Full Paper

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Atmospheric CO_2 and VPD alter the diel oscillation of leaf elongation in perennial ryegrass: compensation of hydraulic limitation by stored-growth

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Summary

- We explored the effects of atmospheric CO_2 concentration (C_a) and vapor pressure deficit (VPD) on putative mechanisms controlling leaf elongation in perennial ryegrass.
- Plants were grown in stands at a C_a of 200, 400 or 800 μmol mol^{-1} combined with high (1.17 kPa) or low (0.59 kPa) VPD during the 16 h-day in well-watered conditions with reduced nitrogen supply. We measured day : night-variation of leaf elongation rate (LER_{day} : LER_{night}), final leaf length and width, epidermal cell number and length, stomatal conductance, transpiration, leaf water potential and water-soluble carbohydrates and osmotic potential in the leaf growth-and-differentiation zone (LGDZ).
- Daily mean LER or morphometric parameters did not differ between treatments, but LER_{night} strongly exceeded LER_{day}, particularly at low C_a and high VPD. Across treatments LER_{day} was negatively related to transpiration ($R^2 = 0.75$) and leaf water potential ($R^2 = 0.81$), while LER_{night} was independent of leaf water potential or turgor. Enhancement of LER_{night} over LER_{day} was proportional to the turgor-change between day and night ($R^2 = 0.93$). LGDZ sugar concentration was high throughout diel cycles, providing no evidence of source limitation in any treatment.
- Our data indicate a mechanism of diel cycling between daytime hydraulic and night-time stored-growth controls of LER, buffering C_a and daytime VPD effects on leaf elongation.

Introduction

Leaf growth is an integrating plant process (Van Volkenburgh, 1999): leaves intercept light, transpire H_2O and assimilate CO_2 in photosynthesis, supporting the growth and maintenance requirements of all parts of the plant. Conversely, leaf growth is sensitive to plant water status, which is influenced by transpiration, and depends on adequate supplies of assimilates and nutrients. As photosynthesis and transpiration respond to changing atmospheric CO_2 concentration (C_a) and humidity, one may expect fundamental effects on leaf growth (Fig. 1). Surprisingly, however, the scientific literature does not report general strong effects of atmospheric CO_2 on leaf growth (see paragraph 3 in this section), or other morphological features, particularly in conditions with growth-limiting nitrogen fertilizer availability (Reich *et al.*, 2014), a typical situation for many terrestrial ecosystems, including grassland (LeBauer & Treseder, 2008).

Grasses provide a convenient model for studies of leaf growth, as the leaf growth-and-differentiation zone (LGDZ) is physically separated from the photosynthesizing and transpiring leaf tissues. The LGDZ is non-transpiring and entirely dependent on assimilate import, as it is enclosed within the whorl of sheaths of older fully-expanded leaves (Fig. 1). Continuous cell production, expansion and differentiation in the LGDZ generate an efflux of near-fully mature and photosynthetically competent tissue from the enclosing sheath (Fig. 1). Leaf elongation rate is equivalent to the integral of (axial) cell expansion rates within a cell file spanning the length of the leaf growth zone (Schnyder *et al.*, 1990). Cell expansion depends on a close coordination of several processes and state variables, including (1) cell turgor pressure that causes a tensional stress in the primary cell wall, which stretches irreversibly when a given yield threshold is exceeded, (2) passive water flow into the expanding cell driven by the water potential gradient between the cell and the water source, (3) maintenance of this gradient by continuous adjustment of the cell osmoticum, which – in turn – generates turgor pressure, and (4) metabolic processes involved in cell wall deposition and loosening, as well as synthesis of intracellular constituents (e.g. Lockhart, 1965; Ray *et al.*, 1972; Barlow, 1986; Passioura & Fry, 1992; Martre *et al.*, 1999; Tardieu *et al.*, 1999; Pantin *et al.*, 2012; Cosgrove, 2018).

Photosynthesis of C_3 plants is not saturated with CO_2 at sub-ambient and present-day C_a (Bowes, 1993; Ainsworth & Rogers, 2007), raising the question if leaf growth can be limited, in principle, by the availability of assimilates (Ainsworth & Rogers, 2007), at least at the C_a of the Last Glacial Maximum (≈ 200 μmol mol^{-1}; Lüthi *et al.*, 2008). In fact, increased carbon

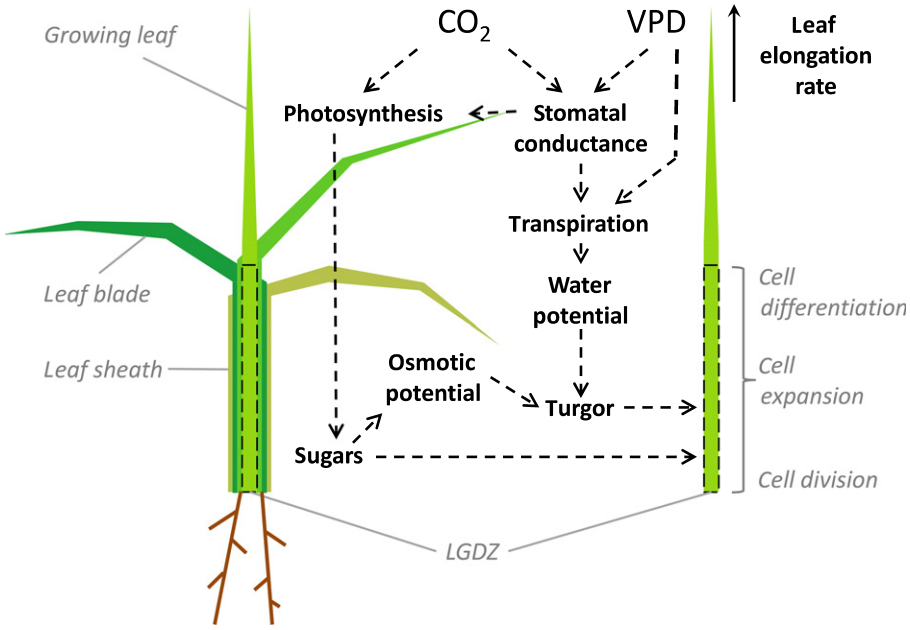

Fig. 1 Schematic illustration of source and hydraulic effects of atmospheric CO_2 concentration and vapor pressure deficit (VPD) on photosynthesis–transpiration–leaf growth physiological relationships in a vegetative grass plant (adapted from Fig. 1 in Liu *et al*., 2017). The leaf growth-and-differentiation zone (LGDZ) is fully enclosed within the sheaths of fully-expanded leaves and comprises successive zones of cell division, expansion and differentiation arranged along the longitudinal axis of the leaf. Leaf elongation is determined by (axial) cell expansion, enabled by turgor pressure-driven water uptake. Turgor pressure is the difference between osmotic potential and water potential. Stomatal conductance is sensitive to VPD and CO_2 and represents the physiological control of transpiration that affects water potential in the growth zone. Photosynthesis is influenced directly by CO_2 and indirectly by the CO_2 effect on stomatal conductance, and may act on assimilate supply to the leaf growth zone (source-limitation) as well as on the contribution of sugars (water-soluble carbohydrates) to the osmotic potential of expanding cells.

supply at elevated C_a tends to enhance leaf elongation via the stimulation of cell expansion, cell production or both (Ferris & Taylor, 1994; Ranasinghe & Taylor, 1996; Gamage *et al*., 2018). However, this response is much weaker than the effect of elevated C_a on leaf photosynthesis, stomatal conductance or water-use efficiency (Ainsworth & Long, 2005), with variability in the response linked to interactions with factors such as nutrient availability (Seneweera & Conroy, 2005), vernalization (Masle, 2000), growing season (Ferris *et al*., 1996), developmental stage (Seneweera *et al*., 1995; Masle, 2000) or genotype (Masle, 2000; Thilakarathne *et al*., 2015). Additionally, very high concentrations of carbohydrates in leaf growth zones were observed even when plants were grown at low irradiance at contemporary C_a (Schnyder & Nelson, 1989), providing no evidence of carbohydrate substrate limitation in tall fescue (*Festuca arundinacea*) in those conditions. Yet, the concentration of monosaccharides, sucrose and low-molecular weight fructans contributed up to > 0.4 MPa to the osmotic potential of leaf growth zone tissue water (Schnyder & Nelson, 1987), suggesting a possibly important role of carbohydrates in osmotic regulation in the leaf growth zone. Whether or not carbohydrate availability in the leaf growth zone can be growth-limiting (as a substrate or osmoticum) over a range of C_a remains unresolved.

Possibly, variation in leaf hydraulics is the most important factor controlling variation of leaf elongation when C_a and atmospheric vapor pressure deficit (VPD) change. A strong and rapid response of leaf elongation rate (LER) to changes in evaporative demand and leaf water status, caused by alterations in temperature, air humidity or light intensity, has been observed in several grasses (Volenec & Nelson, 1982; Parrish & Wolf, 1983; Schnyder & Nelson, 1988; Ben Haj Salah & Tardieu, 1996; Clifton-Brown & Jones, 1999; Bouchabke *et al*., 2006) in addition to effects of edaphic conditions, including drought (e.g. Passioura, 1988; Passioura, 2002). CO_2 and VPD could also indirectly influence the leaf water status of plants through their effect on stomatal conductance (g_s) (Sionit *et al*., 1981; Morrison, 1993; Tyree & Alexander, 1993; Ainsworth & Rogers, 2007; Kimball, 2016; Manderscheid *et al*., 2016; Buckley, 2019). Results from free-air CO_2 enrichment (FACE) and chamber experiments under various environmental conditions show a systematic, significant decrease of g_s in C_3 plants at elevated CO_2, producing an equivalent decrease in transpiration (Leakey *et al*., 2009). Such changes affected water potential, turgor pressure, and osmotic potential in the growing leaves of *Phaseolus vulgaris* (Ranasinghe & Taylor, 1996). However, the combined effect of atmospheric CO_2 and VPD in the growth environment on the mechanisms linking photosynthesis, transpiration and leaf growth at constant temperature have not been investigated in any detail.

One particularly conspicuous feature of leaf elongation rate is its generally strong diurnal variation, with lower rates during the day than during the night when stomata are closed and VPD is low (Bouchabke *et al*., 2006). That variation is clearly linked to hydraulic effects in the growth zone (Tardieu *et al*., 2018), but likely also involves metabolic controls, such as diurnal variation

of wall rheological properties or root hydraulic conductance connected with circadian oscillations (Ben Haj Salah & Tardieu, 1996; Pantin *et al.*, 2011; Pantin *et al.*, 2012; Caldeira *et al.*, 2014b). Possibly, such factors can give rise to a 'stored growth' effect (Pantin *et al.*, 2012), that has been defined as 'the ability of a cell to store up a potential for extension during periods of reduced turgor which can be converted into extra extension upon restoration of normal turgor' (Cleland & Rayle, 1972) and was observed for example by Hsiao *et al.* (1970) and investigated more recently by Proseus & Boyer (2008). One may predict or hypothesize that stored growth would occur at night when turgor is increased due to stomatal closure and decreased VPD. Such an effect would compensate (at least partially) the day-time depression of LER that may be caused by the effect of transpiration on plant and growth zone water potential. It is unknown, at present, if C_a modifies diurnal variation of LER and, if it does, if such variation could be explained by a stored-growth phenomenon.

To address these unknowns, we performed mesocosm experiments with three different CO_2 concentrations: 'half ambient', equivalent to the CO_2 concentration at the Last Glacial Maximum, current 'ambient', and 'double ambient', as projected for the end of this century (IPCC, 2015), combined with high or low VPD during day-time hours, to assess the effects of these environmental drivers on the photosynthesis – transpiration – leaf growth relationships in perennial ryegrass (*Lolium perenne* L.), a major forage crop in temperate climates. Specifically, we asked: (1) Do CO_2 and VPD influence daily-total LER, final leaf length and width, and epidermal cell production and expansion? (2) Does elevated CO_2 decrease the diurnal variation of LER, consistent with predictions of CO_2 effects on stomatal conductance and transpiration? In other words: are relationships between day-time transpiration and day-time LER consistent for CO_2 and VPD effects? (3) Are CO_2 and VPD effects on daytime LER consistent with the effects of these environmental drivers on leaf water potential, osmotic potential and turgor pressure in the LGDZ? (4) What is the effect of CO_2 (and VPD) on the contribution of sugars to (diurnal adjustment of) osmotic potential in the LGDZ? And, (5) do we find evidence for night-time stored-growth effects on LER compensating daytime CO_2 and VPD effects on LER? We studied these relationships with plants growing in sward-like conditions in a culture system with optimal water supply using a nutrient solution with reduced nitrogen concentration, employing previous protocols and experience (Kavanová *et al.*, 2008; Lehmeier *et al.*, 2008, 2013).

Materials and Methods

Experimental design, treatments and growth conditions

The study had a 3 × 2 factorial design with C_a and daytime VPD as factors, and air temperature controlled at 20 °C : 16 °C during the 16 h : 8 h, day : night cycle in all treatments. Three constant CO_2 concentrations (200, 400 or 800 μmol mol^{-1}), corresponding to Last Glacial Maximum (half-ambient), present-day (ambient) and end of the century projections (double-ambient, i.e. elevated) were combined with high (1.17 kPa, 50% relative humidity, RH) or low (0.59 kPa, 75% RH) VPD during the day, corresponding to dry or damp summer days in Central Europe. Night-time VPD was held constant at 0.46 kPa (75% RH) in all treatments. Light was supplied by cool-white fluorescent tubes and warm-white light-emitting diode (LED) bulbs with a photosynthetic photon flux density (PPFD) of 800 μmol m^{-2} s^{-1} at plant height. Treatments were applied 13 d after seed imbibition. Disturbance of atmospheric conditions in the chambers during handling of plants were minimized by installing air-locks in chamber doors (similar to Lehmeier *et al.*, 2008), maintenance of a small overpressure in the chambers relative to the outside atmosphere, and minimizing operations during daylight hours.

Protocols for plant growth followed closely those described by Lehmeier *et al.* (2008). In brief, individual plants of perennial ryegrass (cv. 'Acento') were grown singly in plastic tubes (350 mm height, 50 mm diameter) filled with washed quartz sand (0.3–0.8 mm grain size). Pots were arranged in plastic containers (770 mm × 560 mm × 300 mm) at a density of 383 plants m^{-2}. The close packing of pots (Supporting Information Fig. S1) resulted in a stand-like situation at harvest (leaf area index > 5.5 after canopy closure, in all treatments).

Two containers were placed in each of four growth chambers (see next paragraph). Plants were supplied with a modified 5 mM nitrate-N Hoagland nutrient solution every 6 h by briefly flooding the containers for 9 min followed by draining by gravity (Lehmeier *et al.*, 2008). The composition of the nutrient solution was the following: 1.67 mM KNO_3, 1.67 mM $Ca(NO_3)_2$, 1.0 mM $MgSO_4$, 0.5 mM KH_2PO_4, 0.5 mM NaCl, 134 μM Fe-EDTA, 46 μM H_3BO_3, 9 μM $MnSO_4$, 0.8 μM $ZnSO_4$, 0.3 μM $CuSO_4$, 0.1 μM Na_2MoO_4. This composition corresponded to a nutrient solution with 33% reduced, i.e. two-thirds-strength, nitrate-N concentration relative to normal and nominal concentrations of the other nutrients (compare with Kavanová *et al.*, 2008; Lehmeier *et al.*, 2013).

The experiments were performed inside the four plant growth chambers (PGR15; Conviron, Winnipeg, Canada) that formed part of a modernized version of the gas exchange mesocosm system described by Schnyder *et al.* (2003). Air supply to the chambers was performed by mixing dry CO_2-free air and tank CO_2 (from Linde AG, Unterschleißheim, Germany or Carbo Kohlensäurewerke, Bad Hönningen, Germany) using mass flow controllers. CO_2 and water vapor concentration in each growth chambers were measured every 30 min by an infrared gas analyzer (IRGA, Li-840; Li-Cor, Lincoln, NE, USA). Air temperature and RH in the chambers were measured continuously with the chamber control system (CMP6050, Conviron), which was calibrated before each experimental run (see next paragraph) using external sensors. Light intensity at canopy height was measured with a quantum sensor (LI-190R; Li-Cor) and adjusted periodically. RH gradients between the top and bottom of fully-developed canopies were < 5% and neglected in further analysis.

We performed five sequential experimental runs of 10 to 12 wk duration with four growth chambers, with different treatments in each run, and allocation of treatments to different chambers between sequential runs, as in Liu *et al.* (2016) (Supporting Information Table S1). We observed no chamber effect

on the parameters reported in this study. Measurements of leaf growth, leaf gas exchange and carbohydrate concentration were performed in experimental runs 1–4 and water status measurements in the last run.

Leaf elongation rate (LER)

LER was determined as in Schnyder & Nelson (1988) and Schnyder *et al.* (1990). All plants were in the vegetative stage and had 7 to 11 tillers. Briefly, leaf length was measured on the main tiller of eight randomly selected plants per chamber ($n = 16$–40 per treatment), during 14 d after canopy closure (days 49–62 after imbibition of seed). Measurements were performed every day at the end of the light period on all simultaneously growing leaves (one or two leaves) per main tiller, by recording the distance between the tip of the elongating leaf and the ligule of the youngest fully expanded leaf using a ruler. The daily rate of change in blade length was taken as the measure of the mean daily rate of leaf elongation (LER, in mm h^{-1}). For comparisons among treatments the values obtained during the phase of near-maximum, near-steady growth were used. This corresponded to the phase when the elongating leaf blade had reached ~ 40–65% of its final length (Fig. S2) and all leaf elongation was due to blade elongation. In addition, leaf length was measured at the end of the day and the end of the following night on two successive days, to obtain the LER during day (LER_{day}) and night (LER_{night}).

At the end of the 2 wk-long measurement period plants were sampled for the estimation of additional morphological parameters (leaf length, leaf area) using a similar protocol as Liu *et al.* (2016). The IMAGE J software (Schneider *et al.*, 2012) was used for digital analysis.

Epidermal cell length and number

Two fully developed leaf blades from each of four plants per treatment were sampled at 66 d after the start of the experiment. The selected leaves were cut near the ligule and a 3 cm-long replica of the abaxial epidermis was taken in the basal region of the blade as in Schnyder *et al.* (1990) using a thin layer of 4% (w/w) polyvinylformaldehyde (Formvar 1595 E; Merck Darmstadt, Germany).

Digital images of representative sections of each replica (0.7 mm × 4 mm) were obtained with a fluorescence microscope (BX 61; Olympus Corp., Tokyo, Japan) operated with the CELLSENSE DIMENSION software of the Centre for Advanced Light Microscopy (CALM, Technical University of Munich, Germany), at ×10 magnification. The IMAGEJ software (Schneider *et al.*, 2012) was used to measure cell length in cell files without stomata. Replicas with fewer than 50 identifiable cells were excluded from the analysis.

Epidermal cell number (in a cell file running from the base to the tip of the leaf blade) was estimated as final leaf blade length divided by epidermal cell length. Former work with perennial ryegrass demonstrated epidermal cell length was virtually constant along the leaf blade (Schnyder *et al.*, 1990). Also, we found no differences in cell length densities near the proximal and distal margins of epidermal replicas taken from the region that corresponded to the cells/tissue that expanded during the LER measurements (Fig. S3).

Stomatal conductance and leaf- and canopy-scale transpiration

Chamber-scale canopy transpiration (E_{canopy}) was measured continuously during the 2-wk interval in which LER was determined. For each chamber, canopy transpiration (in mmol H_2O m^{-2} s^{-1}) was calculated every 30 min as the difference between the H_2O fluxes at the inlet (F_{in}, in mmol H_2O s^{-1}) and outlet (F_{out}, in mmol H_2O s^{-1}) of the growth chamber, divided by the chamber ground area (s, 1.5 m^2):

$$E_{canopy} = (F_{in} - F_{out})/s \quad \text{Eqn 1}$$

These measurements were made while the RH of the chamber atmosphere was kept near the nominal level (SD ± 0.9%) by using a high-pressure water vapor generator (FINESTFOG, Ottobrunn, Germany) that added a known amount of water vapor to the chamber air whenever the nominal RH dropped below a specified threshold (1–2% RH lower than the chamber setpoint). The chamber-based humidification system was inactivated during that period. Vapor addition rate by the vapor generator was equated with F_{in}, as the fresh air supplied to the chambers was dry (dewpoint < –70 °C). Before each experimental run, a calibration was performed to obtain the water addition rate of the nozzles. Water vapor losses due to condensation inside the chambers were quantified by collection and weighing of the condensate and accounted for in the calculations.

In parallel, leaf-level measurements of stomatal conductance and transpiration were performed with a LI-6400 (Li-Cor) portable CO_2/H_2O gas exchange system with a clamp-on leaf cuvette on 6–12 plants per treatment. That measurement system was installed in a separate plant growth chamber (E15, Conviron). For measurements, individual plants were removed from their growth chamber, and the midsection of the youngest fully developed leaf blades of four tillers was enclosed in the 2 cm × 3 cm leaf cuvette. Stomatal conductance to water vapor (g_s, in mol H_2O m^{-2} s^{-1}) and leaf transpiration (in mmol H_2O m^{-2} s^{-1}) were measured at a leaf temperature of 21 °C and a PPFD of 800 µmol m^{-2} s^{-1}. CO_2 concentration and RH in the leaf cuvette were set equal to the conditions in the growth environment. Measurements were logged once steady-state conditions for stomatal conductance and water vapor concentration were reached.

Osmotic potential, leaf water potential and turgor

Total osmotic potential in the LGDZ, leaf water potential, and turgor pressure were estimated in the treatments with C_a of 200 and 800 µmol mol^{-1} at both high and low VPD. Samples for osmotic potential measurements were collected 2 h before the end of the day and night, on two sampling days between days 61

and 74. Six plants were randomly selected from each chamber and the LGDZ of two fully developed tillers pooled into one sample, sealed in paper bags, frozen in liquid nitrogen and stored at −18°C. The frozen samples ($n = 4$ per treatment) were thawed at room temperature and sap was extracted under mechanical pressure. Osmotic potential was then measured with a vapor pressure osmometer (5100C; Wescor Inc., Logan, UT, USA). On day 76, leaf water potential of eight plants per treatment was determined with the pressure chamber technique (Scholander *et al.*, 1965): 2 h before the end of the day individual plants were taken from a growth chamber, the youngest fully expanded leaf blade of a major tiller was cut near the ligule and immediately placed in a pressure chamber (Model 1002; PMS Instrument Company, Albany, OR, USA), following the recommendations by Turner (1981). Plants were returned to the same chamber and the protocol repeated on another tiller 2 h before the end of the night. Turgor was estimated as the difference between osmotic potential and leaf water potential, neglecting (1) the water potential gradient between the youngest fully expanded leaf and the LGDZ and (2) the fraction of apoplastic water, that were assumed to be sufficiently small or similar between treatments (Passioura, 1980; Martre *et al.*, 2001). As they comprised the entire LGDZ and youngest fully-expanded leaf blade, our measurements ignored the turgor gradients between expanding and fully-expanded tissue in the LGDZ, and the water potential gradient between the expanding tissue and the water source (e.g. Nonami & Boyer, 1993; Martre *et al.*, 1999; Fricke & Peters, 2002). In detailed investigations of hydraulic conductivities in vegetative plants of tall fescue – a closely related species – Martre *et al.* (2001, Table 1) found a water potential-gradient of 0.19 MPa between the transpiring, youngest fully-expanded leaf and the leaf growth zone, a difference that corresponded to 10% to 16% of the leaf water potential of the youngest fully-expanded leaf blade in light, in our investigations.

Water-soluble carbohydrates

LGDZ tissue was excised from two mature tillers of six plants per chamber on day 62 after imbibition and pooled together into one sample, both at the end of the day and night period, during experimental runs 1–4 ($n = 4$ per treatment). Fresh weight of the samples was determined and samples were frozen in liquid nitrogen and stored in a freezer at −18 °C until freeze drying. Dry samples were ball-milled to a fine powder and stored again at −18 °C until extraction of water-soluble carbohydrates as in Schnyder & de Visser (1999). The water-soluble carbohydrates components fructose, glucose, sucrose and fructan were separated by high-performance liquid chromatography (HPLC, Shodex Sugar KS 801 and 802; Showa Denko, Tokyo, Japan) and carbohydrates detected with a refractive index detector (Shodex RI-101). Analytical grade fructose, glucose, sucrose and inulin (all from Merck) were used as standards.

To obtain the mean degree of polymerization of fructans, fructans were separated from other water-soluble carbohydrates components by HPLC, hydrolyzed by heating in 0.02 M hydrochloric acid (HCl) for 1 h (Wolf & Ellmore, 1975), and fructose and glucose in the hydrolysate separated by HPLC and quantified as earlier. The mean degree of polymerization of fructan was obtained as fructose : glucose + 1, and used to calculate the mean molecular weight of fructan in the LGZD.

The osmotic potential of water-soluble carbohydrates in the LGDZ was estimated from the molar concentration of the individual water-soluble carbohydrates per unit tissue water in the LGDZ, assuming 40 mM = 0.1 MPa (Schnyder & Nelson, 1987).

Statistics

Linear mixed models were fitted to analyze the effect of CO_2, VPD, diel period (day or night) and their interactions on LER, morphometric traits, stomatal conductance, transpiration and carbohydrate concentration. Growth chamber, experimental run and multiple measurements on individual plants were included in the models as random effects. Type III Wald test was used for determining the significance of the fixed effects and the *post hoc* Tukey's HSD test was performed for pairwise comparisons among treatments. Additionally, linear models were used to test the effect of the explanatory variables on leaf water potential, osmotic potential and turgor. Finally, treatment averages were calculated and linear regression analysis was performed to determine the relationship between the different target variables. All statistical analyses were conducted in R v.3.6.1 (R Core Team, 2019). The R-packages NLME (Pinheiro *et al.*, 2019), emmeans (Lenth, 2018) and ggplot2 (Wickham, 2016) were used for fitting linear mixed models, performing the *post hoc* tests and for data plotting, respectively. The number of replicates varied between measured parameters and treatments and is indicated in the figure legends and table captions.

Results

LER, final leaf length and width, and epidermal cell length and number

Daily mean LER showed no statistically significant responses to CO_2, daytime VPD or their interaction (Fig. 2a; Table 1). Likewise, morphometric traits associated with the leaf growth process, i.e. final leaf blade length and width and epidermal cell length and number (Fig. 2e–h), leaf appearance interval (Fig. S4) and the time from leaf appearance to cessation of leaf elongation (not shown) revealed no significant differences between treatments.

Table 1 Results of a linear mixed model, testing the response of daily mean leaf elongation rate of *Lolium perenne* to atmospheric CO_2 concentration, daytime vapor pressure deficit (VPD) and their interaction ($n = 16$–40).

Factor	df	*F*-value	*P*-value
CO_2	9	1.47	0.26
VPD	9	1.12	0.32
$CO_2 \times$ VPD	9	1.87	0.21

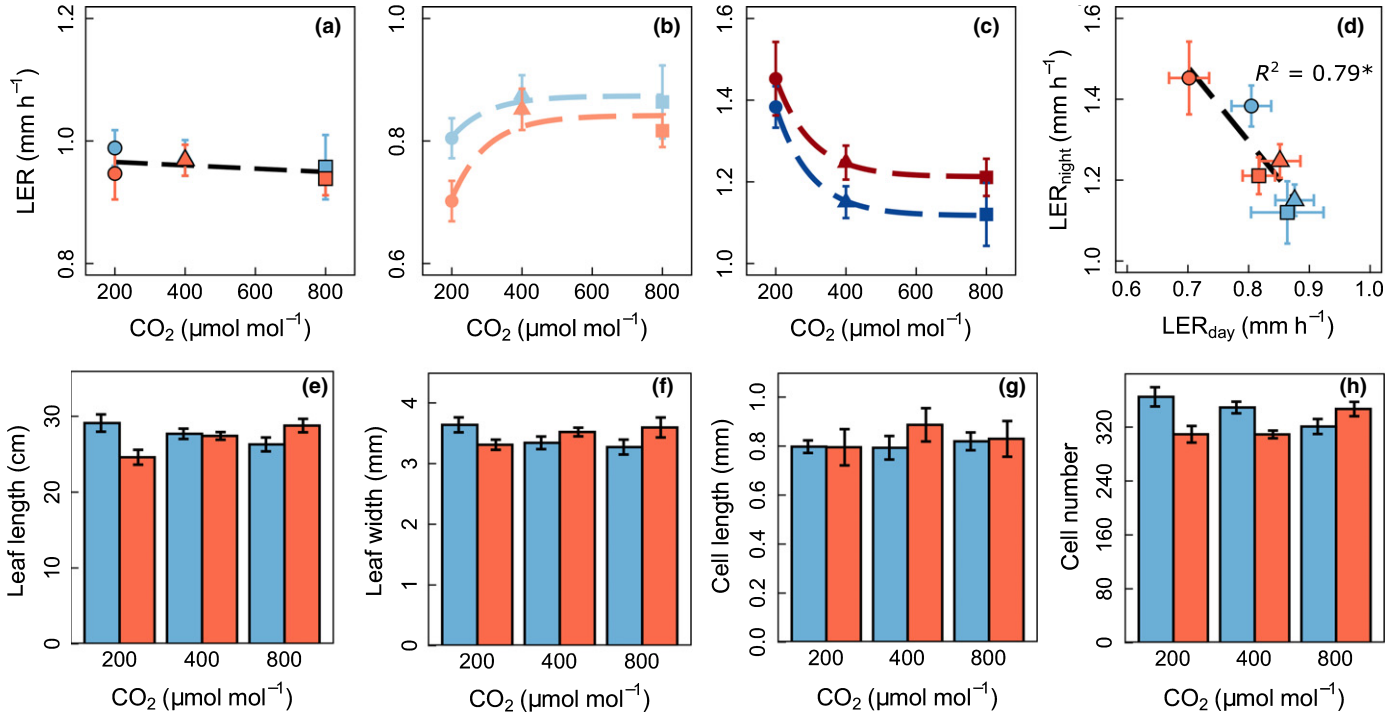

Fig. 2 Morphometric traits of *Lolium perenne* as influenced by atmospheric CO_2 concentration at low (0.59 kPa; blue color) and high daytime vapor pressure deficit (VPD) (1.17 kPa; red): daily mean leaf elongation rate (LER) (a), LER during day (b), LER during night (c), the relationship between LER during day (LER_{day}) and during night (LER_{night}) (d), final leaf length (e), mean leaf width (f), epidermal cell length (g), and epidermal cell number (h). VPD at night was kept the same in all treatments (0.46 kPa). Symbols: circles, 200 µmol mol^{-1} CO_2; triangles, 400 µmol mol^{-1} CO_2; squares, 800 µmol mol^{-1} CO_2. Significance level of the linear regression in (d): *, $P < 0.05$. Data points and error bars represent the mean ± SE. For details, see the Materials and Methods section.

Conversely, LER exhibited pronounced diel variation, with higher rates at night in all treatments ($LER_{night} > LER_{day}$, $P < 0.001$). The amplitude of the diel variation differed between treatments, due to a significant interaction of LER with daytime VPD (night-time VPD was the same in all treatments) and CO_2 (Table 2, $P < 0.001$; Figs 2b,c, S5). LER_{day} increased and LER_{night} decreased exponentially with C_a. Even though daytime VPD had no statistically significant effect (Tables 2, S2), a systematic difference in LER between VPD levels was evident. High (relative to low) daytime VPD reduced LER_{day} (−0.04 mm h^{-1}) and enhanced LER_{night} (+0.07 mm h^{-1}, averaged over all treatments) throughout the range of CO_2 levels. The greatest divergence between LER_{day} and LER_{night} occurred at low C_a and high daytime VPD ($LER_{night} : LER_{day} = 2.0$) and the smallest at high C_a and low daytime VPD (1.3). The net result of CO_2 and daytime VPD effects was a close negative and virtually-fully compensating relationship between LER_{night} and LER_{day} across all six treatments (Fig. 2d, $R^2 = 0.79$, $P < 0.05$).

Stomatal conductance, transpiration and relationship with LER_{day}

The g_s and transpiration were measured on the youngest fully-expanded leaf blade (Fig. 3a,b) under the same conditions of light intensity, C_a and daytime VPD as in the growth environment. The g_s strongly reacted to CO_2 concentration, VPD and their interaction (Table 3): g_s significantly decreased with both C_a (according to a negative exponential function, $P < 0.001$) and VPD ($P < 0.01$), with a VPD-sensitivity that decreased with C_a (Fig. 3a). Accordingly, g_s was highest when VPD and C_a were low, and lowest when VPD and C_a were high; but the enhancement of g_s by low VPD (relative to high VPD) was greater at a C_a of 200 µmol mol^{-1} (2.6-fold) than at 800 µmol mol^{-1} (1.5-fold) (Table S3).

Leaf transpiration decreased exponentially with increasing C_a, dropping by more than 55% between 200 and 800 µmol mol^{-1} ($P < 0.001$; Table 3) at both VPD levels. We also noted a greater leaf transpiration at high relative to low VPD level (> 10% for all

Table 2 Results of a linear mixed model, testing the response of leaf elongation rate of *Lolium perenne* to diel period (leaf elongation during day or night), atmospheric CO_2 concentration (exponential function; see Fig. 2), daytime vapor pressure deficit (VPD) and two-way interactions ($n = 16$–40).

Factor	df	*F*-value	*P*-value
Day/night	100	47.2	< 0.001
CO_2	9	5.6	0.04
Daytime VPD	9	2.2	0.17
Day/night × CO_2	100	60.5	< 0.001
Day/night × daytime VPD	100	12.9	< 0.001

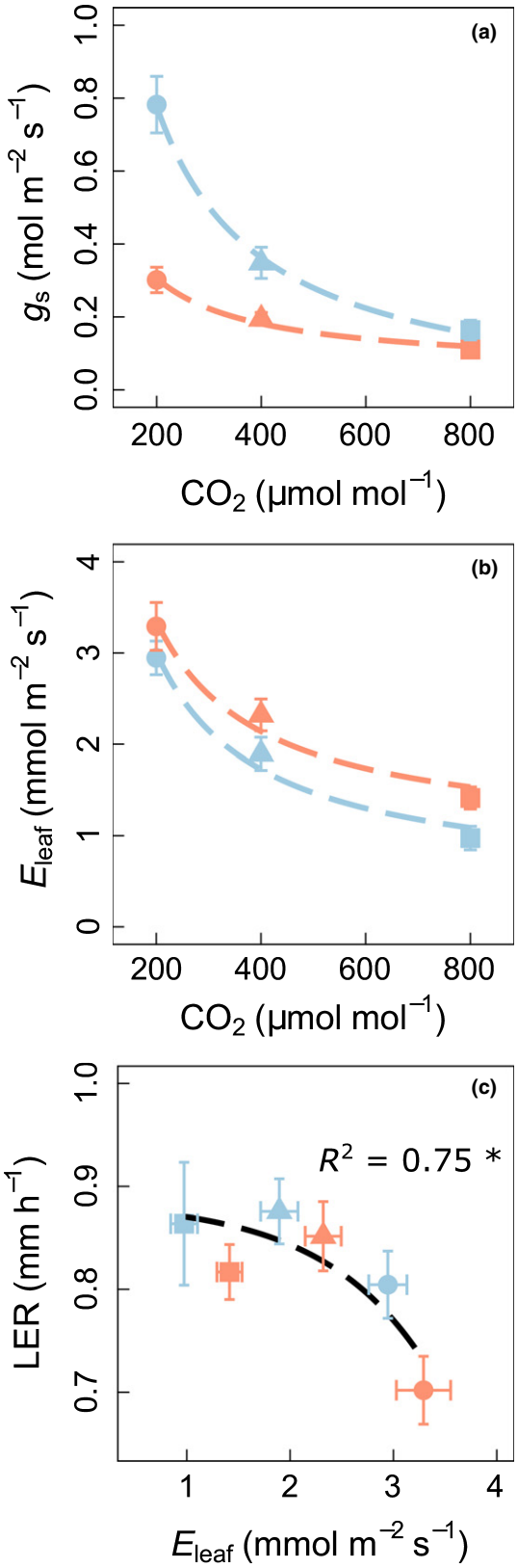

Fig. 3 Daytime stomatal conductance (g_s) (a) and leaf transpiration (E_{leaf}) (b) of the youngest fully-expanded leaf of *Lolium perenne* as affected by atmospheric CO_2 concentration and daytime vapor pressure deficit (VPD) ($n = 6$–12) (low VPD, 0.59 kPa, blue color; high VPD, 1.17 kPa, red) in the growth environment. Night-time VPD in the growth environment was kept the same in all treatments (0.46 kPa). Measurement conditions were the same as in the growth environment during the day (PPFD, 800 µmol m^{-2} s^{-1}; leaf temperature 21 °C, and CO_2 and VPD as indicated earlier). (c) The relationship between leaf elongation rate during day (LER_{day}) (Fig. 2b) and E_{leaf}. Significance level of the exponential function in (c): *, $P < 0.05$. Data points and error bars represent the mean ± SE.

CO_2 levels); but this effect was not statistically significant due to the variability of leaf transpiration (Fig. 3c; Table S3). Simultaneously, a significant curvilinear relationship ($R^2 = 0.75$; $P < 0.05$) existed between leaf transpiration and LER_{day}. LER_{day} decreased sharply with leaf transpiration when leaf transpiration increased above approximately 2.5–3.0 mmol m^{-2} s^{-1} in plants grown at a C_a of 200 µmol mol^{-1} (Fig. 3c).

Water-soluble carbohydrates

The concentration of the water-soluble carbohydrates components fructose, glucose, sucrose and fructan in the LGDZ was determined at the end of the day and of the night, in all treatments. Very high water-soluble carbohydrate concentrations (with total water-soluble carbohydrate > 53% of dry matter content) were observed in all treatments at the end of both day and night. Differences between treatments and throughout the diurnal cycle were generally small, however treatment effects were observed for the molar concentrations of the individual carbohydrates. This was the case for: (1) sucrose, which showed systematic diurnal variation with concentration decreasing by 25% on average between the end of the day and the end of the night (Fig. 4c, $P < 0.001$) and strongest relative decreases at low C_a; (2) a greater fructose concentration in the low C_a treatments relative to the other C_a treatments (+21% on average); (3) a greater fructan concentration at high VPD (+43% in average, due mainly to a lower degree of polymerization relative to low VPD), which determined a greater total water-soluble carbohydrate concentration at high VPD (+16%); (4) a lower fructan concentration at 200 µmol mol^{-1} compared with the other CO_2 levels (−25% on average) and (5) a small decrease of total water-soluble carbohydrate concentration during the night (−5% on average of all treatments), due mainly to decreases of sucrose and fructan concentration (Tables S4, S5). Conversely, glucose and fructose concentrations did not show signs of depletion during the night (Fig. 4a,b). Together, the monosaccharides accounted for 64 to 77% of the total contribution of all water-soluble carbohydrates to the osmotic potential in the LGDZ. The latter was 26–34% of the total osmotic potential, and did not differ systematically between treatments or diel periods, although the relative contribution was, on average, slightly higher at the end of the night (Fig. 4f).

Leaf water potential, osmotic potential, turgor and relationships with LER_{day} and LER_{night}

The water potential of the youngest fully-expanded leaf blade and the osmotic potential of tissue water in the LGDZ were

Table 3 Results of a linear mixed model, testing the response of stomatal conductance (g_s) and leaf transpiration of *Lolium perenne* to atmospheric CO_2 concentration (exponential function; see Fig. 3), daytime vapor pressure deficit (VPD) and their interaction ($n = 6$–12).

	g_s		Leaf transpiration	
Factor	*F*-value	*P*-value	*F*-value	*P*-value
CO_2	75.7	< 0.001	39.8	< 0.001
Daytime VPD	15.6	< 0.01	0.42	0.53
CO_2 × daytime VPD	13.4	< 0.01	0.04	0.84

Conditions in the leaf cuvette during measurement of g_s and leaf transpiration were the same as in the growth chamber of origin of the respective plants.

determined at the end of the day and at the end of the night in the extreme C_a treatments (200 and 800 µmol mol^{-1} CO_2) with low or high daytime VPD (Fig. 5a,b). Turgor pressure in the LGDZ (Fig. 5c) was estimated as the difference between leaf water potential and osmotic potential, neglecting possible water potential-gradients between the LGDZ and the youngest fully expanded leaf blade.

Treatments had no effect on leaf water potential, or osmotic potential or turgor at the end of the night. However, leaf water potential ($P < 0.001$), osmotic potential ($P < 0.01$) and turgor ($P < 0.001$) changed significantly between the end of the night and the end of the day in each of the four treatments (Fig. 5a–c; Table 4). These changes were inversely related to CO_2 concentration ($P < 0.001$); that is, changes of leaf water potential, osmotic potential and turgor between end of the night and end of the day were greater at low than at high C_a (Fig. 5a–c; Table 4). On the contrary, we found a statistically significant effect of VPD on leaf water potential ($P < 0.001$), but not on osmotic potential or turgor at the end of the day (Table 4).

Leaf water potential was negatively related with canopy transpiration, following a virtually identical relationship across treatments when day and night measurements were pooled (Fig. 5d; $R^2 = 0.98$; $P < 0.001$). Leaf water potential and osmotic potential exhibited a close proportionality (Fig. 5e; $R^2 = 0.89$; $P < 0.001$) that also implied a linear (and proportional) increase of turgor with leaf water potential, represented as the difference between the 1 : 1 line and the data points in Fig. 5(e). LER_{day} responded linearly to leaf water potential measured at the end of the day (Fig. 5f, $R^2 = 0.81$). That relationship was determined primarily by the effect of CO_2 on LER_{day} ($P < 0.05$).

We found no relationship between LER_{night} and leaf water potential or osmotic potential or turgor estimates obtained at the end of the night (Table S6, $P > 0.05$). But, we observed a tight relationship between the enhancement of LER_{night} relative to LER_{day} and the increase in turgor between the end of the day and the end of the night ($R^2 = 0.93$, $P < 0.05$; Fig. 6), with the intercept of this relationship not being significantly different from zero ($P > 0.05$).

Discussion

Our work demonstrates a strong diurnal oscillation of LER in *L. perenne* that was determined by (1) a variable hydraulic limitation of daytime LER driven by atmospheric CO_2 level (C_a) and

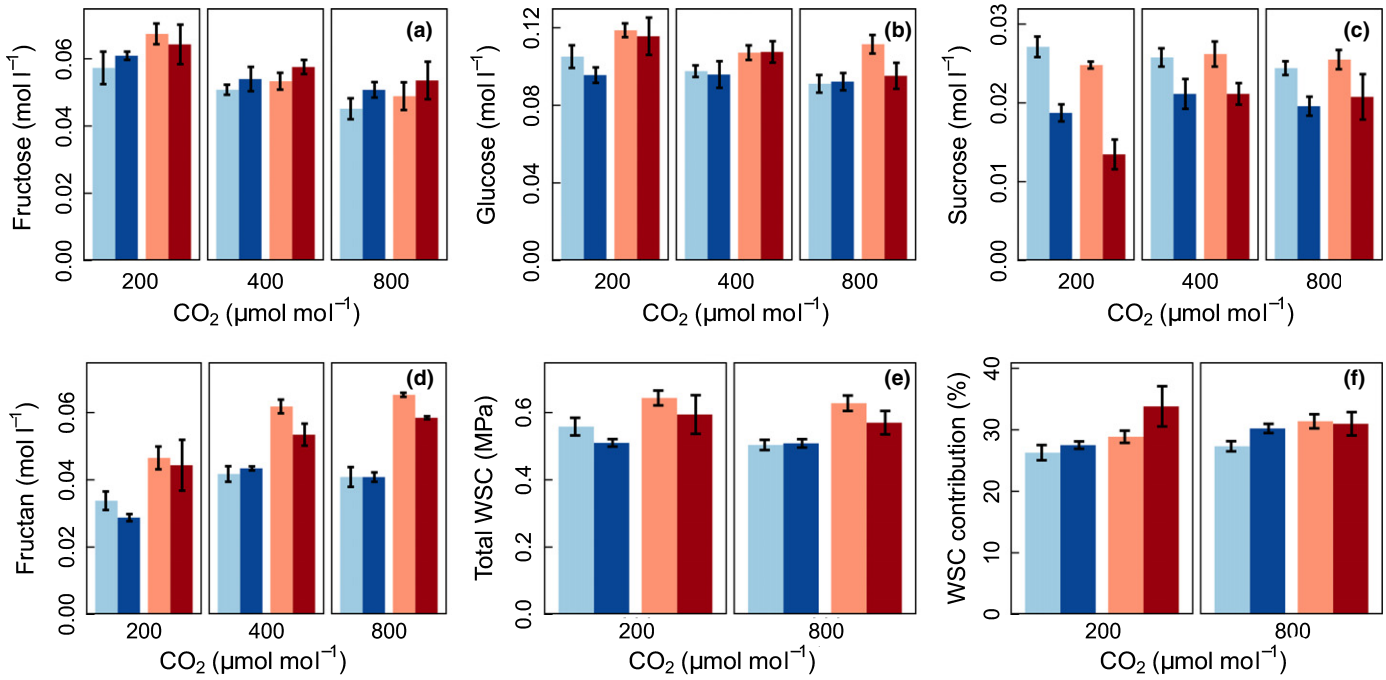

Fig. 4 Molar concentration of fructose (a), glucose (b), sucrose (c) and fructan (d) in the leaf growth-and-differentiation zone (LGDZ) of *Lolium perenne*, and osmotic potential of the total water-soluble carbohydrate (WSC) (e), and its contribution to the total osmotic potential measured in the LGDZ (f) as influenced by atmospheric CO_2 concentration at low (0.59 kPa, blue color) and high daytime vapor pressure deficit (VPD) (1.17 kPa, red), for measurements at the end of the day (light colored bars) and at the end of the night (dark colored bars). VPD at night was kept the same in all treatments (0.46 kPa). Data points and error bars represent the mean ± SE ($n = 4$). Notice the different scales in plots (a)–(d).

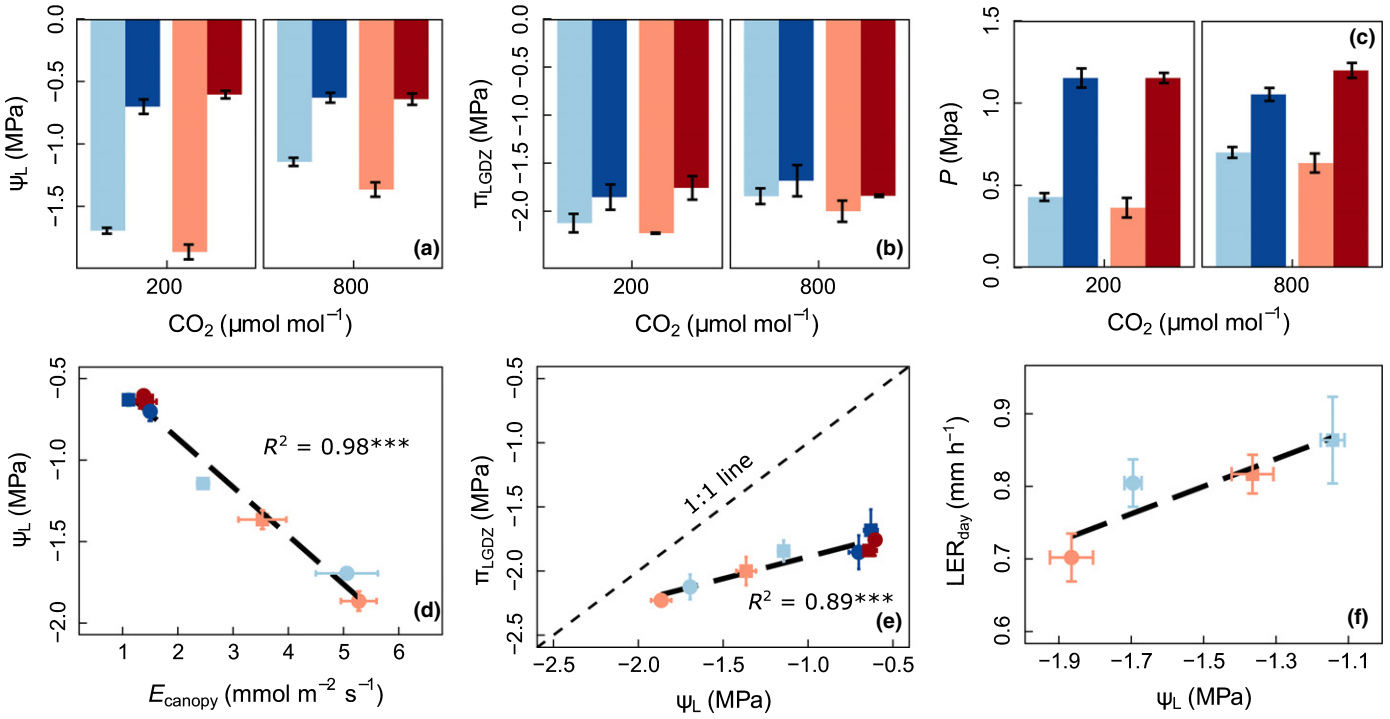

Fig. 5 Water potential of the youngest fully expanded leaf (Ψ_L) of *Lolium perenne* ($n = 8$) (a), osmotic potential of tissue water in the leaf growth-and-differentiation zone, LGDZ (π_{LGDZ}) ($n = 4$) (b), turgor pressure (P) in the LGDZ, estimated as the difference between π_{LGDZ} and Ψ_L ($n = 4$) (c), and relationship between Ψ_L and canopy transpiration (E_{canopy}) (d), between Ψ_L and π_{LGDZ} (e) and between Ψ_L and leaf elongation rate (LER) (f) for measurements at the end of the day (light colored bars or symbols) and at the end of the night (dark colored bars or symbols). In (e) the difference between the 1 : 1 line and the data points represents the estimated turgor. Plants were grown in the presence of half-ambient (200 μmol mol^{-1}, circles) and double-ambient CO_2 (800 μmol mol^{-1}, squares) with low or high daytime vapor pressure deficit (VPD) (low VPD, 0.59 kPa, blue color; high VPD, 1.17 kPa, red) in their growth environment. Night-time VPD in the growth environment was kept the same in all treatments (0.46 kPa). Significance level of the linear regression in (d) and (e): ***, $P < 0.001$. Data points and error bars represent the mean ± SE. For details, see the Materials and Methods section.

Table 4 Results of a linear model (*t*-values) testing the response of leaf water potential (Ψ_L), osmotic potential in the leaf growth-and-differentiation zone, LGDZ (π_{LGDZ}) and turgor pressure of *Lolium perenne* to growth during day or night (a); and effect of atmospheric CO_2 concentration, daytime vapor pressure deficit (VPD) and their interaction on Ψ_L, π_{LGDZ} and P during growth in the day (b).

Factor	Ψ_L	π_{LGDZ}	Turgor
(a) Day/night	12.4***	3.0**	7.4***
CO_2	126.8***	7.9*	5.6*
(b) Daytime VPD	20.1***	2.2^{ns}	0.11^{ns}
CO_2 × daytime VPD	0.3^{ns}	0.08^{ns}	0.0^{ns}

No significant effects were detected during night growth ($P > 0.05$). Significance levels: ns, not significant ($P > 0.05$); *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

(daytime) VPD, and (2) a full compensation of the daytime hydraulic limitation of elongation by a night-time enhancement of LER, that was consistent with a stored-growth effect. As a result, at daily timescale, LER and a suite of leaf morphogenetic parameters (leaf blade length and width, epidermal cell length and number) were insensitive to C_a (and daytime VPD), explaining, in part, the less-than expected growth response towards elevated CO_2. However, we did note a decrease of specific leaf area

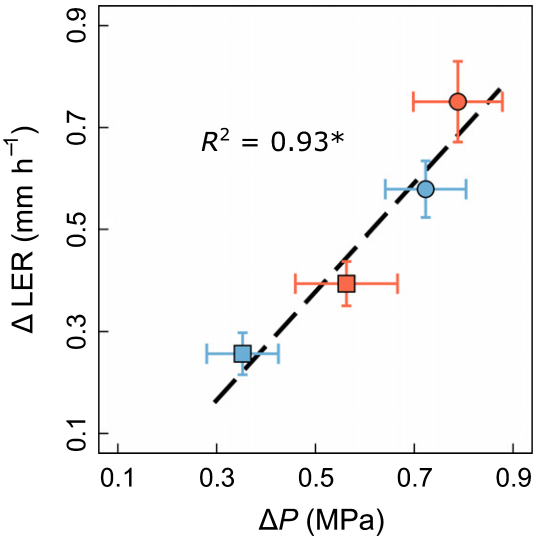

Fig. 6 Enhancement of nocturnal leaf elongation rate (LER_{night}) over diurnal leaf elongation rate (LER_{day}) ($\Delta LER = LER_{night} - LER_{day}$) of *Lolium perenne* as related to the turgor change (ΔP) between end of night and end of day. Symbols: circles, 200 μmol mol^{-1} CO_2; squares, 800 μmol mol^{-1} CO_2; blue, low vapor pressure deficit; red, high vapor pressure deficit. Significance level of the linear regression: *, $P < 0.05$. Data points and error bars represent the mean ± SE.

(SLA) with increasing CO_2 level (Fig. S4), a typical CO_2 growth-response (Poorter & Navas, 2003; Ainsworth & Long, 2005). Furthermore, notably, epidermal cell length was close to that observed by Kavanová *et al.* (2008) in nitrogen-limited conditions with the same cultivar of perennial ryegrass.

The presence of a strong hydraulic limitation of LER_{day} in all treatments in this work was supported by (1) the strong decreases of leaf water potential between the end of the night and the end of day, and low leaf water potential (< -1.0 MPa) at the end of the day, that was combined with (2) strong decreases of osmotic potential and turgor towards the end of the day, and (3) a significantly lower LER_{day} than LER_{night}, despite of the 4 °C lower air temperature at night. These relationships were unrelated to soil drying during the day as stands were watered frequently, resulting in a near-constant volumetric water content in the soil (water content > 20%, data not shown) and a virtually constant canopy transpiration throughout the light periods in all treatments (Fig. S6). Also, source-limitation was highly unlikely, as water-soluble carbohydrate concentration was greater than 53% of dry mass in the LGDZ in all treatments throughout the day–night cycle. Hydraulic limitation of LER_{day} occurring independently of soil water deficit has been observed repeatedly (Tardieu *et al.*, 2010; Pantin *et al.*, 2012; Caldeira *et al.*, 2014a) and was related to high irradiance (Gallagher & Biscoe, 1979), high air VPD (e.g. Parrish & Wolf, 1983; Table 2) or low nitrogen nutritional status (Radin & Boyer, 1982). Radin & Boyer (1982) observed greater reductions of daytime relative to night-time leaf expansion in sunflower, when nitrogen nutrition was limiting. We used a Hoagland-type nutrient solution with reduced nitrogen concentration (−33% relative to the standard solution and nominal concentrations of other elements) which could have contributed to the observed reduction of LER_{day} : LER_{night}. But, between-treatment variation of LER_{day} was unrelated to the effect observed by Radin & Boyer (1982) as nitrogen concentration of the LGDZ was highest (Table S7) in the treatment with the greatest disparity between LER_{day} and LER_{night}. However, we did grow plants at a high irradiance (16 h of 800 µmol m^{-2} s^{-1} PPFD at canopy height), causing a high daytime evaporative demand particularly in the treatments with high daytime VPD. The effect of high irradiance (and high air VPD) on LER_{day} is generally associated with strong decreases of leaf water potential and turgor in the leaf growth zone (Tardieu *et al.*, 2010; Pantin *et al.*, 2012).

The C_a or its interaction with VPD strongly modified the hydraulic limitation of LER_{day}, as shown by (1) the negative relationship between LER_{day} and transpiration, principally caused by a negative response of transpiration to C_a, (2) the close negative relationship between leaf water potential and transpiration that depended mostly on variation of C_a, (3) the covariation of leaf water potential and osmotic potential that was primarily driven by variation of C_a, and finally (4) the drastic decrease in turgor and LER during daytime compared to night-time. Accordingly, the effect of C_a on LER_{day} conformed with that expected for hydraulic limitation, predicted based on its effect on stomatal conductance and transpiration during daytime (Fig. 1), although the effect did not persist at the daily level due to compensating night-time effects on leaf elongation (see 'stored growth' in the following paragraphs). Although hydraulic limitation of LER_{day} caused by low C_a has not been reported before, a strong negative relationship between (ABA mediated) stomatal conductance and leaf elongation during daytime was demonstrated earlier (Tardieu *et al.*, 2010). It is worth noting that the applied treatments did not alter the water relations in our system. The relationship between canopy transpiration and leaf water potential was extremely tight ($R^2 = 0.98$), suggesting a constant hydraulic conductivity and a lack of acclimation of this parameter to the different C_a and VPD environments. Also, the treatments followed a very similar leaf water potential vs osmotic potential relationship which remained unaltered throughout the day : night cycle. Similarly, sugar (and by difference, non-sugar) contributions to osmotic adjustment remained relatively constant in the diel cycle, and accounted for 26 to 34% (and 66 to 74%) of osmotic potential, independently of C_a or VPD.

Remarkably, none of the treatments showed diel variations in the concentration of fructose and glucose, the most important sugar osmoticum (64–77% of the total osmotic potential attributable to water-soluble carbohydrates). Night-time depletion of sucrose may have resulted (at least in part) from enhanced sucrose hydrolysis by invertase to generate fructose and glucose in the LGDZ (Koch, 2004; Lunn, 2008). Also, the night-time reduction of sucrose concentration in the LGDZ may have contributed to sustain carbohydrate import into the LGDZ (Schnyder & Nelson, 1988) as total water-soluble carbohydrate concentration did not decrease markedly during the night, despite of the greatly enhanced LER_{night} (particularly at low C_a) and associated enhanced growth-related water deposition (nocturnal decreases in water content of the LGDZ were not observed, data not shown). Again, these observations support the view that neither LER_{day} nor LER_{night} were source limited.

In contrast with daytime LER, nocturnal LER showed no evidence of hydraulic limitation, as LER_{night} was not significantly related with any of the hydraulic parameters assessed at the end of the dark period. But, we did observe a negative correlation between atmospheric CO_2 concentration and LER_{night} that was – however – non-causal, as reciprocal transfer of plants between CO_2 environments at the beginning of the night period did not alter their subsequent LER_{night} (Fig. S7; Table S8), effectively demonstrating that LER_{night} was insensitive to night-time CO_2. Clearly, therefore, differences between treatments in LER_{night} were a consequence (carryover) of daytime atmospheric conditions of CO_2 and daytime VPD. Indeed, the enhancement of LER_{night} over LER_{day} was closely related to the nocturnal recovery of turgor. This LER_{night} response conforms with the 'stored growth' phenomenon that is reflected in above-normal growth when turgor recovers after a period of turgor loss and inhibited growth (Serpe & Matthews, 1994; Proseus & Boyer, 2008; Pantin *et al.*, 2012). The mechanism of stored growth is not fully understood, but the phenomenon has been associated with adjustments in wall yielding properties (e.g. Serpe & Matthews, 1994). Studies with *Chara corallina* cells demonstrated an accumulation of unused cell wall precursors in the cytoplasm during the phase of low turgor, which were used after turgor recovery

and led to an enhancement in cell growth (Proseus & Boyer, 2006). The stored growth effect, however, may not be ubiquitous among grasses (see e.g. the independent response of LER_{night} and LER_{day} to VPD in maize, Bouchabke *et al.*, 2006) or may perhaps interact with other factors, such as source or nutritional limitation.

Although we did not analyze the kinetics of LER during the night, it is well known that abrupt changes from full light to darkness or from darkness to light (as used here and often employed in diurnal 'cycles' in growth chamber experiments), typically produce transient (< 1 h-long) enhancements or inhibitions of LER (Durand *et al.*, 1995), that are indicative of an elastic component of expansion. These short-lived transients are often followed by near constant LER during the remainder of the day or night periods, with the transient enhancements or inhibitions accounting for a relatively small portion of the overall differences between daytime and night-time elongation in well-watered conditions and constant temperature at the growth zone (Schnyder & Nelson, 1988; Durand *et al.*, 1995).

Clearly, the most remarkable result of this work was the virtually complete compensation of reduced daytime leaf expansion by night-time leaf expansion, as LER averaged over a 24 h period did not differ significantly between treatments. As the photoperiod was 16 h, a unit decrease of LER_{day} actually required a two-unit increase of LER_{night} for full compensation. These results are also consistent and indicative of a mechanism governing the daily mean or integral rate of cell wall loosening independently of the variable daytime depressions of turgor that were controlled by atmospheric conditions of CO_2 and VPD. The nocturnal enhancement of cell wall expansion must have been closely proportional to the nocturnal turgor increase, with a very similar proportionality factor (cell length increment per unit increase of turgor) for all treatments, as the enhancement of LER_{night} over LER_{day} followed the same proportionality with the nocturnal turgor increase. Although we did not observe the processes of cell division and elongation directly, our observations of virtually constant final leaf length, leaf appearance interval, leaf elongation duration post emergence, and final epidermal cell length and number must have some implications for the underlying patterns of cell division and elongation, as LER is a function of cell division (production) and elongation rate along contiguous cell files (Schnyder *et al.*, 1990; Kavanová *et al.*, 2006, 2008), also with respect to CO_2 effects (Masle, 2000). It is well established for perennial ryegrass (and related species such as tall fescue), grown in a wide range of environmental conditions, that the duration of individual epidermal cell expansion, from the emergence from the cell division zone to the time when it attains its' final length, is about 3 to 4 d in similar thermal environments (MacAdam *et al.*, 1989; Schnyder *et al.*, 1990; Kavanová *et al.*, 2006, 2008). This would imply that individual elongating epidermal cells experienced several diurnal perturbations of cell expansion rate resulting from the observed treatment-dependent diurnal variations of LER. The simplest, most parsimonious hypothesis that can explain the relationship between the diurnal LER dynamics and diurnal epidermal cell elongation dynamics is, therefore, that epidermal cell elongation rate exhibited proportionally the same diurnal perturbations as LER. We cannot rule out, however, based on our data alone, that there is perhaps a more complex mechanism, e.g. some complementary compensation between cell elongation rate and duration dynamics that could modify to some extent the exact relationship between LER and epidermal cell elongation rate dynamics (Masle, 2000). In addition, other works have found some CO_2 effect on cell division and expansion (e.g. Ferris *et al.*, 1996; Masle, 2000), and it is not clear what mechanism(s) caused divergence of those results from the present. Certainly, the diel elongation responses found in this work warrant more detailed mechanistic studies with spatio-temporal detail at the level of expanding tissue, including the kinematics of growth, cell division, expansion and associated metabolic processes (e.g. Green *et al.*, 1971; Nonami & Boyer, 1993; Martre *et al.*, 1999; Fricke & Peters, 2002; Moulia *et al.*, 2019).

In conclusion, this work demonstrated a close integration of daytime and night-time leaf elongation in an important forage grass under contrasting atmospheric CO_2 and VPD conditions in a controlled environment mesocosm. LER_{day} was under hydraulic control, in agreement with expected effects of atmospheric CO_2 and VPD on stomatal control, and ensuing effects on transpiration and hydraulic effects on cell wall expansion. Conversely, a compensatory growth mechanism (stored growth) controlled LER_{night} in such a way that daily LER remained unaffected by atmospheric conditions of CO_2 and VPD. Source limitation was not a factor under the conditions of this experiment as water-soluble carbohydrate levels in the LGDZ were high even when atmospheric CO_2 concentration was only half-ambient. Absence of source limitation may have resulted, in part, from a relatively limiting nitrogen fertilizer supply and the high radiation received by the stands. Water supply was unlimiting, thus putative feedbacks of eventual soil drying on the leaf elongation process – that would occur more readily with high transpiration at low C_a – had no effect, but are important factors to be considered. So, building on the present findings, studies of the diel oscillation of leaf elongation and underlying mechanism should be expanded to a greater range of plant functional groups, environmental conditions (including photoperiod length, irradiance, and thermal and nutritional conditions) to further improve our understanding of the plant physiology of climate change adaptation.

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Author contributions

JCBC and HS designed the experiment. JCBC performed the research, analyzed the data and wrote the first draft. RTH, JZ and RS helped with gas exchange measurements. All authors contributed to the revision of the manuscript.

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Supporting Information

Additional Supporting Information may be found online in the Supporting Information section at the end of the article.

Fig. S1 Pot arrangement in the growth chamber.

Fig. S2 Daily leaf elongation rate of *Lolium perenne* at different leaf developmental stages as influenced by atmospheric CO_2 concentration at low and high daytime VPD.

Fig. S3 Epidermal cell length density at the margins of an individual leaf blade replica in *Lolium perenne* plants grown at different atmospheric CO_2 concentrations at low or high daytime VPD.

Fig. S4 Leaf appearance interval and specific leaf area of *Lolium perenne* as influenced by atmospheric CO_2 concentration and VPD.

Fig. S5 Time course of leaf elongation rate of *Lolium perenne* as influenced by atmospheric CO_2 concentration and VPD.

Fig. S6 Canopy transpiration of *Lolium perenne* during day-light hours as influenced by atmospheric CO_2 concentration, VPD and irrigation events.

Fig. S7 Nocturnal leaf elongation rate of *Lolium perenne* as influenced by nocturnal transfers to contrasting atmospheric CO_2 concentration.

Table S1 Experimental design.

Table S2 Response of daytime and night-time leaf elongation rate of *Lolium perenne* to atmospheric CO_2 concentration, daytime VPD and their interaction.

Table S3 Stomatal conductance and leaf transpiration of *Lolium perenne* during the day at different atmospheric CO_2 concentration and daytime VPD.

Table S4 Response of water-soluble carbohydrate concentration in the leaf growth-and-differentiation zone of *Lolium perenne* to atmospheric CO_2 concentration, daytime VPD and diel period.

Table S5 Concentration of water-soluble carbohydrates in the leaf growth-and-differentiation zone of *Lolium perenne* at the end of the day and night at different atmospheric CO_2 concentration and daytime VPD.

Table S6 Response of nocturnal leaf elongation rate of *Lolium perenne* to water potential of the youngest fully-expanded leaf, osmotic potential of the leaf growth-and-differentiation zone and turgor.

Table S7 Nitrogen concentration in the leaf growth-and-differentiation zone of *Lolium perenne* for combinations of atmospheric CO_2 concentration and daytime VPD levels.

Table S8 Response of nocturnal leaf elongation rate of *Lolium perenne* to transfer/swapping of plants between contrasting CO_2 environments.

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