Last-Century Increases in Intrinsic Water-Use Efficiency of Grassland Communities Have Occurred over a Wide Range of Vegetation Composition, Nutrient Inputs, and Soil pH[1][OPEN]

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Last-century climate change has led to variable increases of the intrinsic water-use efficiency (W_i; the ratio of net CO_2 assimilation to stomatal conductance for water vapor) of trees and C_3 grassland ecosystems, but the causes of the variability are not well understood. Here, we address putative drivers underlying variable W_i responses in a wide range of grassland communities. W_i was estimated from carbon isotope discrimination in archived herbage samples from 16 contrasting fertilizer treatments in the Park Grass Experiment, Rothamsted, England, for the 1915 to 1929 and 1995 to 2009 periods. Changes in W_i were analyzed in relation to nitrogen input, soil pH, species richness, and functional group composition. Treatments included liming as well as phosphorus and potassium additions with or without ammonium or nitrate fertilizer applications at three levels. W_i increased between 11% and 25% ($P < 0.001$) in the different treatments between the two periods. None of the fertilizers had a direct effect on the change of W_i (ΔW_i). However, soil pH ($P < 0.05$), species richness ($P < 0.01$), and percentage grass content ($P < 0.01$) were significantly related to ΔW_i. Grass-dominated, species-poor plots on acidic soils showed the largest ΔW_i (+14.7 μmol mol^{-1}). The ΔW_i response of these acidic plots was probably related to drought effects resulting from aluminum toxicity on root growth. Our results from the Park Grass Experiment show that W_i in grassland communities consistently increased over a wide range of nutrient inputs, soil pH, and plant community compositions during the last century.

The intrinsic water-use efficiency (W_i) of plants is controlled by photosynthetic carbon assimilation and stomatal conductance via the leaf-level coupling of CO_2 and water fluxes. A general, but variable, increase of W_i under rising atmospheric CO_2 has been observed in long-term studies (Peñuelas et al., 2011; Franks et al., 2013; Saurer et al., 2014), but little is known about other environmental or ecosystem factors, which may interact with the effect of increasing CO_2 on W_i. An improved understanding of putative interactive mechanisms is important because changes in W_i may have significant effects on the global terrestrial carbon and water cycles (Gedney et al., 2006; Betts et al., 2007). This study explores the interactive effects of the increase in atmospheric CO_2 (observed over the last century), nutrient loading, and soil pH together with other related effects on plant species richness and functional group composition on the coupling of plant CO_2 and water fluxes in a seminatural grassland in southeastern England.

W_i is a leaf-level efficiency that has also been termed potential water-use efficiency or physiological water-use efficiency, as it excludes the direct influence of vapor pressure deficit (VPD), a parameter determined by environmental conditions, on leaf-level water-use efficiency (Farquhar et al., 1989; Franks et al., 2013). W_i reports the relationship between net CO_2 assimilation rate (A_n) and stomatal conductance for water vapor (g_{H2O}):

$$W_i = A_n/g_{H2O} \tag{1}$$

According to the first law of Fick, A_n can be given as the product of the stomatal conductance for CO_2 (g_{CO2}) and the concentration gradient between the atmosphere (c_a) and the leaf internal gas space (c_i): $A_n = g_{CO2} (c_a - c_i)$. Using $g_{CO2} (c_a - c_i)$ instead of A_n in Equation 1, replacement of g_{H2O}/g_{CO2} by the numerical value of g_{H2O}/g_{CO2}

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H.S. conceived the original project; I.H.K. conducted the sampling and analyzed the data; I.H.K. and H.S. wrote the initial draft of the article; A.J.M. supervised the sampling and complemented the writing.

[OPEN] Articles can be viewed without a subscription.

www.plantphysiol.org/cgi/doi/10.1104/pp.15.01472

(1.6) and rearrangement yields the following alternative expression of W_i:

$$W_i = c_a(1 - c_i/c_a)/1.6. \qquad (2)$$

Equation 2 reveals that past changes of W_i must have been controlled by two parameters: the change of c_a and the concurrent change of $1 - c_i/c_a$, the relative gradient for CO_2 diffusion into the leaf (Franks et al., 2013). A change in the relative gradient is determined by the changes in A_n relative to g_{H2O}, as leaves respond to changing c_a and other environmental factors. In particular, Equation 2 shows that any variation in the climate change response of W_i is determined by the c_i/c_a response, if the comparison is made for vegetation at the same location and in the same period of time.

Studies with C_3 vegetation, including trees/forests and C_3 grasslands, have revealed a general increase of W_i in the last century (Bert et al., 1997; Duquesnay et al., 1998; Feng, 1999; Arneth et al., 2002; Saurer et al., 2004; Barbosa et al., 2010; Köhler et al., 2010; Andreu-Hayles et al., 2011). In many cases, c_i/c_a, estimated by ^{13}C discrimination (Farquhar et al., 1989), varied relatively little. Indeed, it has been suggested, based on theoretical grounds and empirical evidence from studies over geological/evolutionary to short time scales, that adaptive feedback responses will tend to maintain c_i/c_a approximately constant (Ehleringer and Cerling, 1995; Franks et al., 2013), as plants optimize carbon gain with respect to water loss (Cowan and Farquhar, 1977). Yet, c_i/c_a-dependent variation in the W_i response to climate change has also been noted (Peñuelas et al., 2011; Köhler et al., 2012) over the last century, indicating that additional factors, perhaps including other global change drivers, can modify the W_i response over this time scale, at least transiently. A meta-analysis by Peñuelas et al. (2011) reports c_i/c_a-dependent increases of W_i for different forests between 6% and 36% from the early 1960s to 2000s. A recent study by Saurer et al. (2014) on European forest trees found increases in W_i ranging from 1% to 53% during the last century. The strongest increase of W_i was recorded in regions where summer soil-water availability decreased in the last century. For different grassland communities, the c_i/c_a-dependent increases of W_i varied between 13% and 28% at one site (Köhler et al., 2012) from 1915 to 2009. Evidently, such variation can have important repercussions for the coupling of terrestrial CO_2 and water fluxes. Yet, little is known about the mechanism(s) underlying the variation.

At the Park Grass Experiment (PGE) at Rothamsted, England, Köhler et al. (2012) observed a nitrogen supply-dependent enhancement of the W_i response on plots receiving nitrate fertilizer and maintained at a near-neutral soil pH by liming. However, the actual relationship between nitrogen supply and W_i response did not hold when the unlimed control (soil pH approximately 5.2) was included in the comparison. Remarkably, however, there was a significant positive relationship between the grass content of the community

and the W_i response of the experimental plots in the investigation. These results suggested that the effect of nutrient supply on the W_i response of the grassland communities was indirect, perhaps working via effects on soil pH and/or vegetation composition (plant species richness or functional group composition).

The PGE provides a unique opportunity to study century-scale variation in the c_i/c_a-dependent variation of W_i for a wide range of diverse grassland communities. Much of the extant ecosystem-scale variability of plant species richness and soil pH in temperate grasslands of Europe (Ceulemans et al., 2014) is included in the range of plot-scale plant species richness and soil pH at the PGE (which is reported in this investigation). The different long-term applications of fertilizer and lime over the past century have resulted in substantial changes in soil pH, species richness, and grass content on the experimental plots, but in most cases, within-plot changes over the study period considered here (1915–2009) were comparatively small (Crawley et al., 2005; Silvertown et al., 2006). All experimental plots are located at the same site and are exposed to the same weather conditions. Consequently, trends in climate as a direct driver for differences in W_i between plots can be ruled out.

Here, we explore putative mechanisms underlying eventual c_i/c_a-dependent variation of W_i during the last century at the PGE by, first, quantifying the sustained effect of a wide range of contrasting fertilizer treatments ($n = 16$) on the change of W_i during the last century and, second, analyzing the relationships between the observed W_i response of treatments and the respective nutrient status, soil pH, plant species richness, and plant functional group composition of the grassland communities.

RESULTS

Long-Term Changes of W_i and c_i/c_a

For the different treatments, the 15-year average W_i ($\varnothing W_i$) ranged between 49.6 and 62.5 μmol mol^{-1} in the 1915 to 1929 period and between 60.2 and 76.8 μmol mol^{-1} in the 1995 to 2009 period (Table II). The change of W_i (ΔW_i) between the two time periods was positive for all treatments, ranging between +5.9 and +14.7 μmol mol^{-1} (Fig. 1A). This effect was significant for all treatments ($P < 0.001$, adjusted for multiple comparisons) and corresponded to a relative enhancement of W_i ($\varnothing W_{i\ 1995\text{-}2009}/\varnothing W_{i\ 1915\text{-}1929}$) of 11% to 25% between the two periods (Table II).

The treatment-specific mean c_i/c_a varied between 0.67 and 0.74 in the 1915 to 1929 period, the same as in the 1995 to 2009 period (Table II). Treatment effects on changes of c_i/c_a between the 1915 to 1929 and 1995 to 2009 periods were not significant, except for the unlimed control (CONTROL.U; for treatment definitions, see "Materials and Methods" and Table I), where the increase was significant ($P < 0.001$), although very small (+0.028).

Table I. *Summary of studied treatments at the PGE grouped by fertilizer nitrogen form (no nitrogen, nitrate-nitrogen, and ammonium-nitrogen)*

Treatment abbreviations and main plot numbers (subplots after 1965 in parentheses), annual nitrogen, phosphorus (P) and potassium (K) fertilizer application (in kg ha^{-1}; nitrogen includes estimated biologically fixed nitrogen [N_{bf}]), functional group composition (in percentage dry weight for grasses [G], nonlegume forbs [F], and legumes [L] in harvested biomass), species richness, and soil pH (in water) are shown. Limed and unlimed subplots are distinguished by L and U in the treatment abbreviation.

Treatment	Plot No. (Subplot)	Annual Nitrogen Input ($N_{fertilizer}$ + Estimated N_{bf})	Annual P/K Input	Functional Group Composition (G/F/L) in Contribution to Total Harvested Herbage Biomass ± SD [n][a]	Species Richness[b]	Soil pH ± SD [n][c]
		kg ha^{-1}		*%*		
No nitrogen						
CONTROL.L	2 (a), 3 (a)	0	0/0	50 ± 12/40 ± 11/10 ± 5 [58]	43	7.1 ± 0.1 [8]
CONTROL.U	2 (c and d), 3 (c and d)	0	0/0	64 ± 14/32 ± 12/4 ± 4 [81]	41	5.3 ± 0.2 [12]
PK.L	7 (a), 15 (a)	50 (only N_{bf})	35/225	54 ± 15/20 ± 11/26 ± 13 [67]	29	6.7 ± 0.2 [6]
PK.U	7 (d), 15 (d)	25 (only N_{bf})	35/225	60 ± 15/22 ± 9/18 ± 11 [72]	28	4.9 ± 0.2 [6]
Nitrate, NO_3^-						
N*1.L	17 (a)	48	0/0	62 ± 18/36 ± 18/2 ± 2 [21]	36	7.2 ± 0.3 [4]
N*1.U	17 (c and d)	48	0/0	62 ± 13/38 ± 13/0 ± 0 [33]	39	5.9 ± 0.2 [6]
N*1PK.L	16 (a)	79 (48 + 31)	35/225	68 ± 15/20 ± 11/12 ± 10 [16]	26	7 ± 0.2 [4]
N*1PK.U	16 (c and d)	66 (48 + 18)	35/225	75 ± 14/16 ± 9/9 ± 7 [26]	31	5.5 ± 0.2 [6]
N*2PK.L	14 (a)	96	35/225	89 ± 9/7 ± 9/4 ± 4 [40]	26	7 ± 0.3 [4]
N*2PK.U	14 (c and d)	96	35/225	88 ± 11/11 ± 11/1 ± 1 [55]	25	6 ± 0.2 [6]
Ammonium, NH_4^+						
N1.L	1 (b)	48	0/0	69 ± 11/29 ± 11/2 ± 2 [22]	30	6.5 ± 0.6 [4]
N1.U	1 (d)	48	0/0	97 ± 5/3 ± 5/0 ± 0 [23]	6	4.3 ± 0.4 [4]
N2PK.L	9 (b)	96	35/225	93 ± 8/5 ± 6/2 ± 4 [46]	19	5.4 ± 0.7 [4]
N2PK.U	9 (d)	96	35/225	98 ± 4/2 ± 4/0 ± 0 [44]	5	3.9 ± 0.1 [4]
N3PK.L	11 (b)	144	35/225	92 ± 7/8 ± 7/0 ± 0 [16]	16	4.7 ± 1 [4]
N3PK.U	11 (d)	144	35/225	99 ± 3/1 ± 3/0 ± 0 [16]	2	3.7 ± 0.1 [4]

[a]Calculated from botanical separation data available for individual years in the 1915 to 1976 period (data set PARKPARTCOMP, eRA, 2013) and for each year in the 1991 to 2000 period (data set PARKCOMPIC, eRA, 2013). [b]Calculated as total number of species counted during a 10-year period (1991–2000). [c]Soil pH in water was calculated as the average from available measurements in the sampling period (Warren and Johnston 1964; Williams, 1978; Rothamsted Research, 2006).

Treatment Effects on ΔW_i: Nitrogen Input, Nitrogen Form, and Soil pH

The highest increases of W_i were observed in the unlimed NH_4^+ treatments (N1PK.U, N2PK.U, and N3PK.U: +13.8, +14.2, and +14.7 μmol mol^{-1}), and the smallest increase was observed in the limed control (CONTROL.L: +5.9 μmol mol^{-1}). Significant differences between treatments existed between the limed control (CONTROL.L) and two of the unlimed NH_4^+ plots (N2PK.U and N3PK.U; $P < 0.05$, adjusted for multiple comparisons).

In general, no significant effect of nitrogen input on ΔW_i was found at the 5% α level (Fig. 2A). Even within the NH_4^+ treatments with the largest differences in nitrogen input (48, 96, and 144 kg ha^{-1} a^{-1}), no significant effect of the amount of applied NH_4-nitrogen on ΔW_i was evident. Also, there were no significant differences between mean ΔW_i when all treatments were grouped by nitrogen form (ANOVA, $P = 0.06$). However, when the limed and unlimed NH_4^+ treatments were analyzed separately, a significant difference was found between the unlimed NH_4^+ treatments and the NO_3-nitrogen or no-nitrogen treatments (ANOVA, $P < 0.01$).

Increasing soil pH led to a significant decrease of ΔW_i (Fig. 2B; $\Delta W_i = 16.7 − 1.1x$; $r^2 = 0.24$, $P < 0.05$, degrees of freedom [df] = 14). Furthermore, NH_4^+ treatments showed a significant difference between limed and unlimed plots ($P < 0.01$, adjusted for multiple comparisons). But such a difference was not observed in the other treatments. No significant relationships were found when the unlimed NH_4^+ plots were omitted from the analysis.

Relationships of ΔW_i with Grass Abundance and Plant Species Richness

Abundance of Grasses

For the entire data set, ΔW_i was linearly related to the percentage abundance of grasses in the sward (Fig. 2C; $\Delta W_i = 3.6 + 0.1x$; $r^2 = 0.36$, $P < 0.01$, df = 14). Quantitatively similar relationships were observed when only the unlimed ($\Delta W_i = 1.1 + 0.13x$; $r^2 = 0.7$, $P < 0.01$, df = 6) or the NH_4^+ ($\Delta W_i = 2.7 + 0.1x$; $r^2 = 0.37$, $P < 0.05$, df = 8 [no-nitrogen treatments included]) treatments were considered in the regression analysis. The relationship for the NO_3^- treatments ($4.1 + 0.1x$; $r^2 = 0.31$, df = 8 [no-nitrogen treatments included]) was only significant at the 10% α level. No significant relationships were found for the limed treatments alone or when the unlimed NH_4^+ treatments were omitted from the analysis.

Species Richness

ΔW_i was negatively related to species richness (Fig. 2D; $\Delta W_i = 13.6 − 0.1x$; $r^2 = 0.43$, $P < 0.01$, df = 14), with

Figure 1. A, Absolute increases in W_i (μmol mol^{-1}) between the two studied periods ($\Delta W_i = \varnothing W_{i[1995\text{-}2009]} - \varnothing W_{i[1915\text{-}1929]}$) in ascending order B, Annual nitrogen input as the sum of fertilizer nitrogen and biologically fixed nitrogen (N_{bf}) in kg ha^{-1}. C, Soil pH in water (circles) and species richness (bars). D, Contribution of grass biomass to total harvested sward biomass in percentage. Error bars represent SE.
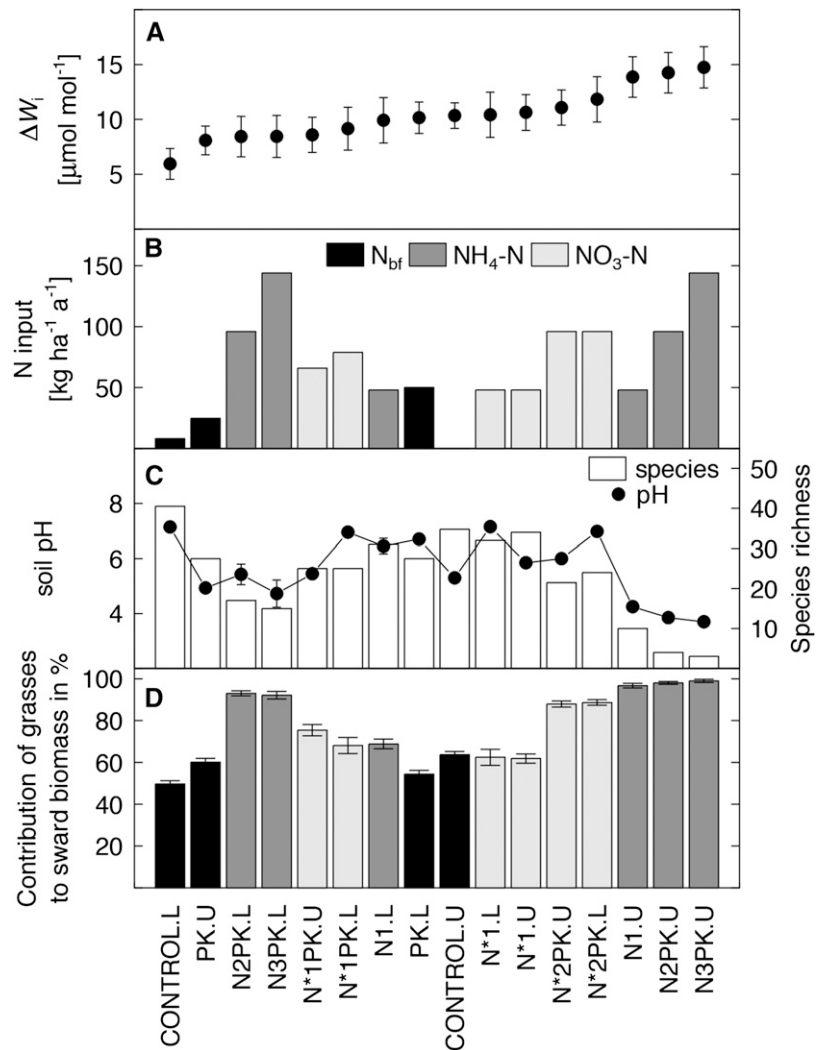
variation in richness explaining 43% of the total variation of ΔW_i. Significant and quantitatively similar relationships between ΔW_i and species richness were also found when only the unlimed ($\Delta W_i = 14.4 - 0.1x$; $r^2 = 0.64$, $P < 0.05$, df = 6) or the NH_4^+ ($\Delta W_i = 13.8 - 0.1x$; $r^2 = 0.55$, $P < 0.05$, df = 8 [no-nitrogen treatments included]) treatments were considered. All the observed regressions were strongly dependent on the inclusion of the species-poor unlimed NH_4^+ treatments (two to six species per plot) in the analysis. No significant relationship with species richness was evident when these treatments were omitted. The other treatments showed a broad spectrum of species richness (16–46), with no significant trend of ΔW_i versus number of plant species.

Correlations between Variables

Correlations between the directly controlled variables (nitrogen input and soil pH) and the indirectly manipulated variables (percentage abundance of grass and plant species richness) were highly significant,

indicating that they were not independent of each other (Table III). Nitrogen input and soil pH were not correlated, as both variables were manipulated separately.

DISCUSSION

W_i Showed a Variable Increase between Different Grassland Communities at a Single Site

In principle, the variability of ΔW_i responses during the last-century CO_2 increase can be attributed either to inherent differential physiological responses (control of A_n or g_{H2O}) of different species and the communities they form (direct effects) or to interacting effects of other environmental factors that modify the aforementioned community-scale physiological responses (indirect effects). The observed range of ΔW_i of the present grassland communities ($n = 16$) was smaller (11%–25%, average 19%) than that reported by Saurer et al. (2014) for a variety of forest trees in a wide range of sites across Europe (1%–53%, average 28%; $n = 35$) in a
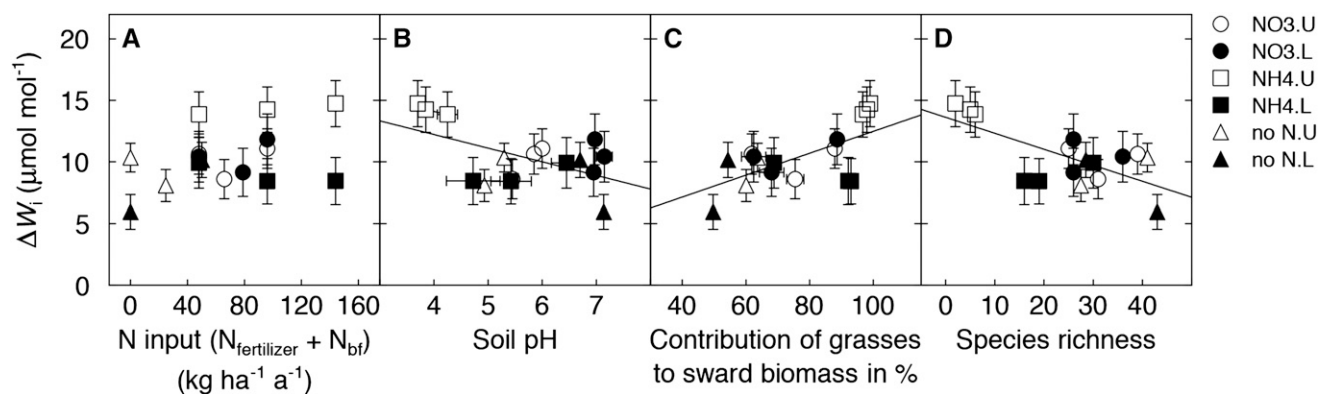
Figure 2. Relationships between ΔW_i (μmol mol^{-1}) and annual nitrogen input as the sum of fertilizer nitrogen and N_{bf} in kg ha^{-1} (A), soil pH in water, $y = 16.7 + 1.1x$, $r^2 = 0.24$, $P < 0.05$ (B), contribution of grass biomass to total harvested sward biomass in percentage, $y = 3.6 + 0.1x$, $r^2 = 0.36$, $P < 0.01$ (C), and species richness, $y = 13.6 + 0.1x$, $r^2 = 0.43$, $P < 0.01$ (D). Simple linear regression lines are only shown where significant ($P < 0.05$). Symbols represent the type of nitrogen addition (triangles = no nitrogen, circles = NO_3^-, and squares = NH_4^+) and liming treatment (white symbols = unlimed and black symbols = limed). Bars denote the SE. Horizontal error bars in B and C are not shown where smaller than the symbols.

similar time period (1901–1910 and 1991–2000). The generally lower W_i and lower ΔW_i of the grasslands at the PGE compared with forests may be related to the observed lower carbon cost per unit of water used in C_3 grasses compared with gymnosperm and angiosperm trees (Lin et al., 2015). The strongest increases in tree ΔW_i were related to decreasing soil water content at the respective sites. Being located at a single site, the observed treatment-specific variation of ΔW_i at the PGE during the past 100 years is unrelated to direct effects of weather factors or other characteristics of geography. Instead, all the evidence for mechanisms controlling the differences in ΔW_i between treatments at the PGE point

to site conditions and plant community characteristics that were modified by the (differential) nutrient and lime inputs. These mechanisms may include the controls of plant community assembly (Silvertown, 1980; Crawley et al., 2005), modifications of the genetic diversity of plant species and niche dimensionality (Harpole and Tilman, 2007), soil microorganism composition (Rousk et al., 2011), plant-soil microbe interactions in processing/sequestration of plant carbon (Fornara et al., 2011), alterations of the relative availabilities of (both fertilized and unfertilized) macronutrients and micronutrients (White et al., 2012), and, possibly, aluminum toxicity (Davies and Snaydon, 1973).

Table II. *Average W_i (derived from carbon isotope discrimination [$^{13}\Delta$]) in the 1915 to 1929 and 1995 to 2009 periods on the studied treatments, corresponding ΔW_i, and average c_i/c_a in the two periods*

The ΔW_i was significantly different from zero in all treatments ($P < 0.001$). A significant increase of c_i/c_a was only observed in the CONTROL.L treatment ($P < 0.001$).

Treatment	Average $W_i \pm$ SD [n]		$\Delta W_i \pm$ SE	$c_i/c_a \pm$ SE	
	1915–1929	1995–2009		1915–1929	1995–2009
	μmol mol^{-1}		%		
CONTROL.L	54.3 ± 4.0 [23]	60.3 ± 3.8 [30]	11 ± 3	0.716 ± 0.004	0.744 ± 0.004
CONTROL.U	50.8 ± 3.7 [29]	61.2 ± 3.5 [58]	20 ± 2	0.734 ± 0.004	0.740 ± 0.002
PK.L	54.5 ± 3.9 [24]	64.6 ± 4.4 [29]	19 ± 3	0.716 ± 0.004	0.726 ± 0.004
PK.U	52.9 ± 4.3 [30]	61.0 ± 4.9 [30]	15 ± 2	0.723 ± 0.004	0.741 ± 0.004
N*1.L	52.3 ± 4.3 [10]	62.7 ± 3.6 [15]	20 ± 4	0.727 ± 0.007	0.734 ± 0.004
N*1.U	49.6 ± 4.6 [15]	60.2 ± 4.3 [30]	21 ± 3	0.741 ± 0.007	0.745 ± 0.004
N*1PK.L	54.8 ± 3.4 [12]	64.0 ± 4.8 [15]	17 ± 4	0.714 ± 0.005	0.729 ± 0.006
N*1PK.U	53.3 ± 4.1 [15]	61.9 ± 5.5 [30]	16 ± 3	0.721 ± 0.006	0.737 ± 0.005
N*2PK.L	52.2 ± 5.0 [10]	64.0 ± 3.8 [15]	23 ± 4	0.728 ± 0.008	0.728 ± 0.005
N*2PK.U	54.9 ± 3.8 [15]	66.0 ± 5.5 [30]	20 ± 3	0.713 ± 0.005	0.720 ± 0.005
N1.L	54.7 ± 3.7 [11]	64.6 ± 3.9 [15]	18 ± 4	0.715 ± 0.006	0.726 ± 0.005
N1.U	57.5 ± 5.5 [15]	71.4 ± 4.8 [15]	24 ± 3	0.699 ± 0.008	0.698 ± 0.005
N2PK.L	58.9 ± 5.1 [15]	67.3 ± 5.5 [15]	14 ± 3	0.692 ± 0.007	0.714 ± 0.007
N2PK.U	62.5 ± 8.0 [15]	76.8 ± 9.2 [15]	23 ± 3	0.673 ± 0.011	0.675 ± 0.010
N3PK.L	60.7 ± 4.7 [13]	69.1 ± 6.4 [15]	14 ± 3	0.683 ± 0.007	0.707 ± 0.008
N3PK.U	58.7 ± 8.2 [14]	73.5 ± 10.5 [15]	25 ± 3	0.693 ± 0.012	0.689 ± 0.011

Table III. *Correlations (Pearson coefficients) for average percentage abundance of grasses in total harvested herbage biomass, species richness, soil pH, and nitrogen input (calculated as fertilizer nitrogen plus N_{bf})*

Asterisks denote the significance level (Pearson's product-moment correlation: ***, $P < 0.001$; **, $P < 0.01$; and n.s., not significant).

	Species Richness	Soil pH	Nitrogen Input
Grasses (%)	−0.862***	−0.660**	0.786***
Nitrogen input	−0.716**	−0.399[n.s.]	
Soil pH	0.748**		

Although the variability of ΔW_i resulted from the diversity of fertilizer and liming treatments, we did not detect any direct, general relationship between a single nutrient factor, such as annual nitrogen inputs (including biologically fixed nitrogen) or phosphorus + potassium additions, on ΔW_i. For every nutrient (or lime) added or not (singly or in combination with other nutrients), the observed relative range of W_i increase varied widely. Moreover, investigations of the plot-specific type of nutrient limitation (phosphorus or nitrogen) for biomass production in the last 50 years revealed no relationship with ΔW_i (R. Hirl, I.H. Köhler, A. Macdonald, and H. Schnyder, unpublished data). Conversely, soil pH, as controlled via targeted amounts of applied lime, was related to ΔW_i, with the highest ΔW_i observed on the most acidic soils. Similar relationships of ΔW_i with species richness and the relative abundance of grasses indicate that this treatment effect may also have worked indirectly via changes in plant community composition.

Does Aluminum Toxicity Lead to Higher ΔW_i via Increased Drought Stress?

The greatest relative increase of W_i occurred on the plots with most acidic soils (pH approximately 3.5–4), which are a result of long-term annual ammonium sulfate additions. The low pH on these plots, which has effected a significant mobilization of aluminum (Gould et al., 2014) and other heavy metals, has also led to a dramatic loss of (acid-sensitive) plant species and the selection of a very small number of acid-tolerant and metal-resistant grasses (e.g. *Holcus lanatus, Anthoxanthum odoratum,* and *Agrostis capillaris*; Blake and Goulding, 2002; Crawley et al., 2005; Silvertown et al., 2006). These species-poor, grass-rich plots have shown much greater interannual variability of biomass yields than species-rich plots, perhaps due to the better buffering of species-rich communities against climatic variability (Dodd et al., 1994b). High aluminum concentrations may have exacerbated the effect of climatic variability by causing greater soil moisture deficits on the acidic plots; aluminum toxicity is known to inhibit root growth (Kochian, 1995; Horst et al., 2010), leading to reduced root length density and shallower rooting depth (Jentschke et al., 2001; Tang et al., 2001) in a variety of

species. Physiological relationships between aluminum toxicity, impeded root growth, and drought sensitivity of plants are well established (Yang et al., 2013). Also, Dodd et al. (1994b) suggested that the large yield variability of the species-poor, acidic plots on the PGE were related (at least in part) to soil moisture deficits. There is no evidence that the dominant species (*H. lanatus, A. odoratum,* and *A. capillaris*) on the acidic plots differ inherently in their reactivity to drought; indeed, their Ellenberg moisture indicator values (Hill et al., 1999) do not differ from the dominant grass species (*Alopecurus pratensis, Arrhenatherum elatius,* and *Dactylis glomerata*) on the other plots. However, effects of soil moisture deficits, resulting from aluminum effects on rooting depth, may interact with increasing atmospheric CO_2 at the level of stomata, enhancing the increase in W_i via a drought stress-induced decrease in g_{H2O}. Accordingly, soil acidification in combination with aluminum toxicity may have a similar effect on ΔW_i to the climate-related decrease in water availability reported by Saurer et al. (2014).

On the Mechanism and Implication of Treatment-Specific W_i Responses

W_i is controlled via c_i/c_a (Eq. 2). This physiological set point seems to have been maintained approximately constant under increasing c_a (Ward et al., 2005; Franks et al., 2013), which leads to a concurrent increase of W_i. Mean atmospheric CO_2 concentration has increased by 23.5% between the first (1915–1929) and second (1995–2009) period of this study. In comparison, W_i increased by 11% to 25% between the two periods. This means that c_i/c_a was approximately maintained constant in the treatments with the highest increase of W_i (the species-poor, grass-rich communities on the acidic plots), whereas c_i/c_a increased to some extent on the plots with the least increase of W_i (the species- and forb-rich communities on plots with neutral pH). This (small but significant) increase in c_i/c_a more than halved the CO_2-driven enhancement of W_i on the limed control treatment.

Despite a large variation in nutrient supply, soil pH, plant species richness, and functional group composition, the majority of the treatments showed intermediate increases in W_i (Fig. 1A). Only the most extreme plots (acidic soil and nutrient poor) differed significantly from the others. This general response suggests that a wide range of European seminatural C_3 grasslands may show increasing W_i under rising atmospheric CO_2 by maintaining c_i/c_a approximately constant or increasing it just slightly. Depending on changes in atmospheric VPD, the increases in W_i could translate directly into a corresponding response of leaf-level transpiration efficiency (W_t), as $W_t = W_i \times VPD^{-1}$ (Farquhar and Richards, 1984; Farquhar et al., 1989). During spring growth (March–June), VPD at the PGE did not change between the two studied periods. If this is also true for other European regions, then, in combination with the observation of constant c_i/c_a, this implies that ecosystem evapotranspiration in European seminatural

C_3 grasslands may be reduced by rising atmospheric CO_2 on a large scale. This could also affect local water balances via increased river runoff, as suggested by Gedney et al. (2006).

CONCLUSION

This study demonstrates significant correlations between the last-century increase of W_i (ΔW_i) and the proportion of grasses in the plant community, species richness of communities, and soil pH, which were all interdependent site-community characteristics resulting from long-term differential fertilizer and liming treatments. Remarkably, however, the correlations depended very strongly on the inclusion of the ammonium sulfate fertilizer treatments, which led to the highest ΔW_i and was associated with low soil pH, a high grass proportion, and low species richness (associated with the selection of aluminum-tolerant grass species). Therefore, drought sensitivity, resulting from the inhibition of root growth by aluminum at low soil pH, seemed to be the most important interactive factor causing an above-average last-century increase of W_i at the PGE.

All grassland communities exhibited a consistent increase in W_i, and this was true for communities with greatly differing plant species richness and functional group composition and nutrient and lime inputs during the last century. The analysis covered virtually the entire range of species richness and soil pH at the PGE, which is representative of a significant proportion of the extensively managed (two cuts per year) European temperate seminatural grasslands. Based on this observation, we suggest that the increase in W_i on the PGE is representative of a large part of the extensively managed temperate grassland in Europe and perhaps other parts of the world. To determine the extent to which this also applies to more intensively managed grassland would require further study.

MATERIALS AND METHODS

The Park Grass Experiment (PGE)

The PGE was established in 1856 at Rothamsted Experimental Station (40 km north of London in Hertfordshire, England, 0°21′W, 51°49′N, 128 m above sea level). Different fertilizer and manure treatments were applied to 20 experimental plots on 2.8 ha of old grassland. In 1903, most of the plots were divided in two to introduce a test of lime on one half; 4 tons ha^{-1} CaCO$_3$ was applied every fourth year. In 1965, most of the plots were further divided into four subplots (a, b, c, and d). The a, b, and c subplots receive lime every 3 years, if necessary, to maintain a target soil pH of 7, 6, and 5, respectively. The d subplots are unlimed (for more details, see Köhler et al., 2012). The experiment is located on a moderately well-drained silty clay loam overlying clay-with-flints. The soil pH was slightly acidic when the experiment began (5.4–5.6), and the nutrient status was poor (Silvertown et al., 2006).

The original vegetation of the PGE has been classified by Dodd et al. (1994a) as a dicotyledon-rich *Cynosurus cristatus-Centaurea nigra* grassland, one of the mesotrophic grassland communities in the British National Vegetation Classification system. Since the start of the experiment, the herbage has been cut around mid-June for hay production; samples were taken from the material dried in situ until 1960. In subsequent years, strips were cut with a forage harvester, and fresh herbage samples were taken for drying and archiving. The herbage remaining on the plot was made into hay, as before. Originally, the following regrowth was grazed by sheep, which were penned on the individual plots, but since 1875, grazing was abandoned and a second harvest cut was removed green. Dried samples from all plots and both cuts have been stored in the Rothamsted Sample Archive since the beginning of the experiment (Silvertown et al., 2006). This work presents data from the first cut of two study periods: 1915 to 1929 and 1995 to 2009.

Rainfall and temperature have been measured at Rothamsted since 1853 and 1878, respectively. In the months preceding the first cut (March–June), mean daily temperature ± SD was 9.2°C ± 0.5°C versus 10.5°C ± 0.6°C and VPD ± SD was 0.32 ± 0.04 kPa versus 0.34 ± 0.04 kPa in the two study periods (1915–1929 versus 1995–2009). The average sum of annual rainfall ± SD in 1915 to 1929 versus 1995 to 2009 was very similar (805 ± 140 mm versus 796 ± 146 mm), and this was also the case for seasonal rainfall from March to June (208 ± 49 mm versus 219 ± 62 mm). For more climatic information, see Köhler et al. (2010).

Fertilizer and Liming Treatments

A previous study (Köhler et al., 2012) investigated a smaller range of treatments ($n = 5$), including nitrate fertilizer inputs, on plots where a near-neutral soil pH was maintained by liming. This work extends the range of treatments ($n = 16$) and now reports on both limed and unlimed treatments combined with or without additions of phosphorus (35 kg ha^{-1} a^{-1} from triple superphosphate) plus potassium (225 kg ha^{-1} a^{-1} from potassium sulfate) and one of two forms of nitrogen fertilizers given at elemental rates of 0, 48, or 96 kg ha^{-1} a^{-1} (as sodium nitrate or ammonium sulfate) or 144 kg ha^{-1} a^{-1} (as ammonium sulfate). The treatments are designated thus: CONTROL denotes unfertilized plots; PK indicates plots receiving phosphorus plus potassium fertilizer; N* refers to nitrate and N to ammonia fertilizer; and 1, 2, or 3 give the dosage (48, 96, or 144 kg ha^{-1} a^{-1}) of nitrogen fertilizer. In the following, limed and unlimed subplots are distinguished by L and U. Thus, for instance, N*1PK. U stands for the treatment receiving 48 kg ha^{-1} a^{-1} nitrogen in the form of sodium nitrate and phosphorus plus potassium, but no lime. For details of treatments and the abbreviations used, see Table I. Most treatments have not been replicated or randomized. However, the meadow was reasonably uniform before the experiment began, and the size of the plots (greater than 100 m^2) compensates to some extent for the lack of replication (Crawley et al., 2005). The experiment included two replicate plots for the CONTROL.U and PK.U treatments for the whole studied period and for the N*1.U and N*1PK.U treatments for the period after 1965.

Fertilizer nitrogen was applied in spring as one dressing except for the ammonium and nitrate treatments with the largest nitrogen inputs (N3PK.U, N3PK.L, N*2PK.U, and N*2PK.L), where the dressing was split. The nitrogen applied to the N3PK.U and N3PK.L plots was split in two, 96 kg ha^{-1} was applied on the first date and 48 kg ha^{-1} on the second, about 4 weeks after the first. Similarly, the nitrogen applied to the N*2PK.U and N*2PK.L plots was also split, 48 kg ha^{-1} being applied on both dates. Since 1980, these split dressings have stopped, and all of the plots given nitrogen received the total amount in one dressing. The other fertilizers were applied in winter. Nitrogen input (Table I; Fig. 1B) was defined as fertilizer-nitrogen supply plus an estimate of biologically fixed nitrogen (N$_{bf}$). The average N$_{bf}$ input for the spring growth was estimated using the model of Høgh-Jensen et al. (2004) with the parameterization for cuts of 1- to 2-year-old mixed grass and red clover (*Trifolium pratense*) systems as described by Köhler et al. (2012). Estimates of N$_{bf}$ were included for treatments with an average nitrogen fixation of greater than 10 kg ha^{-1} a^{-1}, which included treatments PK.L, PK.U, N*1.PK.L, and N*1.PK.U (Table I; Fig. 1B). Results from soil pH measurements (measured in water) were available for the years 1923 and 1959 (Warren and Johnston, 1964), 1974 to 1977 (Williams, 1978), and 1991 to 2000 (Rothamsted Research, 2006). Average soil pH (Fig. 1C; Table I) in the sampling period differed greatly between treatments but was relatively constant within a treatment, except for the limed ammonium sulfate plots, where pH increased over time.

Functional Group Composition and Species Richness

Functional group composition and species richness differed greatly between treatments (Table I; Fig. 1, C and D), but according to Silvertown et al. (2006), functional group composition within treatments has reached a dynamic equilibrium and was relatively constant since 1915. Between years, the contribution of different functional groups has varied widely within many plots, but no general long-term trend was observed (Supplemental Fig. S1). Therefore,

vegetation composition at the functional group level (grasses, nonlegume forbs, and legumes) was assumed to be constant within treatments, and means for each group were calculated from partial botanical separation data available for individual years from 1915 to 1976 (data set PARKPARTCOMP, eRA, 2013) and for each year from 1991 to 2000 (data set PARKCOMPIC, eRA, 2013).

We compared species richness (the number of species present) between the 1915 to 1929 period and the 1995 to 2009 period using available species-level botanical separation data (data sets PARKCOMP and PARKCOMPIC, eRA, 2013). For the first period, however, botanical separation data at the species level were only available for a single year on some treatments. For estimation of the average differences in species richness between the treatments, therefore, we used data from a comprehensive study (Crawley et al., 2005; data set PARKCOMPIC, eRA, 2013), which reflects the total number of species counted during a 10-year period (1991–2000). Slight changes over time between the two studied periods were observed on the PK.U, N1.U, and N2PK.U treatments but were small in comparison with between-treatment variation (Supplemental Fig. S2).

Estimation of W_i from c_a and Carbon Isotope Composition

W_i was obtained from Equation 2. For c_a, we used published data of c_a obtained from measurements of CO_2 in free air (Keeling et al., 2009) and in gas bubbles from ice cores for the time before CO_2 measurements in free air began (Friedli et al., 1986; Francey et al., 1999). c_i/c_a was estimated from the known relationship with carbon isotope discrimination, $^{13}\Delta$ (Farquhar et al., 1982; Farquhar and Richards, 1984) as obtained from the linear model of $^{13}\Delta$:

$$^{13}\Delta = a + (b - a)c_i/c_a, \qquad (3)$$

where a is the fractionation of $^{13}CO_2$ (relative to $^{12}CO_2$) during diffusion through the stomatal pores (4.4‰) and b is the net fractionation by carboxylases (27‰; Eq. 2). $^{13}\Delta$ was obtained from the carbon isotope composition of the archived plant material ($\delta^{13}C_p$) and that of atmospheric CO_2 ($\delta^{13}C_a$; Friedli et al., 1986; Francey et al., 1999; White and Vaughn, 2011) as

$$^{13}\Delta = \left(\delta^{13}C_a - \delta^{13}C_p\right)/1 + \delta^{13}C_p. \qquad (4)$$

The estimates for atmospheric $\delta^{13}C$ and CO_2 concentration were obtained for each year in the two sampling periods as described by Köhler et al. (2010). From 1915 to 2009, atmospheric CO_2 increased by 96 μmol mol^{-1}. The average difference between the two 15-year-long periods was 72 μmol mol^{-1}. The mean atmospheric CO_2 concentration $\pm$ SD during the first period was 306 $\pm$ 1 μmol mol^{-1}, compared with 378 $\pm$ 8 μmol mol^{-1} in the second period.

Carbon Isotope Analysis

Sample preparation and carbon isotope analysis were performed with the same procedures/protocols and instrumentation as described by Köhler et al. (2012). Representative subsamples of plant material were collected in the Rothamsted Sample Archive, dried at 40°C for 48 h, milled to homogeneity, and dried again at 60°C for 24 h. Aliquots of 0.7 $\pm$ 0.05 mg were weighed into tin cups (IVA Analysentechnik) and combusted in an elemental analyzer (NA 1110; Carlo Erba) interfaced (Conflo III; Finnigan MAT) with an isotope ratio mass spectrometer (Delta Plus; Finnigan MAT). Carbon isotope data were obtained as $\delta^{13}C$, with $\delta^{13}C = [(R_{sample}/R_{standard}) -1]$, where R is the $^{13}C/^{12}C$ ratio in the sample or standard (Vienna Pee Dee Belemnite, V-PDB). Each sample was measured against a laboratory working standard CO_2 gas, which was previously calibrated against a secondary isotope standard (IAEA-CH6, International Atomic Energy Agency, Sucrose Reference Material; accuracy of calibration $\pm$ 0.06‰ SD). After every 10th sample, a solid internal laboratory standard with similar carbon-nitrogen ratio to the respective sample material (fine ground wheat [*Triticum aestivum*] flour) was run as a control. The solid internal laboratory standards were previously calibrated against the IAEA-CH6 standard. The precision for sample repeats was better than 0.1‰.

Statistical Analysis

The climate change response of W_i, ΔW_i, of the different plots was obtained as the difference between the average of W_i in the 1915 to 1929 period ($\varnothing W_{i\,1915-1929}$) and the 1995 to 2009 period ($\varnothing W_{i\,1995-2009}$),

$$\Delta W_i = \varnothing W_{i\,1995-2009} - \varnothing W_{i\,1915-1929}. \qquad (4)$$

The length of the two periods was chosen so that it was long enough to absorb the interannual variability of climatic conditions and short enough to still retain a significant difference in CO_2 concentration between the two periods. As a proxy for the interannual climatic variability, we used the plant available soil water for a grass reference crop (PAW$_{ref}$), which has been shown to explain interannual and intraannual variation in $^{13}\Delta$ better than single climatic factors like VPD or precipitation (Schnyder et al., 2006; Köhler et al., 2010). PAW was calculated as $PAW_i = PAW_{i-1} + P_i - AET_i$, where PAW_{i-1} is the modeled plant available water of the previous day, P_i is the precipitation on day i, and AET_i is the modeled actual evapotranspiration on day i. AET_i equaled PET_i as long as $PAW_i/PAW_{capacity} > 0.3$. Otherwise, AET_i was calculated as $AET = \frac{PET}{0.3} \cdot \frac{PAW_i}{PAW_{capacity}}$ (Schnyder et al., 2006). PET was estimated with the FAO Penman-Monteith equation for a standard grass reference crop (Allen et al., 1998). The maximum plant available soil water (PAW$_{capacity}$) of the soil has not been measured directly but was inferred to be 135 mm (for the top 70 cm of the soil) from measurements on similar soils at Rothamsted (Avery and Catt, 1995). For further details on the calculation of PAW$_{ref}$, see Köhler et al. (2012).

We calculated the mean and SD of PAW$_{ref}$ for periods of increasing duration (2–30 years) after 1915 and before 2009 and compared the values between the periods. This procedure led to the choice of a period length of 15 years, for which the mean and SD of both periods were similar (PAW$_{ref1915-1929}$, 55.6 $\pm$ 30.7 mm; PAW$_{ref1995-2009}$, 58.7 $\pm$ 28.3 mm).

For each treatment, we tested if ΔW_i was significantly different from zero using linear regression, adjusting for multiple comparisons. The relationships between ΔW_i and direct and indirect treatment parameters (nitrogen input, soil pH, grass content in percentage, and species richness) were analyzed using simple linear regression. When analysis was conducted on subsets grouped by nitrogen form (NH_4 or NO_3 treatments), the no-nitrogen treatments were included in the regressions as controls. All statistical analysis was done with R (R Core Team, 2014). The multcomp package (Hothorn et al., 2008) in R was used for multiple comparisons of model parameters. Adjusted P values (Holm method) are reported for multiple comparisons.

Supplemental Data

The following supplemental materials are available.

Supplemental Figure S1. Percentage contribution of functional groups to total harvested dry matter on the studied treatments

Supplemental Figure S2. Species counts in individual years on the studied treatments

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