



RESEARCH PAPER

# Variation in acclimation of photosynthesis in *Trifolium repens* after eight years of exposure to Free Air CO<sub>2</sub> Enrichment (FACE)

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## Abstract

The initial stimulation of photosynthesis observed on elevation of [CO<sub>2</sub>] in grasslands has been predicted to be a transient phenomenon constrained by the loss of photosynthetic capacity due to other limitations, notably nutrients and sinks for carbohydrates. Legumes might be expected partially to escape these feedbacks through symbiotic N<sub>2</sub> fixation. The Free-Air Carbon dioxide Enrichment (FACE) experiment at Eschikon, Switzerland, has been the longest running investigation of the effects of open-air elevation of [CO<sub>2</sub>] on vegetation. The prediction of a long-term loss of photosynthetic capacity was tested by analysing photosynthesis in *Trifolium repens* L. (cv. Milkanova) in the spring and autumn of the eighth, ninth and tenth years of treatment. A high and low N treatment also allowed a test of the significance of exogenous N-supply in maintaining a stimulation of photosynthetic capacity in the long-term. Prior work in this Free Air CO<sub>2</sub> Enrichment (FACE) experiment has revealed that elevated [CO<sub>2</sub>] increased both vegetative and reproductive growth of *T. repens* independent of N treatment. It is shown here that the photosynthetic response of *T. repens* was also independent of N fertilization under both current ambient and elevated (600 µmol mol<sup>-1</sup>) [CO<sub>2</sub>]. There was a strong effect of season on photosynthesis, with light-saturated rates ( $A_{\text{sat}}$ ) 37% higher in spring than in autumn. Higher  $A_{\text{sat}}$  in the spring was supported by

higher maximum Rubisco carboxylation rates ( $V_{\text{c,max}}$ ) and maximum rates of electron transport ( $J_{\text{max}}$ ) contributing to RuBP regeneration. Elevated [CO<sub>2</sub>] increased  $A_{\text{sat}}$  by 37% when averaged across all measurement periods and both N fertilization levels, and decreased stomatal conductance by 25%. In spring, there was no effect of elevated [CO<sub>2</sub>] on photosynthetic capacity of leaves, but in autumn both  $V_{\text{c,max}}$  and  $J_{\text{max}}$  were reduced by approximately 20% in elevated [CO<sub>2</sub>]. The results show that acclimation of photosynthetic capacity can occur in a nitrogen-fixing species, in the field where there are no artificial restrictions on sink capacity. However, even with acclimation there was a highly significant increase in photosynthesis at elevated [CO<sub>2</sub>].

Key words: Acclimation, gas exchange, global atmospheric change, global climate change, grassland, nitrogen fixation, photosynthetic electron transport, Rubisco, white clover.

## Introduction

Species capable of symbiotic N<sub>2</sub> fixation often show a larger stimulation of growth and photosynthetic rate to elevated atmospheric carbon dioxide concentration ([CO<sub>2</sub>]) than species or genotypes that do not fix N<sub>2</sub> (Soussana and Hartwig, 1996; Clark *et al.*, 1997; Hebeisen *et al.*, 1997; Schenk *et al.*, 1997; Lüscher *et al.*, 1998, 2000; Ainsworth *et al.*, 2002). One explanation for the

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Abbreviations:  $A_{\text{sat}}$ , light-saturated rate of leaf CO<sub>2</sub> assimilation at growth [CO<sub>2</sub>];  $c_a$ , atmospheric CO<sub>2</sub> concentration;  $c_i$ , intercellular CO<sub>2</sub> concentration; FACE, Free Air CO<sub>2</sub> Enrichment;  $g_s$ , stomatal conductance to water vapour;  $J_{\text{max}}$ , *in vivo* maximum rate of electron transport;  $V_{\text{c,max}}$ , *in vivo* maximum rate of carboxylation.

greater stimulation in the growth of N<sub>2</sub>-fixing species grown at elevated [CO<sub>2</sub>] is a smaller tendency toward photosynthetic acclimation, i.e. down-regulation of photosynthetic capacity in leaves developed in elevated [CO<sub>2</sub>] (Ryle *et al.*, 1992; Davey *et al.*, 1999). Down-regulation of photosynthetic capacity in response to elevated [CO<sub>2</sub>] most commonly involves a decrease in the amount of active Rubisco (Long and Drake, 1992; Rogers and Humphries, 2000). Accelerated growth under elevated [CO<sub>2</sub>] increases the demand for nutrients and, in fertile soils, nitrogen is usually the growth-limiting nutrient (Lüscher *et al.*, 1998). In elevated [CO<sub>2</sub>] and a limiting N-supply, Rubisco content is often reduced (Rogers *et al.*, 1998). It is possible that because Rubisco can account for a large proportion of leaf N, the amount of the enzyme may be reduced under N-limiting conditions (Drake *et al.*, 1997). Clearly, under elevated [CO<sub>2</sub>], N<sub>2</sub>-fixing species would have an added advantage over non-N<sub>2</sub>-fixing species, and may be able to sustain a higher Rubisco concentration and photosynthetic capacity. On the other hand, in the long-term a decrease in photosynthetic capacity may result because of changes at the whole plant and system level. Elevated [CO<sub>2</sub>] may partition resources away from leaves and, through increased production, sequester nutrients into organic matter causing deficiencies which indirectly cause decreased photosynthetic capacity. By modelling these system level feedbacks, Luo and Reynolds (1999) predicted that the initial stimulation of photosynthetic production in grasslands would be lost within nine years of a step increase in [CO<sub>2</sub>], as imposed in FACE experiments.

While plants may face increased N demand under elevated [CO<sub>2</sub>], evidence suggests that the fundamental cause of acclimation is an inability of plants to utilize photosynthate (Stitt and Krapp, 1999; Ainsworth *et al.*, 2003). Therefore, the strong carbon demand of N<sub>2</sub>-fixing species and their ability to adjust symbiotic nitrogen fixation to the growing demand may play an important role in the response to elevated [CO<sub>2</sub>] (Zanetti *et al.*, 1996; Lüscher *et al.*, 1998). *Trifolium repens* (L.) is an important N-fixing species in temperate pastures and grasslands (Parsons *et al.*, 1991). The nodulated root can require up to 60% of the photosynthate produced over a 12 h photo-period (Gordon *et al.*, 1987). In addition to nodules, *T. repens* has a large reserve organ (stolon) for starch and vegetative storage proteins, which adds to the strong sink demand of the species. However, the sink demand of *T. repens* is seasonal. Late in the growing season, when root temperatures drop below 10 °C, shoot growth is greatly reduced, N<sub>2</sub> fixation is inhibited, and vegetative storage proteins begin to accumulate in stolons (Bouchart *et al.*, 1998).

The Free Air CO<sub>2</sub> Enrichment (FACE) experiment at Eschikon in Switzerland, has been investigating the effects of elevated [CO<sub>2</sub>] on grassland vegetation under

completely open-air conditions. It has been the longest running replicated FACE experiment in the world. It therefore provides a unique opportunity to test predictions on how the grassland legume, *T. repens*, responds in the long-term to elevated [CO<sub>2</sub>]. It has already been shown that growth at elevated [CO<sub>2</sub>] in this experiment significantly increased the percentage of plant N derived from symbiotic N<sub>2</sub> fixation in *T. repens* grown under both high and low N fertilization treatments (Zanetti *et al.*, 1996). Early in the growing season, under low fertilization conditions and elevated [CO<sub>2</sub>], over 70% of plant N was derived from symbiosis (Zanetti *et al.*, 1996). However, late in the growing season, the percentage of plant N derived from symbiosis was significantly less, particularly under high N fertilization where it provided less than 10% of total plant N (Zanetti *et al.*, 1996).

In this study, the photosynthetic response of *T. repens* to elevated [CO<sub>2</sub>] in the field at the FACE experiment in Eschikon, Switzerland was investigated after eight years of treatment. Although many studies have examined the effects of elevated [CO<sub>2</sub>] on the acclimation of photosynthesis, few have examined either the effect following such a long period (8 years) under open-air treatment, or considered N-fixing species. The responses of light-saturated photosynthesis ( $A_{\text{sat}}$ ) to intercellular [CO<sub>2</sub>] ( $c_i$ ) were examined at two contrasting periods: early in the growing season when plants were vigorously growing and deriving a significant portion of their N from symbiotic fixation, and late in the autumn, after the first frost, when symbiotic N<sub>2</sub> fixation was low. The aims of this work with *T. repens* were to determine if there was acclimation of photosynthesis in *T. repens* in response to long-term growth at elevated [CO<sub>2</sub>] and, if so, the extent to which this offset the stimulation of photosynthesis that would otherwise have occurred. Specifically, this work examines two contrasting predictions. (1) Despite long-term growth at elevated [CO<sub>2</sub>], there will be no acclimation of photosynthetic capacity because of the N-fixing capacity of the species. (2) Acclimation will occur because of system-level feedbacks that limit the response of grasslands to elevated [CO<sub>2</sub>] (Luo and Reynolds, 1999).

## Materials and methods

### Growth conditions and experimental design

*Trifolium repens* L. (cv. Milkanova) was grown in monoculture plots in the Swiss FACE array (Hebeisen *et al.*, 1997) at Eschikon, Switzerland (8°41' E, 47°27' N), 550 m above sea level. The experiment was designed as a split-plot with three blocks. Blocks were organized according to the crops grown in the year preceding the installation of the FACE facility in 1993. The main plot treatment was carbon dioxide concentration ([CO<sub>2</sub>]), with three 18 m diameter circular plots maintained at ambient [CO<sub>2</sub>] (~360 μmol mol<sup>-1</sup>) and three plots maintained at elevated [CO<sub>2</sub>] (600 μmol mol<sup>-1</sup>). Elevated [CO<sub>2</sub>] was obtained using the FACE technology described by Lewin *et al.* (1992). Fumigation began each year in March when air temperature at 2 m above-ground reached 5 °C; active growth of

**Table 1.** The dates of photosynthesis measurements and the meteorological data, mean air temperature (measured over 24 h), average total daily global radiation, and total precipitation, describing the days are listed

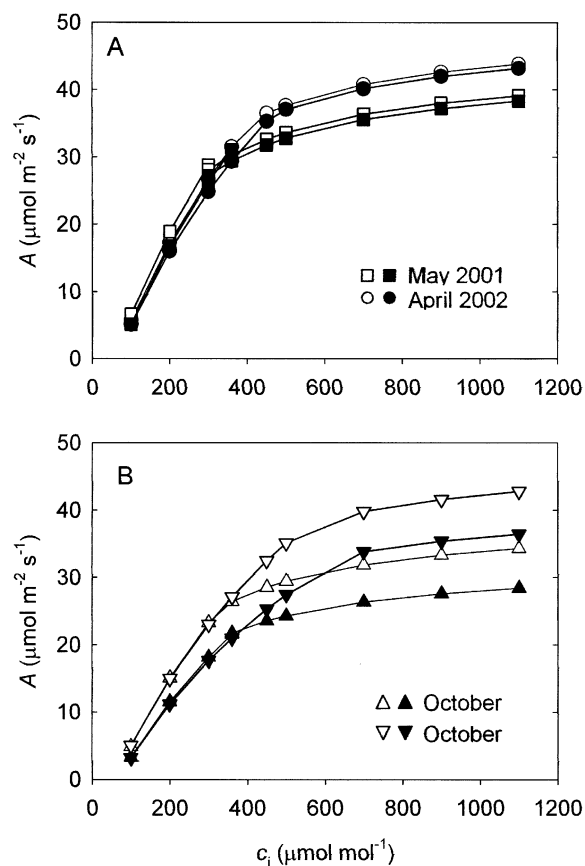
Date	Temperature (°C)	Global radiation (MJ m <sup>-2</sup> )	Rainfall (mm)
8–12 October 2000	7.2	5.8	21.0
1–3 May 2001	14.4	33.5	0
25 April – 1 May 2002	12.2	18.6	17.3
15–18 October 2002	10.4	7.4	48.7

*T. repens* requires temperatures above 5 °C (Hart, 1987). Detailed descriptions of the experimental site and initial field conditions are provided by Zanetti *et al.* (1996) and Lüscher *et al.* (1998). Within each ring, *T. repens* stands were grown under two N fertilization levels, 140 and 560 kg N ha<sup>-1</sup> year<sup>-1</sup> applied as NH<sub>4</sub>NO<sub>3</sub>. Swards were defoliated five times per growing season between April and the beginning of November, and each defoliation removed approximately 80% of the above-ground biomass.

#### Leaf photosynthetic gas exchange

Photosynthetic gas exchange measurements were taken from 8–12 October 2000, 1–3 May 2001, 25 April to 1 May 2002, and 15–18 October 2002. Measurements in April and May (2001 and 2002) were taken before the first harvest, and measurements in October were taken before the final harvest of the season. The average temperature, total precipitation and average sum global radiation during the times of measurement are listed in Table 1. The response of photosynthesis ( $A$ ) to changes in intercellular  $[CO_2]$  ( $c_i$ ) was measured with a portable, steady-state gas-exchange system, incorporating an infrared gas analyser (LI-6400, Li-Cor, Lincoln, NE, USA). Photosynthesis was initially induced at the growth  $[CO_2]$ . The reference  $[CO_2]$  was reduced stepwise to a lower concentration of 50  $\mu\text{mol mol}^{-1}$  and then increased stepwise to an upper concentration of 1200–1400  $\mu\text{mol mol}^{-1}$ . Light-saturated photosynthetic rate ( $A_{\text{sat}}$ ) and stomatal conductance to water vapour ( $g_s$ ) at growth  $[CO_2]$  were extrapolated from  $A/c_i$  response curves. Three to four leaves were measured for each N treatment within each ring. The objective was to infer changes in the underlying photosynthetic capacity of leaves. Transient decrease in water potential, decrease in chloroplast inorganic phosphate concentration, and decrease in maximum photosystem II efficiency can all occur after a few hours of sunlight, and may all alter the  $A/c_i$  response. To avoid this complication, gas exchange analysis was conducted on cut leaves, sampled pre-dawn and kept with petioles in water and at low light prior to measurement. Leaves were sampled from the canopy and removed by cutting the petiole under water.  $A/c_i$  response curves were measured within 5 h of cutting the leaves. Rates of photosynthesis measured on leaves, sampled in this way, equalled or exceeded those measured *in situ* suggesting that this procedure did not lower photosynthetic capacity. In total, 184  $A/c_i$  curves, each of 10–11 data points were determined. A Peltier cooling system maintained leaf temperature at 25 °C and an integral red/blue LED light source provided saturating light of 1250  $\mu\text{mol m}^{-2} \text{s}^{-1}$ .

Photosynthetic parameters were calculated by fitting the equations of Farquhar *et al.* (1980) and by maximum likelihood regression (Sigmaplot, Jandel Scientific, Erkrath, Germany). The maximum *in vivo* Rubisco activity ( $V_{c,\text{max}}$ ) was determined from points below the inflexion of the  $A/c_i$  plot, and the maximum *in vivo* electron transport rate ( $J_{\text{max}}$ ) was determined from values above the inflexion (Fig. 1).



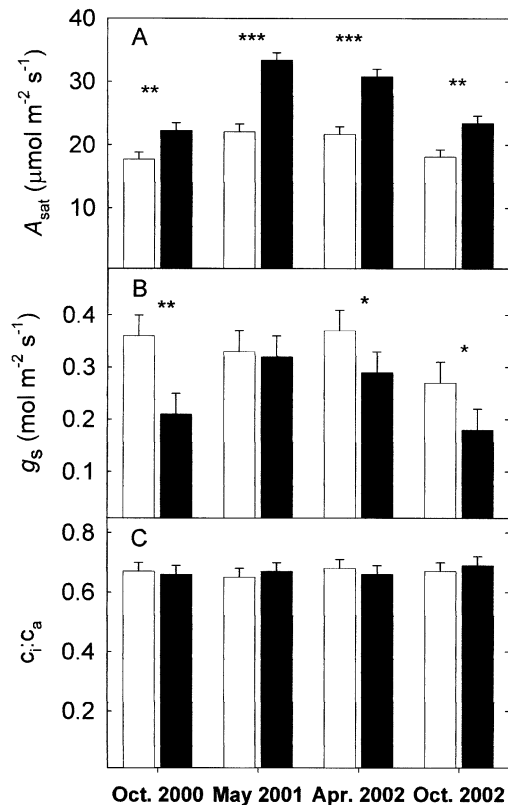
**Fig. 1.** Average plots of  $A/c_i$  response curves for *T. repens* grown (A) at the beginning of the growing season and (B) at the end of the growing season. The modelled values of the maximum RuBP-saturated rate of carboxylation *in vivo* ( $V_{c,\text{max}}$ ) and the maximum *in vivo* rate of electron transport contributing to RuBP regeneration ( $J_{\text{max}}$ ) are fitted for each plot. White symbols represent control  $[CO_2]$  and black circles represent FACE.

#### Statistical analysis

The statistical analysis was carried out using the general linear model (GLM) procedure of the SAS package (SAS Institute, 1996). The original model was a split-plot in time with  $[CO_2]$  as the main plot factor and N as the split-plot factor. However, there was no significant effect of N on any of the variables, so N treatments were pooled for the analysis. The  $[CO_2]$  effect was tested with the  $[CO_2] \times$  block interaction as the error term. Preplanned comparisons of means within measurement periods were carried out using linear contrasts.

#### Results

Growth of *T. repens* at elevated  $[CO_2]$  (600  $\mu\text{mol mol}^{-1}$ ) significantly stimulated the light-saturated photosynthetic rate ( $A_{\text{sat}}$ ) by 37%, when averaged across all measurement periods ( $P < 0.05$ , Fig. 2A). Comparisons within  $[CO_2]$  treatments revealed that  $A_{\text{sat}}$  was higher in the spring (May 2001 and April 2002) than in the autumn (October 2000 and October 2002) ( $P < 0.01$  for ambient  $[CO_2]$ ,  $P < 0.001$  for elevated  $[CO_2]$ ). The difference in spring and autumn

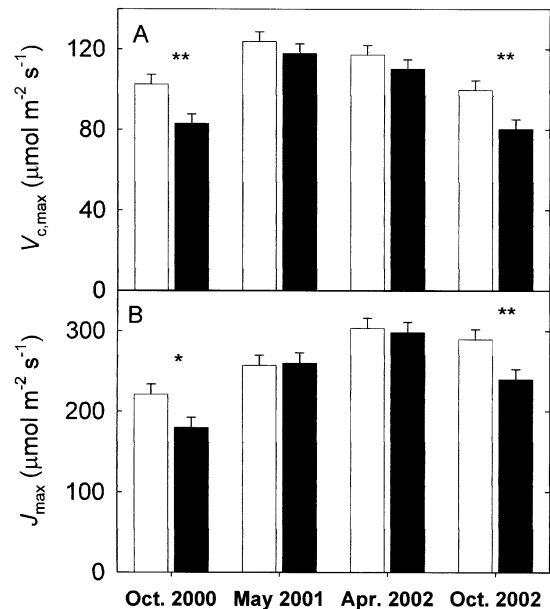


**Fig. 2.** (A) Light-saturated photosynthetic rate at growth  $[\text{CO}_2]$  ( $A_{\text{sat}}$ ), (B) stomatal conductance to water vapour ( $g_s$ ), and (C) ratio of intercellular  $\text{CO}_2$  concentration ( $c_i$ ): atmospheric  $\text{CO}_2$  concentration ( $c_a$ ) for *T. repens* grown under control ( $360 \mu\text{mol mol}^{-1}$ , white bars) and elevated (FACE  $600 \mu\text{mol mol}^{-1}$ , black bars)  $[\text{CO}_2]$  ( $\pm 1$  SE). Mean control and FACE values within time periods ( $n=6$ ) were separated using linear contrast statements. \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ .

photosynthetic rates was greater for plants grown at elevated  $[\text{CO}_2]$  (40% higher in spring) than plants grown at ambient  $[\text{CO}_2]$  (22% higher in spring).

The average  $A/c_i$  response for plants grown at ambient and elevated  $[\text{CO}_2]$  during each measurement period is shown in Fig. 1. There was no change in the photosynthetic capacity of *T. repens* with growth at elevated  $[\text{CO}_2]$  in May 2001 and April 2002 (Fig. 3), but there was significant down-regulation of photosynthetic capacity in October 2000 and October 2002 (Fig. 3). In October 2000 and October 2002,  $V_{\text{c,max}}$  was 19% lower in plants grown under elevated  $[\text{CO}_2]$  (Fig. 3A), and  $J_{\text{max}}$  was 18% lower (Fig. 3B). The ratio of  $V_{\text{c,max}}:J_{\text{max}}$  was 0.41, when averaged over all treatments and measurement periods, and was not significantly affected by  $[\text{CO}_2]$  or season.

Growth at elevated  $[\text{CO}_2]$  caused a 25% decrease in stomatal conductance ( $g_s$ ) when averaged over all measurement periods (Fig. 2B). There was no effect of season on  $g_s$  of plants grown at ambient  $[\text{CO}_2]$ ; however,  $g_s$  was significantly lower in the autumn than the spring for plants grown at elevated  $[\text{CO}_2]$  ( $P < 0.01$ , Fig. 2B). The



**Fig. 3.** (A) Maximum *in vivo* Rubisco activity ( $V_{\text{c,max}}$ ), and (B) maximum *in vivo* electron transport rate ( $J_{\text{max}}$ ) of *T. repens* grown under control ( $360 \mu\text{mol mol}^{-1}$ , white bars) and elevated (FACE  $600 \mu\text{mol mol}^{-1}$ , black bars)  $[\text{CO}_2]$  ( $\pm 1$  SE). Mean control and FACE values within time periods ( $n=6$ ) were separated using linear contrast statements. \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ .

ratio of intercellular  $[\text{CO}_2]$  ( $c_i$ ) to atmospheric  $[\text{CO}_2]$  ( $c_a$ ) for *T. repens* was not affected by growth  $[\text{CO}_2]$  or season (Fig. 2C).

## Discussion

*T. repens* was exposed to elevated  $[\text{CO}_2]$  and two N fertilization treatments in the field since 1993. There was no effect of N fertilization on *T. repens* photosynthesis, and prior work has revealed that the growth of *T. repens* is also unaffected by N fertilization in this experiment (Hebeisen *et al.*, 1997). Long-term growth at elevated  $[\text{CO}_2]$  is associated with consistently higher photosynthetic rates (Fig. 2A) and lower rates of stomatal conductance (Fig. 2B). The 40% increase in  $A_{\text{sat}}$  in the spring and 22% increase in  $A_{\text{sat}}$  in the autumn is in close agreement with previously published data for this species (Greer *et al.*, 2000; Laing *et al.*, 2002). The measured 25% reduction in  $g_s$  closely agrees with the 24% decrease in  $g_s$  reported for 13 prairie grassland species (Lee *et al.*, 2001). However, no change in the  $c_i:c_a$  at elevated  $[\text{CO}_2]$  was observed, indicating that reductions in  $g_s$  did not affect photosynthesis any more than at ambient  $[\text{CO}_2]$  (Drake *et al.*, 1997). These results do not support the prediction that the response of grassland species to elevated  $[\text{CO}_2]$  will be short-lived as the demand for nutrients increases (Luo and Reynolds, 1999). Increased  $\text{N}_2$  fixation per plant under elevated  $[\text{CO}_2]$  may have allowed for sustained stimulation of photosynthetic capacity, enhancing the

response of the ecosystem to elevated [CO<sub>2</sub>] (Zanetti *et al.*, 1996).

In the spring, there was no change in photosynthetic capacity of plants with growth at elevated [CO<sub>2</sub>]. However, acclimation of photosynthesis to elevated [CO<sub>2</sub>] was apparent in October 2000 and October 2002 (Fig. 3). While the cause of acclimation of photosynthesis was not directly measured, the timing of acclimation suggests that sink limitation plays a major role. Observational evidence from many field experiments with white clover in this region showed that the leaf area index of white clover was rapidly decreasing in October (Hebeisen *et al.*, 1997). In addition, the rate of leaf emergence was very low at temperatures below 10 °C (Stäheli Posch, 1998). Acclimation occurred late in the growing season, when the 24 h mean temperature had dropped below 10 °C, and nightly frosts were occurring (Table 1). Under these conditions, shoot growth is limited and the sink for carbohydrate is small (Bouchart *et al.*, 1998), and acclimation of photosynthesis to elevated [CO<sub>2</sub>] would be expected (Stitt and Krapp, 1999). While spring temperatures were also cool (Table 1), daylength and radiation were higher and growth of the *T. repens* sward was approximately four times greater in the spring than the fall, under both ambient and elevated [CO<sub>2</sub>] (M Schneider, personal communication). In the spring mean leaflet size rapidly increased, the extension growth of the petioles was enhanced and flowering was also beginning, so plants were unlikely to be sink-limited, and acclimation of photosynthesis to elevated [CO<sub>2</sub>] was not expected.

In conclusion, an instantaneous increase in [CO<sub>2</sub>] caused a 57% increase in photosynthetic rate of *T. repens* (Fig. 1). Despite acclimation of photosynthetic capacity in leaves that developed under elevated [CO<sub>2</sub>], the long-term effect of growth at elevated [CO<sub>2</sub>] was a 37% increase in photosynthesis. Thus, contrary to the belief that the response of grassland species to elevated [CO<sub>2</sub>] will be short-lived, stimulation of photosynthesis in *T. repens* remained after eight years of exposure to elevated [CO<sub>2</sub>].

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