
WUE	*warm*	2.57		3.58	†↓	2.13	1.51		0.85		0.09		7.38	**↓↑
	cold	0.54		0.43		0.29	0.02		0.03		0.61		1.51	
N%	*warm*	12.62	***↓	0.19		0.63	1.97		0.08		0.45		0.01	
	cold	48.17	***↓	0.64		0.64	3.27	†↓↑	0.04		0.01		0.07	
C/N	*warm*	9.52	**↑	0.59		1.98	2.82	†↓↑	0.10		1.42		0.47	
	cold	52.06	***↑	1.01		0.28	4.12	*↓↑	0.00		0.10		0.17	
SLW	*warm*	5.89	*↑	0.41		0.27	0.24		0.96		0.31		0.00	
	cold	5.75	†↑	0.00		0.08	0.84		2.31		0.46		0.38	

Discussion:

Seasonal temperature acclimation hides the down-regulation on V_{cmax} in elevated CO_2

Calluna showed strong seasonality in most of the evaluated photosynthetic parameters and leaf traits across the year. The seasonal cause was not only seen in the high temporal resolution (HTR) plots, but also within experimental plots, confirming the general feature that growing at lower air temperature in colder seasons reduces the maximum carboxylation rate of (V_{cmax}) and consequently lower light-saturated photosynthesis (A_{sat}), as also found by other studies (e.g. Vogg et al., 1998). Normalization of V_{cmax} to a common reference temperature at 25°C, using non-treated seasonal data, removes most of the systematic variation that is caused by seasonal temperature variation. The significant differences between the normalized photosynthesis parameters are interpreted as treatment effects. The effects of V_{cmax} down-regulation in the warm and the cold season can be compared better because of the normalization, Treatment effects on V_{cmax} can, without the normalizarion, be overlooked due to the strong natural, seasonal down-regulation (figure 2). To evaluate such seasonal changes in temperature responses, temperature response curve for each sample time and treatments would be required.

During the warm season (July-September) regrown *Calluna* leaves increased the light-saturated photosynthesis (A_{sat}) significantly in elevated CO_2 due to increased intercellular CO_2 concentration (c_i) (figure 2, $p < 0.01$). This finding was in line with a previous study from the CLIMAITE experiment, where *Calluna* has been exposed to two years of treatment (Albert et al., 2011a/b). However in the 6th year, as it was seen after two years of treatment, *Calluna* did not show any down-regulation in either V_{cmax} or J_{max}. Neither in the previous nor in the present study treatment effect on stomatal conductance (g_s) or water use efficiency (*WUE*) was found during warm season. However, in the previous study Albert et al. (2012) showed how natural drought periods reduce g_s and *WUE* resulting in lower c_i, A_{sat} and lower V_{cmax}. In the present study drought strongly reduced A_{sat} and in some degree V_{cmax} (not significant). In combination with a ca. 50% higher soil water availability across the growing season after six years of experimentation compared to the second season of the CLIMAITE experiment. Thus, the lack of CO_2-induced down-regulation can be seen as the result of overall higher g_s, *WUE* and V_{cmax} in the measurements during the sixth year. In line with that, when drought was combined with elevated CO_2 $V_{cmax}^{25}$was lower than in plants only exposed to drought. Thus it can be assumed that down-regulation of V_{cmax}/V_{cmax}^{25} in the warm season strongly depends of water status of the system, where in wet years no down-regulation is present. During the cold season (October- March) *Calluna* goes through an acclimation process reducing A_{sat}, V_{cmax} and

leaf N %. The V_{cmax}^{25} values were found to be significantly down-regulated in elevated CO_2, where the non-temperature normalized V_{cmax} only tended to be down-regulated during the cold season. Despite the down-regulation of V_{cmax}^{25} in elevated CO_2 in the cold season, A_{sat} is significantly increased indicating that a potential higher carbon uptake is maintained. The maximal rate of electron transport (J_{max} or J_{max}^{25}) did not show any response to elevated CO_2 in either of the seasons, which is in line with earlier findings (e.g. Ainsworth and Long, 2005; Ainsworth and Rogers, 2007; Leakey et al., 2009). The stronger signal in cold season on down-regulation of V_{cmax}^{25} is consistent with our hypothesis, that the dual impact of lower temperature and elevated CO_2 both reduce photosynthetic capacity.

The N-content in leaves is naturally decreased during the cold season due to nitrogen re-translocation (e.g. Bryant et al., 1983). Additionally the N % in the leaves in elevated CO_2 treated plots is further decreased. The N-content per unit leaf area does however not differ between the elevated and ambient CO_2 plots. The leaves in the CO_2 treated plots are thicker, demonstrated by their higher SLW, but do not contain the proportional, additional amount of N. Looking at the mass ratios (N%) this can be interpreted as a dilution of nitrogen by carbon and other elements that constitute the additional tissue. The phenomenon that neither the N content per unit leaf area nor the photosynthetic capacity (V_{cmax}/V_{cmax}^{25}) differed between the CO_2 treated and non CO_2 treated plots in warm season, indicates the functional relationship between these two parameters rather than A_{sat} with N%. N dilution in leaves grown at elevated CO_2 has been described earlier at the site, within the same magnitude (e.g. Albert et al., 2011a/b; Andresen and Michelsen, 2005) and also in other studies (e.g. Ainsworth et al., 2003). Up to 25 % of leaf nitrogen have been shown to be incorporated in Rubisco, thus several studies relate the degree of down-regulation of V_{cmax}^{25} in elevated CO_2 the reduction in N % (e.g. reviewed in Nowak et al., 2004; Leakey et al., 2009). After 2 years no indication of N-limitation or changes in N availability was found at the experimental site (Larsen et al., 2011). In the cold season *Calluna* re-translocate leaf nitrogen and other nutrients from the leaves to other organs, thus the response on the V_{cmax}^{25} partly can be explained by the decreased available N in the leaves, or vice versa that the reduction of the N content is a consequence of the breakdown of the enzymes that drive photosynthetic capacity and the transport, e.g. as amides such as asparagine and glutamine into the plant corpus such as shown for trees (Cantón et al. 2005).

Independently of season the relationship between c_i and the atmospheric CO_2 (c_a) was increased in all CO_2 treated plots and this relation was constant over time, as the background CO_2 concentrations were higher at all time. Despite the higher CO_2/O_2 sensitivity for Rubisco at low

temperature and the down-regulated V_{cmax} the higher c_i in elevated CO_2 enabled *Calluna* to maintain a ca. 15 % higher A_{sat} in cold season.

Photosynthetic performance in a climate change future

Recent findings at the experimental site showed that *Calluna* increased photosynthetic performance in response to nighttime warming before and after smaller frost-events in late season (Albert et al.2013). However, in this study conducted before the frost took hold, then night-time warming did not influence any of the investigated photosynthetic parameters or leaf traits. This is partly in line with hypothesis II, that nighttime warming increases carbon uptake in cold seasons. In years where *Calluna* did not experience die-back, the significantly higher soil temperature (0 and 5 cm depth) in early-spring can have resulted in an earlier onset of the growing season, as indicated at the CLIMAITE experimental site in the year 2007 (Albert et al., 2011a; Kongstad et al., 2012). Albert et al. (2011a) found significantly increased photosynthetic performance in *Calluna* in the early summer directly related to the nighttime warming, however the effect disappeared later in the warm season. In summer, when air temperature is high the nighttime warming can course increased evaporation in warmed plots as a result of higher night time temperatures. Within most of the time during the CLIMAITE experiment the soil water content was significantly lower in plots with nighttime warming (data not shown), which in periods with low precipitation can lead to water shortage limiting A_{sat}, V_{cmax} via g_s. However an overall high soil water content (more than 10 vol. %) in the experimental period did not lead to any water shortage effects on photosynthesis. Single factor experiments with increased temperature have shown a strong stimulation of photosynthesis and biomass, especially during cold season and with sufficient water availability (e.g. Schmidt et al., 2004; Beier et al., 2004; Peñuelas et al., 2007). Thus, in the present study we expected a positive effect on A_{sat} in the autumn and early winter, however the expected stimulation was not found in our study. Passive nighttime warming increases the nighttime temperature and thus minimizes the periods of sub-zero temperatures in cold periods. The temperature regime in the period where measurements were conducted had only to a limited degree such sub-zero periods, and therefore the influence of the temperature treatment was modest. However, in late season where freeze events occurred, a higher photosynthetic performance was observed in response to warming (Albert et al., 2013).

The dry winter-spring transition in 2011 combined with the die-back of *Calluna* led to an overall delay of the onset of the growth, seen as a reduced NEE in the spring period compared to the following more normal year 2012 (personal communication, Klaus S. Larsen). The combination of a

dry spring and exclusion of 57.8 mm rainfall during the experimental drought was expected to have a strong negative effect in the period following the drought (also see SWC % in figure 1). This was confirmed with a significant reduction of A_{sat} during the warm season, when the soil water contents were still lower in the drought exposed plots (Table 2), although the treatment was ended before these photosynthesis campaigns began. However analyzing single campaigns separately revealed that the negative effect on photosynthesis, mainly was driven by the response in July, all other campaigns showed no drought treatment effects. The SWC% (0-20 cm) was significantly lower during the drought in May and also in the following months (June- August) leading not only to a significantly lower A_{sat}, but also to the trend of decreased water use efficiency (WUE) which confirms single factor experiments (e.g. Ogaya and Peñuelas, 2003; Prieto et al. 2009a,b). The lower A_{sat} in the warm season was not a result of decreased stomatal conductance (g_s) or changed c_i, but V_{cmax}^{25} tended to be lower (p=0.07). In the cold season the drought treated plots did not show any responses, however in a previous study at the site showed carry over effect in relation to drought in *Calluna* (Albert et al., 2011b). In both the previous and the present study *Calluna* reestablished the photosynthetic capacity after rewetting, thus a carry-over effect is more likely related to the reduced vitality caused by the negative influence of drought and the frost induced dieback in early season that caused a slow regeneration. In the present study the experimental drought was performed during the phase of *Calluna* regrowth and the time after the drought was wet and regrown *Calluna* within drought treated plots did not suffer more, thus no delayed onset of growing season was found.

The tree-factor treatment of elevated CO_2, nighttime warming and drought (TDCO2) is more realistic in relation to future climate change than the two-factor treatments (TD, TCO2, DCO2) or the single factor treatments, because these represent the full future climate scenario (Mikkelsen et al., 2008). The responses of the full TDCO2 treatment were in general due to additive effects of single factors responses. No synergistic effects were found and only a few antagonistic interactions resulting in dampening effects were observed in the full combination. Defining additive response only to be present if both single factors are significant, the result is in line with earlier findings from the CLIMAITE project (e.g. Kongstad et al., 2011; Larsen et al., 2011; Albert et al., 2011a,b), where antagonistic effects were more frequent in multifactor treatments than synergistic and additive.

In combination with nighttime warming, elevated CO_2 showed an increase in A_{sat} on 6% in warm and 15% in cold season. However the additive response in combination with drought and elevated CO_2 led to a decrease in A_{sat} of -8% during the warm season, but in cold season A_{sat} was still found to be increased by 15%. The 6% stimulation of A_{sat} in TCO2 during the warm season was counterbalanced of drought in TDCO2 resulting in an overall reduction in A_{sat}. In the cold season no

effects of drought were found to influence TDCO2, in line with other treatments including drought (D, TD and DCO2). Across the whole measurement period (July – February) A_{sat} was only increased with 1% in TDCO2 compared to the none-treated control, related to the additive response of drought in the summer. The effects in warm season are in line with responses detected during the 2th year of treatment (Albert et a., 2011b), where the positive stimulation of elevated CO_2 and in this year also warming on A_{sat}, was counterbalanced by the experimental drought treatment (that year July) in the month after. Here A_{sat} was found to be lower in DCO2 and TDCO2 compared to CO2 and TCO2 treatment, respectively (Albert et al., 2011a).

Despite the counterbalancing effects of drought on the elevated CO_2 stimulation on A_{sat} the increased SLW and diluted N across all times in elevated CO_2 indicated that the overall C-uptake is still stimulated in TDCO2and also DCO2. The stimulation of A_{sat} are maintained in cold season despite the strong down-regulation in V_{cmax}^{25}. In the warm season V_{cmax}^{25} is not significantly down-regulated by elevated CO_2, explained as a result of no N-limitation, thus N reduction in leaves during cold season leads to a significant down-regulation in V_{cmax}. This pattern is in line with Ainsworth and Long (2005), who reviewed that elevated CO_2 is more pronounced to reduce V_{cmax} in plants under N-limiting conditions.

Acknowledgements

The work was carried out within the CLIMAITE project, financially supported by the Villum Kann Rasmussen Foundation. Economic support has also been received from Air Liquide and DONG energy. Thanks to Preben Jørgensen, Poul T. Sørensen, Nina W. Thomsen, Andreas Fernqvist and Svend Danbæk for keeping the experiment running. Special thanks Nina W Thomsen always to be helpful in the field, also during rain and frost. Additional thank to Anja C. Nielsen and Sabine Reinsch for the chemical analysis of leaf compounds.

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PAPER III

Title:

Leaf level ecophysiological responses to climate change are consistent over six years of *in-situ* manipulations

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Keywords:

Net photosynthesis, dark respiration, V_{cmax}, CLIMAITE, elevated CO_2, warming, drought.

List of bbreviations :

CO2, experimental elevated CO_2; FACE, free air carbon enrichment; D, experimental drought; C, leaf carbon concentration; N, leaf nitrogen concentration; C/N-ratio, leaf carbon to nitrogen ratio; and J_{max} , maximal velocity of RuBP regeneration;; A_{sat}, light saturated net photosynthesis at ambient CO_2; A_{max}, potential maximum light- and CO_2 saturated net photosynthesis; ϕ, apperent quantum yield; c_i, intercellular CO_2 concentration; E, leaf transpiration rate; WUE, instaneus water use efficiency = A_{sat}/E; ; T, passive nighttime warming; V_{cmax}.maximal carboxylation rate of Rubisco at ambient temperature;; *PI*, performance index based on chlorophyll-a fluorescence induction curve analysis; F_v/F_m, maximal quantum yield of PSII in dark.

In preparation

Abstract

Plant physiological responses to climate change drivers; elevated CO_2, warming and periodic drought and in full combinations were investigated in an *in-situ* multi-factorial experiment on a Danish heathland after 6 years of treatment. The two dominated plants at the experimental site are the grass *Deschampsia flexuosa* and the dwarf scrub *Calluna vulgaris*. In earlier studies, the species responded differently to climatic treatments. We compare short term physiological responses to climate change manipulations on physiological parameters to long term responses.

Elevated CO_2 was the main driver for plant physiological changes in both *Deschampsia* and *Calluna*. Different growth strategies defined the physiological response to elevated CO_2 and to drought of the two species. *Deschampsia* induced leaf-dieback when exposed to low soil water content and leaves the remaining leaves with high photosynthetic capacity and stomatal conductance. In contrast, *Calluna* maintained leaf biomass significantly by decreasing photosynthesis via reduced photosynthetic capacity. Combined treatments resulted in additive responses, thus prolonged drought counterbalanced the stimulation of photosynthesis in *Calluna* under elevated CO_2. After 6 years of treatments, similar physiological responses were found but the magnitudes of responses were different.

The differences in soil water controlled the magnitude of physiological responses to the climate treatments. This study demonstrates that leaf level responses are stable upon a wide range of seasonal and inter-annual variation. Long term ecosystem feedback after 6 years of treatments was not detected at the leaf level, indicating a strong robustness in Danish heathland ecosystems to moderate climate change.

Introduction:

Climate change affects all levels of ecosystems (e.g. Walther, 2003; Kirschbaum, 2004). Plant physiological responses to rapid (seconds- minutes) and short term (days-month) changes in environmental conditions, such as elevated CO_2, temperature, precipitation and UV-B radiation and have been studied for decades (e.g. reviewed in Ainsworth & Long, 2005; Ainsworth & Rogers, 2007; Newsham & Robinson, 2009). Responses to environmental changes at one level of an ecosystem can have excessive indirect impact in other levels as the result of complicated feedback mechanisms (e.g. illustrated for carbon fate in Körner, 2006). Physiological adjustment to environmental changes enables plants to grow in all kind of habitats and even under high disturbance. Long term plant physiological response to climate change factors are indirect the result of ecosystem feedbacks. Long term studies of the impact of single environmental changes, such as elevated CO_2 are available (e.g. Ainsworth et al., 2003; Ellsworth et al., 2012). However, as predicted climate change do not only influencing single environmental parameters (e.g. IPCC, 2007). Ecosystem and global models

provides sufficient tools to predict the impact of multiple factors based on knowledge from single factor experiments (e.g. Norby & Luo, 2004). So far the responses to climate change factors are additive, models are useful to predict future carbon balance combining more than on climate factor, (Leuzinger et al., 2011). However, experiment including more than one factor have pointed to the complexity in the interactions to different single factors (e.g. Larsen et al., 2011). Leuzinger et al. (2011) explained how upscaling and multifactor experiments reduces single factor responses, thus models easily can overestimate the magnitude of responses. But it also emphasizes the importance of multifactor experiments.

Changes in atmospheric CO_2 concentration is a major climate change driver and in short time scale higher partial pressure of CO_2 increasing photosynthesis directly as an effect of increased CO_2/O_2 at the active site Rubisco (von Caemmerer, 2000). Growth in elevated CO_2 have been found to stimulated photosynthesis, despite a reduced stomatal conductance (g_s), down-regulated photosynthetic capacity, mainly the maximal velocity of Rubisco carboxylase (V_{cmax}) and a nitrogen dilution in the leaf tissue (e.g. Long et al., 2004; Ainsworth & Long , 2005; Leakey et al., 2009, Albert, 2011 a/b/c). Reduced stomatal conductance can affect the water status of the plants and studies have indicated that elevated CO_2 reduced the water consumption reducing soil water depletion, so called water saving (Leuzinger & Körner, 2007; Robredo et al., 2007). However, studies have also indicated that water saving as a result of reduced stomatal conduction, probably only occurs in severe drought periods and is strongly depended of species growth-strategies (e.g. Robredo et al., 2007; Albert et al., 2011a & 2012). In contrast to photosynthetic stimulation to elevated CO_2 leaf respiration has only been found to be influence marginal to elevated CO_2 (e.g. Tjoelker et al, 2001). Thus, in a longer perspective elevated CO_2 may sustain increased carbon uptake, as reported from long term studies (e.g. Ainsworth et al., 2003; Ellsworth et al., 2012). However, a potential larger leaf biomass as a result of increased plant carbon uptake can potential increases the overall ecosystem respiration. Feedback process of increase biomass with elevated CO_2 potential moves the ecosystem toward a new equilibrium, where essential elements and water shortage may insensitive the photosynthetic down-regulation resulting in an overall reduction in plant carbon uptake (e.g. Reich et al., 2006).

Temperature is controlling most enzymatic processes and plants are capable to acclimate rapid to new growth temperatures within seasons (e.g. Sage & Kubien, 2007). Direct effect of photosynthesis and leaf respiration of increased temperature is well documented. However in the light of climate change; it is argued that effects of warming on the plant physiology are more related to effects on other levels of the ecosystem (Kirschbaum, 2004). In Europe increased mean temperatures during the last decades has resulted in an overall earlier onset of the spring and summer of ca. 2.5days/°C (Menzel *et al.*, 2006). Furthermore temperature increase are documented to increase soil mineralization and ecosystem evaporation (e.g. Rustad et al., 2001; Schmidt et al., 2004, Kattge et al.,

2009). Indirectly, changed length of the growing season, nutrient and water availability can be of great importance for ecosystem carbon sink capacity (e.g. Llorens & Penuelas, 2005; Peñuelas et al., 2007). Increased temperature can stimulate plant productivity directly by increasing photosynthesis. But daytime respiration or changes in the temperature responses of respiration can occur, resulting in an overall reduction in carbon uptake (e.g. Campbell et al., 2007). Climate change warming is expected to be diurnal asymmetric and most pronounce during nighttime (Easterling *et al.*, 1997). Studies with nighttime warming have showed that increased nighttime respiration resultin stimulated daytime photosynthesis the following day, increasing the carbon sink strength(e.g. Griffin et al., 2002; Turnbull et al. 2002 & 2004). Further increased soil mineralization and thereby increase the nitrogen availability for plants resulting in an increasing photosynthetic capacity (Kattge et al., 2009). Thus, positive responses of warming in combination with elevated CO_2 can be expected.

Additionally to elevated CO_2 and warming, precipitation patterns are expected to change dramatically (IPCC, 2007). In temperate ecosystems no changes in the amount of precipitation are expected, however heavier rainfall is expected to be more frequent increasing the amount of longer drought periods (Boberg, 2010; Christensenet al., 2010) By definition water limitation are one of the strongest stress factors at all levels of terrestrial ecosystem. Drought reduces most physiological processes, such as g_s, V_{cmax}, electron transport (J_{max}) and maximal light- and CO_2- saturated photosynthesis (A_{max}); negatively influencing the plant carbon uptake (e.g. Nogués & Baker, 2000; Wilson et al., 2000; Midgley et al., 2004). Reduced functionality due to water shortage have be shown to delay phenological process related to growth and germination (e.g. Signarbieux & Feller, 2011; Albert et al. 2011 a/b; Jentsch et al., 2009; Prieto et al., 2008; Llorens & Penuelas, 2005). Additional plants growth strategies have been shown strongly to influence the physiological processes, both during drought and especially in reestablishment after rewetting (Albert *et al.*, 2012). Carry over effects of strong water shortage in later seasons have been shown in different species minimizing photosynthetic capacity (e.g. Ogaya & Peñuelas, 2003; Albert et al., 2013). In combination with elevated CO_2 severe drought in the drought-tolerant dwarf scrub *Calluna vulgaris*, have been shown to synergistic degreasing g_s, but carbon uptake are maintained by higher partial pressure of CO_2 (Albert et al., 2011a).

Long term ecophysiological responses on temperate heathland ecosystem to elevated CO_2, passive night-time warming and periodic summer drought and the full combinations were investigated in a multi-factorial setup, CLIMAITE. The experiment was designed according to the climate change scenario in Denmark 2075 (Mikkelsen et al., 2008). Different physiological responses to treatments in the two dominated plants at the site, the grass *Deschampsia flexuosa* and the evergreen dwarf scrub *Calluna vulgaris* were found after 2 years of treatment (Albert, 2011 a/b/c). Natural environmental fluctuations forces plants to adjust physiological in relative short term, thus direct effect of treatment after 6 year are expected to be the same as in the second year. We suggest that long term ecosystem

acclimation can influence the physiological process indirectly e.g. via feedback from changed soil properties or by a structural change in the plants. Thus, 6 years of treatment can have moved the ecosystem against a new equilibrium causing further plant physiological acclimation. Here we report long term responses on plant physiology, water relation and leaf trait in the Deschampsia and Calluna to 6 years of climate change manipulations.

Method and Material

Experimental site and metrological observation

The data was collected in the period from May - October 2011 at the experimental study site of the long-term climate change experiment CLIMAITE (www.climaite.dk) situated in North Zealand, Denmark (55°53'N, 11°58'E). The ecosystem is a temperate heathland on a nutrient poor and sandy soil, with a pH of 4.5 in the topsoil. The vegetation is dominated by a grass, Wavy- hairgrass (*Deschampsia flexuosa L.*) and the evergreen dwarf scrub Heather (*Calluna vulgaris L.*). The annual precipitation sum was 692.9 and the annual mean temperature was 10.0 °C in 2011. Further information of the study site can be found in Mikkelsen et al. (2008).

The climate change experiment are including treatments of un-treated control (A), elevated CO_2 (CO2), passive nighttime heating (T), periodic summer drought (D) and all combination (TD, TCO2, DCO2, TDCO2) replicated in six blocks in a complete split-plot design. Each block includes two octagons of 6.8 m diameter, divided in four plots. With the technique of Free-Air-CO_2-Enrichement (FACE) one octagon in each block are exposed to 510 ppm CO_2 during daylight hours. The passive nighttime heating is performed by automated infrared reflective curtains covering one half of each octagon. Experimental drought is performed, during a period of two-five weeks in the spring or summer each year of the experiments, by automated rain-excluding curtains, activated by rain, covered one half of each octagon. Drought treatment is stopped if the soil water in the top 20 cm is reduced to 5 %. Together with the nighttime curtains the rain-excluding curtains creates the split-plot design. The experimental treatment of CO_2 and warming was started in October 2005. In each experimental plot soil water content over two depths (0-20cm and 0-60 cm) has been continually recorded using time domain reflectometry (TDR). Simultaneous temperature where measured in 20 cm height, 0 and 5 cm depth. For further technical detail of the setup, see Mikkelsen et al. (2008).

Table 1. Precipitation and minimum daily mean SWC (in %) in 0-20 and 0-60 cm soil depths during the employed extended drought periods between 2006 and 2011.

				0-20 cm (vol %)				0-60 cm (vol %)			
Year	Experimental drought period	Annual precipitation	Precipitation excluded	D	DCO2	TD	TDCO2	D	DCO2	TD	TDCO2
2006	3/7-4/8	676.4 mm	11 %	4.7	5	4.6	5.1	4.5	7.5	4.7	6.5
2007	21/5-21/6	894.1 mm	8 %	5.4	5.2	4.6	4.4	5.5	6.8	5	5.3
2008	5/5-26/5 + 16/9-1/10	665.8 mm	5 %	5.4	5.7	4.8	5.2	5.8	7	5	5.4
2009	18/5-24/5 + 26/6-12/7	732 mm	5 %	7.7	6.8	6.3	6.6	8.4	8	5.8	6.6
2010	4/5-2/6	882.2 mm	8 %	8.7	9.4	9.1	8	9.6	10.4	8.5	8.7
2011	2/5-31/5	692.9 mm	8 %	8.4	16.7	8.2	7.3	8.8	10.2	7.4	7.8

Leaf Gas exchange

Leaf level gas-exchange of CO_2 and H_2O were measured *in situ* using an open portable photosynthetic system using leaf adaptor frames (Boesgaard et al., 2013). Three identical instruments (LI-6400LI-COR Biosciences, Lincoln, Nebraska, USA connected to a standard 2*3cm chamber/cuvette with a LED light source (6400-02B)) were used for measurements in campaigns during May – October 2011. Monthly campaigns within treatments were performed on *Deschampsia* in 18-19 May, 20-22 June, 11-12 July, 8-14 August, 12-14 September and 5-7 October and on *Calluna* 13-21 July, 16-18 August, 19-22 September, 25-27 October 2011.

Healthy shoots of *Calluna* with similar structures, from the upper part of the canopy and a small bundle of 10 – 15 green leaves of *Deschampsia* were selected for every measurement. To improve the precision of measurements such as leaf dark respiration, shoots or leaves were placed in leaf adaptor frames (LAF) as recommended in Boesgaard et al. (2013) and sealed using blue tack (Lyreco, 59770 Marly, France).

Leaves were acclimatized to cuvette conditions for 6 min at 390 ppm CO_2 under ambient CO_2 and 510 ppm in FACE plots, before net photosynthesis and stomatal conductance were stabilized (CV < 1%). Using the Li-6400 auto-program 'ACi-curves' (Open 6.2) CO_2 response-curve was measured stepping down the CO_2 concentration from 390 ppm (510 ppm) to 50 ppm CO_2 and then brought back to the 390/510 ppm level again, for at least 3 min. After the reestablishment of the CO_2 level the concentration of CO_2 was stepped up to complete saturation at 1400 ppm CO_2. Light in the cuvette was set at 1500 µmol photosynthetically active photons m^{-2} s^{-1} (PAR). To reflect the true ambient temperature of the day, block temperature was set to the expected mean temperature of the measuring day (controlled to be ± 5 °C the target value, due to limitation of the equipment). Relative humidity was adjusted to 45-55% during measurements. Maximum carboxylation (V_{cmax}) and electron transport (J_{max}) rates were calculated from curve-fitting to the Farquhar-von Caemmerer-Berry (FvCB) model equations (Dubois et al., 2007 and Bernacchi et al., 2001). When no minimum between

the Rubisco-limited and RuBP-regeneration-limited phases could be found by the fitting procedure, V_{cmax} was fitted using data with C_i<500 ppm and J_{max} was fitted using data with c_i > 550 ppm. Following the measurements of ACi-curve the auto program "Light-curves" on the Li-6400 was used to detect the light response. The light response was done stepping down the light from 2000 µmol photosynthetic photons m^{-2} s^{-1} (PAR) in 9 steps to zero. The photosynthesis saturating reference CO_2 concentration was set to 1400 µmol m^{-2} s^{-1}. From the light-response curve the potential maximum light-saturated rate of photosynthesis (A_{max}) and apparent quantum yield (ϕ) was estimated using a non-rectangular hyperbola as regression model (Lambers et al., 1998). In a last step, leaf dark respiration (R_D) was measured directly in the dark at 390/510 ppm in none-FACE and FACE plot, respectively. R_D was estimated from the 2 sec data during 6 minutes.

The light-saturated net photosynthesis (A_{sat}), intercellular CO_2 concentration (c_i), stomatal conductance (g_s) and transpiration (E) were extracted from the first point in the CO_2 response-curves at CO_2 reference = 390 ppm CO_2 in non-FACE plots and at 510 ppm in FACE plots. The water use efficiency (WUE) was calculated as $WUE = A_{sat}/E$. All data was corrected with empty-chamber measurements as recommended, for data collected with leaf adaptor frames (for detailed protocol see Boesgaard et al., 2013).

Leaf characteristics, area, weight, nitrogen and carbon

After each measurement the leaf adaptor frame containing the leaves were cut off and plant material inside the frame is cut out in the laboratory. Leaf adaptor frames containing *Deschampsia* leaves were scanned with the frames against a known distance of 3 cm. *Calluna* leaves and stems were detached from each other and placed on a paper, with a square of the fixed dimension of 3x3 cm, a photo was taken placing the paper with leaves and stems on top of a light table. Area estimations were quantified using the image processing program (ImageJ, National Institute of Health, USA). The side of the square (3 cm) was in all cases the reference length, and area was determined from 8-bit colour pictures with the threshold approach. The scanned *Deschampsia* leaf area and the photographed *Calluna* leaf areas are given as projected leaf areas, after Smith et al. (1991). Dry weight of the plant material was measured after drying in 48 hours at 60°C. The specific leaf area (*SLA*) was determinate from the weight and area as *SLA*= weight (g) /area (cm^2). The dried leaf material was analyzed for carbon, nitrogen concentration and ^{13}C natural abundance ($\delta^{13}C$) on an elemental analyzer (CE 1110, Thermo Electron, Milan, Italy) coupled to a Finnigan MAT Delta PLUS isotope ratio mass spectrometer (Thermo Scientific, Bremen, Germany). Expression of the $^{13}C/^{12}C$ isotope ratios as delta notation ($\delta^{13}C$) is the relative measurement against the $^{13}C/^{12}C$ ratio in an international standard (Vienna Pee Dee Belemnite): $\delta^{13}C = \frac{R_{sample} - R_{standard}}{R_{standard}} * 1000‰$.

Data analysis and Statistics

Treatments effects per monthly campaign were analyzed with a linear mixed effect model using the lmer function in the lme4-package of the statistical free-software R (R Develpment Core Team, 2010). The lmer function provides the option to incorporate both fixed-effect parameters (main factors) and random effects (random factors). Main effect factors of the model were the treatments elevated CO_2 (CO2), warming (T) and drought (D) and their full factorial interactions (T x CO2, T x D, D x CO2, T x D x CO2), while block were set as a random factor. F-values and p-values were extracted with the MixMod-package using the SAS-algorithm. Normal distribution of data was tested with Levene's test and was either log(x) or 1/x transformed if necessary. Homogeneity of variance was inspected visual with residual plot. Differences between regressions were tested using pairwise t-test and Tukey's grouping test.

Level of significance were set to be p-values < 0.05 and trends were notes if p-value < 0.1. The simplest interaction that can occur between two or more single factors are additive. An additive effect is when two or three factors effects are simply added to each other. Statistically additive effect will not always be shown as significant due to lack of single factor significance. Watching interaction plots produced by the MixMod-package significant interactions were categorized as synergistic if effects of single factors have a stronger impact together, bigger than the expected additive effect, or as antagonistic if the combined interaction is dampening the expected additive effect.

Results

Climate and experimental control measurements

A major dieback of the standing population of *Calluna vulgaris* was seen after the following weather situation: A period with snow cover from November 2010 to mid-January 2011 was followed by 1-2 weeks of warm sunny weather and in end January to end February 2011 there was snow free conditions, many clear sky days and repeatedly hard frost events (below zero during day-time).. In April 2011 only around 5-10% of all *Calluna* stands had functioning green shoots. New shoots regrew from the dried stands during May-July 2011, with an approximate 75% regrowth (personal observation, Kristine S. Boesgaard & Nina W. Thomsen). The dieback was not quantified but observations indicated no differences between treatments (personal observation, Johannes….) and *Deschampsia flexuosa* was not directly affected by the of weather situation. Slow regrowth in spring, homogenous leaf structures across the whole experimental area was first established in early July (personal observation, Kristine S. Boesgaard & Nina W. Thomsen).

The dry winter/spring, causing the dieback of *Calluna*, was followed by, to the Danish normal from 1960-1999 (http://www.dmi.dk), a comparable warmer and wetter summer. In 2011 the

experimental drought was performed during one month starting on May 2nd and excluded 57.8 mm of rain, corresponding to 8.3% of the annual precipitation in 2011. The minimum daily mean soil water content (SWC %) reached in drought plots were within the period found to be 8.8 vol % in 0-20 cm depth and 8.4 vol % in 0-60 cm depth. Compared to earlier years in the experiment the minimum SWC% reached with drought was high, ex. in 2006 the lowest value was below the critical threshold of 5 vol % (4.7 vol % in 0-20 cm and 4.5 vol % in 0-60 cm) and can be explored in Table 1. During the present study daily mean air temperature was varying between 5.0-21.0 C with highest mean temperatures in June - August.

Within treatments SWC % in 0-20 cm depth was significantly reduced by drought during the period and did never meet the ambient level either before or after (figure 1). Warming (T) have as the experimental drought (D) resulted in a significantly lower SWC % in 0-20 cm depth across the entire experiment (2005 -2011, data not shown) and the full-factorial combination (TDCO2) follows a similar pattern, where DCO2 and TCO2 is significantly increased compared to D and T, respectively. A similar pattern is seen in 0-60 cm depth (not shown). Soil temperature within treatments was significantly increased in 5 cm depth across the period, with strongest increase in early summer (May).

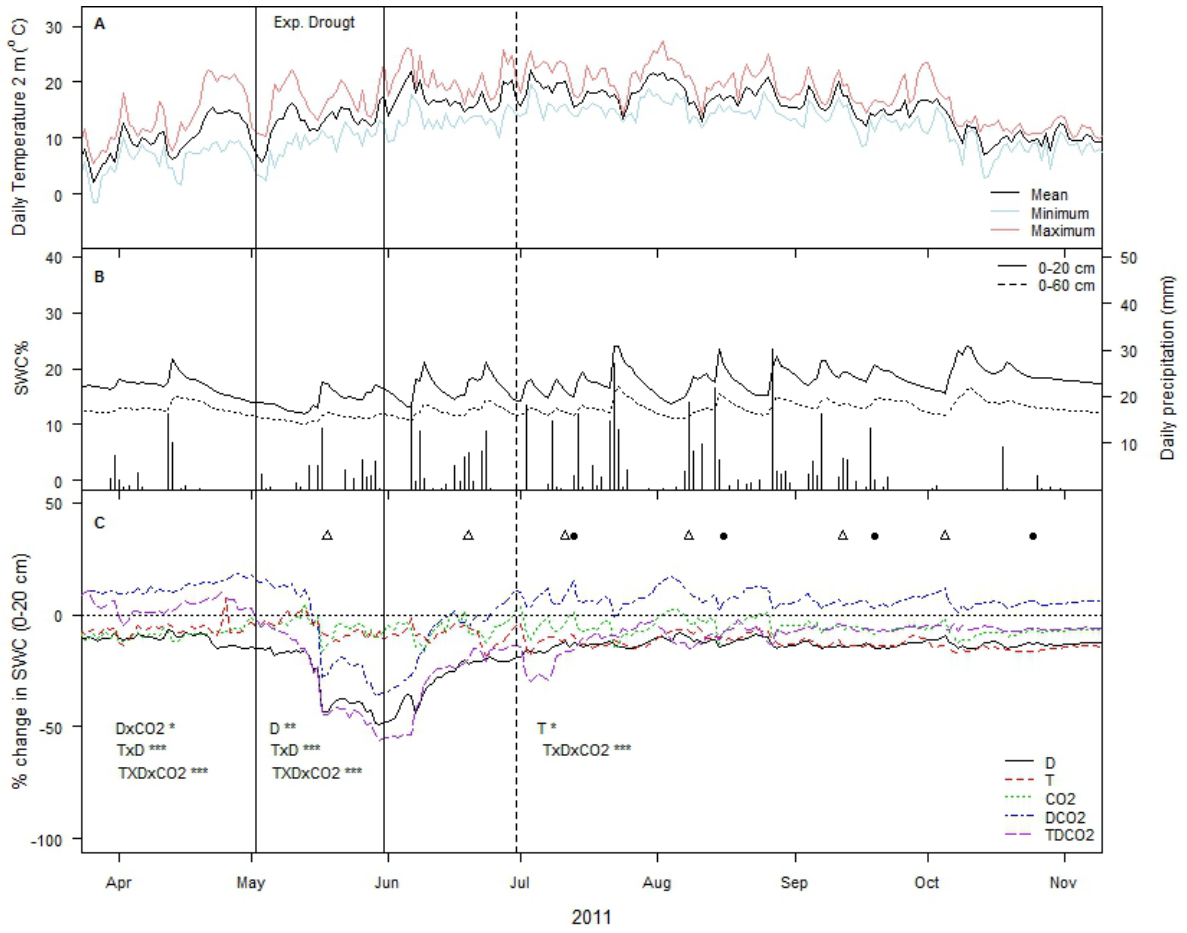

Figure 1. Air temperature, precipitation and soil water content. A) Daily mean (black), minimum (blue) and maximum (red) temperature in 2 m height at the CLIMAITE experimental site. B) Daily mean soil water content (SWC %) in 0-20 (—) and 0-60 cm depth (- - -). Bars are daily precipitation in mm. C) % change in SWC (0-20 cm) from non-treated control (A). The treatments are; extended drought (D, —), passive nighttime warming (T, - - -), elevated CO_2 (CO2, ····), combination between drought and elevated CO_2 (DCO2, ·¯·¯) and the full combination (TDCO2, - - -). Experimental drought period are indicated on each panel by vertical black lines and lack-phase where soil water is still lower in 0-60 cm depth after drought is stated by a vertical dotted black line. Triangles (Δ) in C) indicated state date for campaigns measuring leaf gas exchange on *Deschampsia flexuosa* and closed circle (●) *Calluna vulgaris*.

Photosynthetic performance in Deschampsia flexuosa and Calluna vulgaris

Across the study period light-saturated photosynthesis (A_{sat}) was higher in *Deschampsia* than *Calluna*, only during September campaign *Calluna* in generally had an overall higher A_{sat} (figure 2), and varied between 12.8± 3.1– 29.6±5.0 µmol CO_2 m^{-2} s^{-1}. *Deschampsia* had a continuous lower dark respiration (R_D), from highest in May and lowest in October (5.0 ±0.8 and 1.5±0.4 µmol CO_2 m^{-2} s^{-1}, respectively). *Calluna* R_D varied between 2.5± 0.5– 8.7±0.4 µmol CO_2 m^{-2} s^{-1}. *Deschampsia* maintained higher water use efficiency (*WUE*) (figure 3) and stomatal conductance (g_s) across the period compared to *Calluna*. The two species had different significant responses to treatments, e.g. elevated CO_2 significantly stimulated A_{sat} in *Calluna* ($p<0.001$), while this could only be seen as a trend in *Deschampsia* ($p=0.09$) as seen in figure 2. Warming did not influence A_{sat} in either of the species, drought significantly reduced A_{sat} in *Calluna* ($p<0.04$), mainly driven by the strong impact in

July (single campaign statistic p<0.001). Multi-factor treatment overall followed responses found in single-factor treatments, with an increased A_{sat} for *Calluna* and no change for *Deschampsia*. R_D responses correlate with A_{sat} for both species, resulting in a significantly increased R_D in Calluna within elevated CO_2, where no responses was seen on *Deschampsia*.

Elevated CO_2 in *Deschampsia* was found to be related to a significantly lower V_{cmax} and A_{max} (p<0.05), where either seasonality or treatment effect was found on J_{max} (table 2). *Deschampsia* did not have significantly higher c_i or g_s, but significantly increased *WUE* in the plants (p<0.001). *Calluna* did increase c_i in elevated CO_2 and also increased A_{max} (p<0.001 and p<0.05, respectively). Neither V_{cmax}, J_{max} nor *WUE* was affected by elevated CO_2 (figure 3), but V_{cmax} trended to be reduced in drought plots (p=0.08). No other single factor effect was found and interactions different from additive, was all found to be antagonistic, and was only seen for *Calluna* in g_s (DxCO2 and TxDxCO2, p<0.05) and *WUE* (TxDxCO2, p<0.01). Campaigns means ± SE of A_{sat}, R_D, V_{cmax}, J_{max}, A_{max}, g_s, *WUE* and c_i can found in table 3.

Table 2. F-values and significance levels for single factor effects of elevated CO_2 (CO2), night time warming (T) and drought (D), and their interactions on light-saturated photosynthesis (A_{sat}), leaf dark respiration (R_D), maximal rate of Rubisco carboxylation (V_{cmax}), maximal rate of RuBP regeneration (J_{max}), maximum light- and CO_2-saturated photosynthesis (A_{max}), stomatal conductance (g_s), water use efficiency (WUE), intercellular CO_2 concentration (c_i), specific leaf area (SLA), leaf nitrogen concentration (N %) and leaf carbon to nitrogen concentration (C/N).
Statistics is including repeated measurements and is considered significant as * p<0.05, ** p<0.01 and *** p<0.001, trends are denoted as p<0.1 with increased (↑), decrease (^) and antagonistic interactions as (↑↓). If not noted seasonality was found significant. [A)] No seasonality in the variable was found.

		Single factor effect			Multi-factor interactions			
Variable		**CO2**	**T**	**D**	**TxCO2**	**DxCO2**	**TxD**	**TxDxCO2**
A_{sat}								
	Deschampsia	4.17 ⁻↑	0.01	0.02	0.79	0.08	0.01	1.24
	Calluna	16.9 ***↑	0.00	4.33 *↓	3.21 ⁻↑↓	1.62	1.69	0.90
R_D								
	Deschampsia	0.43	2.11	3.40 ⁻↓	0.08	0.00	1.20	0.07
	Calluna	1.31	0.45	1.08	0.27	0.37	0.02	0.02
V_{cmax}								
	Deschampsia	6.16 *↓	0.01	0.04	0.01	0.77	0.15	0.19
	Calluna	0.37	0.67	3.12 ⁻↓	2.6	2.18	2.46	1.44
J_{max}								
	Deschampsia[A)]	1.73	0.01	0.05	0.08	0.38	0.61	0.62
	Calluna	0.18	0.22	0.48	2.48	1.48	0.43	2.05
A_{max}								
	Deschampsia[A)]	5.71 *↓	0.03	0.87	1.63	0.05	0.00	1.89
	Calluna	5.35 *↑	2.79 |↑	0.85	1.80	0.15	0.50	0.24
g_s								
	Deschampsia	3.30 ⁻↓	0.23	0.08	0.01	0.71	0.04	0.95
	Calluna	0.29	0.18	1.09	0.14	4.75 *↑↓	2.38	7.09 **↑↓
WUE								
	Deschampsia	28.2 ***↑	0.00	0.00	2.11	0.60	0.02	0.52
	Calluna	3.18 ⁻↑	1.61	1.28	0.45	1.90	0.30	7.65 **↑↓
c_i								
	Deschampsia	1.19	0.00	0.93	0.42	0.65	0.19	0.67
	Calluna[A)]	284 ***↑	0.09	0.55	0.29	0.88	0.01	3.04 ↑↑↓
SLA								
	Deschampsia	23.1 ***↓	0.03	4.53 *^	0.85	0.75	3.39 ↑↑↑	0.54
	Calluna	6.34 *↓	0.00	0.46	1.48	0.69	0.02	0.69
$N\ mgcm^{-2}$								
	Deschampsia	3.52 ⁻↓	0.01	0.02	0.55	0.33	3.90 *↑↓	0.12
	Calluna	1.12	0.09	0.44	0.06	0.98	0.01	0.1
C/N								
	Deschampsia	22.8 ***↑	0.17	1.31	0.44	0.59	0.52	0.05
	Calluna	9.69 **↑	1.17	0.94	0.06	5.07 *↑↓	1.84	0.53

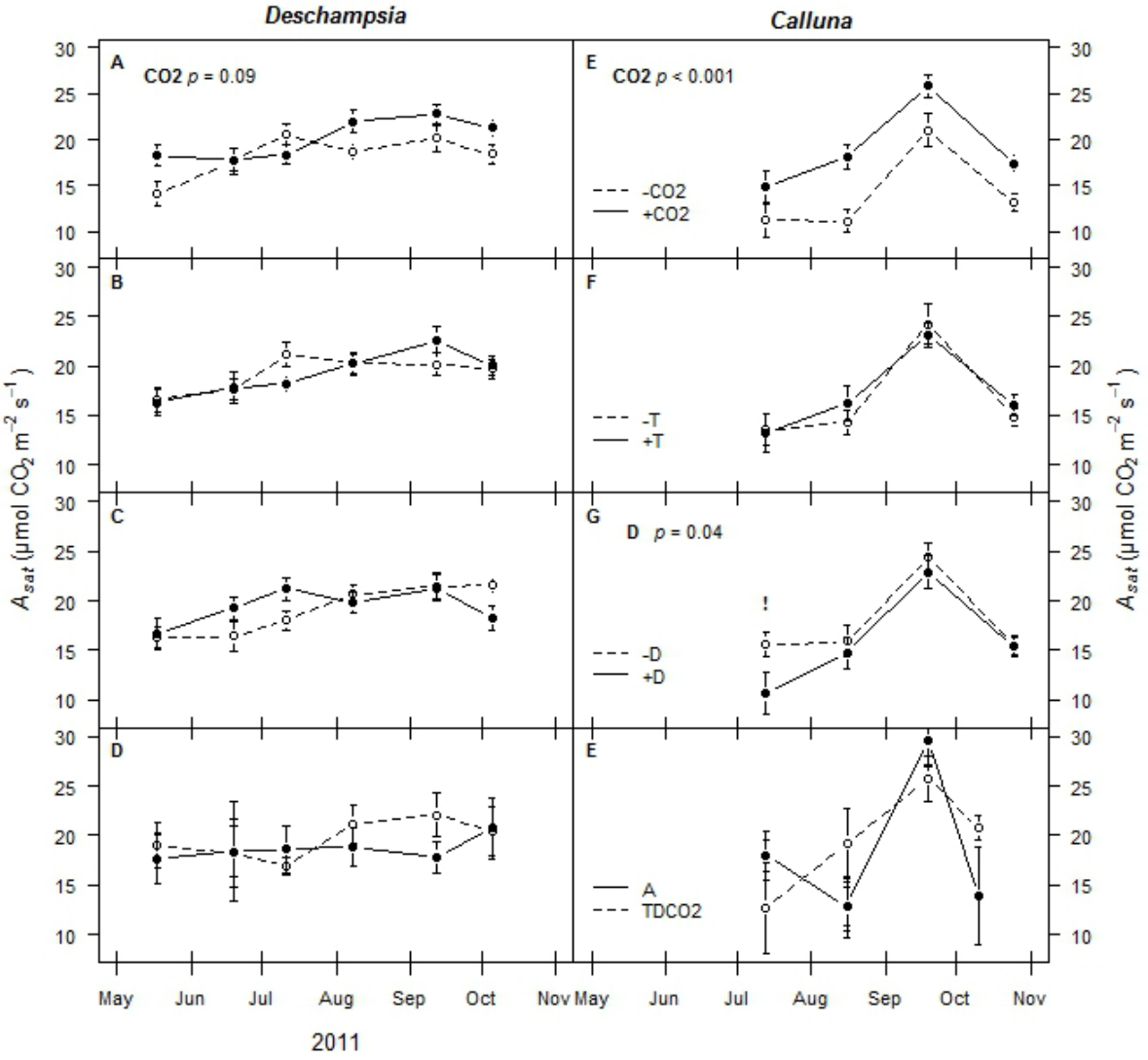

Figure2. Mean A_{sat} ±SE across the measuring period. A-C) and E-G) mean of all ambient CO_2 (dashed line) and elevated CO_2 plot (solid line), all warming (dashed line) and none warmed (solid line) and all drought treated (dashed line) and none drought plot (solid line), for *Deschampsia* and *Calluna* respectively (n=24). D) and H) ambient plot (solid line) and the multi-factor treatment (dashed line), for *Deschampsia* and *Calluna*, respectively (n=6). Single factor differences are denoted on directly above the measurement, where interactions are denoted in top left corner of each graph. Difference is indicated as significant with * p< 0.05 and **p<0.01, trends on p<0.1 is noted †.

Leaf chemistry and structure

The species differed also in leaf chemistry and structure responses to treatments (table 2). *Deschampsia* did not change in leaf thickness (*SLA*) but significantly reduced both leaf nitrogen and increased the C to N ratio ($p<0.001$) to growth in elevated CO_2. Calluna in elevated CO_2 not only reduced N and increased C/N in the leaves ($p<0.01$), but also increased leaf thickness (reduced SLA, $p<0.05$). $\delta^{13}C$ in the leaves was more depleted in the elevated CO_2 treatment ($p<0.001$) for both species due to another isotopic signature in the added CO_2. No other treatments effect was found. Campaign means and standard errors can be found in table 4.

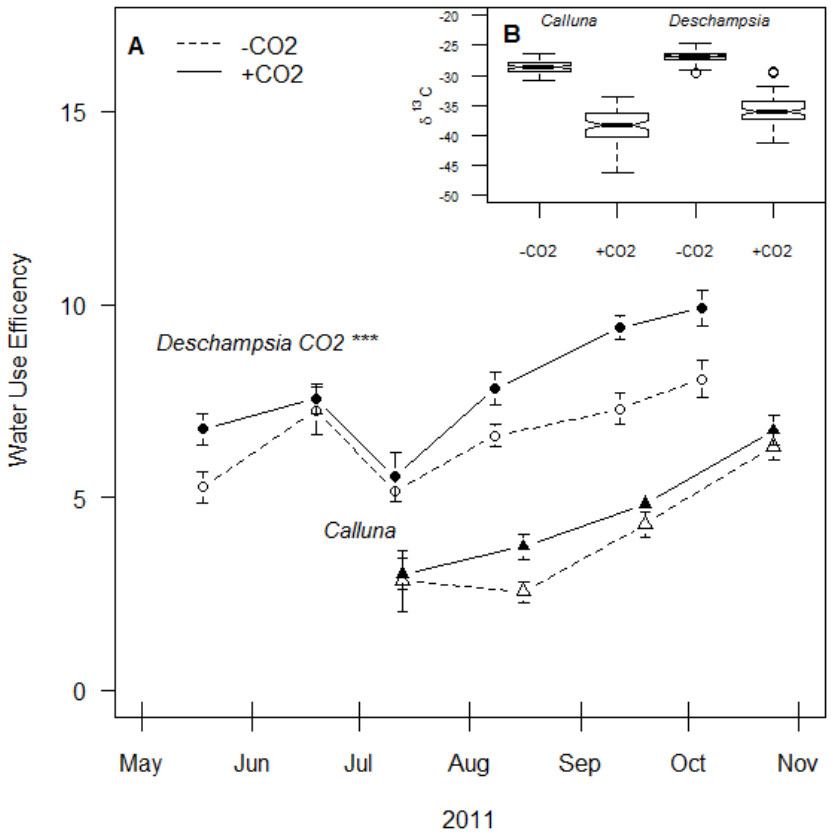

Figure 3. Seasonal responses to elevated CO_2 treatment on water use efficiency (WUE) and $\delta^{13}C$ in both *Deschampsia flexuosa* and *Calluna vulgaris*. A) Mean ± SE of all elevated CO_2 (solid line) and ambient CO_2 plot (dotted line) in *Deschampsia* (circles) and *Calluna* (triangles), n=24. B) Means of all $\delta^{13}C$ in elevated CO_2 and ambient CO_2 plot, in *Deschampsia* and *Calluna*, respectively.

Table 3. For each month, the mean SE (n=6) for the two species *Deschampsia flexuosa* and *Calluna vulgaris* in elevated CO_2 (CO2), night time warming(T), drought (D) and their combinations. Specific leaf area (SLA; g cm^{-2}), leaf nitrogen content (N %), leaf carbon to nitrogen ration (C/N) and leaf $\delta^{13}C$(‰).

Variable		A	CO2	T	D	TCO2	DCO2	TD	TDCO2
Deschampsia flexuosa									
SLA	May	7.57 ± 0.30	6.51 ± 0.37	7.69 ± 0.43	7.32 ± 0.25	5.83 ± 0.26	6.26 ± 0.67	8.26 ± 0.74	6.51 ± 0.19
	June	7.45 ± 1.19	6.37 ± 0.48	5.54 ± 0.59	6.86 ± 0.59	6.63 ± 0.45	5.93 ± 0.20	5.51 ± 0.46	6.55 ± 0.41
	July	5.78 ± 0.18	5.73 ± 0.20	5.94 ± 0.43	6.73 ± 0.40	5.70 ± 0.32	5.94 ± 0.31	7.20 ± 0.69	6.21 ± 0.49
	August	7.10 ± 0.27	6.10 ± 0.36	5.63 ± 0.50	6.57 ± 0.18	6.54 ± 0.49	6.50 ± 0.37	7.77 ± 0.76	7.37 ± 0.50
	September	8.60 ± 0.97	7.17 ± 0.49	8.34 ± 0.90	8.23 ± 0.49	7.16 ± 0.18	7.45 ± 0.57	8.21 ± 0.88	6.97 ± 0.32
	October	8.23 ± 0.38	7.71 ± 0.48	8.60 ± 0.56	9.36 ± 0.60	7.72 ± 0.58	7.32 ± 0.25	9.78 ± 0.24	8.12 ± 0.47
N %	May	1.86 ± 0.11	1.84 ± 0.12	1.99 ± 0.20	1.98 ± 0.15	1.68 ± 0.10	1.60 ± 0.16	1.96 ± 0.23	1.54 ± 0.16
	June	1.91 ± 0.25	1.35 ± 0.13	1.55 ± 0.17	1.94 ± 0.21	1.37 ± 0.08	1.69 ± 0.17	1.56 ± 0.13	1.53 ± 0.11
	July	1.69 ± 0.17	1.43 ± 0.06	1.76 ± 0.13	2.36 ± 0.19	1.44 ± 0.12	1.55 ± 0.10	2.01 ± 0.13	1.71 ± 0.16
	August	1.88 ± 0.21	1.46 ± 0.09	1.60 ± 0.12	1.73 ± 0.07	1.63 ± 0.10	1.66 ± 0.11	1.94 ± 0.07	1.76 ± 0.14
	September	1.90 ± 0.07	1.59 ± 0.09	2.23 ± 0.11	1.91 ± 0.10	1.65 ± 0.04	1.53 ± 0.09	2.04 ± 0.20	1.54 ± 0.06
	October	2.10 ± 0.13	1.91 ± 0.19	2.20 ± 0.14	2.11 ± 0.15	1.94 ± 0.18	1.75 ± 0.15	2.63 ± 0.18	1.62 ± 0.17
C/N	May	25.25 ± 1.39	25.52 ± 1.91	24.34 ± 2.40	24.02 ± 1.67	27.78 ± 1.53	29.79 ± 2.57	25.42 ± 3.29	31.30 ± 3.17
	June	26.39 ± 1.27	31.51 ± 2.86	31.02 ± 2.81	24.78 ± 2.55	33.67 ± 2.31	27.80 ± 2.12	30.55 ± 2.27	30.18 ± 2.22
	July	28.47 ± 2.65	31.90 ± 1.18	26.93 ± 1.96	20.04 ± 1.45	32.22 ± 2.14	30.05 ± 1.99	22.91 ± 1.22	27.58 ± 2.47
	August	25.55 ± 2.11	31.83 ± 2.16	29.72 ± 2.42	26.52 ± 1.02	28.41 ± 1.45	27.95 ± 1.63	24.03 ± 0.82	26.58 ± 1.86
	September	21.52 ± 0.81	29.25 ± 1.58	21.02 ± 1.11	24.60 ± 1.36	27.81 ± 0.75	30.37 ± 1.77	23.67 ± 2.36	29.87 ± 1.26
	October	22.30 ± 1.34	25.86 ± 3.27	21.54 ± 1.42	19.72 ± 1.34	24.70 ± 2.16	27.37 ± 2.57	18.02 ± 1.18	29.98 ± 3.03
$\delta^{13}C$	May	-27.40 ± 0.33	-35.11 ± 0.82	-27.66 ± 0.43	-27.66 ± 0.09	-34.81 ± 0.91	-34.85 ± 0.53	-28.15 ± 0.33	-35.42 ± 0.57
	June	-26.91 ± 0.26	-35.56 ± 0.87	-26.96 ± 0.23	-26.56 ± 0.20	-36.89 ± 1.21	-35.07 ± 0.98	-27.02 ± 0.20	-35.55 ± 1.67
	July	-26.22 ± 0.28	-35.99 ± 1.07	-26.96 ± 0.42	-27.00 ± 0.29	-36.22 ± 0.86	-34.30 ± 0.69	-27.16 ± 0.16	-35.42 ± 1.70
	August	-26.97 ± 0.26	-35.44 ± 0.64	-26.45 ± 0.32	-26.48 ± 0.35	-36.98 ± 0.80	-35.65 ± 1.28	-27.02 ± 0.48	-35.83 ± 1.51
	September	-26.92 ± 0.36	-36.47 ± 0.89	-26.77 ± 0.26	-27.05 ± 0.37	-37.49 ± 0.60	-35.63 ± 0.52	-27.11 ± 0.52	-38.10 ± 1.11
	October	-27.10 ± 0.21	-36.92 ± 0.64	-27.42 ± 0.32	-28.03 ± 0.33	-38.14 ± 0.72	-37.14 ± 0.81	-28.34 ± 0.47	-36.11 ± 1.00
Calluna vulgaris									
SLA	July	5.73 ± 0.31	5.24 ± 0.58	5.89 ± 0.34	5.55 ± 0.31	4.54 ± 0.16	5.58 ± 0.46	8.41 ± 2.17	5.11 ± 0.57
	August	6.44 ± 0.39	7.14 ± 0.76	7.34 ± 0.32	6.60 ± 0.38	6.02 ± 0.39	6.00 ± 0.46	7.28 ± 0.85	5.80 ± 0.52
	September	5.50 ± 0.43	5.25 ± 0.36	5.29 ± 0.46	6.45 ± 0.36	5.92 ± 0.39	6.11 ± 0.69	5.18 ± 0.62	5.56 ± 0.42
	October	5.32 ± 0.69	4.26 ± 0.15	4.62 ± 0.27	4.84 ± 0.63	4.92 ± 0.30	4.65 ± 0.07	4.37 ± 0.18	4.22 ± 0.15
N %	July	1.74 ± 0.16	1.42 ± 0.07	1.48 ± 0.05	1.74 ± 0.10	1.23 ± 0.05	1.60 ± 0.14	1.81 ± 0.29	1.19 ± 0.08
	August	2.10 ± 0.10	2.08 ± 0.16	2.19 ± 0.14	2.18 ± 0.14	2.32 ± 0.04	2.01 ± 0.14	2.14 ± 0.13	1.88 ± 0.21
	September	1.99 ± 0.13	1.88 ± 0.10	2.02 ± 0.12	2.12 ± 0.09	1.75 ± 0.04	1.69 ± 0.10	1.88 ± 0.07	1.92 ± 0.14
	October	1.52 ± 0.06	1.51 ± 0.05	1.57 ± 0.06	1.60 ± 0.05	1.58 ± 0.05	1.40 ± 0.04	1.62 ± 0.04	1.40 ± 0.04
CN ratio	July	29.89 ± 2.67	36.07 ± 1.90	35.00 ± 0.99	29.51 ± 1.80	41.60 ± 1.69	32.63 ± 2.59	29.70 ± 3.78	44.63 ± 3.57
	August	21.83 ± 1.26	25.12 ± 1.81	23.93 ± 1.39	23.92 ± 1.58	21.88 ± 0.44	25.69 ± 1.60	21.50 ± 1.53	30.00 ± 5.18
	September	25.95 ± 1.78	27.24 ± 1.23	25.39 ± 1.62	23.91 ± 0.99	29.11 ± 0.67	30.10 ± 1.57	27.56 ± 1.15	27.34 ± 2.07
	October	33.31 ± 1.43	33.42 ± 1.16	32.49 ± 1.25	31.42 ± 0.87	32.08 ± 1.06	36.17 ± 1.09	31.29 ± 1.01	36.13 ± 1.28
$\delta^{13}C$	July	-29.47 ± 0.51	-37.52 ± 1.34	-28.99 ± 0.21	-28.50 ± 0.37	-38.72 ± 2.15	-37.85 ± 1.64	-30.03 ± 0.50	-37.10 ± 0.87
	August	-29.05 ± 0.33	-38.81 ± 1.28	-28.85 ± 0.29	-28.72 ± 0.54	-40.65 ± 1.45	-38.77 ± 0.81	-29.45 ± 0.61	-39.09 ± 0.55
	September	-29.49 ± 0.34	-40.08 ± 0.97	-28.96 ± 0.40	-29.47 ± 0.26	-41.55 ± 1.39	-38.93 ± 0.79	-29.43 ± 0.46	-39.50 ± 1.47
	October	-29.09 ± 0.50	-38.28 ± 1.12	-28.98 ± 0.52	-29.59 ± 0.44	-40.24 ± 1.85	-38.02 ± 0.60	-29.36 ± 0.37	-38.90 ± 1.34

Table 4. For each month, the mean SE (n=6) for *Deschampsia flexuosa* in elevated CO_2 (CO2), night time warming (T), drought (D) and their combinations. Light-saturated photosynthesis (A_{sat}), dark respiration rate (R_D), maxima carboxylation rate (V_{cmax}), maximal rate of RuBP regeneration (J_{max}), light- & CO_2-saturated photosynthesis (A_{max}), stomatal conductance (g_s), water use efficiency (WUE), intercellular CO_2 concentration (c_i).

Variable		A	CO2	T	D	TCO2	DCO2	TD	TDCO2
A_{sat}	May	17.67 ± 2.47	15.46 ± 2.09	13.16 ± 2.73	11.85 ± 1.44	17.83 ± 1.85	20.94 ± 2.33	14.23 ± 4.56	18.97 ± 2.29
	June	18.39 ± 2.53	17.21 ± 1.88	13.27 ± 3.39	16.94 ± 2.16	18.07 ± 3.56	17.72 ± 0.42	21.91 ± 1.63	18.23 ± 3.49
	July	18.61 ± 2.35	19.63 ± 3.90	17.65 ± 1.34	25.18 ± 1.53	16.93 ± 1.15	19.89 ± 2.62	20.40 ± 1.90	16.90 ± 0.93
	August	18.83 ± 1.58	22.86 ± 3.35	19.93 ± 1.45	17.26 ± 0.75	21.41 ± 2.08	22.32 ± 2.81	18.50 ± 2.50	21.21 ± 1.96
	September	17.85 ± 2.55	21.84 ± 1.68	22.32 ± 3.65	18.34 ± 1.53	23.91 ± 1.47	22.90 ± 2.98	22.05 ± 3.16	22.09 ± 2.25
	October	20.71 ± 0.69	21.86 ± 1.22	21.69 ± 1.24	14.77 ± 2.41	21.81 ± 1.51	20.90 ± 1.68	16.25 ± 2.10	20.47 ± 2.51
R_D	May	5.08 ± 0.80	3.95 ± 0.20	4.76 ± 0.82	4.38 ± 0.87	4.54 ± 0.31	3.99 ± 0.18	4.94 ± 0.91	3.47 ± 0.80
	June	2.11 ± 0.46	1.97 ± 0.79	2.18 ± 0.47	2.12 ± 1.03	1.79 ± 0.27	0.82 ± 0.54	2.51 ± 0.58	1.90 ± 0.74
	July	2.86 ± 0.59	2.68 ± 1.29	2.82 ± 0.38	2.59 ± 0.45	2.94 ± 0.21	2.52 ± 0.65	2.36 ± 0.43	2.96 ± 1.92
	August	2.38 ± 0.17	2.64 ± 0.38	2.87 ± 0.38	2.43 ± 0.31	2.79 ± 0.56	2.66 ± 0.27	2.08 ± 0.62	2.27 ± 0.25
	September	1.77 ± 0.18	1.68 ± 0.30	2.20 ± 0.28	1.77 ± 0.22	1.76 ± 0.11	1.68 ± 0.23	1.89 ± 0.35	1.84 ± 0.21
	October	1.45 ± 0.37	1.50 ± 0.12	1.69 ± 0.24	1.29 ± 0.07	1.93 ± 0.16	1.68 ± 0.17	1.41 ± 0.12	1.52 ± 0.19
V_{cmax}	May	98.47 ± 19.50	93.94 ± 7.59	85.09 ± 15.06	66.44 ± 4.29	71.93 ± 8.56	90.45 ± 9.43	71.55 ± 12.00	75.11 ± 8.54
	June	84.35 ± 6.12	69.99 ± 4.99	65.62 ± 10.99	84.25 ± 7.00	91.64 ± 8.88	69.39 ± 9.19	84.92 ± 9.75	98.59 ± 18.07
	July	105.47 ± 2.77	71.27 ± 13.77	107.98 ± 17.15	122.01 ± 13.63	72.89 ± 4.45	88.85 ± 8.91	111.56 ± 2.82	62.70 ± 18.33
	August	86.78 ± 8.05	81.90 ± 14.23	93.43 ± 4.79	84.23 ± 7.57	76.92 ± 13.33	87.08 ± 9.19	85.01 ± 19.83	85.24 ± 4.74
	September	72.63 ± 9.47	60.61 ± 6.11	72.73 ± 11.12	71.39 ± 6.80	66.08 ± 3.14	65.61 ± 1.29	79.11 ± 10.31	66.40 ± 3.99
	October	71.17 ± 4.56	56.71 ± 2.84	69.91 ± 6.05	57.38 ± 5.47	57.36 ± 3.32	54.57 ± 2.81	56.10 ± 5.00	55.27 ± 6.42
J_{max}	May	172.97 ± 28.36	184.41 ± 21.09	171.18 ± 22.37	146.02 ± 8.74	136.25 ± 20.82	175.77 ± 15.45	154.80 ± 3.97	139.70 ± 38.53
	June	166.10 ± 17.56	113.98 ± 13.35	132.23 ± 23.43	183.03 ± 11.55	164.02 ± 19.60	137.50 ± 7.75	176.61 ± 26.11	180.60 ± 33.57
	July	178.22 ± 13.57	142.63 ± 31.37	167.39 ± 18.31	172.92 ± 25.62	135.15 ± 2.30	157.41 ± 20.88	173.14 ± 7.30	119.44 ± 66.68
	August	153.45 ± 9.01	153.91 ± 9.84	175.42 ± 18.23	162.01 ± 13.68	163.16 ± 12.37	169.36 ± 22.27	149.43 ± 16.72	153.79 ± 9.83
	September	159.11 ± 8.18	169.95 ± 15.54	174.02 ± 18.71	161.25 ± 4.75	172.15 ± 5.68	174.06 ± 8.53	173.04 ± 15.63	163.85 ± 10.71
	October	186.90 ± 10.28	151.84 ± 4.92	187.43 ± 15.77	165.34 ± 11.47	166.88 ± 17.55	148.04 ± 11.19	155.46 ± 6.01	146.99 ± 12.20
A_{max}	May	35.08 ± 5.12	32.89 ± 4.12	31.00 ± 4.02	28.40 ± 2.02	31.29 ± 3.78	33.42 ± 3.16	29.24 ± 2.31	26.39 ± 5.74
	June	37.45 ± 4.56	26.25 ± 2.12	28.72 ± 6.60	37.26 ± 3.35	31.79 ± 4.29	29.45 ± 1.66	43.82 ± 3.02	33.73 ± 6.67
	July	41.60 ± 3.79	29.67 ± 6.53	31.48 ± 4.80	38.55 ± 1.39	26.55 ± 1.09	30.78 ± 3.61	41.09 ± 4.22	20.49 ± 9.07
	August	32.50 ± 2.03	36.16 ± 4.42	39.80 ± 4.17	33.01 ± 3.27	34.17 ± 2.31	35.16 ± 4.91	36.07 ± 5.50	31.94 ± 2.35
	September	33.86 ± 2.67	35.19 ± 2.50	40.77 ± 5.19	34.72 ± 1.73	37.27 ± 1.33	33.47 ± 4.26	38.21 ± 3.92	33.84 ± 2.64
	October	39.39 ± 2.00	32.86 ± 1.24	40.92 ± 3.21	30.85 ± 5.32	35.85 ± 3.87	32.44 ± 2.14	33.25 ± 1.78	31.30 ± 3.07
g_s	May	0.23 ± 0.06	0.12 ± 0.03	0.20 ± 0.05	0.13 ± 0.02	0.14 ± 0.02	0.19 ± 0.02	0.16 ± 0.06	0.16 ± 0.01
	June	0.23 ± 0.03	0.16 ± 0.03	0.16 ± 0.05	0.17 ± 0.03	0.16 ± 0.04	0.16 ± 0.02	0.28 ± 0.04	0.17 ± 0.05
	July	0.21 ± 0.03	0.19 ± 0.03	0.19 ± 0.02	0.33 ± 0.03	0.22 ± 0.03	0.27 ± 0.10	0.26 ± 0.03	0.30 ± 0.17
	August	0.26 ± 0.02	0.25 ± 0.05	0.24 ± 0.03	0.24 ± 0.01	0.21 ± 0.02	0.25 ± 0.05	0.21 ± 0.03	0.21 ± 0.03
	September	0.25 ± 0.02	0.27 ± 0.03	0.32 ± 0.06	0.25 ± 0.02	0.32 ± 0.05	0.28 ± 0.05	0.32 ± 0.07	0.23 ± 0.05
	October	0.46 ± 0.08	0.30 ± 0.06	0.40 ± 0.05	0.31 ± 0.07	0.28 ± 0.05	0.29 ± 0.05	0.23 ± 0.02	0.26 ± 0.05
WUE	May	5.43 ± 0.29	7.28 ± 1.00	3.96 ± 0.33	5.81 ± 0.89	6.85 ± 1.04	6.49 ± 0.39	5.44 ± 1.34	6.21 ± 1.02
	June	6.84 ± 1.41	7.91 ± 0.20	7.82 ± 1.10	9.15 ± 2.45	7.51 ± 1.18	8.03 ± 0.58	6.09 ± 0.33	6.91 ± 0.80
	July	5.31 ± 0.44	6.18 ± 0.93	5.30 ± 0.49	5.21 ± 0.50	4.45 ± 0.80	6.48 ± 1.50	4.83 ± 0.63	4.98 ± 1.28
	August	6.06 ± 0.29	8.95 ± 1.01	7.14 ± 0.68	6.11 ± 0.64	7.82 ± 1.01	7.08 ± 0.86	7.08 ± 0.64	7.44 ± 0.42
	September	6.56 ± 0.47	9.75 ± 0.54	7.75 ± 1.37	7.13 ± 0.28	8.85 ± 0.77	9.63 ± 0.34	7.76 ± 0.85	9.51 ± 0.80
	October	7.51 ± 0.73	9.22 ± 1.11	7.94 ± 0.53	7.11 ± 1.09	10.27 ± 0.74	9.63 ± 1.03	9.48 ± 1.23	10.45 ± 0.98
c_i	May	235.37 ± 17.95	230.09 ± 50.36	262.93 ± 18.56	226.17 ± 8.97	251.38 ± 45.73	306.41 ± 4.08	225.77 ± 14.98	299.74 ± 16.00
	June	243.67 ± 7.04	307.45 ± 16.49	208.85 ± 26.82	207.97 ± 7.44	292.98 ± 25.42	308.87 ± 20.69	235.23 ± 10.46	299.88 ± 17.53
	July	224.33 ± 5.82	322.55 ± 9.73	223.05 ± 2.46	237.92 ± 8.09	350.68 ± 24.22	315.32 ± 38.13	235.77 ± 9.38	330.10 ± 54.62
	August	250.89 ± 10.54	328.76 ± 13.59	233.03 ± 9.46	250.58 ± 10.41	322.18 ± 5.21	332.83 ± 17.87	229.90 ± 8.37	315.23 ± 14.79
	September	257.13 ± 15.90	358.98 ± 7.52	251.63 ± 6.17	252.21 ± 10.09	355.36 ± 13.13	345.13 ± 12.61	244.72 ± 13.58	323.53 ± 13.38
	October	287.40 ± 13.76	357.81 ± 11.11	274.41 ± 12.98	287.78 ± 22.80	341.91 ± 22.58	359.51 ± 15.44	255.16 ± 14.40	346.78 ± 11.66

Table 5. For each month, the mean SE (n=6) for ***Calluna vulgaris*** in elevated CO_2 (CO2), night time warming (T), drought (D) and their combinations. Light-saturated photosynthesis (A_{sat}), dark respiration rate (R_D), maxima carboxylation rate (V_{cmax}), maximal rate of RuBP regeneration (J_{max}), light- & CO_2-saturated photosynthesis (A_{max}), stomatal conductance (g_s), water use efficiency (*WUE*), intercellular CO_2 concentration (c_i).

Variable		A	CO2	T	D	TCO2	DCO2	TD	TDCO2
A_{sat}	July	18.00 ± 0.65	13.85 ± 2.56	11.41 ± 2.33	9.78 ± 3.06	18.45 ± 2.28	13.58 ± 5.28	3.81 ± 0.11	12.69 ± 4.64
	August	12.80 ± 3.11	15.39 ± 2.51	10.90 ± 2.78	12.19 ± 2.10	22.87 ± 1.74	15.00 ± 2.00	9.48 ± 1.76	19.23 ± 3.58
	September	29.58 ± 4.97	27.98 ± 2.04	21.43 ± 3.33	17.67 ± 3.21	23.07 ± 2.18	27.77 ± 4.10	21.16 ± 1.02	25.76 ± 2.27
	October	13.96 ± 1.95	17.55 ± 2.13	12.65 ± 2.36	12.63 ± 2.00	16.67 ± 1.63	15.18 ± 1.08	13.66 ± 1.98	20.78 ± 1.19
R_D	July	4.01 ± 1.05	4.15 ± 1.58	4.23 ± 0.49	4.73 ± 0.54	5.80 ± 1.64	3.57 ± 0.06	5.27 ± 1.56	4.16 ± 0.71
	August	8.71 ± 0.38	7.04 ± 0.87	8.16 ± 0.43	6.05 ± 0.34	7.34 ± 1.33	8.29 ± 0.57	5.65 ± 1.26	6.99 ± 1.29
	September	4.52 ± 0.39	4.20 ± 0.70	5.02 ± 0.39	4.43 ± 0.26	4.22 ± 0.91	4.36 ± 1.21	5.03 ± 0.49	4.91 ± 0.81
	October	2.51 ± 0.49	1.63 ± 0.13	2.08 ± 0.46	2.09 ± 0.51	1.18 ± 0.13	1.07 ± 0.13	1.65 ± 0.34	1.57 ± 0.15
V_{cmax}	July	87.62 ± 0.50	63.72 ± 15.13	67.11 ± 14.50	48.34 ± 11.54	80.61 ± 12.03	63.83 ± 24.79	66.58 ± 13.22	48.74 ± 4.47
	August	67.21 ± 18.32	71.23 ± 9.45	70.36 ± 9.84	75.14 ± 15.15	92.88 ± 15.45	79.29 ± 9.84	52.71 ± 6.89	83.56 ± 14.83
	September	100.44 ± 12.58	72.51 ± 4.01	75.12 ± 11.60	75.62 ± 1.27	62.87 ± 7.01	73.77 ± 10.84	80.69 ± 5.60	69.30 ± 5.36
	October	44.19 ± 4.97	33.52 ± 2.76	34.59 ± 4.15	31.92 ± 3.91	29.48 ± 2.88	27.87 ± 2.05	33.67 ± 3.61	36.89 ± 2.98
J_{max}	July	168.71 ± 6.03	134.66 ± 19.73	106.39 ± 3.69	132.83 ± 25.39	168.06 ± 17.91	175.06 ± 50.65	159.98 ± 12.18	141.28 ± 8.57
	August	148.17 ± 35.28	149.94 ± 12.64	164.20 ± 20.66	151.13 ± 25.23	204.88 ± 24.30	181.64 ± 17.02	122.81 ± 11.58	178.71 ± 26.27
	September	228.44 ± 17.84	193.20 ± 11.71	181.78 ± 18.02	180.61 ± 9.85	165.87 ± 15.75	189.91 ± 20.82	195.88 ± 13.18	182.12 ± 13.81
	October	114.72 ± 14.97	110.38 ± 8.13	106.78 ± 22.19	109.87 ± 12.22	100.75 ± 5.04	92.03 ± 5.55	110.36 ± 7.49	121.96 ± 8.44
A_{max}	July	29.95 ± 1.11	21.73 ± 4.26	18.38 ± 2.75	23.12 ± 4.76	30.62 ± 2.46	25.36 ± 10.01	18.39 ± 5.82	25.42 ± 1.94
	August	25.78 ± 7.60	27.84 ± 3.20	29.56 ± 4.66	21.97 ± 1.71	38.10 ± 3.28	29.63 ± 2.05	23.03 ± 2.03	32.51 ± 4.58
	September	N.D.	39.38 ± 2.19	32.95 ± 3.73	30.04 ± 4.09	32.45 ± 2.59	33.22 ± 2.93	36.91 ± 1.98	35.70 ± 2.42
	October	22.90 ± 3.76	25.94 ± 2.40	22.81 ± 4.66	24.38 ± 3.21	23.05 ± 1.79	21.76 ± 1.46	24.07 ± 2.51	27.86 ± 1.61
g_s	July	0.72 ± 0.13	0.49 ± 0.08	0.41 ± 0.05	0.42 ± 0.11	0.45 ± 0.04	0.69 ± 0.16	0.67 ± 0.12	0.52 ± 0.11
	August	0.70 ± 0.12	0.30 ± 0.07	0.56 ± 0.07	0.50 ± 0.13	0.57 ± 0.06	0.53 ± 0.09	0.58 ± 0.12	0.52 ± 0.08
	September	0.79 ± 0.20	0.75 ± 0.06	0.74 ± 0.06	0.57 ± 0.08	0.64 ± 0.07	0.69 ± 0.12	0.69 ± 0.17	0.68 ± 0.06
	October	0.50 ± 0.14	0.45 ± 0.11	0.39 ± 0.12	0.32 ± 0.08	0.54 ± 0.09	0.41 ± 0.07	0.41 ± 0.09	0.51 ± 0.12
WUE	July	2.65 ± 0.81	3.47 ± 0.98	2.20 ± 0.44	4.25 ± 2.59	3.19 ± 0.59	2.02 ± 0.58	1.02 ± 0.00	3.11 ± 1.31
	August	2.49 ± 0.63	5.17 ± 0.76	2.81 ± 0.63	2.47 ± 0.19	3.77 ± 0.38	2.51 ± 0.29	2.35 ± 0.73	3.46 ± 0.61
	September	5.31 ± 0.71	5.40 ± 0.15	4.10 ± 0.58	4.32 ± 0.70	4.51 ± 0.52	4.85 ± 0.21	4.02 ± 0.52	4.76 ± 0.43
	October	6.10 ± 0.96	7.32 ± 1.01	6.13 ± 0.81	7.04 ± 0.71	6.23 ± 0.96	6.53 ± 0.35	5.96 ± 0.57	7.08 ± 0.55
c_i	July	330.49 ± 8.78	429.28 ± 22.88	326.87 ± 17.24	297.54 ± 59.37	414.97 ± 14.87	460.17 ± 13.14	372.18 ± 2.99	434.29 ± 34.09
	August	341.90 ± 14.04	400.74 ± 9.32	341.04 ± 11.18	334.32 ± 5.31	417.36 ± 10.87	441.83 ± 5.55	340.98 ± 17.10	423.68 ± 12.85
	September	308.23 ± 4.31	428.95 ± 1.03	325.06 ± 8.85	323.89 ± 13.28	430.10 ± 4.26	422.25 ± 1.67	314.27 ± 15.87	428.00 ± 3.51
	October	324.43 ± 12.63	415.33 ± 21.80	286.02 ± 42.41	309.31 ± 7.62	436.25 ± 14.42	428.44 ± 12.11	319.11 ± 7.94	415.52 ± 14.47

Discussion

Experimental and methodological considerations

Outdoor experiments running over long period demands a high level of maintaining and therefore treatment functionality should always be questioned. At the time where the present study was carried out the CO_2 enrichment (FACE) had been running for more than six years with only few unintended and planned down-periods. CO_2 enrichment was shot off during nighttime and in period with high wind and snow cover (Miglietta *et al.*, 2001). During the present study plants within CO_2 enrichment experienced the target CO_2 concentration of 510 ppm as hourly median during daytime (data not shown).

Passive nighttime warming performed indeed increased the average nighttime temperature, however less than expected. Across the entire experiment the warming on average increased the temperature with +1.2°C with highest increases during spring and summer. When solar input are high and minimum temperature during nighttime go just below 0°C the warming treatment increased the nighttime air temperature with up to 4 °C and reduced the amount of night-frost events. Under certain conditions air temperature was increased 3-5 hours after sunrise, however often less (Mikkelsen *et al.*, 2008) and a possible stimulating of photosynthesis could happen in such periods (Sage & Kubien, 2007). Methods for improving the performance of nighttime warming systems are given in Bruhn et al., (2013).

The warming treatment increased the amount of growing degree days (GDD) due to a reduction of nights with frost (Mikkelsen et al. 2008). In the spring of the third year of treatment (April-May 2007), significantly higher biomass of *Deschampsia* in combination with an increased photosynthetic capacity within warmed plot, support that warming treatment influenced the onset of growing season. Nevertheless, the earlier growing onset did not increased the total biomass later in the season and the effects on photosynthetic capacity also disappeared (Kongstad et al., 2011; Albert et al., 2011c). *Calluna* was on the other hand not stimulated to initiate early growth in the warmed plots and in general nighttime warming did only influence *Deschampsia* minimally (Kongstad *et al.*, 2012). In the present study measurement were conducted from May – October 2011 and none stimulation of the photosynthetic capacity was found, thus it can be argued that the early onset of the growing season have been present earlier or lacked due to dry spring condition observed in February- May 2011 (discussed later).

Beside direct effect of warming increased night-time temperature has been reported to increase night-time leaf respiration and even decreasing the net photosynthesis in the hours after sunrise (e.g. Griffin et al., 2002; Turnbull et al. 2002 & 2004). In the present study all measurement were conducted in the timespan two hours after sunrise and two hours before sunset, thus neither

negative nor positive stimulation on respiration or photosynthesis was expected or found. Only in combination with elevated CO_2 or/and experimental drought warming was found to influence the response. Thus, single factor effects of warming will not be discussed further.

The third treatment within the CLIMAITE experiment is exclusion of precipitation in selected periods. The first experimental drought was performed in July - August within the second year and resulted in an exclusion of c.11% of the total annual precipitation. Following year's precipitation exclusion was mainly performed in spring and the soil water content never went down to minimum as within the first period (Table 1). Natural heavy rain events with in the experimental drought (2008 and 2009) forced treatments to be stopped in risk of material destruction of the setup. In these years drought periods performed twice in the season and both time treatment resulted in an exclusion of c. 5% of annual precipitation. Summarizing, drought treatment was successful in all years of the experiment.

At a shorter time scale (day – months) plant reallocation of nutrients can occur to longer environmental changes; like the experimental treatment in the present study, resulting in a physiological acclimation (Albert et al., 2011a/b/c; Ainsworth & Long, 2005; Ainsworth & Rogers, 2007). At a longer time scale (months- years) ecosystem feedbacks (as described by Körner, 2006) to long term environmental changes can reduce soil water and nutrient availably causing further plant physiological acclimating. . Ecosystem responses are slow and even though they in many cases are induced by faster plant physiological processes, feedback process from the ecosystem back to the leaf can be expected to be slow and first be seen in long term perspective (e.g. Körner, 2006: Ainsworth et al., 2003).

Long term responses on the plant physiology to climate change experiment, CLIMAITE, was in general found to be similar to findings within the second year of treatments. However, inter-annual variation within the two study periods influences the magnitude of the leaf level responses on both *Deschampsia* and *Calluna*.

Physiological performance of Deschampsia

The two co-occurring species at the experimental site, *Deschampsia* and *Calluna* belong to two different function types and respond differently to environmental changes (Albert et al., 2012). *Deschampsia* is an opportunistic grass, with a variable number of leaves per shoot and a maximal leaf lifespan less than a year (Gimingham, 1960; Aerts et al., 1990; Jackson et al., 1999). *Deschampsia* can adjust leaf nitrogen and redistribute nitrogen between leaves and rhizomes (Andresen & Michelsen, 2005; Andresen et al. 2009; Nielsen et al, 2009). The opportunistic life strategy includes the capability fast to reallocate importance nutrient and water resource (Jackson et al., 1999).

Effects of elevated CO_2

Growth in elevated CO_2 is known to effect grass physiology more and aboveground biomass less compared to slow growing scrubs and trees (Ainsworth & Long, 2005; Nowak et al., 2004). After six years of treatment *Deschampsia* still meet the hypothesis that growth in elevated CO_2 led to down regulated photosynthetic capacity; as a reduction in the maximum carboxylation rate (V_{cmax}) and in the maximum light and CO_2 saturated photosynthesis (A_{max}). However, as it was found within the second year (Albert et al., 2011b) photosynthetic stimulation was no longer significant in elevated CO_2, but only tended to be higher. Stomatal conductance was not found to be reduced in the second year (Albert et al., 2011b), but in the present study we saw a tend towards lower stomatal conductance. Reduced stomatal conduction can lead to homeostasis, where plants grown at elevated CO_2 has similar intercellular CO_2 concentration (c_i), as plants growth at ambient atmospheric CO_2 (Campbell *et al.*, 2007). During the study conducted in the second year soil water content was ca. 50% lower across the growing season compared to the year of the present study. In low soil water content, stomatal conductance is decreased in all treatments and the differences to the treatment of elevated CO2 becomes smaller (Albert et al., 2011b). This might explains the absence of response on stomatal conductance in elevated CO_2 within the second year. Water use efficiency was significantly improved in elevated CO_2 across the sixth growing season of the experiment. The improvement of WUE was not the result of a slightly higher photosynthesis (n.s., p=0.09) and reduced stomatal conductance (n.s., p=0.07), conflicting with the results from the second year (Albert et al., 2011b),.WUE continually increased across the growing season and can partly be explained by a higher stimulation of photosynthesis in late season and new emerging leaves from the second leaf flush after flowering and increased soil water in July (see Albert et al., 2012).

Reduced amount of leaf nitrogen is in line with other studies and was also found with in the second year (e.g. Albert et al., 2011 a/b/c; Larsen et al., 2011). However as pointed in Albert et al. (2011b) the reduced N was not found per leaf area indicating a reallocation more than a dilution, consistent with the opportunistic growth form (Andresen & Michelsen, 2005; Andresen et al. 2009; Nielsen et al, 2009). Leaves in elevated CO_2 were thicker after six years of treatments contradicting finding from the second year (Albert et al., 2011b), which we suggest can be explained by the long term extra carbon available and buildup of energy over the extra treatment years.

Effects of prolonged drought

It is a general opinion that water shortage induces down-regulation of photosynthesis, stomatal conductance and photosynthetic capacity and *Deschampsia* shows the responses in the second year of treatment (Albert et al 2011b). However, in the 6 year of treatment *Deschampsia* did not down-regulate the photosynthetic capacity in relation to the experimental drought. Albert et al. (2012) points out that under mild to modern drought, *Deschampsia*, are able to remain both stomatal conductance

and photosynthetic capacity, reducing the aboveground leaf biomass. As water shortage increases within a dry period, leaf dieback induces osmotic adjustment in remaining leaves and strongly improving WUE (Verslues et al., 2006). In line, WUE is increased within experimental drought period, causing the higher WUE seen in May and June (figure 3). On the other hand, the magnitude of photosynthesis was higher than the decrease in stomatal conductance increasing the WUE in later season. Under severe drought as the first experimental period, the reduced leaf biomass was sufficient enough to main photosynthesis, thus photosynthetic capacity were reduced via decreased stomatal conductance (Albert et al 2011b). In the sixth year the experimental drought was not as severe as in the second year. Thus leaf biomass reductions caused maintain photosynthetic capacity, stomatal conduction and net carbon uptake during the drought.

Interactive effects of warming, drought and elevated CO_2

In the second treatment year different interaction between the single treatments has been reported for several ecophysiological parameters and the interactive response have mainly been found to be in relation to water shortage and other environmental variables (Albert et al., 2011b/c). However in the present study mainly additive responses to treatments was found. Looking at none significant additive responses it is important to have in mind, that the magnitude of the additive response is not the true additive mean and often the effect of responses is dampened when added to each other (Leuzinger etal., 2011). As explained above warming treatment did not influence the physiological responses reported here. Only in combination with experimental drought warming synergistically decreased *Deschampsia* leaf thickness, explained by the overall lower soil water content in warmed plots and as drought as a factor decreased leaf thickness. In general, within multifactor treatments including elevated CO_2 (TCO2, DCO2 and TDCO2) *Deschampsia* responded similar as to single factor treatment of elevated CO_2.

Physiological performance of Calluna

Where *Deschampsia* is a grass with opportunistic life strategy, *Calluna* is considered of be a stress tolerant competitor, with traits adapted to periodically drought and nutrient poor habitats (Grime et al., 1988). *Calluna* are characterized having small xeromorphic leaves with a long life-span and low nitrogen. Additional *Calluna* are known to have high nutrient reabsorption and low photosynthesis (Aerts et al., 1990; Gimingham, 1960; Jackson et al., 1999). Evergreens are often stress tolerance and are likely to constrain the ability to maximize photosynthetic capacity in relation to stimulating environmental variables (Warren & Adams, 2004) .

Effects of elevated CO_2

Shrubs and trees are less responsive in optimizing their photosynthetic machinery in relation to environmental changes, thus adjustment to growth in elevated CO_2 is less pronounced in these functional types (Nowak *et al.*, 2004). Both within the presented year and the second year of the CLIMAITE experiment *Calluna* showed increases photosynthesis in elevated CO_2 (Albert et al., 2011a). However, as observed in the certain periods within the second year (Albert et al 2011c), no significant down-regulation of the photosynthetic capacity was found during the present study period. Only in single campaigns in end of the growing season photosynthetic capacity tended to be down-regulated. In the sixth year a dry and sunny winter/spring caused a giant dieback of *Calluna*. New shoots were morphological different from the old ones e.g. they were smaller, with a ca. 10% higher SLA, than leaves analyzed in the second year (Albert et al. 2011a). More over leavesprobably contained less lignin and lower shoot root ration (more root biomass available per above ground biomass). This difference might have stimulated a demand for extra carbon and energy in the new shoots and partly explaining why no down regulation was seen here compared to the second year.

Calluna leaves in elevated CO_2 grew significantly thicker and N per area leaf was unchanged. Evaluated in combination with an increased C/N ratio the reduced N is not reallocated in the system but simply diluted within thicker leaves, in line with other findings (e.g. Kongstad et al., 2012; Andresen et al. 2005; Kattge et al., 2009). The maintained N per leaf area can also explain part of the absent of down regulation of photosynthetic capacity in elevated CO_2.

Water use efficiency is often found to be increased in elevated CO_2 (e.g. Ainsworth & Long, 2005; Nowak et al., 2004) and in line *Calluna* had an improved WUE in the second year of treatment, mainly due to increased photosynthesis but also in minor degree degreased stomatal conductance (Albert et al. 2011a). Integrated soil water status as $\delta^{13}C$ were as described for *Calluna,* significantly related to WUE, which was not the case for *Deschampsia* (Albert et al., 2012). As mention earlier the soil water content in the campaigns in the second year was only half of the content in the present's year. At high soil water content stomatal conduction are high and despite stimulation in photosynthesis WUE is not significantly improved in elevated CO_2, within the present study (figure 3). However, as it was argued for *Deschampsia* increased WUE in late season is related to a smaller increase in stomatal conductance compared to the decrease in photosynthesis.

Effects of prolonged drought

The significantly reduced photosynthesis in *Calluna* in the campaign closest to the experimental drought was in accordance to the expectation from drought tolerant species. The lower photosynthesis in drought plot could not only be explained by reduced stomatal conductance as it was found in the second year of treatment (Albert et al., 2011a). The experimental drought in the present study year

was started on top of an already natural dry period, which caused a giant dieback of the *Calluna*. The dieback was not observed to differ between the different treatments, but a significantly lower green fraction of *Calluna* biomass was observed post-drought, increasing to the same level late summer (personal communication with J. Ransjin et al., unpublished). Thus a slightly decreased photosynthetic capacity observed in combination with decreased photosynthesis in July indicated a later onset of growth after the dieback.

Interactive effects of warming, drought and elevated CO_2

Combination of single factors ecophysiological responses on *Calluna* was, as for *Deschampsia*, explained by various different interactive ways in the second year of treatments. In single campaigns across the experimental period significantly interactions was found, mainly antagonistic and in line with findings explained in Albert et al. (2011a). However across season interactions was found mainly to be additive (no significant in statistic) and similar to responses to thoughts in elevated CO_2. Only during in single campaign of July photosynthetic of both DCO2 and TDCO2 was related to an additive effect of both D and CO2 counterbalance the reduced photosynthesis ending at a level close to none-treated control. Mainly driven from the response of the single campaign in July antagonistic effects of DxCO2 and TxDxCO2 were found on the stomatal conductance. The lower, not significant, stomatal conductance in elevated CO_2 and in drought neutralizes each other resulting in the same stomatal conductance as in none-treated plots. Warming treatment did in general not influence the responses we have detected during this study, and the full combinations are acting like the combination of only D and CO2.

Conclusions

After 6 years of treatment the leaf physiology data showed the same reactions as seen in the beginning of the experiment (within second year) for both of the two co-occurring species at the site. This suggests that none long term ecosystem responses were acting on leaf level physiology and this confirm our hypothesis. The investigated heathland ecosystem can be considered to be fully developed and the dieback of *Calluna* in the sixth winter of the experiment only influences aboveground biomass. Thus, long -term belowground responses was not canceled. Feedbacks from ecosystem changes that strongly can affect plant physiology are related mainly to changes in nutrient and soil water availability. Increased biomass production as a result of photosynthetic stimulation of elevated CO_2 and warming can in long term change the resource supply in the system, e.g. leading to progressive nitrogen limitation (PNL, Reich et al., 2006) or severe water shortage. Moreover, within the first three years no changes in the aboveground biomass were found in elevated CO_2 (Kongstad et al., 2012), only belowground biomass was significantly increased (M. Arndal et al. not published).

Thus, elevated CO_2 within the first years of the experiment did not induce biomass expansion much. In line Larsen et al. (2011) did not see any changes in the nitrogen cycle in elevated CO_2 and within the first years CO_2-induced stimulation of photosynthesis did not induce strong ecosystem changes. Nor or less it can be expected that after longer time the stimulated root growth in elevated CO_2 can have result in an aboveground biomass increase. Within the sixth year normalized difference vegetation index (NDVI) showed a higher green biomass of *Calluna* in elevated CO_2 plots, indicating a higher above ground biomass of *Calluna* (J. Ransjin et al, not published). In line, *Calluna* was found to be significantly stimulated in elevated CO_2 and since leaf respiration was not changed their indicating a potential higher carbon uptake. *Deschampsia* did also not show any respiratory change nor a stimulation of photosynthesis in elevated CO_2, thus no increases biomass was expected here, in line NDVI over *Deschampsia* was not found be changed (J. Ransjin et al, not published).

Climatic manipulative treatments in the CLIMAITE experiment showed that on top of seasonal and inter-annual variability elevated CO_2 were the most effective treatment influencing leaf level physiology. Extended drought strongly reduced the CO_2-induced stimulation of *Calluna* photosynthesis and caused strong leaf dieback in *Deschampsia*. Thus species growth strategies are strongly important for ecosystem responses (also evaluated in Albert et al., 2012). Warming at c. 1°C was in the present study not found to influence ecophysiology, however in the beginning of the experimental manipulation observations for an earlier on set of growing season was done (e.g. Albert et al. 2011a/b/c; Kongstad et al., 2012). Ecophysiological responses in both the second and sixth year, to combinations of treatments were mainly found to be additive and antagonistic interactions was dominating over synergistic (also in line with Larsen et al., 2011).

Leaf physiology respond rapid to environmental changes and this enable plants to acclimate fast to new environmental conditions. Daily, seasonal and inter-annual variability control the ecophysiological responses more than the climate change manipulations increases the robustness of response. In long term ecosystem feedbacks of climate change induced responses can lead to changed ecophysiology. However, six years of *in-situ* climate change manipulation have not changed a Danish heathland ecosystem strong enough to be evaluated at a leaf level scale.

Acknowledgements

The work was carried out within the CLIMAITE project, financially supported by the Villum Kann Rasmussen Foundation. Economic support has also been received from Air Liquide and DONG energy. Thanks to Preben Jørgensen, Poul T. Sørensen, Nina W. Thomsen, Andreas Fernqvist and Svend Danbæk for keeping the experiment running. Special thanks Nina W. Thomsen and Mette Flodgaard for helping in the field during the warm and wet summer. Additional thank to Anja C. Nielsen and Sabine Reinsch for the chemical analysis of leaf compounds.

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