Last-century changes of alpine grassland water-use efficiency: a reconstruction through carbon isotope analysis of a time-series of *Capra ibex* horns

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Abstract

The ecophysiological response of an alpine grassland to recent climate change and increasing atmospheric CO_2 concentration was investigated with a new strategy to go back in time: using a time-series of *Capra ibex* horns as archives of the alpine grasslands' carbon isotope discrimination ($^{13}\Delta$). From the collection of the Natural History Museum of Bern, horns of 24 males from the population of the Augstmatthorn–Brienzer Rothorn mountains, Switzerland, were sampled covering the period from 1938 to 2006. Samples were taken from the beginning of each year-ring of the horns, representing the beginning of the horn growth period, the spring. The horns' carbon ^{13}C content ($\Delta^{13}C$) declined together with that of atmospheric CO_2 over the 69-year period, but $^{13}\Delta$ increased slightly (+ 0.4‰), though significantly ($P < 0.05$), over the observation period. Estimated intercellular CO_2 concentration increased (+ 56 µmol mol^{-1}) less than the atmospheric CO_2 concentration (+ 81 µmol mol^{-1}), so that intrinsic water-use efficiency increased by 17.8% during the 69-year period. However, the atmospheric evaporative demand at the site increased by approximately 0.1 kPa between 1955 and 2006, thus counteracting the improvement of intrinsic water-use efficiency. As a result, instantaneous water-use efficiency did not change. The observed changes in intrinsic water-use efficiency were in the same range as those of trees (as reported by others), indicating that leaf-level control of water-use efficiency of grassland and forests followed the same principles. This is the first reconstruction of the water-use efficiency response of a natural grassland ecosystem to last century CO_2 and climatic changes. The results indicate that the alpine grassland community has responded to climate change by improving the physiological control of carbon gain to water loss, following the increases in atmospheric CO_2 and evaporative demand. But, effective leaf-level water-use efficiency has remained unchanged.

Keywords: alpine meadows, carbon isotope discrimination, climate change, CO_2, C-13, intrinsic water-use efficiency, stomatal conductance, transpiration

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Introduction

The concentration of atmospheric CO_2 has increased strongly in the last century (Forster *et al.*, 2007). Projecting the impacts of this change on ecosystems is complicated by a poor understanding of the combined effects of rising CO_2 and associated climatic changes at long time scales (Norby & Luo, 2004; Fischlin *et al.*, 2007). To overcome the temporal limitations of ecological experiments, the identification of vegetation responses to past or recent climate changes can contribute qualitatively and quantitatively to better projections.

Plants respond to short-term increases of ambient CO_2 by two mechanisms: increasing photosynthetic carbon assimilation rate (A) and decreasing stomatal conductance (g_s) (Long *et al.*, 2004), which combined potentially enhance actual water-use efficiency. Multi-year free-air CO_2 enrichment (FACE) experiments applying elevated CO_2 concentrations (550–600 µmol mol^{-1}) in different ecosystems revealed, by meta-analysis, an average

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approximately 30% increase of daily photosynthesis and a about 20% decrease of stomatal conductance (Long *et al.*, 2004). However, it is not clear whether these effects prevail on a longer term or if down-regulation of photosynthesis or acclimatation of stomatal conductance occurs (Long *et al.*, 2004; Ainsworth & Rogers, 2007).

Integrated water-use efficiency can be reconstructed from the carbon isotopic composition of plants, $\delta^{13}C$ (Farquhar *et al.*, 1989). During photosynthesis, C_3 plants discriminate against the ^{13}C isotope, physically and biochemically, causing a ^{13}C depletion in photosynthetic products relative to the atmospheric CO_2 source. This effect is termed carbon isotope discrimination ($^{13}\Delta$) and is defined as:

$$^{13}\Delta = \frac{\delta^{13}C_a - \delta^{13}C_p}{1 + \delta^{13}C_p/1000},\qquad(1)$$

where $\delta^{13}C_a$ refers to atmospheric CO_2 and $\delta^{13}C_p$ to plant carbon. Farquhar *et al.* (1989) described the dependence of $^{13}\Delta$ on plant physiological properties, in particular c_i/c_a, the ratio of intercellular to ambient CO_2 concentrations for plants, as:

$$^{13}\Delta = a + (b - a)\frac{c_i}{c_a},\qquad(2)$$

where a is the fractionation during diffusion in air (4.4‰) and b is the net fractionation caused by carboxylations (27‰). The relationship between $^{13}\Delta$ and c_i/c_a has been classically used to estimate water-use efficiency. Thus, instantaneous water-use efficiency, W_t, i.e. the ratio between assimilation (A) and transpiration (E) rates is defined as:

$$W_t = \frac{A}{E} = \frac{g_{CO_2}(c_a - c_i)}{g_{H_2O}v} = \frac{c_a\left(1 - \frac{c_i}{c_a}\right)}{1.6v}\qquad(3)$$

where g_{CO_2} is the leaf conductance for CO_2, g_{H_2O} is the leaf conductance for water vapour, the 1.6 factor is the ratio of diffusivities of water vapour and CO_2 in air, and v is the water vapour concentration gradient, given by:

$$v = \frac{e_i - e_a}{P},\qquad(4)$$

with e_i the water vapour pressure in the leaf, e_a that of the air, $e_i - e_a$ the vapour pressure deficit (VPD), and P atmospheric pressure. Because g_{H_2O} determines how leaves potentially transpire water, the ratio A to g_{H_2O}:

$$W_i = \frac{A}{g_{H_2O}} = \frac{(c_a - c_i)}{1.6} = \frac{c_a\left(1 - \frac{c_i}{c_a}\right)}{1.6},\qquad(5)$$

is termed intrinsic water-use efficiency, W_i (Ehleringer *et al.*, 1993). W_i can be regarded as the physiological leaf-level component that controls water-use efficiency, assuming a constant evaporative demand (v). W_i is of particular interest when comparing the water-use efficiency of different genotypes under the same environmental conditions. However, under variable environmental conditions, W_i should be regarded as the potential rather than the actual water-use efficiency, and W_t should be used instead.

Using Eqn (5) several studies have reconstructed long-term responses of C_3 plants to the atmospheric CO_2 fluctuations which occurred in the last glacial maximum and during the last century. Such studies have used time-series of different conserved plant materials like fossil plants (Beerling & Woodward, 1993), herbarium samples (Peñuelas & Azconbieto, 1992), tree rings (Francey, 1981; Marshall & Monserud, 1996; Bert *et al.*, 1997; Duquesnay *et al.*, 1998; Feng, 1999; Saurer *et al.*, 2004) and nondecomposed litter profiles (Ehleringer & Cerling, 1995). In the majority of these studies W_i increased in response to atmospheric CO_2, in agreement with short-term experiments (e.g. FACE), meaning that adjustment on the demand (A) or the supply (g_s) of CO_2 occurred when c_a increased. However, the adjustment varied between the studies. On the other hand, these studies covered a small part of the diversity of plant life forms, focusing mostly on trees and missing different strategies of carbon–water relations. In addition they focussed on the response of individuals which may not scale linearly to ecosystem-level behaviour. Hence, there is a need to study different life forms and ecosystems. The present study contributes to this need, by providing the first quantitative evidence of $^{13}\Delta$ responses of natural alpine grassland to the recent atmospheric changes.

To overcome the limited availability of temporal series of plant material from grassland and to include community rather than species individual responses, we applied a new strategy to assess the community-level $^{13}\Delta$ of an alpine grassland, making use of the principle 'You are what you eat isotopically' (DeNiro & Epstein, 1978). For instance, the hair of grazers integrates carbon isotopic variations of diet temporally and spatially (West *et al.*, 2004; Schnyder *et al.*, 2006; Männel *et al.*, 2007). Similarly, the horns of alpine ibex (*Capra ibex*), also made of keratin, can be used as spatial and temporal archives of alpine grassland $^{13}\Delta$ variations (Barbosa *et al.*, 2009). These horns grow throughout the life of the animal, offering long records, ~ 13 years (Toigo *et al.*, 2007), they have distinct rings that separate the different years of animal life (Ratti & Habermehl, 1977; Giacometti *et al.*, 2002), and a considerable number of horns can be found in private or museum collections, due to active hunting and patrolling of the populations of the European Alps (Lüps & Zuber, 1986). After the almost extinction of the species in 16–19th centuries, the Augstmatthorn–Brienzer Rothorn population was one of the first successfully reestablished populations in Switzerland. Founded in

1921, it soon achieved a great number of effectives (Nievergelt, 1966; Lüps & Zuber, 1986). The long history of the population, together with a considerable high and complete record of horns in the Natural History Museum of Bern, made this population a suitable choice for this study.

The aims of the study were to (1) determine if and how the $\delta^{13}C$ of the *C. ibex* horns from the Augstmatthorn–Brienzer Rothorn population changed between 1938 and 2006; (2) assess how alpine meadows ^{13}C discrimination and intrinsic water-use efficiency in these mountains changed during this period; (3) discuss and compare these changes to other observed responses (in trees) and to three hypothetical plant physiological responses to increased atmospheric CO_2 concentrations (Marshall & Monserud, 1996; Saurer *et al.*, 2004); and finally (4) determine the effect of last century changes of atmospheric evaporative demand on the instantaneous water-use efficiency of alpine meadows.

Materials and methods

The Augstmatthorn–Brienzer Rothorn C. ibex population

A time-series of *C. ibex* horns from the Natural History Museum of Bern archives was sampled, covering the period from 1938 to 2006. The horns of 24 males from the population of Augstmatthorn–Brienzer Rothorn were selected from the Museum's collection as the most representative record with the longest period of one population.

This population is located on the Augstmatthorn (2137 m a.s.l.) and Brienzer Rothorn (2350 m a.s.l.) mountains of the Bernese Alps, Switzerland (Fig. 1). The grassland in this habitat is dominated by *Seslerion albicantis*, *Caricion ferrugineae* and *Eu-Nardion* communities (Prof. O. Hegg, Geobotanical Institute, Universität Bern, personal communication).

During the 16–18th centuries the overexploitation of the species severely decreased its numbers throughout the European Alps until just one population remained with <200 animals on the hunting grounds of the King Victor Emanuel II in the Valle d'Aosta, Italy (Stüwe & Nievergelt, 1991). In 1906 captive breeding programmes started in Switzerland, and in the early 1920s repopulation of the Augstmatthorn area started. This population successfully established on the south side of the mountain, achieving 60–69 animals in only 10 years. The Brienzer Rothorn is a neighbour mountain, where some animals from Augstmatthorn migrated and founded another population, but individuals still move between the two mountains and so Augstmatthorn–Brienzer Rothorn are considered to be just one population (Lüps & Zuber, 1986). The archived horns from Augstmatthorn–Brienzer Rothorn in the museum collection de-
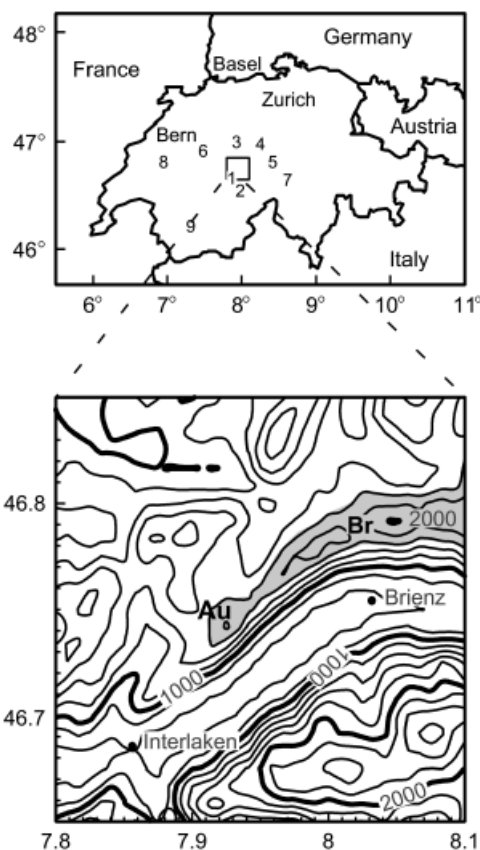

Fig. 1 Location of *Capra ibex* population at the Augstmatthorn (Au) and Brienzer Rothorn (Br) mountains of the Swiss Alps, Canton of Bern (upper panel); topography of the mountains and potential habitat of *C. ibex* shaded area (lower panel). Surrounding weather stations are marked: (1) Interlaken 579 m a.s.l., beginning of record 1982; (2) Jungfraujoch 3576 m a.s.l., beginning of record 1973; (3) Napf 1406 m a.s.l., beginning of record 1982; (4) Pilatus mountain 2110 m a.s.l., beginning of record 1989; (5) Engelberg 1035 m a.s.l., beginning of record 1990; (6) Bern-Belp 510 m a.s.l., beginning of record 1990; (7) Guetsch ob Andermatt 2284 m a.s.l., beginning of record 1955; (8) Payerne 491 m a.s.l., beginning of record 1964; (9) Sion 481 m a.s.l., beginning of record 1955.

rive from 3 to 17-year-old animals that were found dead or killed (for illness or bad condition) or game hunted.

Horn sampling

For each horn, one sample per year-ring was collected on the backside of the horn. The first year-ring was not sampled as it was formed when the animal was still feeding on the mothers' milk, complicating the interpretation of the isotope data due to the trophic level effect (Jenkins *et al.*, 2001).

Horn carbon isotope composition varies in a recurring pattern within the horn year-rings: the $\delta^{13}C$ is more

variable in the middle section of the year-ring than at the beginning and end, so that multi-annual trends are much more 'noisy' when constructed from middle section isotope data (Barbosa *et al.*, 2009). In order to minimize such intra-annual variations in the long-term trend of $\delta^{13}C$, we sampled the distal third of each year-ring (sections of 5×5 mm). This corresponded to the beginning of the horn growth period, the spring (Ratti & Habermehl, 1977; Giacometti *et al.*, 2002), when soil water availability is generally high in the alpine meadows (Körner, 2003) and, hence, drought effects on plant ^{13}C discrimination are uncommon. A total of 233 year-rings were sampled from the 24 horns. Thin layers of horn were removed with a wood carving knife. The surface layer (about 1 mm thick) was discarded to avoid contamination (Barbosa *et al.*, 2009), and the subsurface was collected. The samples were dried at 60 °C for 24 h.

Carbon isotope analysis

Dried samples (0.3–0.4 mg) were enclosed in tin cups and combusted in an elemental analyser (NA 1110; Carlo Erba, Milan, Italy) interfaced (modified ConFlo III; Finnigan MAT, Bremen, Germany) to an isotope ratio mass spectrometer (Delta Plus; Finnigan MAT). The isotope data are presented as $\delta^{13}C$ (‰) relative to PDB standard and were calculated as follows:

$$\delta^{13}C = \left[\frac{R_{sample}}{R_{standard}} - 1 \right] \times 10^3, \qquad (6)$$

where R represents the abundance ratio $^{13}C/^{12}C$ in the sample and in the standard. All samples were measured against a laboratory working standard CO_2 gas, which was previously calibrated against the secondary isotope standard IAEA-CH6 with an accuracy of ± 0.13‰. A solid internal laboratory standard (SILS) with similar C/N ratio as the horn samples, cattle horn powder (C/N 2.91), was also calibrated against this international standard and run regularly after every tenth sample as a control. The external precision during the measurements (SILS standard deviation) was 0.22‰ for $\delta^{13}C$.

Basic assumptions

Reconstructing alpine meadows $\delta^{13}C$ through the *C. ibex* horns was based on four main assumptions. (1) The carbon isotopic 'diet–horn shift' was constant – to our knowledge there is no evidence for climate effects on carbon isotope fractionation in metabolism of mammals. (2) There were no ontogenetic effects on horn $\delta^{13}C$ – yet, we have found a small ontogenetic effect (0.035‰ yr^{-1}) in this population (Barbosa *et al.*, 2009). This was not significant at the level of individuals. The age range of the animals was small (3–17 years) compared to the period of

this study, so that the age effect levelled out in the long-term data, which were composed of animals of different age. (3) Animals grazed in groups and on the same grounds every year providing a temporal record of vegetation $\delta^{13}C$ – indeed *C. ibex* graze in groups and annual migration patterns are quite conservative. Although some spatial segregation occurs between age classes of *C. ibex* males, no habitat segregation occurs between males older than 2 years (Bon *et al.*, 2001; Abderhalden, 2004). A negligible effect of such segregation was also supported by the small age effect on horn $\delta^{13}C$. Furthermore, wardens and experts of this population reported that the seasonal movements of the male groups during foraging are repeated every year. (4) *C. ibex* males did not change their selection or diet preferences – there is no supporting evidence of the contrary. On these grounds, the $\delta^{13}C$ of horn samples should reflect the community-integrated $\delta^{13}C$ of the same meadows visited by these *C. ibex* males during spring.

Estimating alpine grasslands ^{13}C discrimination and water-use efficiency

Vegetation $\delta^{13}C$ was derived from horn $\delta^{13}C$, assuming an isotopic enrichment (ε^*) between horn and vegetation of 3.1‰, as shown for the horns of different ungulate species by Cerling & Harris (1999), as follows:

$$\varepsilon^* = \frac{\delta^{13}C_{horn} - \delta^{13}C_{vegetation}}{1 + \frac{\delta^{13}C_{horn}}{1000}}. \qquad (7)$$

Rearranging, the $\delta^{13}C$ of the alpine grassland vegetation ($\delta^{13}C_{vegetation}$) can be estimated as

$$\delta^{13}C_{vegetation} = \frac{1000(\delta^{13}C_{horn} - 3.1)}{1003.1}. \qquad (8)$$

Vegetation carbon isotopic discrimination ($^{13}\Delta$), intrinsic water-use efficiency (W_i) and instantaneous water-use efficiency (W_t) were then estimated using Eqns (1)–(5).

Atmospheric CO_2 concentration and $\delta^{13}C$

The atmospheric CO_2 concentration and $\delta^{13}C$ are needed for the calculation of $^{13}\Delta$ in Eqns (1) and (2), and water-use efficiency in Eqns (3) and (5). The progressive increase of atmospheric CO_2 concentration and concomitant decrease of $\delta^{13}C$ was calculated using annual data from measurements of free air and air bubbles separated from ice cores. Data from Antarctica ice cores covered the periods 1938–1978 at Law Dome (Francey *et al.*, 1999) and 1943–1953 at Siple (Friedli *et al.*, 1986). Free air samples data were used from the

following stations: Mauna Loa, Hawaii; Hegyhatsal, Hungary and Black Sea, Romania; covering the periods: 1959–2007 for CO_2 concentration data (Conway et al., 2008; Keeling et al., 2008;) and the period 1981–2006 for $\delta^{13}C$ data (Keeling et al., 2005; White et al., 2009). The resulting CO_2 concentration model, c_a (μmol mol^{-1}), was a double two-order polynomial, for the periods 1938–1962 and 1963–2006:

$$c_a = m_2 t^2 + m_1 t + m_0, \qquad (9)$$

where t is time in years; m_2 was 0.0013, m_1 -4.841 and m_0 4642.3 ($R^2 = 0.99$) for the period 1938–1962 and 0.0195, -75.827 and 73938.0, respectively, for the period 1963–2006 ($R^2 = 0.99$). The root mean squared error of the double model for the studied period was 1.8 μmol mol^{-1}.

The atmospheric $\delta^{13}C_a$ (‰) model was a double three-order polynomial for the periods 1938–1962 and 1963–2006:

$$\delta^{13}C_a = m_3 t^3 - m_2 t^2 + m_1 t + m_0, \qquad (10)$$

where t is time in years; m_3 was $-5.5E{-}08$, m_2 $2.90E{-}4$; m_1 -0.517 and m_0 301.23 ($r^2 = 0.84$) for the period 1938–1958; and $-3.249E{-}06$, 0.019; -36.71 and 23763.0 ($R^2 = 0.92$), respectively, for the period 1959–2006. The root mean squared error of the double model for the studied period was 0.14‰.

Estimation of the VPD at Augstmatthorn–Brienzer Rothorn

To calculate instantaneous water-use efficiency, W_t, the water vapour concentration gradient between the leaf and the air, v, must be known (see Eqns (3) and (4)). Assuming e_i is close to saturation and leaf temperature is close to air temperature; leaf VPD can be estimated as the air VPD: e_i being the saturation vapour pressure of the air, and e_a the actual vapour pressure in the air; divided by the atmospheric pressure (see Eqn (4)). VPD was calculated for nine meteorological stations surrounding the Augstmatthorn–Brienzer Rothorn (Fig. 1) as described in Allen et al. (1998) from the daily maximum, minimum and dew-point temperatures when applying the Magnus Teten equation. Data were taken from the NOAA database (NOAA NCDC Climate Data Online database, 2008) from 1955 to 2006 (earlier data were not available). The monthly means of VPD during the growing period at the weather stations correlated closely with altitude ranging from 481 to 3576 m a.s.l. [$y = -0.0001x + 0.966$, where y is $\sqrt[4]{VPD}$ (kPa) and x is altitude (m a.s.l.); $R^2 = 0.70$; $P < 0.0001$; $n = 1325$] indicating a decrease in VPD with altitude. Similar regressions for every year (R^2 between 0.53 and 0.99; mean 0.76) were used to estimate the year-specific growing period VPD at the potential habitat of

the C. ibex population (Fig. 1), which was at a mean (area-weighted) altitude of 1660 m a.s.l.

Results

The horn time series ($n = 233$) represented by about 3.5 animals per year showed an average standard deviation of the $\delta^{13}C$ values per year of 0.47‰ ($\pm$ 0.26‰).

The horn $\delta^{13}C$ followed a similar pattern as the $\delta^{13}C$ of atmospheric CO_2, decreasing over time, particularly steeply from 1960 on (Fig. 2). Both, the mean $\delta^{13}C$ of the individual animals and the $\delta^{13}C$ of the individual year-rings showed a highly significant trend over time ($P < 0.001$) and the regression parameters of the two data sets were very similar (legend Fig. 2).

The $\delta^{13}C$ life record of individual animals also followed the long-term trend of the population and of the atmosphere (Table 1): the majority of individuals that lived from 1938–1960 did not experience consistent changes in horn $\delta^{13}C$. Conversely, most of the individuals that lived from 1960 to 2006 exhibited a significant decrease of horn $\delta^{13}C$ with time.

The horn-estimated alpine grassland ^{13}C discrimination ($^{13}\Delta$) increased slightly but significantly over time ($y = 0.006x + 6.96$; $P < 0.001$), about 0.4‰ over the 69-year period (Fig. 3a). This increase of $^{13}\Delta$ indicated a small increase of the ratio of CO_2 concentration in the leaf intercellular space relative to the atmosphere (c_i/c_a) of about 0.02 (Fig. 3a). According to the $^{13}\Delta$ and c_i/c_a
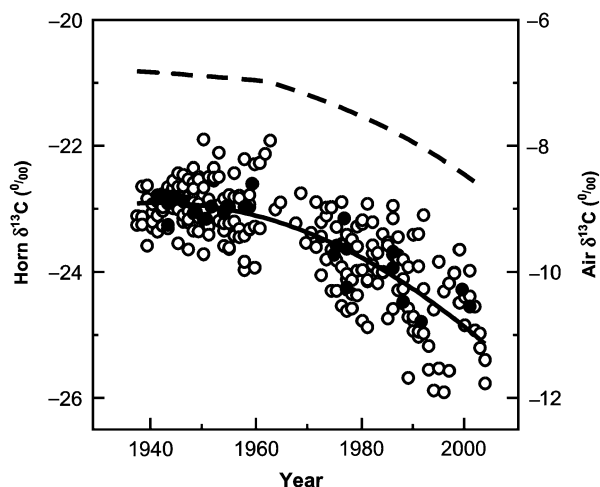
Fig. 2 Carbon isotope composition ($\delta^{13}C$) of the atmospheric CO_2 (dashed line) and of the Capra ibex horns ($\circ$, $\bullet$) from the population Augstmatthorn – Brienzer Rothorn from 1938 to 2006. A two-order polynomial model ($-$) was fitted to the $\delta^{13}C$ values of individual year-rings of the animals' horns ($\circ$), $y = -5.074e^{-4}x^2 + 1.968x - 1933.0$. Mean $\delta^{13}C$ values of individual animals ($\bullet$), the model fitting this data set was $y = -4.334e^{-4}x^2 + 1.680x - 1648.1$.

trends, the alpine grassland c_i increased by about 56 $\mu mol\,mol^{-1}$ ($y = 0.0142x^2 - 54.96x + 53541$; $R^2 = 0.82$; $n = 233$), but not sufficiently to match the 81 $\mu mol\,mol^{-1}$ increase of atmospheric CO_2 concentration (Fig. 3b). In consequence, the intrinsic water-use efficiency rose

by 11.4 $\mu mol\,mol^{-1}$ ($y = 0.003x^2 - 11.5388x + 11278$; $R^2 = 0.30$) during the 69-year period of the study (Fig. 3c).

The estimated vapour pressure deficit at Augstmatthorn–Brienzer Rothorn increased by about 0.1 kPa from 1955 to 2006 ($y = 0.0014x - 2.505$; $R^2 = 0.20$; $P < 0.001$) (Fig. 3d). This increase was strong enough to offset the potential water-use efficiency response (W_i), so that the estimated instantaneous water-use efficiency, W_t, did not change significantly over time (Fig. 3d).

Table 1 List of studied *Capra ibex* horns from the Bern Natural History Museum archives and their carbon isotopic signature changes with time.

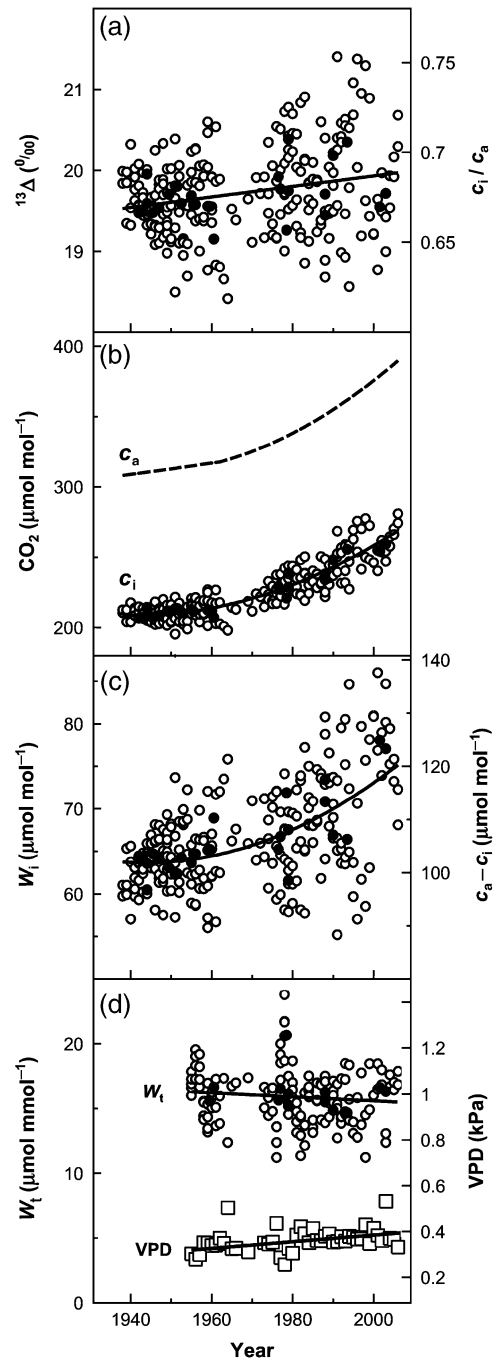| Animals					$\delta^{13}C$ variation over time				
Code	Origin	From	To	Age	Slope	Intercept	R^2	P-value	n
1031659	Au	1938	1948	12	**0.055**	−130.2	0.54	**0.010**	11
1031614	Au	1938	1950	14	−0.004	−14.8	0.00	0.828	13
1031447	Au	1939	1945	8	0.021	−63.6	0.09	0.515	7
1031452	Au	1941	1947	8	**0.102**	−221.3	0.67	**0.025**	7
1031467	Au	1942	1949	9	0.040	−99.8	0.26	0.192	8
1031470	Au	1945	1949	6	0.035	−91.8	0.05	0.715	5
1031633	Au	1947	1956	11	−0.014	4.0	0.01	0.803	10
1031618	Au	1948	1951	5	0.031	−82.9	0.06	0.762	4
1031645	Au	1945	1960	17	−0.053	79.8	0.47	**0.003**	16
1031653	Au	1949	1961	14	−0.069	112.7	0.28	0.065	13
1031646	Au	1949	1962	15	−0.076	125.9	0.33	**0.033**	14
1031667	Au	1955	1966	13	0.040	−101.4	0.13	0.251	12
1038507	Au	1969	1984	17	−0.092	157.6	0.45	**0.004**	16
1038483	Br	1973	1981	10	**−0.126**	226.3	0.53	**0.026**	9
1038501	Au	1974	1984	12	−0.019	14.4	0.02	0.672	11
1038494	Br	1976	1982	8	0.007	−37.2	0.00	0.896	7
1022069	Br	1982	1994	14	−0.069	114.2	0.11	0.268	13
1015429	Br	1983	1993	12	−0.036	48.0	0.08	0.401	11
1047665	Au	1984	1996	14	**−0.097**	169.4	0.57	**0.003**	13
1048975	Au	1988	1999	13	**−0.215**	404.7	0.74	**0.000**	12
1946	Au	1997	2006	11	**−0.170**	316.0	0.59	**0.010**	10
1991	Au	2000	2006	8	**−0.172**	319.5	0.69	**0.021**	7

Two individuals of the dataset were not included as the table just contains individuals older than 3 years.
Code museum's identification code, *origin* place where animals were shot or found dead: Au, Augstmatthorn; Br, Brienzer Rothorn; *from* and *to* living period under study (from the second year to the last), *age* of the animal, *slope*, *intercept*, R^2, *P-value* and *n* of the linear relation of horn $\delta^{13}C$ with time for each individual.

Fig. 3 Alpine meadows ecophysiological parameters from 1938 to 2006, reconstructed by the $\delta^{13}C$ time-series of the *Capra ibex* horns from Augstmatthorn–Brienzer Rothorn population: (a) ^{13}C discrimination ($^{13}\Delta$) and CO_2 concentration in the leaf intercellular spaces relative to that in the atmosphere (c_i/c_a; $\circ$, $\bullet$); (b) CO_2 concentration in the atmosphere (c_a), and in the intercellular space (c_i); (c) intrinsic water-use efficiency (W_i) and CO_2 concentration difference between external CO_2 and that in the intercellular space (c_a–c_i); and (d) instantaneous water-use efficiency (W_t; $\circ$, $\bullet$) along with atmospheric vapour pressure deficit (VPD; $\square$). The parameter trend lines were calculated using the values derived from $\delta^{13}C$ of all individual year-rings ($\circ$), $n = 233$. Parameters calculated from the mean $\delta^{13}C$ values per animal, $n = 24$ ($\bullet$).

All the horn-derived vegetation parameters were calculated using both the mean $\delta^{13}C$ of the individual animals (Fig. 3, closed symbols) and individual year-ring $\delta^{13}C$ values (Fig. 3, open symbols). As for the horn $\delta^{13}C$, animal means and year-ring values gave very similar trends for the vegetation parameters, showing that, although derived form the same animal, all year-ring samples along a horn could be treated as independent, and differences between individual animals ignored.

Discussion

Horn ^{13}C content of different individuals and generations of C. ibex followed the changes of atmospheric $\delta^{13}C$ from 1938 to 2006

The carbon isotope composition of the time-series of horns from the Augstmatthorn–Brienzer Rothorn population paralleled the changes of the atmospheric CO_2 of the last 69 years: horn $\delta^{13}C$ decreased slowly between 1938 and 1960 and rapidly from 1960 on. This was evident at the level of individual animals and the whole dataset. This finding corroborates the conservative behaviour of the C. ibex males of the Augstmatthorn–Brienzer Rothorn population (see Basic Assumptions in Materials and Methods): little spatial or individual effects on horn $\delta^{13}C$ must have occurred between different animals spanning different generations, so that the long-term trend of atmospheric CO_2 $\delta^{13}C$ was the most evident effect on population horn $\delta^{13}C$ (the correlation between the $\delta^{13}C$ of individual horn segments analysed and atmospheric $\delta^{13}C$ was highly significant: $y = 1.22x - 14.54$, $R^2 = 0.61$, $n = 233$, $P < 0.001$). The particularities of C. ibex behaviour, group grazing (Bon et al., 2001) and revisiting the same grounds every year, together with the strong correlation between the $\delta^{13}C$ of the population horns and the atmosphere, support the view that herd grazers provide an nearly unbiased record of upstream carbon isotopic processes. Hence, grazers' tissues are potential records of habitat and environment related isotopic processes, such as the increasing CO_2 in the atmosphere or ecophysiological trends of the plant community. Such grazing behaviour helps to overcome constraints of animal longevity or available plant material and to extend the periods of ecosystem reconstruction.

Alpine meadows $^{13}\Delta$ increased slightly in the last 69 years

The estimated alpine grasslands' ^{13}C discrimination and hence the estimated c_i/c_a ratio increased slightly between 1938 and 2006, meaning that there was an adjustment of intercellular leaf CO_2 (c_i) to the increase
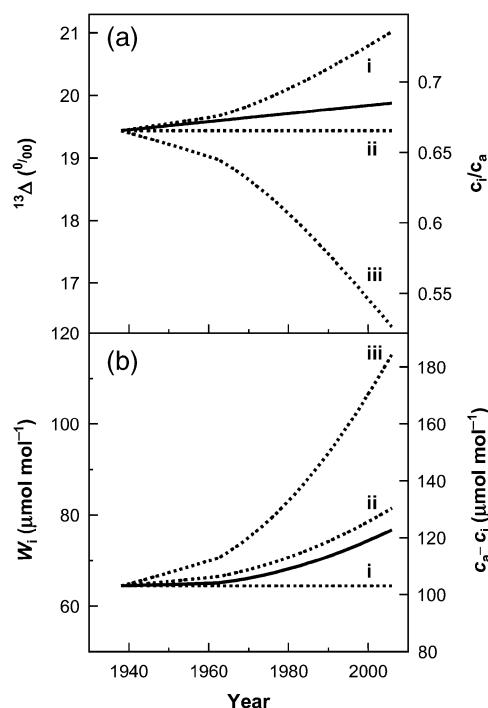

Fig. 4 Responses of carbon isotope discrimination ($^{13}\Delta$) and c_i/c_a (a); and intrinsic water-use efficiency (W_i) and c_a–c_i (b) of alpine meadows at Augstmatthorn–Brienzer Rothorn in the last 69 years (solid lines). These responses are compared with three theoretical scenarios [dashed lines in (a) and (b)]: c_i increases as much as c_a, i.e. c_a–c_i stays constant (i); c_i increases proportionally to c_a, i.e. c_i/c_a stays constant (ii); or c_i stays constant (iii).

of atmospheric CO_2 (c_a). However, c_i did not augment as much as c_a, as the c_a–c_i gradient, and hence, the intrinsic water-use efficiency actually increased.

To illustrate the way by which the alpine grasslands' c_i changed relative to c_a and its consequences on $^{13}\Delta$ and W_i, the alpine grasslands' trends of $^{13}\Delta$ can be compared with three theoretical responses to increasing c_a as shown in Fig. 4: (i) c_i increases as much as c_a, keeping c_a–c_i constant; (ii) c_i increases proportionally to c_a, keeping c_i/c_a constant; or (iii) c_i stays constant. The response-types ii and iii imply that intrinsic water-use efficiency increases with c_a whereas in i it remains constant. Although estimated values of alpine meadows $^{13}\Delta$ vary between the response-types i and ii (Fig. 3a); the general trend corresponds more closely to the response ii than to response i, which would have required an increase of $^{13}\Delta$ of $\sim 1.5‰$ (Fig. 4). Hence, the results indicate that alpine meadows have responded to the recent atmospheric CO_2 and climatic changes, by increasing the CO_2 concentration inside the leaf in such a way that the ratio c_i/c_a remained nearly constant, consequently increasing W_i. This implies that on the gas-exchange level the CO_2 uptake, A, must have

Table 2 Last century changes in intrinsic water-use efficiency for trees, calculated from tree-ring carbon isotopes

Species or systems	Local	Period	c_a increase (μmol mol^{-1})	Increase of W_i (%)		Reference
				Total	Per 10 μmol mol^{-1} of c_a increase	
Pseudotsuga menziesii, *Pinus ponderosa* and *Pinus monticola*	Rocky Moutains, United States	1910–1991	58	0	0	Marshall & Monserud (1996)
Pinus sp., *Picea sitchensis,* *Quercus lobata,* *Fitzroya cupressoides,* *Juniperus phoenicea*	Western North America and Chile (Fitzroya)	1800–1990	72	5–45	0.70–6.25	Feng (1999)
Abies alba	Jura Mountains, eastern France	1930–1980	33	30	9.10	Bert *et al.* (1997)
Larix sp., *Pinus* sp. *Picea* sp.	Northern Eurasia	1861–1990	67	19.2 ± 0.9	2.87 ± 0.13	Saurer *et al.* (2004)
Fagus sylvatica Coppice-with-standards High forest	North-eastern France	1850–1990	69 69	23 44	3.33 6.38	Duquesnay *et al.* (1998)
Fagus sylvatica 1610–1665 m a.s.l. 1105–1170 m a.s.l. 940–1040 m a.s.l.	Montseny Mountains, Spain	1978–2003	50 50 50	0 6 10	0.00 1.20 2.00	Peñuelas *et al.* (2008)

Local of the sampled trees; *Period* of the study; c_a *increase* during the respective period (μmol mol^{-1}); *increase of W_i* expressed as total percentage of the W_i in the beginning of the studied period (Total) and as the percentage change per increase of 10 μmol mol^{-1} CO_2 in the atmosphere (*per 10 μmol mol^{-1} of c_a increase*). The increase of W_i values are mean or range (Feng, 1999) or mean and standard error (Saurer *et al.*, 2004).

increased and/or the stomatal conductance, g_s, must have decreased in the last 69 years.

These findings agree with the results of FACE experiments reviewed by Long *et al.* (2004), which demonstrated increases in photosynthesis and decreases in stomatal conductance, keeping c_i/c_a approximately constant when plants were exposed to elevated CO_2. Our results cannot separate whether the increase in W_i resulted from higher assimilation rates or reduced stomatal conductance. However, short-term studies with open-top chamber-based CO_2 enrichment of an alpine community of Körner *et al.* (1997) and Diemer (1994) indicated that both occurred: under elevated CO_2 there was ecosystem-level stimulation of net CO_2 uptake and reduction of evapotranspiration.

In comparison with other long-term $^{13}\Delta$ reconstructions, made with tree-rings, our findings do not differ considerably from observed trends (Table 2). Estimated alpine meadows W_i increased about 17.8%, from 63.78 μmol mol^{-1} in 1938 to 75.15 μmol mol^{-1} in 2006, when c_a increased 81 μmol mol^{-1}. So the increase of W_i relative to c_a was 2.2% per 10 μmol mol^{-1} c_a increase. This value is in the range of the observed trends, but it is in general inferior to the mean values of trees (Table 2). Some differential stimulation on carbon up-

take between herbaceous and woody plants have been observed in FACE experiments (Nowak *et al.*, 2004). However, our results of the first natural grassland response to last-century CO_2 increase do not support the view that the long-term W_i responses of forests and grasslands differ fundamentally. However, further research of different life forms (especially herbaceous vegetation) responses to past climate and atmospheric changes are needed before more general conclusions can be drawn about effects of life forms on W_i and its implications for ecosystem composition and functioning.

There is still some uncertainty in the use of ^{13}C discrimination as a quantitative measure of integrated plant gas-exchange performance, which arises from the simplifying assumption in Eqn (2) that mesophyll conductance (g_m) is infinite (for a discussion see Warren & Adams, 2006; Seibt *et al.*, 2008). Measurements in several species, have shown that CO_2 concentration at the site of carboxylation (c_c) is less than in the intercellular spaces (c_i) and that this difference may vary in response to environmental factors, thus showing that g_m, is finite and may be variable (Flexas *et al.*, 2008). However, there is evidence that g_s and g_m are correlated, often increasing and decreasing in parallel across

different plant species (Loreto *et al.*, 1992), genotypes (Lauteri *et al.*, 1997), and salt and drought stress (Centritto *et al.*, 2003; Flexas *et al.*, 2004). This coordination of g_s and g_m would mean that the conclusions based on Eqn (2) (concerning intrinsic water-use efficiency) were at least qualitatively supported, particularly if the animals did not change diet, as appeared to be the case.

Contrasting trends of intrinsic and instantaneous water-use efficiency

Intrinsic water-use efficiency provides a measure of the physiological control on the water-use efficiency but does not account for the role played by the environment on transpiration (Ehleringer *et al.*, 1993); therefore an increase of W_i may not directly reflect an improvement of the plant carbon–water balance. Indeed, the estimated vapour pressure deficit at Augstmatthorn for the growing period months (April–August) increased by about 0.1 kPa from 1955 to 2006. This effect was large enough to completely compensate the improvement of W_i: W_i and vapour pressure deficit increased in such a way that instantaneous water-use efficiency (W_t) remained unchanged. These results call for a careful interpretation of past W_i reconstructions, as concurrent changes in vapour pressure deficit could greatly dampen (or enhance) the actual leaf-level water-use efficiency responses to past CO_2 and climatic changes.

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