**The Photosynthetic Acclimation Response of *Lolium perenne* to Four Years
Growth in a Free-air CO₂ Enrichment (FACE) Facility**

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MASTER

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The Photosynthetic Acclimation Response of *Lolium perenne*
to Four Years Growth in a Free-Air CO₂ Enrichment
(FACE) Facility

By

Rebecca Creasey

Report of original research submitted in partial fulfillment of the requirements for the degree of MSc in Crop Production in the Changing Environment, Department of Biology, University of Essex; and Writtle College.

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ABSTRACT

In this study, the photosynthetic responses of field grown *Lolium perenne* to ambient ($354 \mu\text{mol mol}^{-1}$) and elevated ($600 \mu\text{mol mol}^{-1}$) C_a were measured. The experiment utilised the FACE facility at Eschikon, Switzerland; here the *L. Perenne* swards had been grown at two nitrogen treatments, with six cuts per year, for 4 years.

The study revealed a significant decrease in Rubisco activity (V_{cmax}) in the low nitrogen FACE plots; this is consistent with the theories of source-sink imbalance resulting in feedback inhibition and down-regulation. Such negative acclimation was not wholly supported by diurnal investigations which revealed an average stimulation of 53.38% and 52.78% in the low and high nitrogen, respectively. However, light response curves and AI investigations also suggested down-regulation, especially in the low nitrogen. SI is expected to decrease in response to elevated C_a , if any change is seen. This was indeed observed in the high nitrogen plots but for the low nitrogen a significant increase was found.

Conclusions drawn from this project centre around the implications of negative acclimation to future crop productivity. For instance, inter-specific differences in response to elevated C_a may result in ecosystem changes and new management techniques may be necessary. However, real predictions cannot be made from leaf level studies alone as these may not represent the overall changes at the whole plant level.

KEYWORDS

Elevated CO_2 , *Lolium perenne*, FACE, photosynthesis, acclimation.

ABBREVIATIONS AND SYMBOLS

A	CO ₂ Assimilation/Uptake Rate per unit area ($\mu\text{mol m}^{-2} \text{s}^{-1}$)
AI	Acclimation Index
C_a	Atmospheric CO ₂ Concentration ($\mu\text{mol mol}^{-1}$)
C_i	Leaf Internal CO ₂ Concentration ($\mu\text{mol mol}^{-1}$)
ETH	Eidgenossische Technische Hochschule (Technical University)
FACE	Free-Air Carbon Dioxide Enrichment
J_{max}	Max. light saturated rate of electron transport ($\mu\text{mol m}^{-2} \text{s}^{-1}$)
LAI	Leaf Area Index
Pi	Inorganic Phosphate
PPFD	Photosynthetic Photon Flux Density ($\mu\text{mol m}^{-2} \text{s}^{-1}$)
Rubisco	Ribulose-1,5 biphosphate carboxylase-oxygenase
RubP	Ribulose-1,5 biphosphate
SI	Stomatal Index
V_{cmax}	Max. Rate of carboxylation ($\mu\text{mol m}^{-2} \text{s}^{-1}$)

WORD COUNT

Excluding References = 9158

Total (including References, excluding Appendices) = 11037

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1. INTRODUCTION

1.1 Rising Atmospheric Carbon Dioxide

Fossil fuel burning, deforestation and many other human activities are resulting in an unprecedented rise in global atmospheric carbon dioxide (C_a) (Amthor, 1995). Such a compositional change is likely to result in climatic modifications; in particular average global temperature increases and increased variation in weather systems (IPCC, 1996). The significance of these changes for global plantlife is enormous, particularly in terms of ecosystem changes and effects on crop productivity. However, the interactive effects of such changes is too complex and uncertain a subject for this investigation; indeed, the only certainty of atmospheric change is a rise in C_a from current levels of $350 \mu\text{mol mol}^{-1}$ to about $700 \mu\text{mol mol}^{-1}$ in 2100. Consequently, this project aims to understand the long-term effects of such a rise in C_a on photosynthesis.

1.2 Effects of Elevated C_a

Plants which use the C_3 photosynthetic pathway are limited by the current ambient CO_2 concentration (Arp, 1991; Reining 1994). Consequently, it is realistic to expect a stimulation of photosynthesis under elevated C_a ; the mechanisms behind this are saturation of the primary carboxylation enzyme (Rubisco) and competitive inhibition of photorespiration (for a more detailed account of this stimulation effect see Stitt, 1991, and Webber *et al*, 1994). Farquhar and his co-workers (1980) derived equations to explain that, as C_a rises, the factors limiting photosynthesis change. Farquhar describes a shift from

limitation by Rubisco activity to control by RubP-regeneration capacity instead. At ambient C_a these processes actually co-limit photosynthesis (Webber *et al*, 1994).

1.3 Long-Term Response to Elevated C_a

Plants exposed to elevated C_a in the long-term (weeks to years [Amthor, 1995]) typically show an 'acclimatisation' effect (Long *et al*, 1993). The direction of this acclimatisation remains a somewhat contentious issue within the literature; negative responses have been observed by many authors (Clough *et al*, 1981; DeLucia *et al*, 1985; Larigauderie *et al*, 1988; Sage *et al*, 1989) whilst others have seen a positive stimulation (Campbell *et al*, 1990; Ziska *et al*, 1990; Arp & Drake, 1991).

Negative acclimation (a term which will be used here to describe a 'down-regulation' under elevated C_a) manifests itself as either an inability to maintain previously high photosynthetic rates under elevated C_a , or a decrease in A once returned to ambient conditions; there is considerable theoretical support for this response. In simplistic terms, it is believed that in enriched C_a , photosynthesis becomes solely limited by the capacity for RubP regeneration and that this represents a sub-optimal allocation of resources, compared to the co-limitation described earlier which exists under ambient conditions. Many authors, therefore, suggest that the negative acclimation seen is a result of the reallocation of resources in order to restore optimisation to the system (Sage *et al*, 1989; Bowes, 1991; Webber *et al*, 1994; Woodrow, 1994)). This is supported by evidence of a decline in Rubisco, sometimes by as much as 60%, suggesting that

valuable resources such as nitrogen are being re-partitioned into more limiting areas (Rowland-Bamford *et al*, 1991).

1.4 Source-Sink Theory

Of course not all authors agree with the aforementioned theory (Campbell *et al*, 1988; Campbell *et al*, 1990; Lloyd & Farquhar, 1996) and with so many inconsistencies within the experimental evidence it is best to retain an open mind at this time. However, with so many convincing arguments behind the hypotheses it is essential that the mechanisms behind them are examined in more detail with a view to testing their fundamental basis experimentally. There is a general consensus, amongst advocates of the negative acclimation theory, that under elevated C_a an imbalance between the increased photosynthate source at the leaves and the sinks is created and that this results in the observed down-regulation through feedback inhibition (Arp, 1991; Stitt, 1991; Long *et al*, 1993; Webber *et al*, 1994; Woodrow, 1994).

The source-sink mechanisms proposed include sucrose accumulation at the leaves (DeLucia *et al*, 1985; Ehret & Joliffe, 1985; Long & Drake, 1992), which may influence photosynthetic gene expression (Sheen, 1994; Webber *et al*, 1994). In addition, starch grain build-up may directly damage the chloroplast apparatus or stomatal closure may be induced. Further, Woodward (1987) has observed that stomatal density can be controlled by C_a and, therefore, it is possible that this may contribute to the negative acclimation seen in photosynthetic response to an enriched CO_2 environment. Ryle and Stanley (1992) found no conclusive stomatal response to elevated C_a in *Lolium perenne*, but this project aims to re-investigate this issue.

1.41 Experimental Issues

Negative acclimation appears to be less apparent in field studies compared with laboratory experiments (Arp & Drake, 1991). This led Arp (1991) to the important conclusion that small rooting volumes and low nitrogen supply are strongly correlated to down regulation at elevated C_a ; thus, it seems that artefact effects of experimental work could cause sink limitation and, therefore, feedback inhibition, as previously described. Consequently, it is important to study responses to C_a in field-grown plants, using apparatus such as open-top chambers or Free-Air Carbon dioxide Enrichment (FACE). This study uses such a FACE system which allows fumigation of the plants at the desired gas concentration without the use of chambers. Therefore, for this study the problem of restricted rooting volumes is avoided, and also there is less disruption of plant microclimate than would occur in a chamber.

1.42 Nutrient Supply

The supply of nutrients, particularly nitrogen, has emerged as a very important factor in the response of plants to elevated C_a (Sage *et al*, 1989; Bazzaz, 1990; Long *et al*, 1993; Woodrow, 1994; Rogers *et al*, 1996). Webber *et al* (1994) hypothesise that at a low nitrogen supply there is even more need for a reallocation away from Rubisco and into the parts of the plant where nitrogen is limiting. Essentially this is supportive of a 'functional balance' hypothesis (Acock & Pasternak, 1986); that is, resources are allocated to the source of the most limiting material. Therefore, it is reasonable to expect root growth to increase under elevated C_a as plants strive to obtain more nitrogen; some

evidence supports this hypothesis (Jongen *et al*, 1995) but, further investigation is not feasible within the scope of this project.

Despite the apparent role of nitrogen in negative acclimation suggested by the above authors, one should not ignore the fact that many writers disagree with this hypothesis (Bowes, 1991; Hocking & Meyer, 1991; Bunce, 1992; Rowland-Bamford *et al*, 1991). In particular, Lloyd and Farquhar (1996) argue that this theory centres around the 'Law of Limiting Factors' approach (Liebig, 1851) which is now superseded by 'Simultaneous Limitation' (Sinclair, 1992).

This study will examine in detail the role of nitrogen because it is vital that the disparities are solved if scientists are to provide advice for future farmers on fertiliser rates which will maximise any potential benefit from an elevated C_a environment.

1.43 Cutting Regime

This study is concerned with the forage crop *Lolium perenne* (perennial ryegrass) and, therefore, the effect of cutting regime on photosynthesis at elevated C_a is extremely significant. Other studies have found evidence to suggest that cutting the crop sooner and more frequently maintains it in the younger growth stage for longer. This means it is able to make greater use of the increased CO_2 concentration and may yield higher seasonal increases in production in the future environment (Nijs *et al*, 1988; Nijs & Impens, 1993).

1.44 Light Level

The available literature contains many inconsistencies on the interactive effects of light level and elevated C_a ; some workers believe that high light is

disadvantageous under CO₂ enrichment (Ehret & Joliffe, 1985) whilst others found high PPFD is necessary for a beneficial effect of elevated C_a (Sionit *et al*, 1982; Nijs *et al*, 1989). The question remains as to whether elevating C_a actually decreases the light compensation point of photosynthesis; evidence shows that this is the case over the short-term (Long, 1995 - Personal Communication) and possibly long-term (Arp & Drake, 1991, carried out a three year experiment) but more research is still needed to answer this question.

Very little work has been carried out on how photosynthetic responses to elevated C_a vary diurnally. Nijs and his co-workers (1992) indicate that high C_a could actually alleviate the afternoon depression of A, which is often observed under ambient conditions. The implications of this for future productivity are significant and, therefore, this issue will be further investigated in the following study.

1.5 *Lolium Perenne*

This study is concerned with the response of the grassland forage crop, *Lolium perenne*, to sustained growth at elevated C_a. This species is found naturally in temperate ecosystems and is also extremely important to the lowland pasture production of these areas (Nijs *et al*, 1988; Ryle *et al*, 1992). Indeed *L. perenne* is a widely grown crop in the Northern region of Switzerland, hence its presence in the Eschikon FACE experiment. However, in addition to its economic importance, it is also a useful plant to base this particular study on because a considerable amount of other work has been carried out on it (Nijs *et al*, 1988; Ryle & Stanley, 1992; Nijs & Impens, 1993), therefore, providing useful background and comparable information.

1.6 Aims of this Project

This introductory section has highlighted particular areas of study, within the general subject of photosynthetic response to elevated C_a , in which current knowledge is lacking or serious inconsistencies exist amongst the available literature. The most important of such topics is the very question of the direction of photosynthetic response to an enriched CO_2 environment. In the past, both positive adjustment (Hymus, 1995) and negative acclimation (Bryant, 1994) have been observed on the very *Lolium perenne* plots which will be used in this investigation. Therefore, the overall aim of this project is to establish the direction of acclimatisation, if it occurs, on these plants which are now in their fourth year of growth under the FACE system.

Within this investigation, other factors will also be studied; these are summarised below:-

- **SOURCE-SINK CHANGES**

- Is acclimatisation influenced by nitrogen supply?
- Does cutting regime effect response to elevated C_a ?

- **DIURNAL CHANGES**

- Is any acclimation seen maintained to the same degree at all times of the day?
- How does light level interact with elevated C_a ?

- **STOMATAL CHANGES**

- How does prolonged growth at elevated C_a influence the stomatal density of *Lolium perenne*?

2. MATERIALS AND METHODS

2.1 The Site

This investigation utilises a Free-Air Carbon dioxide Enrichment (FACE) system as designed by the Brookhaven National Laboratory (Lewin *et al*, 1994). This particular FACE experiment is situated at Eschikon (the field station of the ETH) 20km north-west of Zurich, in Switzerland. The six rings at Eschikon were established at the beginning of 1993, thus the *Lolium perenne* (cv. Bastion) plots, around which this investigation is based, are now in their fourth year of growth. The soil pH of these plots, prior to fertilisation, is 6.9-7.6 and the texture is characterised by 25% clay, 35% silt and 37% sand; therefore, the soil is classified as a 'clay loam' according to US Soil Taxonomy (Jongen *et al*, 1995). The photograph in Appendix 1(a) shows the *L. Perenne* plots which measure 6m².

Three of the rings act as controls and are maintained at ambient C_a (approximately 355 $\mu\text{mol mol}^{-1}$), the other three rings are at elevated C_a (600 $\mu\text{mol mol}^{-1}$); these rings were paired according to past cropping history. In 1995 the elevated C_a rings were maintained within 10% of the target concentration (540-660 $\mu\text{mol mol}^{-1}$) for 89-94% of the time and within 20% of the target for 98% of the time (when measured over one minute intervals) (Blum, 1996 - Personal Communication). Fumigation of the FACE rings was initiated on April 5th this year and is continuous throughout the daytime.

Each FACE ring is constructed using a 14m diameter toroidal plenum under the ground; from this 32 vertical pipes protrude to release pre-diluted CO₂ all around the ring. Control of the gas release is maintained by a central computer

(IBM PC) which takes into account wind speed, wind direction and air temperature.

Each block contains one FACE and one control ring, however, the rings cannot be sampled in block order due to their position at the site (see map in Appendix 1 [b]); experiments were usually carried out in the following sequence:-

C2 , F1, C1, F2, F3, C3

Each ring has the same basic layout (see Figure 2.1) although the *L. perenne* manipulation plots, containing several different treatments, are arranged randomly in each ring. These treatments include high (420 kg ha^{-1}) and low (140 kg ha^{-1}) nitrogen treatments which were used in this study. This investigation, from 8th May until 31st July, used plots which were under a six-cuts per year regime and this season cuts were carried out on 13th May, 24th June and 28th July.

FIGURE 2.1: Diagram of the FACE and Control Ring Lay-outs at ETH Eschikon

2.2 Materials

Leaf gas exchange measurements were made, non-destructively, using an Open Combined Infra-red Gas Analysis System (CIRAS 1 model 1.4, PP Systems, Hitchin Herts, UK). In association with this, two Parkinson Leaf Curvettes (model 1.1, PP Systems, Hitchin, Herts, UK) were used; one with a small, circular chamber of 2.5 cm² and one with a larger, rectangular chamber of 9.9 cm². In addition, a palm-top PC (Hewett Packard 1000CX) was used to control the CIRAS.

The CIRAS was calibrated regularly for CO₂ (at 600 μmol mol⁻¹) using a gravimetric CO₂ concentration (Carbagas, Zurich) and for water vapour, at 10mb, using a water vapour generator (Type WE 600, Analytical Development Co., Hoddesden, UK). For each investigation, measurements were only made if the C_a value was within 20 μmol mol⁻¹ of the target concentration.

The leaf gas exchange measurements required a calculation of leaf area. When using the small leaf chamber (area 2.5 cm²), area was determined by multiplying the width of the leaf by the diameter of the chamber and assuming a rectangle is formed. For the large leaf chamber (area 9.9 cm²) the same method was applied but this was indeed rectangular (with a length of 4.5 cm) so perhaps provided a slightly more accurate measurement. However, as the CIRAS can only accept leaf areas to an accuracy of 0.1cm both the above methods were considered sufficient.

For many of the investigations saturating light was required. This was achieved using stabilised quartz-iodide light units which fitted above the window

of the leaf chamber. A diffuse filter was used to achieve saturating PPFD of 750 $\mu\text{mol m}^{-2} \text{s}^{-1}$ for the smaller unit and 800 $\mu\text{mol m}^{-2} \text{s}^{-1}$ for the larger one.

The leaves used in all experiments are the youngest-fully expanded; that is, the leaves nearest the top of the canopy bearing a ligule. The leaf curvette was generally positioned mid-way along the leaf unless stated otherwise in the method. The leaves were always positioned with their adaxial surface upwards and in the centre of the chamber.

2.3 Methods

2.3.1 Acclimation Indices

The 'Acclimation Index' (AI) is a useful summary tool for studying the response of photosynthesis to elevated Ca; it utilises the following formula:-

$$A_{\text{grown, measured}} : \quad AI = \frac{A_{600,600} - A_{360,360}}{A_{360,600} - A_{360,360}}$$

- 1 = no acclimatory change under elevated Ca.
- 0 = complete down-regulation of photosynthesis.
- >1 = up-regulation of photosynthesis.

Pilot studies of AI values were made using the data obtained from the A/C_i investigations, however, these showed that such variation in temperature, as occurs during A/C_i measurements, made the results of the AI invalid. Consequently, a new method was devised to remove such errors and it is this which was utilised in this project.

The AI investigations were carried out indoors to remove temperature variation, consequently, cut leaves had to be used. To maintain the leaves in a healthy condition they were cut with a sharp scalpel blade, as low as possible on the stem, and then placed immediately into a plastic bag moistened with wet

tissue. Leaves kept for a long time before being measured were placed in a refrigerator. On measuring the leaves the stems were cut once more with the scalpel but this time under water to avoid air pockets forming in the xylem vessels. The leaf curvette was then positioned in a clamp so that the cut stem could be held under water throughout the experiment.

To fit into the equation shown above, the A values of FACE plants were measured at $600 \mu\text{mol mol}^{-1}$ whilst control plants were recorded at both 360 and $600 \mu\text{mol mol}^{-1}$. Each reading takes approximately 15 minutes to fully stabilise, therefore, due to time restriction it was only usually possible to measure 2 leaves from each treatment on one sampling day. To achieve light-saturation the light unit was used in this experiment. See time line in Figure 2.2 for dates of the experiments.

For each value of AI, the combined standard errors were calculated and the results from the three measurement days were combined in a single-factor ANOVA to test for variation in acclimation between the nitrogen treatments.

2.3.2 A/C_i Investigations

Using the equations of Farquhar and his co-workers (1980), the A/C_i response curve acts as an *in vivo* measure of the maximum capacity for RubP regeneration (J_{max}) and the activity of Rubisco within the leaf (V_{cmax}). To investigate this response the leaves are exposed to varying levels of CO₂ under saturating light. Therefore, for these experiments the light unit was used to give a PPFD of approximately $800 \mu\text{mol m}^{-2} \text{s}^{-1}$. C_a was changed according to the

**FIGURE 2.2: Time-Line Showing the Dates of all Experiments and how they
Relate to Cutting Times.**

following defined sequence so that the activation state of Rubisco was not raised above normal levels:-

CONTROL RINGS 360, 150, 100, 50, 600, 900 ($\mu\text{mol mol}^{-1}$)

FACE RINGS 600, 150, 100, 50, 360, 900 ($\mu\text{mol mol}^{-1}$)

Measurements began at 8.00 hrs and continued no later than 15.00 hrs to avoid the possibility of "end-product" inhibition. Each recording session involved measuring one leaf from both the low and high nitrogen treatments in all six rings; that is, a total of 12 A/C_i responses.

Whilst tillers were chosen at random within the plot, it was ensured that the measurement was taken on the youngest, fully expanded leaf. The CIRAS leaf curvette was held approximately 5cm from the leaf axil. To investigate the effect of source-sink changes following canopy removal it was essential to measure A/C_i curves at all stages before, after and within the cutting period. The dates of the experiments are shown on the time-line in Figure 2.2, the time before and after cut can clearly be seen by this representation.

For each A/C_i curve, values for J_{max} and V_{cmax} were fitted using a linearised curve-fit program (Long & Fischer, 1996 - unpublished). The results of this investigation were analysed statistically using a 4-way ANOVA model with split-split plot design (see Appendix 2 for details of this analysis design).

2.3.3 Diurnal

To investigate the effect of elevated C_a over the whole day requires photosynthesis measurements continuously from sun rise to sun set. For reasons

of practicality, such as distance around the site, in this study all six rings were measured every 2 hours from 6.00 hrs until 20.00 hrs.

In each ring, three leaves per nitrogen treatment were sampled and the measurements were made at the particular growth C_a (360 or 600 $\mu\text{mol mol}^{-1}$). The leaves chosen were always the youngest, fully-expanded, and the leaf chamber was positioned half-way along the leaf and held horizontally.

Ideally, diurnal experiments should be carried out on days with a minimum of cloud, but, due to poor weather conditions this was rarely possible. Consequently, the first diurnal investigation, 2nd July, was undertaken on a cloudy day and had to be terminated at 16.30 hrs due to rain. The second diurnal, 14th July, is complete but the third experiment, 20th July, is also truncated due to technical problems. For an indication of how these measurement days relate to the cutting period see the time-mine in Figure 2.2.

Data from all three diurnals, including Assimilation rates, transpiration and microclimate information, is analysed using one- and two-way ANOVA. The complete diurnal is used in analysis of relative enhancement during the feedback inhibition period and also when temperature and light are at their highest.

2.3.4 Light Response Curves

Light response curves were taken for both the high and low nitrogen treatments in Block 1 on 15th and 17th July respectively. These were measured using a series of light filters, of known effect, which fitted to the quartz iodide light unit. The plants assimilation rate was recorded at the different light levels at

both 360 and 600 $\mu\text{mol mol}^{-1}$. The experiment was repeated on three different leaves for each C_a value. Results were analysed using two-way analysis of variation with replication represented by the different light levels.

2.3.5 Stomatal Index

Stomatal index was investigated by using acetone to soften perspex microscope slides so that permanent stomatal impressions could be made. Using a light microscope stomatal and epidermal cells could be counted and then combined in the following formula to give a value for stomatal index (SI). Guard cells were not counted as epidermal cells:-

$$\text{SI} = \frac{\text{no. of stomata}}{\text{no. of epidermal cells} + \text{stomata}} \quad (\text{Salisbury, 1927})$$

Two leaves from each treatment were collected on three sampling days and from each samples of abaxial and adaxial surfaces were taken from halfway along the leaf blade. The impressions were made using three drops of acetone to soften the perspex and then holding the leaf sample firmly in place for approximately 30 seconds. From each impression three fields of view were recorded. On the abaxial surface the fields of view were chosen entirely at random. However, on the adaxial surface of *L. perenne* this is not possible due to the arrangement of the stomata along the flanks of the longitudinal grooves which lie between the vein ribs. Therefore, to overcome the problem of excessive vein material in the measurement area, fields of view were chosen at random but along the line of a groove.

The microscope (Ortholux II, Leitz, wetzlar, Germany) was calibrated using a stage micronmeter and eyepiece graticule. It gave a total magnification of x312.5 and each field of view measured 0.1128mm^2 .

The time-line in Figure 2.2 illustrates when measurements were taken. Results were analysed using one-way analysis of variance models to test for significant differences between treatments.

3. RESULTS

3.1 Acclimation Indices

Table 3.11 shows the results of the investigations of Acclimation Index.

Table 3.1: Results of Acclimation Index Experiment

DATE	No. of Days After Cut	Nitrogen Treatment	AI	SEM
10/07/96	16	Low	0.21	0.17
10/07/96	16	High	0.56	0.13
16/07/96	22	Low	0.55	0.37
16/07/96	22	High	1.34	0.21
28/07/96	34	Low	0.27	0.14
28/07/96	34	High	0.62	0.25

All values of AI, except for the high nitrogen treatment on 16/07/96, were found to be between 0 and 1, therefore, indicating negative acclimation. The value of 1.34 obtained in the high nitrogen treatment on 16/07/96 suggests up-regulation. There is no clear pattern of change in AI with days into the regrowth period but, on all three sample days the AI of low nitrogen was lower than for high nitrogen. However, when the average of the three experiments was taken, there was found to be no significant difference between the low and high nitrogen; the means and ANOVA results are as follows-

Low Nitrogen Mean = 0.342

High Nitrogen Mean = 0.840

ANOVA between low and high: $F=3.38$, $p=0.139$

The standard error of the mean values (shown in Table 3.1) are very high but this is partially caused by an artefact effect of the AI formula itself .

3.2 A/C_i Response

Figure 3.21 shows the V_cmax results obtained from the A/C_i responses measured in at FACE and control in both nitrogen treatments over a range of measurement days (error bars are SEM); the split-split plot ANOVA results of V_cmax and J_{max} are shown in Table 3.21. Nitrogen alone was not found to be a significant source of variation for V_cmax, the same is true of CO₂ when tested as a single variable (although with a CI of 90% this would be a significant factor). However, the interaction of these two factors was found to be statistically significant (95% CI) in explaining variation of V_cmax. A Bonferroni post hoc test of pairwise comparison found the source of this significant variation to be between Control and FACE plots when measured at low nitrogen (mean difference = -23.45, pairwise comparison probability = 0.033). This result is clearly understood through the graphs on Figure 3.21; graph (a) shows the low nitrogen results, illustrating large reduction in V_cmax of the FACE, relative to Control, plants. The high nitrogen results, graph (b), do not show such a clear difference and, therefore, the results do not contribute to the significant variation explained by the CO₂ and nitrogen interaction.

The number of days after cut was found to be a significant variable in the V_cmax investigation; in addition, the interaction of days with nitrogen was also significant. Although , the interaction of days and CO₂ did not significantly explain the variation in V_cmax. However, when days, nitrogen and CO₂ were interacted together a significant result was obtained. This may be explained by looking at Figure 3.21; on the low nitrogen graph there is a very obvious decrease in FACE V_cmax, relative to Control, as time into the regrowth period increases. After cut 1 only a decrease of 8.82% exists between the two CO₂ treatments but

FIGURE 3.21 Graphs showing Variations in V_{cmax} measured in the *L. perenne* FACE and Control plots at various times in the cutting regime: (a) Low Nitrogen, (b) High Nitrogen.

FIGURE 3.22 Graphs showing Variations in $J_{\max}$ in *L. perenne* measured at various times in the cutting regime in the FACE and Control plots: (a) Low Nitrogen, (b) High Nitrogen

by 04/06/96 and 12/06/96 this increases to 33.22% and 66.01% respectively. Once more no such obvious pattern can be identified in the high nitrogen graph which may explain why the interaction of days and CO₂ only becomes significant when nitrogen is also included.

Figure 3.22 illustrates the changes in Jmax between treatments and over time. In this case, CO₂ was found to be a significant source of variation but nitrogen and the nitrogen/CO₂ interaction were not significant. However, days were again found to be significant on their own, and when combined with nitrogen or CO₂. Once more the interaction of days, nitrogen and CO₂ was a significant source of variation. This is supported by Figure 3.32 which clearly shows an increasing down-regulation of Jmax in the FACE plots of the low nitrogen treatment as time into the regrowth period increases. Just as with the Vcmax results, the pattern on graph (b), high nitrogen, is far less clear. Block was not a significant source of variation for Vcmax or Jmax.

Table 3.21: results of Split-Split Plot ANOVA for Vcmax and Jmax
(Significant results are shown in bold)

SOURCE OF VARIATION	Vcmax	Jmax
CO ₂	F=8.675, p=0.099	F=110.89, p=0.009
Block	F=0.216, p=0.822	F=1.238, p=0.447
Nitrogen	F=0.988, p=0.376	F=0.459, p=0.099
Nitrogen x CO ₂	F=8.514, p=0.043	F=0.519, p=0.511
Days	F=5.543, p=0.002	F=110.89, p=0.009
Days x Nitrogen	F=5.701, p=0.002	F=7.769, p=0.000
Days x CO ₂	F=2.311, p=0.082	F=9.124, p=0.000
Days x Nitrogen x CO ₂	F=3.244, p=0.026	F=4.346, p=0.007

3.3 Diurnals

Figures 3.31, 3.32 and 3.33 show the diurnal variations in assimilation rate between the Control and FACE plots on the three diurnal days for both the high and low nitrogen treatments in graphs (a) and (b) respectively. Graph (c) on each of these three figures illustrates changes in the relative % enhancement of A for each of the diurnal days and the average of this data is summarised in Table 3.31. This data demonstrates a clear stimulation of A in the FACE, relative to the Control, plots. This finding is confirmed by the results of two single-factor ANOVA tests:-

LOW NITROGEN: **F=5.668, p=0.023**

HIGH NITROGEN: **F=6.064, p=0.019**

The results are presented in bold to highlight the statistically significant findings; that is, in both nitrogen treatments there is a significant enhancement of A in the FACE plots at the 95% confidence interval (CI).

Table 3.31: Relative % Enhancement of Assimilation and Transpiration Rates

DATE	No. of days after cut	Relative % Enhancement of Assimilation Rate		Relative % Enhancement of Transpiration Rate	
		Low N	High N	Low N	High N
02/07/96	8	64.91	49.68	-1.56	5.09
14/07/96	20	59.65	52.66	-8.87	-12.9
20/07/96	26	35.58	56.00	29.43	15.24

Analysis of data of the relative % enhancement of A found no significant differences between low and high nitrogen; this was the case if diurnals were tested separately or all together. Looking at the high nitrogen alone on Table 3.31 shows very little difference in the relative enhancement of A across the three

FIGURE 3.31 Graphs showing Diurnal Variations in *L.perenne* Assimilation Rates between FACE and Control Plots for (a) Low and (b) High Nitrogen as Measured on 02/07/96. Graph (c) shows the Relative % Enhancement for each Nitrogen treatment

FIGURE 3.32 Graphs showing Diurnal Variations in Assimilation Rate between FACE and Control plots measured on 14/07/96: (a) Low Nitrogen, (b) High Nitrogen. Graph (c) shows the Relative % Enhancement for both Nitrogen treatments.

FIGURE 3.33 Graphs showing Diurnal Variations in Assimilation Rate between FACE and Control plots measured on 20/07/96: (a) Low Nitrogen, (b) High Nitrogen. Graph (c) shows the Relative % Enhancement for both Nitrogen treatments.

diurnal days; this is supported by ANOVA findings of insignificant differences between the measurements days for the high nitrogen treatment ($F=0.074$, $p=0.928$). However, for the low nitrogen treatment there appears to be a decline in the relative enhancement of A as days into the regrowth period increase, however, this change was also found to be insignificant at the 95% CI ($F=0.897$, $p=0.429$).

To test whether there is a difference in stimulation of A when light level and temperature are at their greatest (11.00-15.00 hrs) and the rest of the day data from the full diurnal of 14/07/96 was used. Single-factor ANOVA reveals that there is no significant difference between the relative % enhancement of A at this time of day and the rest of the day (Low N: $F=1.204$, $p=0.308$; High N: $F=1.145$, $p=0.321$). This is despite a large difference in the means for the low nitrogen treatment; that is, an average relative enhancement of 26.93% during the high light period compared to 66.4% over the rest of the day.

In order to compare the relative % enhancement of A during a time of likely feedback inhibition with the rest of the day, the full diurnal record, taken on 14/07/96, was used. Relative enhancement figures for the period 15.30-17.30 hrs were averaged and compared to the average figures from the rest of the day; the results of this, and single-factor ANOVA for each nitrogen treatment, are shown in Table 3.32. For both the high and low nitrogen treatments there is lower relative % enhancement of A during the feedback inhibition period than for the rest of the day, however, these differences are not found to be significant at a 95% CI.

Table 3.32: Relative % Enhancement of A during the Feedback Inhibition Period Compared to the Rest of the Day (14/07/96)

Nitrogen	Period of Day	Relative % Enhancement of A	ANOVA Results
LOW	Feedback period	58.43	F=0.002, p=0.970
	Rest of Day	60.2	
HIGH	Feedback period	48.9	F=0.029, p=0.87
	Rest of Day	52.82	

Table 3.31 also summarises information of relative % enhancement of transpiration between the Control and FACE plots; graphical display of actual transpiration data is shown in Figure 3.34 for each nitrogen treatment within each diurnal day. From Figure 3.34 it is clear to see that no great differences between transpiration rates in the Control and FACE plots were found.

The actual direction of relative % enhancement of transpiration between the control and FACE plots is rather unclear (as shown by the summary data in Table 3.31). There seems to be a greater positive enhancement on the third diurnal day (20/07/96) whereas on previous days the overall enhancement has generally been negative. Single-factor ANOVA revealed that these differences between diurnal days were not statistically significant for either nitrogen treatment (Low N: F=3.629, p=0.054; High N: F=2.231, p=0.144). ANOVA also showed that the differences in relative % enhancement of transpiration between nitrogen treatments were not significant (F=0.271, p=0.606).

The results of leaf microclimate measurements on each diurnal day are shown in Figure 3.35. Leaf temperature appears to be higher in the FACE relative to the control plots, but there is very little variation between the two. One-way ANOVA tests for both nitrogen treatments reveal no significant differences (Low N: F=0.0288, p=0.8661; High N: F=0.0452, p=0.833). In addition, there was

FIGURE 3.34 Graphs showing Diurnal Variations in Transpiration rates between *L.perenne* grown in Control and FACE plots for both Nitrogen Treatments: measured on (a) 02/07/96, (b) 14/07/96, (c) 20/07/96.

FIGURE 3.35 Graphs showing microclimate data for the Diurnals on (a) 02/07/96, (b) 14/07/96 and (c) 20/07/96

found to be no significant difference in the leaf temperature relative % enhancement between nitrogen treatments when all diurnals were tested together ($F=0.165$, $p=0.687$) or for each diurnal day separately (Diurnal 1: $F=0.289$, $p=0.605$; Diurnal 2: $F=0.776$, $p=0.395$; Diurnal 3: $F=0.008$, $p=0.932$).

3.4 Light Response Curves

Figure 3.41 shows the light response curves obtained for the FACE and control low nitrogen plots on 19/07/96 at (a) $360 \mu\text{mol mol}^{-1}$ and (b) $600 \mu\text{mol mol}^{-1}$. At $360 \mu\text{mol mol}^{-1}$ the control plants produced consistently higher A values than the FACE plants at almost all light levels. At $600 \mu\text{mol mol}^{-1}$ there is a less clear difference between the CO_2 treatments and this is supported by the results of two-way ANOVA (shown in Table 3.41); the difference between FACE and Control is statistically significant, at the 95% CI, when measured at $360 \mu\text{mol mol}^{-1}$ but not at $600 \mu\text{mol mol}^{-1}$.

For the high nitrogen treatments the light response curves are shown in Figure 3.42; here there is an even more distinct stimulation of A in the control rather than the FACE plots when measured at $360 \mu\text{mol mol}^{-1}$. The inability of the FACE plots to maintain A values as high as the controls appears to be even greater in the higher light levels; Table 3.41 shows this difference to be highly significant. In addition, for the high nitrogen, there is a statistically significant difference between the light response curves when measured at $600 \mu\text{mol mol}^{-1}$. However, Figure 3.42 shows clearly that the pattern of higher values in control

FIGURE 3.41 Graphs showing Light Response Curves for *L.perenne* in Block 1 FACE and Control Low Nitrogen plots; measured on 19/07/96 at (a) 360 $\mu\text{mol mol}^{-1}$ and (b) 600 $\mu\text{mol mol}^{-1}$.

FIGURE 3.42 Graphs Showing Light Response Curves for *L.perenne* in Block 1 FACE and Control High Nitrogen Plots; measured on 17/07/96 at (a) 360 $\mu\text{mol mol}^{-1}$ and (b) 600 $\mu\text{mol mol}^{-1}$

rather than FACE grown plants only persists in the lower PPFD range; over 500 $\mu\text{mol m}^{-2} \text{s}^{-1}$ any significant differences disappear.

Table 3.41: Results of 2-Way ANOVA on Light -Response Curves
(Significant results are shown in bold)

Date	Days after cut	Nitrogen	ANOVA results when measured at:-	
			360 $\mu\text{mol mol}^{-1}$	600 $\mu\text{mol mol}^{-1}$
19/07/96	25	LOW	F=18.82, p=0.002	F=0.70, p=0.79
17/07/96	23	HIGH	F=102.76, p=7.12x10⁻¹¹	F=12.73, p=0.0013

3.5 Stomatal Index

Graphs (a) and (b) on Figure 3.51 show the results of the stomatal index investigation, Table 3.51 summarises the data and Tables 3.52 & 3.53 demonstrate whether the differences seen graphically are statistically significant at the 95% CI. On the adaxial leaf surface (graph (a) on Figure 3.51) the SI for plants grown at low nitrogen does not vary between the control and FACE plots. However, for the high nitrogen treatment, plants grown at FACE have statistically lower SI values for the adaxial surface. Further analysis reveals that in the control plots there is a statistically significant difference between nitrogen treatments but this difference is not mirrored in the FACE-grown plants.

Table 3.51: SI Means and SEM for Ad- and Abaxial Leaf Surfaces

Leaf Surface	Control, low N	Control, high N	FACE, low N	FACE, high N
Adaxial	9.93±0.37	13.37±0.86	9.95±0.43	9.13±0.49
Abaxial	0.67±0.30	3.89±0.75	1.79±0.52	0.31±0.18

FIGURE 3.51 Graphs (a) and (b) show changes between CO₂ treatments in SI for the adaxial and abaxial surfaces, respectively, for both nitrogen levels. Graph (c) shows changes in the ad-/abaxial ratio between FACE and Control.

Observations of the abaxial surface of the leaves also reveal a decrease in SI for the FACE plants in the high nitrogen relative to their counterparts in the control plots. However, in the low nitrogen treatment there is the opposite effect on abaxial SI - a significant increase in the FACE relative to control. In addition, on the abaxial surface there are significant differences in SI between high and low nitrogen for both the CO₂ treatments.

Table 3.52: ANOVA Results for SI Values on the Adaxial Leaf Surface
(Values in bold are statistically significant at the 95% CI)

	Control, High Nitrogen	FACE, Low Nitrogen
Control, Low Nitrogen	F=14.24, p=0.0003	F=0.102, p=0.750
FACE, High Nitrogen	F=16.719, p=8.77x10⁻⁵	F=1.533, p=0.219

Table 3.53: ANOVA Results for SI Values on the Abaxial Leaf Surface
(Values in bold are statistically significant at the 95% CI)

	Control, High Nitrogen	FACE, Low Nitrogen
Control, Low Nitrogen	F=16.80, p=7.73x10⁻⁵	F=4.789, p=0.031
FACE, High Nitrogen	F=19.52, p=2.3x10⁻⁵	F=7.09, p=0.009

Graph (c) on Figure 3.51 shows changes in the ratio of adaxial/abaxial SI between control and FACE for both nitrogen treatments. Whilst there is very little difference between the ratios of the two nitrogen treatments in the control plots, the FACE plants show a large relative increase in the ratio of the high nitrogen plots and a large decrease in the low nitrogen.

4. DISCUSSION

The AI experiments carried out in this study provide a useful indication of the impact of 4 years growth at elevated C_a on the photosynthetic behaviour of *Lolium perenne*. Both nitrogen treatments showed an almost consistent down-regulation response on the three measurement days; only the high nitrogen plants on 16/07/96 showed evidence of up-regulation. However, this method of investigating acclimatisation direction in response to long-term CO_2 elevation has several flaws. These include the experimental difficulties of maintaining open stomata on cut leaves and also the high standard errors obtained. It is an artefact nature of the AI formula which causes higher standard errors when the AI value is low (Morison, 1996 - Personal Communication) but, nevertheless, this reduces the confidence placed in the AI results. Consequently, it is necessary to look closely at the other investigations to see if this initial suggestion of negative acclimation is supported.

It is the A/C_i curves which provide information on the biochemical responses to growth at elevated C_a ; the values of V_{cmax} and J_{max} obtained can show us how Rubisco activity and RubP regeneration rates, respectively, are affected. Both factors were found to decrease in the FACE relative to the Control plots when an overall average was taken. However, such average values do not really tell about the source of the variation - for a fuller picture the interactive effects of all the relevant variables must be considered.

Of particular interest is the nitrogen effect as the AI results had shown consistently more down-regulation in the low, relative to the high, nitrogen plots

(although this difference was not found to be significant). The V_{cmax} results did find a significant effect of the CO_2 and nitrogen interaction; further analysis revealed that the source of this significance was the variation in V_{cmax} between FACE and Control but only in the low nitrogen plots. For J_{max} the same interaction was found to be insignificant. This result is consistent with many authors who believe that plants grown at elevated C_a but with sub-optimal nitrogen will reallocate some nitrogen from Rubisco into more limiting areas (Sage *et al*, 1989; Bazzaz, 1990; Arp 1991; Long *et al*, 1993; Webber *et al*, 1994; Woodrow, 1994; Rogers *et al*, 1996). The lack of significance of the $\text{CO}_2 \times$ Nitrogen interaction for J_{max} is also consistent with this idea because the enzymes involved in RubP regeneration are locking up far less nitrogen than Rubisco does and, therefore, would not be expected to show reallocation under low nitrogen conditions.

The question of why J_{max} shows a significant decrease under elevated C_a remains. Regression analysis found V_{cmax} and J_{max} to be strongly positively correlated to each other ($R = 0.7$) which supports the theory of co-limitation being most optimal; that is, when V_{cmax} declines in response to CO_2 enrichment, J_{max} will also decline so that the two processes remain in joint control of the photosynthetic rate (Long *et al*, 1993; Webber *et al*, 1994). Alternatively, Webber *et al* (1994) suggest that in a low nitrogen situation there may be insufficient resources to reallocate into the now limiting process of RubP regeneration. Hymus (1995) reported that by the third year of growth this initially limiting capacity had been up-graded but the evidence presented here suggests that in the fourth year of growth this is not the case.

The A/C_i experiments also allowed further testing of the source-sink theory through interaction of crop regrowth stage and elevated C_a . The AI results revealed no consistent pattern with respect to days from cut, but for both V_{cmax} and J_{max} this was found to be a significant source of variation. Post hoc examination of the ANOVA results was not possible but from the graphs produced it appears that down-regulation of the FACE plots, in the low nitrogen treatment, increases into the regrowth period. Such increasing down-regulation as regrowth continues agrees with Nijs & Impens (1993) who suggested that cutting a sward sooner and more frequently could yield higher benefits from an enriched C_a environment. The reasoning behind this is that immediately after the cut the plant has a high sink demand as it grows new leaves; when the leaves are developed the source becomes higher than the sink, photosynthate levels increase and feedback inhibition occurs. The sink limitation is clearly exaggerated in a low nitrogen situation as the plant does not have enough resources, to match carbohydrates, for growth.

The feedback mechanism is thought to centre around carbohydrate inhibition of photosynthetic genes (Sheen, 1990); Webber *et al* (1994) explain this mechanism for the *rbcS* gene which codes for the Rubisco small-sub unit. What is especially interesting about these results is that the days effect is also seen for J_{max} suggesting that other genes are also being inhibited; Stitt (1991) reviews some evidence of this but generally this whole area still remains rather unclear and is in need of much further research.

The results discussed so far seem to point clearly to negative acclimation; the same conclusion that Bryant (1994) drew on working with these plots two years previously. However, they do strongly disagree with Hymus (1995) who found no evidence of down-regulation and also with the work of Lloyd & Farquhar (1996) and Sinclair (1992) who consider the source-sink theory a too simplistic and old-fashioned approach. In addition, the results of the diurnal investigations made in this study are also rather contradictory.

All the diurnals demonstrate a stimulation of A in the FACE relative to the control plots; this stimulation is of the proportions used by other authors to demonstrate an up-regulation effect of elevated C_a (Cure & Acock, 1986; Hymus, 1995). However, there are several features of the diurnal work done here which make such conclusions less sensible. Firstly, in the low nitrogen plots the average relative % enhancement of A decreases with time into the regrowth period; a finding which would support the conclusion of sink limitation developing in a sub-optimal nutrient supply. However, this decline was not found to be statistically significant. In addition, there were no significant differences between the relative enhancement of the high and low nitrogen plots, yet to agree with the aforementioned theory, the low nitrogen plants would be expected to show less stimulation.

Secondly, the incompleteness of two diurnal data sets makes them of little practical use to this investigation. With better weather conditions and less technical problems several complete diurnals could have been recorded which would have made understanding of these issues far easier.

The data from the complete diurnal is used to attempt to answer other questions which were posed in the introduction. Analysis revealed no significant differences in the relative % enhancement of A when the light was at its greatest and over the rest of the day; although on average the stimulation of A was actually lower during the lightest period for both nitrogen treatments. This is in opposition with the findings of Hymus (1995) who found significantly larger increases in A when the light and temperature were at their highest.

The light response curves can also be used to unravel the interaction of CO₂ and light. In the high nitrogen plots there was a substantial down-regulation of A throughout the PPFD range when measured at 360 $\mu\text{mol mol}^{-1}$. The difference appears to be greater at the highest light levels tested ($>500 \mu\text{mol m}^{-2} \text{s}^{-1}$) suggesting that the high nitrogen plots reach their light saturation at much lower irradiance when the CO₂ supply is low. The low nitrogen plots show a similar down-regulation when measured at 360 $\mu\text{mol mol}^{-1}$ but to a lesser extent; also, at near-saturating irradiance there is little difference between the low nitrogen CO₂ treatments. When measured at 600 $\mu\text{mol mol}^{-1}$ there is no significant difference in light response between FACE and Control in the low nitrogen plots but the high nitrogen exhibits significant down-regulation in the lower PPFD range ($< 500 \mu\text{mol m}^{-2} \text{s}^{-1}$). The inability of the high nitrogen FACE plots to cope with low light levels may actually be linked to their lack of negative acclimation. These plant have been growing at high light, high nitrogen and high CO₂ and, because they are not sink-limited, the photosynthetic apparatus has developed to exploit all the available resources to the full. This finding agrees

with several authors who conclude that CO_2 is of the greatest benefit at high light (Sionit *et al*, 1982; Nijs *et al*, 1989; Ryle *et al*, 1992; Wilks *et al*, 1995).

The low nitrogen plots react differently because they have, apparently, undergone negative acclimation in response to growth at FACE. At ambient C_a levels these plants do not become light saturated as quickly as their high nitrogen counterparts because some Rubisco has probably been reallocated to other parts of the plant. This means the CO_2 is not being used at a quicker rate than the light energy can support.

These findings disagree with Long & Drake (1992) who predicted that a stimulation of photosynthesis will occur even at lower light intensities under elevated C_a . However, it must be remembered that the leaves used in the light response investigations were growing at the top of the canopy in full sunlight, the question remains of how the response would have been different if leaves acclimatised to a lower position in the canopy were used. Perhaps leaves used to growing in shaded conditions would not have experienced the down-regulation seen in the unshaded leaves; this was the finding of Ehret & Joliffe (1985) working with bean plants. It is crucial to know how leaves at different positions in the canopy will respond to rising C_a if future productivity is to be predicted. Nijs *et al* (1989) predict that increased structural components, such as LAI, associated with growth at elevated C_a will be disadvantageous due to the impact of increased shading. The results obtained here cannot be used to predict a response because the leaves were acclimatised to high PPFD levels - further light response curves of leaves lower in the canopy would have allowed conclusions to be drawn on whether the shade-acclimatised leaves were still able to produce a stimulation of A at low light levels.

In the introduction questions of feedback inhibition were posed with the hypothesis that FACE grown plants may be better able to alleviate afternoon depression of A due to a change in the temperature optimum of photosynthesis (Nijs *et al*, 1992). However, when this was investigated on the full diurnal day there was actually found to be a decrease in the relative % enhancement of A during the likely feedback inhibition period when compared to the rest of the day (although this difference was not significant). Perhaps the lack of a significant effect of the feedback inhibition period can be linked to the investigations into leaf microclimate.

Long (1991) predicts that there will be proportionate increases in photosynthesis at elevated C_a as temperature rises; the findings of Hymus (1995) on the same *L. perenne* swards are in agreement with this hypothesis not only with higher A enhancement when temperature was higher but also a significant increase in temperature in the FACE relative to Control plants. However, the results of this study found no such obvious relationships. There were no significant differences in temperature of the leaves from the different treatments and, as previously mentioned, the period of the day with the highest temperature had the lower relative enhancement of A. These findings are supported and explained by a lack of significant differences in transpiration between the Control and FACE plots. Long (1985) predicts a reduction in the evaporative latent heat loss from leaves at elevated C_a due to decreased stomatal conductance; both Bryant (1994) and Hymus (1995) confirm this theory but, again, no such effect was seen in this study. Perhaps this discrepancy is due to an inadequacy of the CIRAS in providing accurate temperature and transpiration

readings. Investigations into stomatal index made in this project may provide some indications to actual changes in transpiration.

Stomatal Index (SI) may be expected to decrease after prolonged growth in elevated C_a because the plant can take in less air but still obtain enough CO_2 and decreasing SI may confer greater water use efficiency (Woodward, 1987; Radoglou & Jarvis, 1990; Boetsch *et al*, 1996). In this study of *L. perenne* such a decrease, on both the adaxial and abaxial leaf surfaces, was found in the FACE grown plants of the high nitrogen treatment.

However, for the low nitrogen FACE plots no change in SI was seen on the adaxial surface but on the abaxial side of the leaf a significant increase in SI, relative to the Control plants, was found. The results of this is that in the low nitrogen the abaxial side of the leaf becomes relatively more important following prolonged growth at elevated C_a but in the high nitrogen it is the adaxial surface which becomes more dominant.

It is difficult to understand how down-regulation of the photosynthetic apparatus in the low nitrogen plots results in a need for more stomata. Is it possible that the apparatus could have acclimated, through reallocation of nitrogen, to such an extent that in order to maintain a reasonable photosynthetic rate much more air must be taken in? This seems highly unlikely as it is the high levels of CO_2 which cause the observed down-regulation in the first place. If there is a genuine reason for increasing SI then it would indeed be expected that the extra stomata would be found on the lower leaf surface where risk of high water loss is lower. However, it seems that very few authors, if any, have reported such a finding. Ryle & Stanley (1992) found no significant changes in

stomatal distribution under elevated C_a but on closer examination of their results increases in several relevant parameters are found (such as stomata per mm^{-2}). Ryle & Stanley do, however, attribute their insignificant result to the highly variable stomatal index of *L. perenne*, an out-breeding species. It is possible that this high variability could explain the differences found in this study. Also, the abaxial surface of *L. perenne* tends to have very few stomata and, therefore, the changes in SI conferred by just one or two stoma makes a great deal of difference.

The SI results are not supported by the transpiration measurements made on diurnal days as here no significant differences were seen. However, transpiration is also effected by many other factors, such as stomatal aperture, so the lack of a significant link does not necessarily point to reduced confidence in the SI results. Stomatal conductance readings may have been useful in either supporting the SI results or indicating other effects of elevated C_a on gas exchange from a leaf.

5. CONCLUSIONS

This investigation provides clear evidence of negative acclimation in the low nitrogen plots of *Lolium perenne* following four years of growth at elevated C_a . The significant down-regulation of V_{cmax} found in the low nitrogen FACE plots is consistent with the sink limitation theories of Arp (1991) and many others. The associated down-regulation of J_{max} in the FACE plots, particularly (although not significantly) at low nitrogen, suggests that sufficient reallocation of resources has not yet occurred to make this process non-limiting.

The diurnal results rather confuse these findings, with stimulation of A in the FACE rings reaching proportions which other authors have described as up-regulation (in particular Hymus, 1995, on these same plots). Explanations for this include the possibility that stimulation with no evidence of negative acclimation would actually be much higher or even that these results are unreliable due to the technical problems experienced.

Other experiments within the study have also pointed to a negative acclimation effect. The AI results revealed down-regulation consistently for the low nitrogen and the light response curves demonstrated that the high nitrogen plots, which are thought not to have down-regulated, could not produce a stimulation of A when light levels are low. The low nitrogen plots did not become light saturated at such low PPFD levels and this suggests that the photosynthetic apparatus had acclimated to the stage that carboxylation could not occur at a faster rate than the light available could support.

The implications of negative acclimation in response to an elevated C_a environment cause much concern. There is an apparent need for additional fertiliser

in order to avoid down-regulation has serious economic and environmental consequences, such as the increasing problem of eutrophication in freshwaters. However, such advice should not be given to farmers on the strength of photosynthetic measurements alone. It is very unfortunate that due to technical problems this study could not include planned experiments of canopy photosynthesis, because without knowing the whole plant response to elevated C_a it is impossible to draw conclusions concerning future plant productivity. It is possible that while negative acclimation is seen at the leaf level, canopy changes, such as an increase in LAI, may compensate for this at the plant level. In addition, rising C_a may be the only certainty of future global change but changes in other factors such as temperature are also very likely. Previous work has suggested that increased temperature will enhance the benefit of elevated C_a yet this study found no such result.

There are further problems in using this study to predict future plant productivity. This study has dealt solely with a perennial grass species whose response of partitioning relatively more photosynthate from elevated C_a has been implicated in causing sink limitation (Wardlaw, 1982). Some species may respond differently and, therefore, benefit from this environmental change. A leguminous plant may not experience sink limitation and farm management may have to change in response to this, perhaps with the introduction of more intercropping. Inter-specific, and possibly intra-specific, differences in response to CO_2 enrichment may result in ecosystem changes, these could be particularly significant in agriculture as weed-crop interactions may change.

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APPENDIX 1

(a) Photograph Showing the *Lolium perenne* plots in a Control Ring at ETH Eschikon FACE Experiment

(b) Map Showing the Site Lay-out of FACE and Control Rings at ETH Eschikon

APPENDIX 2: Design of Split-Split Plot ANOVA for Vcmax and Jmax Results

The data for the Vcmax and Jmax results could not be analysed using a simple 4-way ANOVA design because some variables are 'within' others. This effect is explained by the diagrammatic representation of the experiment below:-

The experiment consists of three block like the one shown above. Within each block there the two CO₂ treatments are represented and, therefore, blocks and CO₂ form the 'main' plot. However, nitrogen is not part of this main plot because the two nitrogen treatments are within the CO₂ rings and not all the possible replication is achieved by this design; thus nitrogen forms the 'split' plot. To add a further complication the variable of days after cut is actually within each nitrogen treatment, therefore, representing a 'split-split' plot. The table below shows how these different plot levels can be combined in an ANOVA model.

SOURCE OF VARIATION		DEGREES OF FREEDOM
MAIN PLOT	CO ₂	1
	Blocks	2
	Main Plot Error	2
	Total for Main Plot	5
SPLIT PLOT	Nitrogen	1
	Nitrogen x CO ₂	1
	Nitrogen x Rings{CO ₂ }	6
SPLIT-SPLIT PLOT	Days	4
	Days x Nitrogen	4
	Days x CO ₂	4
	Days x Nitrogen x CO ₂	4
	Split-Split Plot Error	32
TOTAL		59

APPENDIX 3: PROJECT PLAN

TITLE 'Does Field Elevation of carbon Dioxide (CO₂) Effect a Sustained Increase in Leaf Photosynthetic C-assimilation in Ryegrass (*Lolium perenne*)?'

INTRODUCTION

The primary concern of this project is the effect of increasing levels of atmospheric CO₂ on agriculturally important plants. CO₂ has already risen from 275 - 345 $\mu\text{mol mol}^{-1}$ since the industrial revolution and is predicted to increase to 700 $\mu\text{mol mol}^{-1}$ by the year 2100 (Watson et al, 1990).

The effect of this increase on C3 crop plants is currently under a great deal of investigation and many theories surrounding this issue have been put forward. It would seem probable that increased CO₂ would stimulate photosynthesis within the plants and, therefore, increase productivity (See Kimball, 1993). Yet, some experiments have demonstrated an 'acclimation' or down-regulation in photosynthesis after the initial enhancement (Webber et al, 1994). The cause of this apparent acclimation is hypothesised to be due to the inability of the plant to utilise the excess carbohydrate produced; thus an imbalance between sources and sinks is created (Arp, 1991; Sheen, 1994).

However, more recent work, especially in the field, has indicated that acclimation is merely an artefact effect of the controlled environments in which previous experiments have been carried out. For instance, in a field investigation rooting volume is not controlled so 'sink limitation' effects may not be seen (Lawlor & Mitchell, 1991).

The 'Free-Air Carbon-Dioxide Enrichment' (FACE) apparatus provides a unique way to investigate the effects of elevated CO₂ on plants as no alteration of their microclimate occurs. At the ETHZ Eshikon site in Switzerland Ryegrass (*L. perenne*) is now in its fourth year of growth; therefore, long term effects of elevated CO₂ can be studied to investigate whether photosynthetic enhancement is sustained or whether acclimation is observed.

A (leaf net CO₂ uptake, assimilation)/ C_i (internal CO₂ concentration of the leaf) curves are the most useful tool in analysing the plants' responses to changes in CO₂. These are derived from the equations of Farquhar et al (1980) and, in the simplest terms, provide a measure of the maximum capacity of the leaf to regenerate RubP (J_{max}) and its carboxylation efficiency (V_{c max}).

Changes in source-sink dynamics could be extremely important to the impact of increased CO₂. Consequently, different nitrogen treatments and cutting regimes are employed within the FACE rings. It is important to know the implications that management techniques may have in future, CO₂ enriched, agricultural environments. Ryegrass is studied because it is a widely spread pasture grass, particularly in Western Europe, and is likely to be important well into the future.

AIMS AND HYPOTHESES

Overall Aim: To investigate whether enhancement of leaf and canopy photosynthesis, in ryegrass, is continuing into a fourth year within the FACE rings.

Hypothesis: No evidence of acclimation will be seen.

Secondary Aims:

- (a) To investigate whether enhanced photosynthetic rates are attained at all times, throughout the day, within the FACE rings. It is possible that as photosynthates accumulate in the leaf during the day, some feedback inhibition of photosynthesis could occur. Any differences in such a feedback mechanism between a plant grown at ambient and elevated CO₂ will be studied.
- (b) To investigate whether acclimation is removed by source-sink changes. Ryegrass in the FACE and Control rings is cut approximately every four weeks; the impact of the change in sink size before and after each of these cuts will be investigated. In addition, the interaction of different nitrogen treatments with elevated CO₂ will be examined.
- (c) To investigate whether changes in photosynthesis at the leaf level reflect changes at the canopy level. The relationship between enhanced photosynthesis in the leaves and the overall Leaf Area Index (LAI) of the canopy will be studied.

MATERIALS AND METHODS

Site: ETHZ Eshikon FACE System, near Lindau, Switzerland.

This system consists of 6 rings in three blocks; each block contains one FACE ring, with CO₂ elevated at 600 $\mu\text{mol mol}^{-1}$, and one Control ring, with ambient CO₂ levels (355 $\mu\text{mol mol}^{-1}$).

Materials: The leaf gas analysis measurements will be made using an 'Open Combined Infra-red Gas Analysis System'. A quartz iodide lamp will be used to provide a constant light saturating photon flux density of 750 $\mu\text{mol m}^{-2} \text{s}^{-1}$.

Methods:

(a) Diurnal

Sample days will be chosen to coincide with the cutting regime employed at the site; that is, about one week before and one week after the cuts. Gas analysis measurements will be performed every 2 hours from 06.00 hours until 20.00 hours. Three leaves from each nitrogen treatment will be sampled; those leaves chosen will be the youngest, fully-expanded.

The data from this experiment will be analysed using 2-way ANOVA as the blocks between the FACE and control rings are paired.

(b) A/C_i Determination

By tagging leaves, A/C_i measurements can be taken before and after cuts using the gas analyser. Stomatal conductance (g_s) will be determined from A; and light response curves (A/Q) will also be measured in order to determine maximum quantum efficiency and the light level required for light saturation. From the response curves, values for J_{max} and $V_{c\ max}$ will be fitted; a three-way ANOVA analysis can be carried out on these results as three treatments will be studied - CO_2 , cutting regime and nitrogen treatment. Also, this data will be used to calculate the 'Acclimation Index' for the ryegrass plants.

(c) Leaf/Canopy Comparison

Measurements of A/C_i will be taken from additional ryegrass plot which are also being investigated for canopy effects of elevated CO_2 by another research group. Both sets of results will be combined, via the WIMOVAC computer model, to predict potential interactive effects on canopy C-uptake.

(d) Canopy Effects

A/C_i measurements at different positions in the canopy will be taken in order to determine their interaction with elevated CO₂. In addition to this, investigations into the influence of elevated CO₂ on different parts of the leaf will be carried out. Both these experiments will be conducted under both nitrogen treatments and at different times within the cutting regime.

(See Long & Hallgren, 1993, for precise details of methods).

TIMETABLE

30/4 - 7/5	:	Learn how to use equipment(e.g. gas analyser) at Essex.
8/5	:	Fly to Zürich.
May	:	Research & write literature review. Carry out diurnal and A/C _i measurements 1 week before and after cuts. Synchronise canopy experiment with other research group.
June	:	Hand in literature review - 7/6 Continue with experiments as in May.
July	:	Continue with experiments as in May. Commence writing-up. Use of WIMOVAC leaf to canopy model. Return to Essex - 31/7?
August	:	Writing-up.

APPENDIX 4: LITERATURE REVIEW

1. INTRODUCTION

Atmospheric carbon dioxide (CO₂) concentrations are likely to reach 550 vpm by the middle of next century due to emissions from fossil fuels, deforestation and other sources (IPCC, 1996). Such a rapid rise from present levels of around 360 vpm is unprecedented in the Earth's history, although vast variations have been experienced over geological time (Amthor, 1995). Consequently, there is much interest in the effect that this increase may have on global plantlife; for instance, climate change, mediated through an elevation of atmospheric CO₂ concentration (C_a), is likely to influence plants but such an issue is too wide for the scope of this review. Instead this analysis is concerned with the direct impact of elevated C_a on plant (especially crop) productivity, in particular the response of photosynthesis.

Of special interest is the work done on *Lolium perenne* (perennial ryegrass), a species found in the natural ecosystems of temperate areas, such as north-west Europe (Nijs *et al*, 1988). The ryegrasses are extremely important in the lowland pasture production of these areas, and as they will continue to be so into the foreseeable future, their response to an elevated C_a environment is extremely interesting and important (Ryle *et al*, 1992).

It is well known that plants, such as ryegrass, which use the C₃ photosynthetic pathway are limited by CO₂ at its ambient concentration (Arp, 1991; Reining, 1994), therefore, a stimulation of photosynthesis under elevated C_a may be expected. Indeed, in the 1980's many studies predicted that a future doubling of C_a would result in general yield increases of around 30-40% for C₃ crops (Kimball, 1983; Cure & Acock, 1986). However, more recent work reveals that while a short-term stimulation is observed, longer-term exposure to elevated C_a (usually set at around 600 vpm) can result in a negative photosynthetic response (Bowes, 1991; Rowland-Bamford *et al*, 1991; Webber *et al*, 1994). The mechanisms behind such a 'down-regulation' are at the centre of much discussion in the recent literature; as indeed is the very question of

whether the observed decline is a real or experimental effect. The plant's source-sink balance emerges as a major factor in the photosynthetic response to elevated C_a . Therefore, following an analysis of the down-regulation theories, studies concerned with changes in sources or sinks (in particular nitrogen levels, cutting regime and light difference, as these are especially relevant to *L.perenne*) will be reviewed.

Towards the end of the discussion a review of leaf and canopy interactions will be made as it is of the author's opinion that to unravel the mystery of a plants adjustment to elevated C_a , studies of leaf photosynthesis alone are inadequate. Under conditions of elevated C_a structural changes at the canopy level are likely to result; Bowes (1991) summarises these morphological and anatomical changes and gives examples such as increases in Leaf Area Index (LAI), leaf thickness and branching or tillering. Changes in root size are also likely and, therefore, extrapolation to the whole plant level is necessary if we are ever to begin to predict the future productivity of crops.

2. SHORT-TERM RESPONSE TO ELEVATED C_a

A rapid increase in leaf photosynthesis has been shown to occur following transference to an elevated C_a environment (Cure & Acock, 1986); the reasons for this stimulation are two-fold. Firstly, the enzyme ribulose-1,5-bisphosphate carboxylase-oxygenase (Rubisco) is involved in both photosynthesis and photorespiration; the latter process may depress the rate of carbon assimilation (A) by 20-50% in temperate climates (Long, 1985). In the elevated C_a environment, photorespiration is reduced because CO_2 actually acts as a competitive inhibitor of the oxygenation of ribulose biphosphate (RuBP). Secondly, the Michaelis constant of Rubisco for CO_2 is high relative to ambient C_a ; in other words the enzyme is not saturated with CO_2 . Thus, under elevated C_a a further increase in carboxylation can result by increased substrate binding (Stitt, 1991; Webber *et al*, 1994). Amthor (1995) suggest a third factor which may help explain the initial stimulation; CO_2 is required for the activation of Rubisco. However, this final suggestion is unlikely to be as significant as the others in the stimulation of global photosynthesis under enhanced C_a .

There is a general consensus on the change in photosynthesis control factors as C_a rises; this is based on the equations of Farquhar *et al* (1980) and is explained in fairly simplistic terms in Figure 1.

FIGURE 1

(Adapted from Farquhar *et al*, 1980, and Webber *et al*, 1994)

Figure 1 is a graph illustrating the biochemical factors which limit carbon dioxide assimilation rate (A) at varying internal CO_2 concentrations (C_i).

- (a) Represents the concentrations of C_i at which A is limited by Rubisco activity.
- (b) Represents the C_i values when A becomes limited by RubP regeneration capacity.
- (c) Represents possible inorganic phosphate (Pi) limitation at very high C_i values.

Ambient C_a is represented by the dotted line as giving a C_i value of around $200 \mu\text{mol mol}^{-1}$. It is clear that at this C_i , A is co-limited by both Rubisco activity and RubP regeneration. A rise in C_a would result in A being solely limited by the capacity for RubP regeneration.

3. LONG-TERM RESPONSE TO ELEVATED C_a

Long *et al* (1993) use the term 'acclimatisation' to describe the effect of prolonged growth in elevated C_a on the development and maintenance of the photosynthetic apparatus. In many cases the photosynthetic adjustment which occurs in the acclimatisation process is negative, although positive adjustment has also been reported (Campbell *et al*, 1990; Ziska *et al*, 1990; Arp & Drake, 1991). In much of the available literature the term 'acclimation' has come to describe only the former, down-regulation effect (Reining, 1994; Woodrow, 1994); this is rather confusing, and perhaps a little premature considering that the evidence for the acclimatisation direction is not yet conclusive, therefore, in this review prefixes of 'positive' and 'negative' are used for clarity.

Negative acclimation is observed as an inability to maintain previously high photosynthetic rates following prolonged exposure to C_a , or as a decrease in photosynthetic rates once returned to ambient conditions (Clough *et al*, 1981; DeLucia *et al*, 1985; Larigauderie *et al*, 1988; Sage *et al*, 1989). Work done by

Cure and Acock (1986) illustrates this well, they found an initial increase in CO_2 exchange of 52% yet this declined to only 29% more than ambient-grown plants after longer exposure to elevated C_a . It is not the intention of this paper to review the findings of such experiments but rather to critically analyse the theories which surround negative acclimation; this is attempted in the sections below.

3.1 Theoretical Basis of Negative Acclimation

The graph in Figure 1 demonstrates that at current C_a the rate of CO_2 assimilation is co-limited by both Rubisco activity and RuBP regeneration (Farquhar *et al.*, 1980). Several authors have suggested that under elevated C_a , C_i will be raised to a point where A is solely limited by the capacity for RuBP regeneration (Sage *et al.*, 1989; Bowes, 1991; Webber *et al.*, 1994). It is assumed that this disruption of photosynthetic control represents a sub-optimal allocation of resources; consequently, negative acclimation is suggested as the process by which optimal allocation of resources is resumed. Sage *et al.* (1989) explain that according to this theory, perfect photosynthetic adjustment would involve reallocation of nitrogen, and other resources, away from the non-limiting, down-regulated components and into the limiting components. Bowes (1991) explains that this would mean a loss of resources from Rubisco, and other photosynthetic proteins, and a gain in processes such as light-harvesting and electron transport.

There is indeed some substantial evidence to support this hypothesis of reallocation of resources under long-term elevation of C_a . Rubisco, the most widely studied of the photosynthetic enzymes, has been shown to decrease by as much as 60% in some C3 species in response to CO_2 enrichment (Sage *et al.*, 1989; Rowland-Bamford *et al.*, 1991). Certainly there is much evidence of reduced leaf nitrogen concentrations following prolonged exposure to elevated C_a (Wong, 1979; Rogers *et al.*, 1996) and, as Rubisco accounts for a significant amount of global protein, Hilbert *et al.* (1990) were able to present a strong argument that this represents an active reallocation. Wong (1979) actually found in cotton that the decrease in Rubisco under elevated C_a was large

enough to account for the observed inhibition of photosynthesis. However, in some species the photosynthetic decline was larger than the decrease in Rubisco (such as in *Phaseolus vulgaris* L. studied by Von Caemmerer & Farquhar, 1984, and *Brassica oleracea* studied by Sage *et al*, 1989).

In response to such inconsistencies, advocates of the reallocation theory suggest that a decrease in activation state or a decline in specific activity of Rubisco may account for the excess decline in photosynthesis. A decline in activation state has certainly been documented (Sage *et al*, 1989; Rowland-Bamford *et al*, 1991). However, in some experiments no change in activation state was seen (Campbell *et al*, 1988) nor was there any decline in Rubisco activity (Wong, 1979; Campbell *et al*, 1988; Campbell *et al*, 1990). Thus, there is some confusion in the literature concerning the biochemical basis of negative acclimation - a later discussion of the mechanisms behind such adjustment will attempt to review the hypothesis which explain away such disparities.

One other major criticism of the negative acclimation theory, concerns the whole basis of the argument that co-limitation of photosynthesis (as represented in Figure 1) represents an optimal allocation of resources. Lloyd and Farquhar (1996) point out that it is only since 1780 that C_a has risen to its present level of around 350 vpm, before that time there had been fluctuation around 270 ppm for over 10,000 years. Consequently, they suggest evolution is unlikely to have occurred fast enough during this time to achieve an optimal allocation of nitrogen between the different photosynthetic processes. However, some authors firmly believe in the optimal allocation theory; for instance, Woodrow (1994) concludes that C3 plants probably possess the genetic feedback mechanisms required to efficiently 'smooth out' any imbalance within the photosynthetic system caused by a rise in atmospheric C_a . Still, Lloyd and Farquhar's suggestion certainly requires further investigation as it may undermine the whole fundamental basis of the negative acclimation theory.

3.2 Mechanisms Behind Negative Acclimation Theory

Many authors believe that the negative acclimation seen in plants exposed to elevated C_a over the long term may be a result of an imbalance between sources and sinks (Arp, 1991; Stitt, 1991; Long *et al.*, 1993; Webber *et al.*, 1994; Woodrow, 1994). Ehret and Joliffe (1985) explain that an enhanced supply of carbohydrate, from the stimulation of photosynthesis, exceeds the capacity of the sink resulting in some form of feedback inhibition.

Some alternative hypotheses to feedback inhibition are reviewed by Reining (1994). Firstly, he states that high C_a can lead to partial stomatal closure which may reduce the carbon transport to the site of carboxylation (as seen by DeLucia *et al.*, 1985). However, this hypothesis is rather dubious as the causal direction is not clear; is stomatal closure actually being induced by the reduced photosynthesis rather than causing it? Reining goes on to suggest that the reduction of Rubisco in plants grown at elevated C_a could be the cause of negative acclimation, but further analysis reveals that this too may be the result of source-sink feedback inhibition (Webber *et al.*, 1994).

Consequently, the current discussion need only proceed with an analysis of the mechanisms behind source-sink imbalances which may result in feedback inhibition and reduced photosynthesis. Arp (1991) provides a useful analysis which can be summarised as follows. A sucrose build up at the source will lead to reductions in inorganic orthophosphate (Pi) and this induces higher rates of starch synthesis. Such starch accumulation in the leaf can damage the chloroplast resulting in lower photosynthesis. Sharkey (1985) suggests that if Pi levels decline sufficiently then phosphorylation rates in ATP synthesis may become reduced (as indicated in Figure 1). However, Stitt (1991) argues that during this long-term adaptive process, direct Pi limitation does not make a major or sustained contribution.

There is certainly much evidence to support the theory of carbohydrate accumulation in leaves grown under elevated C_a (DeLucia *et al.*, 1985; Ehret & Joliffe, 1985). Long and Drake (1992) found, in a study of photosynthetic adjustment to around double ambient C_a , that on average sucrose concentrations per unit leaf area were

increased by 52% and that, generally, a larger increase in leaf carbohydrate resulted in a greater decrease in photosynthetic capacity. Work such as this has lead Webber and his co-workers (1994) to implicate leaf carbohydrate as a factor determining the level of expression of *rbcS* (the gene which codes for the Rubisco small sub-unit) and other photosynthetic genes. This, therefore, provides the mechanism by which feedback inhibition can lead to decreased Rubisco levels which several authors assumed to be two unconnected phenomena (Arp, 1991; Reining, 1994).

It has already been noted that in some studies a decline in Rubisco was not sufficient to account for the reduced photosynthetic capacity under elevated C_a ; advocates of the resource reallocation theory were able to explain this by changes in activation state of the enzyme. However, there are other disparities in the experimental evidence, such as a lack of a clear association between negative acclimation and carbohydrate build-up in some studies, and sometimes even an inverse relationship has been found (Radin *et al*, 1987; Vu *et al*, 1989). In addition, in some experiments a positive acclimation response to growth at elevated C_a is seen (Campbell *et al*, 1990; Ziska *et al*, 1990; Arp & Drake, 1991). Arp and Drake (1991) found an increase in photosynthetic capacity following 4 years growth of perennial vegetation at 700 vpm and they explain that it is mainly field-grown plants which do not seem to experience the down-regulation effect. So, how do the negative acclimation theorists explain these disparities? Stitt (1991) suggests that in the long-term, the ability of the leaf to maintain higher photosynthetic rates will depend upon the source-sink status of the whole plant and how this balance is regulated. Within his review, he explains how inter-specific variations may be responsible for differences in acclimation response aswell as differences in developmental stage and developmental conditions. The following three sections look in detail at some major ways in which the source-sink balance is effected and try to unravel how these could contribute to the acclimation of plants to elevated C_a and, therefore, help explain the differences between experimental results.

4. THE IMPORTANCE OF SOURCE-SINK CHANGES IN ACCLIMATION

Although this review is primarily interested in the effects of elevated C_a on photosynthesis, it now becomes vital to look beyond the leaf and towards the whole plant response if the processes of photosynthetic adjustment are to be understood. It has been explained that negative acclimation is widely thought to occur when photosynthesis is inhibited by the capacity to generate new site of storage or utilisation (i.e. sinks) (Long *et al*, 1993). The following sections suggest how source-sink could be effected by experimental design, nitrogen supply, cutting regime or light availability.

4.1 Experimental Issues

Arp (1991) brought to notice the influence that certain experimental conditions, in particular rooting volumes, may have on the photosynthetic adjustment of plants to elevated C_a . Through a review of the literature, Arp found that all experiments with a rooting volume of less than 3.5 dm³ obtained negative acclimation results whereas plants grown in the field or in the largest containers had no reduction, or even an increase, in photosynthetic capacity. From his work, Arp concluded that if the capacity of the sink for carbohydrates is reduced by low nitrogen, low temperature or restricted root growth, then an increased supply of photosynthate under elevated C_a will result in feedback inhibition and a subsequent decrease in photosynthetic capacity (the nitrogen issue is reviewed in the next section). Thus, under this theoretical background, negative acclimation is not the response which is likely to be seen in natural or agro-ecosystems in response to a future rise in C_a . Ziska *et al* (1990), and other authors, have also come to the conclusion that field plants are less likely to show down-regulation as they have fewer constraints and can develop stronger sinks.

In addition to these experimental problems, Long *et al* (1993) explain that some past experiments may have been too short to show fully the response to elevated C_a . Over prolonged exposure plants may develop changes in sink sizes, root system size and tissue carbon:nitrogen ratios. Long and his colleagues suggest that even in the field a loss of photosynthetic capacity may occur in the first few months as a

temporary phenomena until the sinks develop to an adequate size.

4.2 Nutrient Supply

Many writers have suggested that negative acclimation will be more apparent in plants grown with a low nutrient supply, especially low nitrogen, under exposure to elevated C_a (Sage *et al.*, 1989; Bazzaz, 1990; Arp, 1991; Long *et al.*, 1993; Webber *et al.*, 1994; Woodrow, 1994; Rogers *et al.*, 1996).

Webber and his co-workers (1994) explain the theoretical basis for this hypothesis; at a low nitrogen supply negative acclimation facilitates the reallocation of the nitrogen contained in Rubisco towards either RuBP regeneration or even to other plant organs to increase sink size. This is supported by experimental findings; for instance, Woodrow (1994) found that reduction of Rubisco under elevated C_a increases the Nitrogen Use Efficiency (NUE) of the leaf. Rogers *et al.* (1996) found that foliar nitrogen concentrations were reduced from 42 to 38 and 36 mg g⁻¹ where CO₂ concentrations were increased from 350 to 500 and 900 μLL^{-1} respectively.

Arp (1991) found that, when grown in large pots or in the field, plants exposed to elevated C_a increased their root:shoot ratio. Such a finding supports the hypothesis that plants can maintain a 'functional balance' between materials essential for growth by allocating resources nearest to the source of the most limiting material (Acock & Pasternak, 1986). In other words, increasing root growth increases the plants' uptake of nitrogen from the soil. Jongen *et al.* (1995) found that root ingrowth of *L. perenne* decreased as nitrogen fertiliser increased, suggesting that the plant did not need to invest so many resources into obtaining nitrogen when the artificial supply was high. It is important to note briefly that the growth of a leguminous species to elevated C_a may be expected to demonstrate an alternative response to nitrogen treatments, as these plants can fix their own nitrogen. Indeed, Jongen and his colleagues (1995) found that root ingrowth in the legume *Trifolium repens* did increase under an enriched CO₂ environment, but this response was not effected by nitrogen supply.

However, despite these convincing arguments, there are some authors

who do not wholly believe in the role of nitrogen in photosynthetic adjustment to elevated C_a (Wong, 1979; Cure & Acock, 1986; Bowes, 1991; Hocking & Meyer, 1991; Bunce, 1992). For instance, Rowland-Bamford *et al* (1991) found that even when nitrogen is not limiting, the Rubisco content and activity still decline in some species. Bowes (1991) states that growth is usually enhanced under C_a elevation, even when the supply of total or specific nutrients is limiting. Thus, the evidence all seems rather contradictory and confusing. In a very recent paper, Lloyd and Farquhar (1996) have actually argued that the whole idea of nutrient limited plants responding less to increases in C_a has no theoretical basis anyway. They suggest that the central thesis for this 'limiting factor' approach seems to be based on 'The Law of the Minimum' devised by Liebig back in 1851. Yet even at that time there were experiments which proved this theory to be wrong (Lawes & Gilbert, 1855). Lloyd and Farquhar (1996) use substantial data to demonstrate that relative growth enhancement under elevated C_a will not necessarily be reduced where N is not optimal. In agreement with this, Sinclair (1992) introduces a framework of 'simultaneous limitation' of plant growth, with respect to nutrients and CO_2 concentration.

Evidence for the arguments against the role of nitrogen in photosynthetic adjustment include two studies which suggest alternative reasons for reduced leaf-nitrogen concentrations under elevated C_a . Conroy and Hocking (1993) explain that lower foliar nitrogen levels could result from increases in the concentration of leaf carbohydrate, or even because elevated C_a may reduce stomatal aperture which could decrease nitrogen uptake through lower transpiration rates. Alternatively, Coleman *et al* (1993) conclude that the reduction in plant nitrogen concentrations may not be due to physiological changes in plant NUE, rather it is probably a size dependent phenomenon resulting from accelerated plant growth.

It is imperative that the association between photosynthetic adjustment under elevated C_a and nitrogen supply is understood because this may greatly influence future agricultural practices. Changes in fertiliser application rates may be required in order to optimise any benefit from increased CO_2 (Rogers *et al*, 1996).

4.3 Cutting Regime

Arp (1991) explains how the removal of sinks, such as through cutting, will decrease the demand for carbohydrate and so aggravate sink limitation, which under his theory would lead to more negative acclimation of photosynthesis under elevated C_a . This mechanism is simple to understand for a crop such as soybean, where removal of pods was found to reduce photosynthesis and so cause more negative acclimation (Clough *et al*, 1981). However, for *L. perenne* this idea is more complicated because when cut the plant is losing not only some of its sink but also its source. This makes understanding the effect under elevated C_a more complicated, even with the simple framework set out by Arp (1991); thus, it becomes necessary to study work done on forage grass species in an attempt to understand the mechanisms involved.

Woledge and Parsons (1986) explain how cutting effects the photosynthesis of grassland crops under ambient conditions; this reference provides useful background information for this topic. Nijs *et al* (1988) found that of the considerable increase in dry matter attained in *L. perenne* grown in elevated C_a , an important fraction was built up in the first few days after cutting. In 1989 the same working group reported that after clipping the extra carbon, from the enriched atmosphere, was invested relatively more into shoots. Then, in the course of the canopy development, the allocation became reversed and with more partitioned to the roots. This clearly highlights the balancing of the sources and sinks under elevated C_a , in agreement with the hypothesis of functional equilibrium introduced in an earlier section. These results also suggest, in answer to the above query, that the leaves of *L. perenne* are extremely important as a source and relatively unimportant as a sink in relation to cutting.

In 1993, Nijs and Impens concluded that cutting stands sooner and more frequently could yield a higher increase in seasonal production from elevated C_a . This is thought to be because the leaves would be in the younger growth stage for most of the time, where they are found to be most efficient at utilising the additional CO_2 . Such a conclusion could have great implications for future grassland management but,

may need additional clarification and quantification if it is to be of practical use to farmers.

4.4 Interactions Between Elevated C_a and Light Level

In keeping with Arp's (1991) ideas on source-sink changes, the influence of varying light levels on photosynthesis under elevated C_a is extremely interesting. Ehret and Joliffe (1985) found that the photosynthetic capacity of shaded bean plants was not effected after growth in elevated C_a , whereas the capacity of unshaded plants was reduced. This is in agreement with Bunce (1992) who worked on soybean; he found that, statistically, there was a larger negative effect on photosynthesis at enhanced C_a when plants were grown at a higher Photosynthetic Photon Flux Density (PPFD). Bunce speculates that this may be related to feedback inhibition but stresses that his results are not wholly supportive of such a hypothesis.

In addition to this inconsistency within Bunce's (1992) work, several other authors have actually found an opposite effect when studying the interactions of light and elevated C_a . For instance, Sionit and colleagues (1982) found that total dry matter production increased in four species studied when exposed to elevated C_a and high PPFD. Wilks *et al* (1995) also calculated the CO_2 fertiliser effect to be greatest at high light intensity. In their work on *L. perenne*, Nijs *et al* (1989) also found that the effect of elevated C_a is strongest at high PPFD, and they concluded that the high LAI, which developed in the elevated C_a canopy, is a disadvantage under low light due to fast light extinction.

Little work has been carried out on the important question of how photosynthesis responds diurnally under exposure to elevated C_a . Nijs *et al* (1992) have studied this on perennial ryegrass; they explain that afternoon depression in photosynthesis can result under normal conditions for some of the following reasons:-

- saturation of sinks for carbohydrate, leading to an inhibition of phloem loading
- temperatures exceeding the optimum for photosynthesis

- stomatal closure
- increased respiration due to increased temperature
- inhibition through lower water potential of the leaf caused by higher transpiration

The results obtained by Nijs and colleagues (1992) indicate that high C_a treatment could actually alleviate the afternoon depression of A. They speculate that this could be due to changes in the temperature optimum for photosynthesis. This is because at higher temperatures the specificity of Rubisco for CO_2 is decreased (Long, 1991); therefore, in a C_a enriched environment would have a greater influence at higher temperatures. Alternatively, Nijs *et al* suggest that under elevated C_a the sink capacity for photosynthates may be relatively larger due to a higher root:shoot ratio developing.

A significant finding from Nijs *et al* (1992) is that they were able to measure photosynthesis on some days when temperature and light were actually the same in the morning as in the afternoon; on these occasions afternoon A was always lower than morning A. This leads to the hypothesis that it is source-sink balance which controls the depression rather than an environmental factor such as temperature. However, Nijs and his colleagues are quick to point out that the evidence for this hypothesis is scarce as environmental conditions are rarely the same in the morning as the afternoon. More work such as this though, could provide the evidence needed to confirm the role of sources and sinks in the negative acclimation theory.

5. CANOPY FACTORS

5.1 Canopy and Leaf Relations under Elevated C_a

Previous sections have concentrated on the effects of elevated C_a at the leaf level but, such investigations are of little practical value unless some extrapolation to the whole plant level can be made. Although leaf level analysis can provide valuable insights into a plant's adaptation to its' environment, integration with the canopy level is necessary in understanding and predicting crop, community and biome level productivity (Norman, 1980; Forseth & Norman, 1993).

In line with this it is important to realise that the process of plants' adjustment to elevated C_a is not restricted to the biochemical processes of the leaves. Some discussion of the role of root changes in acclimatisation have been made, with particular reference to the idea of functional equilibrium (see Jongen *et al*, 1995). However, anatomical and morphological changes at the canopy level can also be extremely important (Bowes, 1991). There are several theories surrounding the relationship between leaf and canopy during acclimation to elevated C_a . Firstly, Bowes (1991) hypothesises that if photosynthetic efficiency per unit area were to decline in response to enriched CO_2 , an increase in other parameters such as LAI could make enhancement of biomass still possible. Bowes goes on to speculate that such canopy changes could provide large enough storage sites for starch that chloroplasts do not become damaged, by the accumulation into starch grains, and thus a high photosynthetic rate could be maintained. This hypothesis is supported by the source-sink theory, for instance, Long *et al* (1993) explain that negative acclimation is most marked in species where the capacity for new sites of storage or utilisation is limited. Also, Rogers *et al* (1996) suggest that species such as grasses, which are more flexible in their sink capacity through their ability to tiller, could experience a more positive response to elevated C_a as they are less likely to be sink limited.

However, a high LAI may actually be disadvantageous due to high light extinction and greater respiration loss (Long *et al*, 1993). Also, there is some evidence of an opposing theory of the link between leaf photosynthesis and canopy parameters. Blum (1996 - Personal communication) has observed a decrease in LAI in *L. perenne* grown for three years at low nitrogen and a C_a of around 600 vpm. Yet in the third year of growth no negative acclimation of leaf photosynthesis was seen (Hymus, 1995). This suggests that the crop may have acclimated negatively at the canopy level to elevated C_a so that a balance between source and sink is achieved but, still a stimulation of leaf photosynthesis can be maintained.

With reference to *L. perenne*, Nijs *et al* (1989) studied the response of both leaves and canopy to elevated C_a . They found that the net canopy CO_2 and H_2O

exchange rate maximum to be 49% at elevated C_a whereas stimulation of leaf photosynthesis was only 46%. They concluded that this indicated the important influence of a higher LAI to increasing productivity under elevated C_a . However, the evidence concerning *L. perenne* is not conclusive, as in a later study Nijs and Impens (1993) found that leaf CO_2 uptake was a more influential factor than LAI in determining photosynthetic differences between the ambient and elevated C_a treatments. Nijs and Impens did find that straight after a cut, the contribution of LAI to the canopy is relatively high although it become progressively less over time; this suggests that the role of growth stage within canopy experiments needs further investigation.

5.2 Models Linking Leaf and Canopy Photosynthesis

There have been several attempts to model, mechanistically, the relationships between leaf and canopy photosynthesis, so that productivity in a future high C_a environment can be predicted. Farquhar *et al* (1980), and later modifications by Sharkey (1985), have developed formulations for examining the direct and interactive effects of CO_2 , light and temperature on the photosynthesis of individual leaves. Wilks (1995) believes that there are too many difficulties in parameterization and differences in scale between photosynthesis biochemistry and whole plant processes; he states that these problems may explain why extrapolation of the Farquhar model to whole canopy photosynthesis has not been more widely used. However, Humphries and Long (1995) disagree, they believe that the mechanistic model of leaf photosynthesis can be effectively used, when combined with physical models of light and gas transport in canopies, to extrapolate from leaf to whole plant scale. Indeed predictions of photosynthetic stimulation under elevated C_a are consistent with the few canopy level exchange measurements made to date (see Drake & Leadley, 1991; Pinter *et al*, 1995).

Norman (1980) and Forseth & Norman (1993) explain a strategy for estimating canopy photosynthesis. They write that, if leaf photosynthetic rate and stomatal conductance are known as a function of the light incident on the leaf, then the canopy photosynthetic rate, and canopy conductance, can be measured from simple

measurements above and below the canopy. This method requires division of the canopy into sunlit and shaded leaves, and then a summing up of their relative contributions. Although simple, this method has been found to give results as accurate as some more complex models.

The very latest models being developed for scaling from the canopy are now very sophisticated (see Aber *et al*, 1996, for forests). The WIMOVAC model developed by Humphries and Long (1995) actually offers three separate models of canopy microclimate; firstly, the simple model of Forseth and Norman (1993), secondly, a model with multiple layers to account for diffuse light absorption (Reynolds *et al*, 1992) and also a model which allows for the canopy to be divided into rows as a crop would be.

Thus, with such developments in canopy modelling, it is likely that scientists will soon be able to predict canopy photosynthesis from leaf photosynthesis with an increasing degree of accuracy. However, what will the use of this be with regard to future plant productivity? The answer to this is unclear as many writers assume that measures of canopy photosynthesis can be used to predict growth. yet, Lloyd and Farquhar (1996) disagree, they state that the CO₂ dependence of plant growth is not a simple quantitative reflection of the CO₂ dependence of photosynthesis. In addition, the calculation of actual yield is an even more complicated subject. Therefore, perhaps it is enough at the moment to use models of canopy photosynthesis to predict future productivity because, despite the criticisms, they may be the best available means at this time.

6. CONCLUSIONS

It is difficult to draw conclusions from a review which has shown that many inconsistencies and disparities still exist within the available literature. Still, a general consensus regarding the long-term acclimatisation of C3 plants to elevated C_a does emerge. It can be summarised as a source-sink imbalance resulting in a reallocation of resources (especially Rubisco) which causes a reduction in photosynthetic capacity.

However, it is rather concerning that many scientists working in this field assume many of the hypotheses surrounding the photosynthetic adjustment to be correct despite evidence to the contrary. These assumptions include acceptance of the term 'acclimation' to describe down-regulation when it should also encompass a possible positive response to elevated C_a . Also, there is overwhelming support for the reallocation theory and the role of nitrogen despite arguments against this. Therefore, one conclusion which can be drawn is that it is essential that workers in this area retain an open mind regarding the mechanisms behind the long-term acclimatisation of plants to elevated C_a .

This review has clearly shown the importance of using a whole plant framework, even when studying just leaf photosynthesis. It is important to remember that the chloroplasts do not operate as separate entities but, rather as integrated parts of the whole plant system. This review has highlighted the necessity of linking with the canopy level but in addition much more work is needed on root changes in an enriched CO_2 environment.

Finally, if studies, such as those reviewed here, are to be used in predicting future productivity, it is essential to know how photosynthetic predictions can be related, not only to growth, but also to yield parameters. Also, the under-researched issue of inter- and intra-specific variation must be addressed as its consequences for competition within natural and agro-ecosystems could be critical.