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## Growth and reproduction of the alpine grasshopper *Miramella alpina* feeding on CO<sub>2</sub>-enriched dwarf shrubs at treeline

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**Abstract** The consequences for plant-insect interactions of atmospheric changes in alpine ecosystems are not well understood. Here, we tested the effects of elevated CO<sub>2</sub> on leaf quality in two dwarf shrub species (*Vaccinium myrtillus* and *V. uliginosum*) and the response of the alpine grasshopper (*Miramella alpina*) feeding on these plants in a field experiment at the alpine treeline (2,180 m a.s.l.) in Davos, Switzerland. Relative growth rates (RGR) of *M. alpina* nymphs were lower when they were feeding on *V. myrtillus* compared to *V. uliginosum*, and were affected by elevated CO<sub>2</sub> depending on plant species and nymph developmental stage. Changes in RGR correlated with CO<sub>2</sub>-induced changes in leaf water, nitrogen, and starch concentrations. Elevated CO<sub>2</sub> resulted in reduced female adult weight irrespective of plant species, and prolonged development time on *V. uliginosum* only, but there were no significant differences in nymphal mortality. Newly molted adults of *M. alpina* produced lighter eggs and less secretion (serving as egg protection) under elevated CO<sub>2</sub>. When grasshoppers had a choice among four different plant species grown either under ambient or elevated CO<sub>2</sub>, *V. myrtillus* and *V. uliginosum* consumption increased under elevated CO<sub>2</sub> in females while it decreased in males compared to ambient CO<sub>2</sub>-grown leaves. Our findings suggest that rising atmospheric CO<sub>2</sub> distinctly affects leaf chemistry in two important dwarf shrub species at the alpine treeline, leading to changes in feeding behavior, growth, and reproduction of the most important insect herbivore in this system. Changes in plant-grasshopper interactions might have significant long-term impacts on herbivore pressure, community dynamics and ecosystem stability in the alpine treeline ecotone.

**Keywords** Elevated CO<sub>2</sub> · Global change · Herbivory · Leaf chemistry · *Vaccinium* sp.

### Introduction

Rising atmospheric CO<sub>2</sub> has been shown to influence insect herbivore performance (Watt et al. 1995; Lindroth 1996; Bezemer and Jones 1998; Whittaker 1999). The CO<sub>2</sub> effects typically result from changes in food quality, such as decreased nitrogen concentration or increased concentrations of carbon-based defense compounds. Insect responses to these changes include compensatory feeding (Fajer 1989; Docherty et al. 1996), slower growth rates (Fajer 1989; Traw et al. 1996; Hättenschwiler and Schafellner 1999), reduced final weight, prolonged development time (Goverde and Erhardt 2003), increased mortality (Fajer 1989), and reduced reproduction (Buse et al. 1998; Brooks and Whittaker 1998). At the level of the individual, these responses may negatively influence fecundity and population dynamics of insect herbivores. Alternatively, compensatory feeding in a CO<sub>2</sub>-enriched atmosphere could lead to greater damage of food plants. Both these responses may alter plant-herbivore interactions, and possibly community dynamics and ecosystem functioning.

However, extrapolations from experiments commonly restricted to particular larval stages under predominantly greenhouse conditions to natural ecosystems are difficult (Goverde et al. 2002). Moreover, different plant species respond distinctly to atmospheric CO<sub>2</sub> enrichment (Cotrufo et al. 1998; Körner 2000), as do herbivores feeding on them (Traw et al. 1996; Kinney et al. 1997; Veteli et al. 2002). A possible consequence of species-specific responses to elevated CO<sub>2</sub> could be a switch to other, more suitable food plants by generalist herbivores, which might induce changes in plant species composition (Hättenschwiler and Schafellner 2004).

Climatic conditions are particularly severe and variable at the alpine treeline, and are hard to simulate in the

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greenhouse. Grasshoppers are the dominant insect herbivores in alpine ecosystems (Illich and Winding 1999; Blumer and Diemer 1996). The alpine grasshopper (*Miramella alpina* Fruhstorfer) is a wide-spread generalist herbivore in the Alps at altitudes between 1,000 and 2,500 m a.s.l., and preferentially feeds on different species of the genus *Vaccinium* (Harz 1957; Illich and Winding 1989). Compared to other Mid-European Acrididae, the genus *Miramella* is known for its particularly long adult phase combined with a high reproductive potential (Asshoff and Köhler 2003; Köhler et al. 1999). However, at high elevations grasshoppers generally experience a shorter growing season and cooler temperatures, meaning that they have to develop, mature, and reproduce in a shorter period of time and under less favorable conditions than grasshoppers at low elevations. An optimal resource availability, and hence high food quality, may thus be of particularly high importance for successful grasshopper development and reproduction in high altitude environments. Changes in plant tissue quality in response to elevated atmospheric CO<sub>2</sub> may therefore have serious consequences for high altitude populations of insect herbivores. To our knowledge the impact of elevated CO<sub>2</sub> on plant-herbivore interactions, however, has never been tested in alpine ecosystems.

The objective of this study was to assess how *M. alpina* might be affected by the projected higher CO<sub>2</sub> concentration in the atmosphere. Specifically, we quantified the direct effects of elevated CO<sub>2</sub> on leaf quality of major host plants of *M. alpina* and determined the impact of leaf quality changes on nymph growth, final adult biomass and reproduction of *M. alpina*. We hypothesized that elevated CO<sub>2</sub> reduces leaf quality and that these changes will negatively affect grasshopper performance leading to a reduced reproductive output under the harsh conditions at the alpine treeline. A second hypothesis was that grasshopper responses to elevated CO<sub>2</sub> depend on plant species-specific changes in leaf chemical composition, which might indicate the potential for compensatory responses of grasshoppers by switching host plant species.

## Materials and methods

### Study site and experimental setup

The experimental area is located at Stillberg, Davos, in the Swiss Central Alps at an elevation of 2,180 m a.s.l. The long-term annual precipitation of the NE

exposed study site is 1,050 mm; the average temperature is  $-5.8^{\circ}\text{C}$  in January and  $9.4^{\circ}\text{C}$  in July (Schönenberger and Frey 1988). Compared to the long-term average, precipitation was somewhat higher during 2002 with a particularly wet August (Table 1). Temperatures were similar to the long-term average during the first part of the vegetation period with a warm June, but tended to be somewhat lower during late summer and early fall. The soil is classified as a Ranker (US system: Lithic Haplumbrept) with 10 cm deep organic topsoil underlain by siliceous bedrock (Paragneis, Schönenberger and Frey 1988). In a large afforestation experiment the tree species *Larix decidua* L., *Pinus cembra* L. and *P. uncinata* Ramond were planted across an area of 5 ha in 1975. At the upper limit of this large-scale plantation (2,180 m a.s.l.), we have chosen a total of 40 individual trees (on average 1.5 m tall) from the two species *L. decidua* and *P. uncinata* together with the accompanying vegetation to establish our experimental plots. Due to their small size and scattered distribution, the trees form an open canopy and the site is actually dominated by the dwarf shrubs *Vaccinium myrtillus*, *V. uliginosum*, and *Empetrum hermaphroditum*. *Gentiana punctata*, *Homogyne alpina*, and *Melampyrum pratense* are the most common herbaceous species. Half of all plots, each of an area of 1.1 m<sup>2</sup>, have been randomly assigned to an elevated CO<sub>2</sub> concentration (550  $\mu\text{mol mol}^{-1}$ ), and the remaining 20 plots served as controls at ambient CO<sub>2</sub>. Elevated CO<sub>2</sub> was applied using free air CO<sub>2</sub> enrichment (FACE) with pure CO<sub>2</sub> release technology from the beginning of the vegetation period in 2001 (Hättenschwiler et al. 2002).

A total of 20 plots showing a good dwarf shrub coverage ( $n=10$  per CO<sub>2</sub> treatment) were used to establish the grasshopper experiment. Mesh bags (volume 520 cm<sup>3</sup>, mesh size: 280  $\mu\text{m}$ , Sefar Propyltex 05 280/41, Sefar Holding, Rüslikon, Switzerland) were constructed to place grasshoppers onto their respective food plants. The relatively small mesh size was chosen because initial tests for light transmission showed the best performance compared to a number of other mesh sizes (85–100% of transmitted light at 280  $\mu\text{m}$  mesh width, depending on the angle of light incidence). Third instar nymphs of *M. alpina* were collected close to the study site on 20 June 2002. Before distributing a total of 160 individuals (average fresh weight of 43 mg per individual) to the mesh bags on 22 June 2002, the initial biomass of each individual was determined. To avoid crowding effects, only two individual nymphs were placed in each of four mesh bags per plot. The mesh

**Table 1** Mean temperature ( $T$ ; 50 cm above ground) and precipitation during the *Miramella alpina* experiment (2002) and the long term average (LTA) at the site (1975–1984; Schönenberger and Frey 1988)

	June		July		August		September		October	
	LTA	2002	LTA	2002	LTA	2002	LTA	2002	LTA	2002
$T$ (air mean; $^{\circ}\text{C}$ )	6.9	10.5	9.4	9.4	8.7	8.5	6.8	4.4	3.5	2.4
Precipitation (mm)	110	105	131	138	125	208	127	92	82	113

bags contained shoots of either *V. myrtillus* or *V. uliginosum* plants (two mesh bags of each plant species per plot). Because mesh bags may alter microclimatic conditions we measured air temperature and air humidity within and outside the bags, but found no overall significant differences over the entire vegetation period. The largest differences were observed on clear days. For example, on a clear day in June we measured temperatures and humidities of 24.8°C and 33.2% within bags compared to 23.7°C and 35% outside bags.

#### Leaf nutritional analysis

From each of the 20 plots (10 elevated CO<sub>2</sub> and 10 ambient CO<sub>2</sub>) leaves of both *Vaccinium* species were collected from different shoots at the beginning (27 June 2002) and at the end (26 August 2002) of the experiment. An intermediate sample was taken on 28 July ( $n = 5$ ). The bulk of this leaf material was dried immediately after sampling, ground and analyzed for starch, sugar (sucrose, glucose and fructose), nitrogen, carbon and lignin. From a subsample of six leaves per *Vaccinium* species, projected area and water content (difference between fresh and dry mass) were determined. Starch and sugar were analyzed with an enzymatic starch digestion and a spectrophotometric glucose test after invertase and isomerase addition (Hoch et al. 2002). Total N and carbon were measured using a CHN analyzer (Model 900, LECO Instruments, St. Joseph, Mich.). Lignin concentration was determined on 50–100 mg samples using the thioglycolic acid method (Bruce and West 1989; Hirschel et al. 1997).

#### Performance of grasshopper nymphs

Grasshopper biomass was measured three times during the experiment, 32 and 52 days after the experiment started, and directly after individuals eclosed to the adult stage. After 32 days most of the grasshoppers were in the fourth instar, and after 52 days most of them had reached the fifth instar. Relative growth rate (RGR) of nymphs was calculated as the biomass gained per gram initial nymphal biomass per day ( $\text{mg g}^{-1} \text{day}^{-1}$ ). Mesh bags were checked every fourth day for dead individuals and to assess food supply. Food shortage was not frequently observed and over the entire duration of the experiment a total of 29 mesh bags had to be switched to a new neighboring shoot to assure adequate food supply. The experiment was terminated as soon as the last individual molted into the adult stage. Biomass of freshly molted adults and development time (from instar 3 to the adult stage) was determined. Relative consumption rate (RCR), defined as milligram leaf biomass ingested per milligram mean nymphal biomass and per day ( $\text{mg mg}^{-1} \text{day}^{-1}$ ), was estimated using specific leaf area (SLA) and the difference of the total leaf area within each mesh bag measured at the start (total

number of leaves per *Vaccinium* shoot multiplied by the average area per leaf) and at the end of the experiment. This relatively crude estimate of consumed leaf biomass was the only possible method in this field experiment. However, the results obtained are quite robust because (1) *Vaccinium* leaves vary little in size within shoots, (2) there is a large number of leaves within a single bag (about 170 leaves) averaging out size differences, (3) there is no current-year regrowth in *Vaccinium*, and (4) *M. alpina* produces very little frass.

#### Reproduction

As soon as individuals from the nymphal trial reached the adult stage, pairs of them were placed into newly installed mesh bags according to their previous CO<sub>2</sub> treatment and plant species for assessment of reproduction. Because the final molt did not occur synchronously among all individuals and treatments, the setup was time-lagged. The reproductive output in female grasshoppers is determined by environmental conditions, feeding activity, and nutritional quality during the adult phase, but may also be influenced by nymphal performance (Ingrisch and Köhler 1999). If, for example, grasshoppers are switched from high to low protein nutrition, oocytes may be resorbed. The number of ovarioles in the ovarian determines the egg number in one egg pod. Each ovariole contributes one egg to the clutch. After the final molt, the ovaries of grasshoppers are still relatively small, and egg maturation starts normally after a further week (Uvarov 1966; Joern and Gaines 1990; Ingrisch and Köhler 1999).

To distinguish the impact of feeding on high-CO<sub>2</sub>-exposed plant material during the adult phase only from that during the entire development, we set up an additional experiment with newly molted adults: 28 female adults were collected in the field and were then placed individually in a total of seven newly installed mesh bags per *Vaccinium* species and CO<sub>2</sub> treatment (a total of 28 mesh bags) in 14 experimental plots (similar to the nymphal trial setup). An individual field-collected male and a vial filled with soil as substrate for egg deposition were added. An additional control with a more diverse diet (*V. myrtillus*, *V. uliginosum*, *V. vitis idaea*, *Adenostyles alliariae*, *Gentiana punctata*) was established in cages adjacent to the study site (five replicates), with individuals collected and treated in the same way as described above. This reproduction trial started on 3 August 2002 and grasshoppers were kept in the mesh bags until they died (end of September). Since this experiment was aimed at understanding how elevated CO<sub>2</sub> and host plant species will affect the quality of egg pods rather than total egg pod production, data evaluation focused on the first egg pod deposited. Most of our grasshoppers died after depositing the first egg pod. Egg pod quality commonly declines with repeated depositions (shown for *Oedipoda germanica* by Wagner 2000; G. Köhler, personal communication), as was observed

in our study, with substantially smaller second egg pods. Egg pods consist of a certain amount of eggs and a glue-like secretion serving as protection from desiccation and mechanical damage. Consequently, we measured total pod mass, egg mass, secretion mass, and number of eggs of the first pod.

### Food preference

An additional cafeteria experiment was designed to test whether adult males and females of *M. alpina* change their food preference in response to plant growth under elevated CO<sub>2</sub>. Adult individuals collected in the field were transferred to cages and were offered four different plant species (*V. myrtillus*, *V. uliginosum*, *V. vitis idaea*, *G. punctata*) to choose from. Similar amounts of leaf material (an area of approximately 10 cm<sup>2</sup>) from each plant species taken from either control plots or CO<sub>2</sub>-enriched plots, were added to Petri dishes and kept moist. Each individual Petri dish contained plant material from one individual plot, and grasshoppers fed either on plant material from CO<sub>2</sub>-enriched or control plots. Leaf consumption was calculated from the difference in leaf area before and after grasshopper feeding and was converted to leaf mass, using the SLA. Individuals were first kept without any food for 24 h until their guts were empty and were then allowed to feed for 24 h. This experiment was repeated three times, twice with females (one individual per Petri dish,  $n = 5$ ) and once with males (two individuals per Petri dish,  $n = 5$ ).

### Statistical analysis

Analyses of variance were applied to test for differences in leaf quality traits, nymphal RGR and RCR using a split-plot design with the factors “CO<sub>2</sub>” varied among plots and “plant species” nested within individual plots. Accordingly, the effect of CO<sub>2</sub> was tested against the plot error term, and the effect of host plant species and its interactions with CO<sub>2</sub> against the residual. Due to grasshopper mortality during nymphal development we lost about 45% of the original ten replicates before the end of the experiment. However, remaining replicates were distributed equally among plant species and CO<sub>2</sub> treatments. Analyses of RGR and RCR were based on plot means. Possible differences in nymphal mortality rates were tested using the likelihood ratio test statistic ( $-2 \log$  likelihood). Simple linear regression was used to test for possible correlations between RGR and leaf quality parameters.

Statistical tests for differences in adult body mass (final weight) and development time included initial biomass as a covariable. According to the split-plot design the covariable and the factor CO<sub>2</sub> were tested against the plot error term, and host plant, sex and interactions were tested against the residual. Because of the reduced replicates for adult grasshoppers, it was not

always possible to test for all possible interactions. Reproduction parameters were also tested using a split-plot design as described above. For the food preference test we used a three-way ANOVA with CO<sub>2</sub>, host plant and sex as factors. All data were tested for normality and when appropriate were log transformed or arcsine-square root (RGR) transformed. All ANOVA were conducted with type I SS (Sequential) using JUMP 3.2.2., SAS Institute.

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## Results

### Leaf chemistry

Leaf water content was significantly lower in *V. myrtillus* compared to *V. uliginosum* at the beginning of the experiment, not different in July, and significantly higher in *V. myrtillus* than in *V. uliginosum* in August. There was no difference between CO<sub>2</sub> treatments, except in the final measurement in August when leaves in high-CO<sub>2</sub> plots had a higher water content compared to those at ambient CO<sub>2</sub> in both species (Table 2). The SLA was significantly lower in *V. uliginosum* than in *V. myrtillus* throughout the entire vegetation period and tended to be lower at elevated CO<sub>2</sub> in both species and at all dates (Table 2).

Nitrogen concentration remained significantly higher in *V. uliginosum* than in *V. myrtillus* during the whole summer. Elevated CO<sub>2</sub> resulted in a significantly lower N concentration in both species, but this effect disappeared in August (Table 2). Likewise, the C/N ratio was significantly higher in *V. myrtillus* than in *V. uliginosum*, and elevated CO<sub>2</sub> increased the C/N ratio in both species (Table 2).

Starch and sugar concentration was higher in *V. uliginosum* than in *V. myrtillus*, except for starch in June and sugar in August. Starch concentration was significantly higher at elevated CO<sub>2</sub> in both species in July and August. There was no CO<sub>2</sub> response in sugar concentration in either species for the June and July sampling, but a significantly increased sugar concentration at elevated CO<sub>2</sub> in August. At start of the vegetation period, lignin concentration tended to be higher in *V. myrtillus* plants compared to *V. uliginosum*. In August, this difference between plant species disappeared. Both species, however, showed a marginally significant reduction in lignin concentration at elevated compared to ambient CO<sub>2</sub> in August (Table 2).

### Grasshopper performance

Grasshopper mortality did not differ significantly among treatments, but tended to be highest on *V. myrtillus* plants growing under elevated CO<sub>2</sub>. Total grasshopper mortality was unexpectedly high, given that we started with instar 3 nymphs. After 25 days we lost half of all animals (Fig. 1).

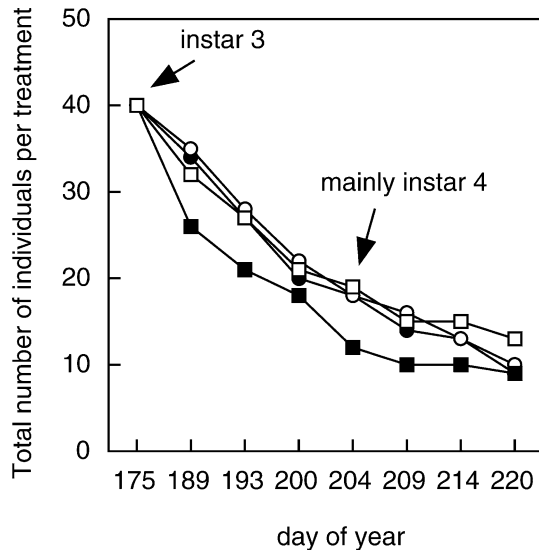
**Table 2** Quality of *Vaccinium* leaves fed on by *M. alpina* nymphs in ambient and elevated CO<sub>2</sub> (mean ± SE, n = 5–10). Sampling dates: 27 June, 28 July, 24 August. SLA Specific leaf area

Host plant	CO <sub>2</sub>	June	July	August
Water (% of fresh mass)				
<i>Vaccinium uliginosum</i>	Ambient	70.2 ± 0.7	44.9 ± 1.4	51.0 ± 1.3
	Elevated	69.0 ± 1.6	45.0 ± 2.3	53.1 ± 0.8
<i>Vaccinium myrtillus</i>	Ambient	67.1 ± 0.8	41.9 ± 3.3	52.4 ± 0.9
	Elevated	64.9 ± 1.8	43.3 ± 1.9	54.9 ± 1.0
ANOVA	Host plant	$F_{1,18} = 6.76^*$	$F_{1,7} = 0.46$	$F_{1,17} = 4.31^*$
	CO <sub>2</sub>	$F_{1,18} = 1.84$	$F_{1,9} = 0.06$	$F_{1,19} = 4.43^*$
	CO <sub>2</sub> × host plant	$F_{1,18} = 0.15$	$F_{1,7} = 0.53$	$F_{1,17} = 0.17$
SLA (cm <sup>2</sup> g <sup>-1</sup> )				
<i>V. uliginosum</i>	Ambient	206 ± 5	128 ± 5	141 ± 4
	Elevated	199 ± 7	126 ± 3	147 ± 6
<i>V. myrtillus</i>	Ambient	238 ± 6	158 ± 7	180 ± 9
	Elevated	233 ± 12	149 ± 6	178 ± 6
ANOVA	Host plant	$F_{1,18} = 24.7^{***}$	$F_{1,7} = 27.5^{***}$	$F_{1,17} = 29.7^{***}$
	CO <sub>2</sub>	$F_{1,18} = 0.46$	$F_{1,9} = 0.46$	$F_{1,19} = 0.08$
	CO <sub>2</sub> × host plant	$F_{1,18} = 0.04$	$F_{1,7} = 0.01$	$F_{1,17} = 0.85$
C/N ratio				
<i>V. uliginosum</i>	Ambient	14.9 ± 0.7	17.1 ± 0.5	19.6 ± 0.5
	Elevated	18.6 ± 0.6	22.2 ± 1.4	23.0 ± 0.6
<i>V. myrtillus</i>	Ambient	18.4 ± 0.4	22.4 ± 0.8	23.4 ± 0.9
	Elevated	22.2 ± 1.3	27.0 ± 2.7	26.7 ± 1.1
ANOVA	Host plant	$F_{1,8} = 28.0^{***}$	$F_{1,6} = 25.0^{***}$	$F_{1,11} = 58.8^{***}$
	CO <sub>2</sub>	$F_{1,17} = 12.2^{**}$	$F_{1,10} = 17.8^{***}$	$F_{1,17} = 4.7^*$
	CO <sub>2</sub> × host plant	$F_{1,8} = 0.00$	$F_{1,6} = 0.05$	$F_{1,11} = 1.04$
Nitrogen (% of dry mass)				
<i>V. uliginosum</i>	Ambient	3.06 ± 0.06	2.69 ± 0.08	2.23 ± 0.1
	Elevated	2.43 ± 0.08	2.02 ± 0.12	2.19 ± 0.12
<i>V. myrtillus</i>	Ambient	2.43 ± 0.06	2.00 ± 0.07	1.96 ± 0.06
	Elevated	2.00 ± 0.12	1.72 ± 0.15	1.75 ± 0.07
ANOVA	Host plant	$F_{1,8} = 36.3^{***}$	$F_{1,6} = 32.3^{***}$	$F_{1,11} = 9.92^{**}$
	CO <sub>2</sub>	$F_{1,17} = 14.8^{***}$	$F_{1,10} = 17.5^{**}$	$F_{1,17} = 1.24$
	CO <sub>2</sub> × host plant	$F_{1,8} = 2.38$	$F_{1,6} = 3.1$	$F_{1,11} = 1.14$
Starch (% of dry mass)				
<i>V. uliginosum</i>	Ambient	5.5 ± 0.6	6.2 ± 0.7	4.9 ± 0.2
	Elevated	7.5 ± 0.7	8.4 ± 0.6	6.6 ± 0.5
<i>V. myrtillus</i>	Ambient	5.1 ± 0.5	4.2 ± 1.1	2.9 ± 0.7
	Elevated	6.0 ± 0.9	6.7 ± 0.8	4.3 ± 0.3
ANOVA	Host plant	$F_{1,18} = 1.71$	$F_{1,6} = 6.34^*$	$F_{1,6} = 8.74^*$
	CO <sub>2</sub>	$F_{1,18} = 2.41$	$F_{1,10} = 8.27^{**}$	$F_{1,16} = 8.47^{**}$
	CO <sub>2</sub> × host plant	$F_{1,18} = 0.77$	$F_{1,6} = 0.3$	$F_{1,6} = 1.88$
Sugar (% of dry mass)				
<i>V. uliginosum</i>	Ambient	7.0 ± 0.5	7.6 ± 0.3	5.6 ± 0.2
	Elevated	7.5 ± 0.7	8.4 ± 0.6	6.6 ± 0.5
<i>V. myrtillus</i>	Ambient	6.3 ± 0.4	7.3 ± 0.4	5.6 ± 0.7
	Elevated	5.7 ± 0.6	6.4 ± 0.7	7.6 ± 0.6
ANOVA	Host plant	$F_{1,18} = 6.76^*$	$F_{1,6} = 7.6^*$	$F_{1,6} = 0.86$
	CO <sub>2</sub>	$F_{1,18} = 0.00$	$F_{1,10} = 0.01$	$F_{1,16} = 5.33^*$
	CO <sub>2</sub> × host plant	$F_{1,18} = 0.46$	$F_{1,6} = 1.81$	$F_{1,6} = 0.65$
Lignin (% of dry mass)				
<i>V. uliginosum</i>	Ambient	7.0 ± 0.6	–	8.1 ± 0.5
	Elevated	7.2 ± 0.2	–	7.3 ± 0.5
<i>V. myrtillus</i>	Ambient	9.5 ± 0.7	–	9.4 ± 0.9
	Elevated	8.2 ± 0.2	–	7.7 ± 0.4
ANOVA	Host plant	$F_{1,4} = 5.25$	–	$F_{1,1} = 0.04$
	CO <sub>2</sub>	$F_{1,11} = 1.72$	–	$F_{1,12} = 3.78$
	CO <sub>2</sub> × host plant	$F_{1,4} = 0.81$	–	$F_{1,1} = 4.51$

\* $P < 0.05$ , \*\* $P < 0.01$ ,  
\*\*\* $P < 0.001$

We did not find a significant influence of host plant species or elevated CO<sub>2</sub> on RGR over the entire period of development (instar 3–5) (Fig. 2a, Table 3). However, there were clear host plant and CO<sub>2</sub> effects during specific stages of nymphal development (Fig. 2b, c; Table 3). During the initial stage, *M. alpina* individuals feeding on *V. uliginosum* showed an overall higher RGR than those feeding on *V. myrtillus*, and CO<sub>2</sub> had a significant negative effect on RGR (Table 3). The negative CO<sub>2</sub> effect was

particularly strong when nymphs were feeding on *V. uliginosum* (Fig. 2b; Table 3). This plant species-specific difference in RGR disappeared at later stages of nymphal development and RGR was even higher, though not significant, in grasshoppers feeding on both high-CO<sub>2</sub> exposed *Vaccinium* species (Fig. 2c). Across all treatment combinations, simple linear regression analyses revealed positive correlations of RGR with nitrogen concentration ( $r^2 = 0.66$ ,  $F_{1,21} = 38.5$ ,  $P < 0.0001$ ), water content



**Fig. 1** Mortality during nymphal development. *Open circles: Vaccinium uliginosum* at ambient CO<sub>2</sub>, *filled circles: V. uliginosum* at elevated CO<sub>2</sub>, *open squares: Vaccinium myrtillus* at ambient CO<sub>2</sub>, *filled squares: V. myrtillus* at elevated CO<sub>2</sub>

( $r^2=0.22$ ,  $F_{1,30}=8.6$ ,  $P<0.01$ ) and marginally negative correlations with lignin ( $r^2=0.20$ ,  $F_{1,12}=3.0$ ,  $P<0.10$ ) from instar 3 to 4. At later developmental stages (instar 4–5) only starch showed a significant (positive) correlation with RGR ( $r^2=0.35$ ,  $F_{1,11}=5.8$ ,  $P=0.03$ ).

Relative consumption rates tended to be higher in grasshoppers feeding on *V. uliginosum* ( $0.28 \pm 0.04$  mg mg<sup>-1</sup> day<sup>-1</sup>) than those feeding on *V. myrtillus* ( $0.17 \pm 0.02$  mg mg<sup>-1</sup> day<sup>-1</sup>, Table 3). Although there was no overall significant CO<sub>2</sub> effect on RCR (Table 3), grasshoppers feeding on *V. uliginosum* showed an average increase of 44% in RCR under elevated CO<sub>2</sub> ( $0.36 \pm 0.04$  mg mg<sup>-1</sup> day<sup>-1</sup> compared to  $0.25 \pm 0.04$  mg mg<sup>-1</sup> day<sup>-1</sup> at ambient CO<sub>2</sub>), whereas this increase was not observed in grasshoppers feeding on *V. myrtillus* under elevated CO<sub>2</sub> ( $0.16 \pm 0.05$  mg mg<sup>-1</sup> day<sup>-1</sup> compared to  $0.18 \pm 0.02$  mg mg<sup>-1</sup> day<sup>-1</sup> at ambient CO<sub>2</sub>). Pooled across all treatments, grasshoppers

consumed an average of 21.8 mg plant biomass (dry mass) per individual and per day, which is in good accordance with other studies using more accurate estimates of RCR (Köhler and Schaller 1981).

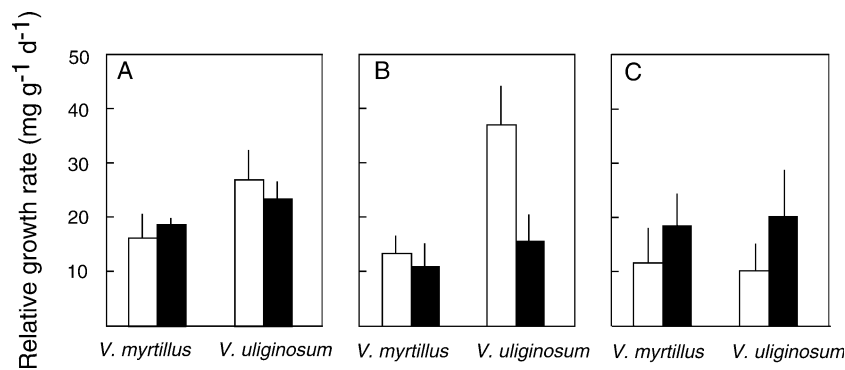
#### Final adult biomass and development time

A total of 22 from an initial 160 individuals molted in the adult stage. Males were significantly lighter than females (males:  $122 \pm 5$  mg, females:  $144 \pm 12$  mg, Table 3). Final biomass was not significantly different among individuals feeding on different host species, but was marginally significantly reduced by elevated CO<sub>2</sub> (Table 3). However, this trend was sex-specific. Newly eclosed females showed a lower final mass at elevated CO<sub>2</sub> than at ambient CO<sub>2</sub> (–18%). In contrast, the newly eclosed males attained a higher biomass when feeding on elevated CO<sub>2</sub>-grown plants (+14%), irrespective of the host plant species. These results of the sex-specific CO<sub>2</sub> response need to be interpreted with caution, because proper statistical analyses were not possible due to the loss of too many replicates. Compared to newly eclosed adults collected in the field, both males and females attained a somewhat lower final biomass under the experimental conditions (females –28%, males –19%).

In our trial, *M. alpina* needed on average  $65 \pm 4$  days from instar 3 to the final molt. Development time was significantly prolonged by elevated CO<sub>2</sub> (Fig. 3, Table 3), and was shortest in grasshoppers feeding on *V. uliginosum* in control plots. There was no difference in development time between males and females of *M. alpina* (Fig. 3, Table 3).

#### Reproduction

Reproduction was poor in females developing in our experimental plots from stage 3 instar. Only females raised on ambient CO<sub>2</sub>-grown *V. uliginosum* produced egg



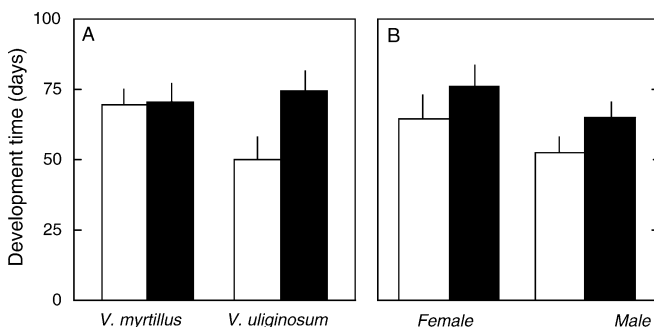
**Fig. 2** Relative growth rates (RGR; mean  $\pm$  SE) of grasshopper nymphs **a** over the entire period of development from 22 June 2002

to 12 August 2002 (instar 3 to instar 5), **b** during the initial phase (instar 3 to instar 4) from 22 June 2002 to 23 July 2002, and **c** during the second phase (instar 4 to instar 5) of development from

**Table 3** Statistical analyses of grasshopper performance and reproduction using a split-plot model. *RGR* Relative growth rate, *RCR* relative consumption rate

Parameter	Factor	<i>F</i>	<i>P</i>	
Grasshopper nymphs	RGR (instar 3–5)	Host plant	$F_{1,6} = 3.3$	0.12
		CO <sub>2</sub>	$F_{1,12} = 0.0$	0.96
	RGR (instar 4)	CO <sub>2</sub> × host plant	$F_{1,6} = 2.5$	0.17
		Host plant	$F_{1,12} = 5.9$	0.03*
	RGR (instar 5)	CO <sub>2</sub>	$F_{1,16} = 4.6$	< 0.05*
		CO <sub>2</sub> × host plant	$F_{1,12} = 4.5$	< 0.05*
	RCR	Host plant	$F_{1,6} = 1.1$	0.33
		CO <sub>2</sub>	$F_{1,12} = 2.3$	0.16
		CO <sub>2</sub> × host plant	$F_{1,6} = 0.4$	0.56
		Host plant	$F_{1,4} = 4.7$	0.10
Grasshopper imagos	Final weight	CO <sub>2</sub>	$F_{1,9} = 0.2$	0.34
		CO <sub>2</sub> × host plant	$F_{1,4} = 5.7$	0.08
		Initial weight	$F_{1,9} = 0.1$	0.77
		Host plant	$F_{1,4} = 1.7$	0.27
		CO <sub>2</sub>	$F_{1,9} = 3.8$	0.08
		Sex	$F_{1,4} = 7.9$	< 0.05*
	Development time	Sex × host plant	$F_{1,4} = 1.5$	0.29
		CO <sub>2</sub> × host plant	$F_{1,4} = 0.0$	0.84
		Initial weight	$F_{1,9} = 4.6$	0.06
		Host plant	$F_{1,4} = 2.7$	0.18
Adult reproduction	CO <sub>2</sub>	$F_{1,9} = 14.4$	0.004**	
	Sex	$F_{1,4} = 0.0$	0.96	
	Sex × host plant	$F_{1,4} = 0.6$	0.49	
	CO <sub>2</sub> × host plant	$F_{1,4} = 3.5$	0.14	
Egg pod mass	Host plant	$F_{1,1} = 1.6$	0.42	
	CO <sub>2</sub>	$F_{1,8} = 7.7$	0.02*	
Mass of secretion	Host plant	$F_{1,1} = 213.2$	0.04*	
	CO <sub>2</sub>	$F_{1,8} = 10.6$	0.01*	
Average egg mass	Host plant	$F_{1,1} = 2.0$	0.39	
	CO <sub>2</sub>	$F_{1,8} = 2.7$	0.14	
Number of eggs per pod	Host plant	$F_{1,1} = 0.1$	0.80	
	CO <sub>2</sub>	$F_{1,8} = 1.0$	0.36	

\* $P < 0.05$ , \*\* $P < 0.01$



**Fig. 3** Development time (mean ± SE) of *Miramella* individuals on two different food plant species (a) and separated for males and females (b). *Open bars* Ambient CO<sub>2</sub>, *filled bars* elevated CO<sub>2</sub> plots

pod, with an average of 6.5 eggs, whereas no egg pods were deposited in any other treatment combinations.

Females of *M. alpina* collected in the field as freshly emerged adults and feeding in experimental plots only as adults produced an average of 0.6 egg pods per individual across all treatments. Of the 28 females, 57% did not produce any egg pods, 32% produced one egg pod, and 11% two egg pods. There were no significant effects of either CO<sub>2</sub> or host plant species on total egg pod production.

Mean egg pod mass, produced by individuals feeding on CO<sub>2</sub>-exposed plants was significantly lower compared to that produced by individuals feeding on control plants (Fig. 4a, Table 3). This difference was due mainly to significantly lower mass of secretion, (Fig. 4b) and, to a lesser extent, to a reduced egg mass (Fig. 4c), with no significant CO<sub>2</sub> effect on egg numbers (Fig. 4d). Females having the choice among different plant species (mixed diet) showed essentially the same reproductive parameters as females feeding on ambient CO<sub>2</sub>-grown *Vaccinium* (data not shown).

#### Food choice experiment

Given a choice of five different plant species, *G. punctata* was preferably consumed by males of *M. alpina*, followed by *V. uliginosum* and *V. myrtillus* ( $F_{2,78} = 2.7$ ,  $P = 0.07$ , Fig. 5). In contrast, females feeding almost three times as much as males ( $F_{1,78} = 22.6$ ,  $P < 0.0001$ ) preferred *V. uliginosum* followed by *G. punctata* and *V. myrtillus*. Interestingly, we found a marginally significant CO<sub>2</sub> × plant × sex interaction ( $F_{2,78} = 2.4$ ,  $P = 0.09$ ). Males consumed similar amounts of the two *Vaccinium* species and 46% more of *Gentiana* compared to *Vaccinium* when the leaves were produced at ambient CO<sub>2</sub>. However, there

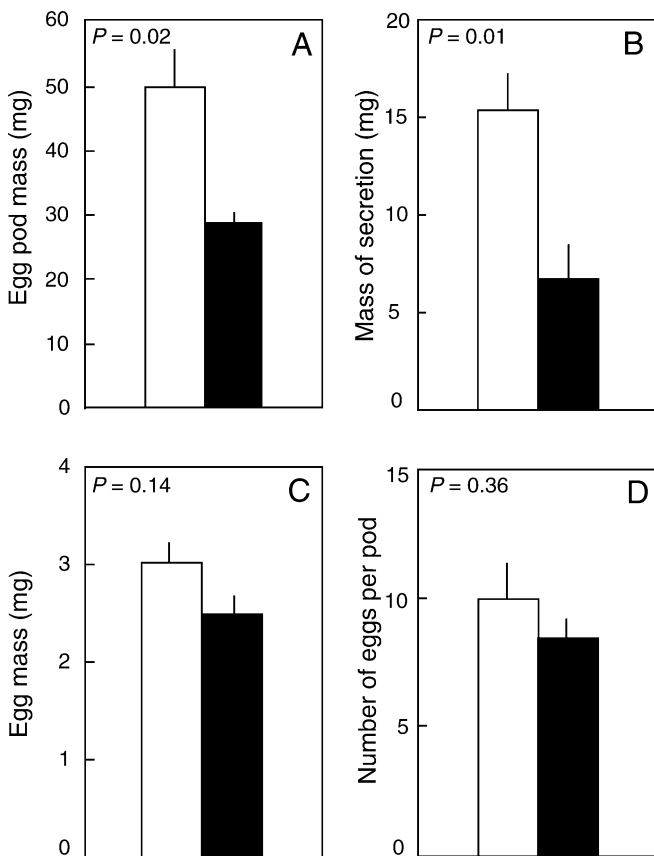
was a shift towards more *G. punctata* and less *V. myrtillus* consumption by males when the leaves were grown at elevated CO<sub>2</sub>. In contrast, females consumed similar amounts of *Gentiana* and *V. uliginosum* from ambient CO<sub>2</sub> plots. *Gentiana* consumption decreased by 42% in elevated CO<sub>2</sub>-grown leaves. Less *Gentiana* consumption by females was accompanied by more *Vaccinium* consumption. *V. vitis idaea* was not consumed at all.

## Discussion

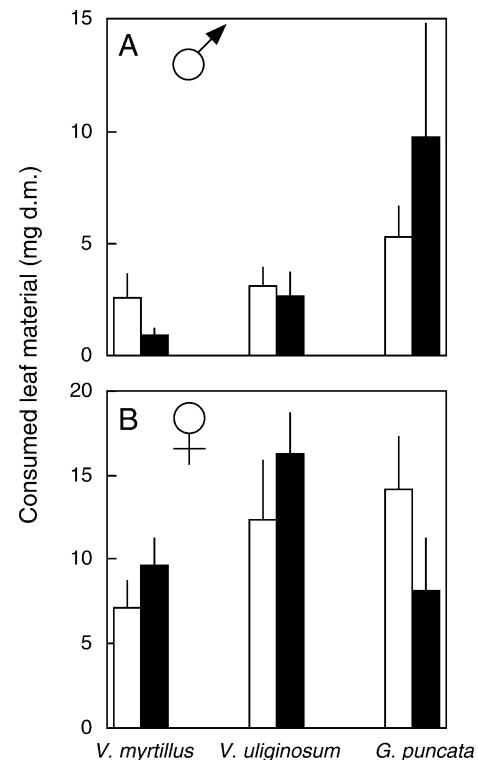
### Grasshopper development

In line with our initial hypothesis, we observed significant changes in leaf chemistry in both *Vaccinium* species in response to a CO<sub>2</sub>-enriched atmosphere at treeline. Although the two dwarf shrub species differed significantly in most leaf quality traits, the CO<sub>2</sub> effect was essentially the same in both species. In general, nitrogen concentration decreased and starch and sugar concentrations increased at elevated CO<sub>2</sub>. Insects faced with a CO<sub>2</sub>-enriched atmosphere, which is commonly associated with lower food nitrogen concentrations, often exhibit slower growth rates (Traw et al. 1996; Williams

et al. 1997; Hättenschwiler and Schafellner 1999). Nitrogen is known to be an important factor for herbivore development (Strong et al. 1984), including grasshoppers (McGinnis and Kasting 1965; Bernays and Chapman 1978). In a previous CO<sub>2</sub> experiment with grasshoppers, it was reported that lower nitrogen concentration at elevated CO<sub>2</sub> had a negative effect on relative growth of fifth instar nymphs of *Melanoplus* sp. (Johnson and Lincoln 1990). In our experiment, RGR of early instar nymphs of *M. alpina* was positively correlated with nitrogen and water, whereas higher lignin concentrations resulted in a lower RGR. These correlations diminished in later instars suggesting that CO<sub>2</sub>-induced leaf quality changes were not as important for late instars as for early instars. Thus, it is relevant to consider the age (instar) of grasshopper nymphs in any evaluation of insect responses to elevated CO<sub>2</sub>. Nutritive demands maybe differ depending on nymphal age, or grasshoppers may incorporate ingested food of a lower quality into body mass in older stages more efficiently. The importance of post-ingestive mechanisms was recently shown in a study with *Melanoplus* grasshoppers feeding on C<sub>3</sub> and C<sub>4</sub> grasses growing at ambient or elevated CO<sub>2</sub> (Barbehenn et al. 2004). Although the nutritional quality of C<sub>3</sub> grasses declined to a greater extent than that of C<sub>4</sub> grasses in elevated CO<sub>2</sub>, no differences in grasshopper growth rates or consumption rates were found.



**Fig. 4** Reproduction parameters of *M. alpina* females (mean  $\pm$  SE). **a** Pod mass [mg dry mass dm], **b** secretion weight (mg dm), **c** egg weight (mg dm), **d** number of eggs per pod. *Open bars* Ambient CO<sub>2</sub>, *filled bars* elevated CO<sub>2</sub> plots



**Fig. 5** Total leaf material consumed (mean  $\pm$  SE) by female (**a**) and male (**b**) *M. alpina* after 24 h of feeding. *Open bars* Leaf material from ambient CO<sub>2</sub> plots, *filled bars* leaf material from elevated CO<sub>2</sub> plots



In our case, it is plausible to argue that lower nitrogen concentration in elevated CO<sub>2</sub>-grown plants impaired the growth of early instar grasshoppers. Given that grasshoppers meet their requirements for water mainly through plant material uptake, the lower leaf water content at elevated CO<sub>2</sub> in June could also have been limiting for early instar growth, especially because June 2002 was rather dry. In line with this reasoning, Schälller and Köhler (1981) reported a clear preference for grasses with higher water content (76–78%) in two Mid-European acridoid species. Apparently, nitrogen concentration did not seem as important for grasshopper growth in later instars. Moreover, the negative CO<sub>2</sub> effect on leaf nitrogen concentration became less pronounced in late summer, and the initially lower water content in high CO<sub>2</sub>-grown leaves actually turned into a higher water content later in the season, which could help explain the higher RGR at elevated CO<sub>2</sub> in older nymphs. Additionally, we found a strong positive correlation of RGR with starch from instar 4 to 5. Starch plays an important role in grasshopper nutrition because carbohydrates are known as phagostimulants and they are easy to digest (Bernays and Simpson 1990). Indeed, grasshoppers exhibited a higher RGR on high CO<sub>2</sub>-grown, starch-rich *V. uliginosum* compared to control plants, and thus might have been able to compensate for lower nitrogen concentrations in later developmental stages.

We did not find any references to verify whether the relatively high grasshopper mortality observed in our experiment is common for *M. alpina* or not. Generally, there are high generation dependent variations in acridoid grasshopper mortality, ranging from 2 to 98% during nymphal development (Ingrisch and Köhler 1999), suggesting that the observed mortality in our experiment might have been a normal phenomenon for *M. alpina* populations. Although we tried to avoid including unhealthy animals in our experiment, we frequently observed grasshoppers infested by pathogenic fungi and parasitic mites in the close neighborhood of our study site (Asshoff and Köhler 2003). Perhaps the unusually wet August in 2002 (Table 1) also negatively influenced survival.

Surviving female grasshoppers showed a higher final biomass than males, irrespective of CO<sub>2</sub> treatment. This size dimorphism is typical for many acridoid grasshopper species. Female grasshoppers, however, achieved a lower final weight in elevated CO<sub>2</sub> plots than in ambient CO<sub>2</sub> plots, whereas the reverse was observed for the males. This result is in agreement with a previous report on the sex-specific CO<sub>2</sub> effect on final pupal weight of the lepidopteran *Lymantria dispar* (Traw et al. 1996). We assume that this result is attributable to a trade-off in female grasshoppers to allow egg production as early as possible (especially in a high elevation ecotone with a short vegetation period), and therefore they eclose earlier in the adult stage at the expense of lower final biomass (Joern and Behmer 1997). The prolonged development time of females in elevated CO<sub>2</sub> plots apparently was not enough to compensate for slower

RGR to attain final weights similar to those of females in ambient CO<sub>2</sub> plots.

## Reproduction

The much lower reproductive success in females developing under experimental conditions from their instar 3 stage compared to females exposed to experimental conditions only in their adult stage, is evidence for a significant influence of nymphal development on total grasshopper egg production. However, exposure to high CO<sub>2</sub>-grown plants during the adult stage only, still had very important consequences for grasshopper reproduction. Two weeks after the start of the experiment with freshly molted adults, all 28 female grasshoppers were alive, and mating was observed frequently. After 3 weeks there were still 18 females equally distributed across the treatments. This time span should normally be sufficient to produce at least one egg pod. Under optimal greenhouse conditions, females of the genus *Miramella* lay on average three egg pods (Asshoff and Köhler 2003). In our field experiment, *Miramella* laid only one egg pod and exceptionally a second, much smaller, egg pod. Our findings of similar egg numbers produced by female grasshoppers in both CO<sub>2</sub> treatments are in agreement with an earlier study of butterfly reproduction under elevated CO<sub>2</sub> (Goverde and Erhardt 2002). The latter study found no significant CO<sub>2</sub> effect on the total number of eggs produced by *Coenonympha pamphilus* (Lepidoptera). In contrast, *Operophtera brumata* (Lepidoptera) produced more eggs in elevated CO<sub>2</sub> than in ambient CO<sub>2</sub> (Buse et al. 1998). Despite similar egg numbers, eggs in our experiment were lighter at elevated CO<sub>2</sub> compared to ambient CO<sub>2</sub> suggesting that they had lower yolk content. Brooks and Whittaker (1998) also found that eggs of *Gastrophysa viridula* (Coleoptera) were 15% lighter under elevated CO<sub>2</sub> conditions, in contrast to Buse et al. (1998) who found no significant CO<sub>2</sub> effect on egg mass. It is noteworthy that the strongest effect we observed was a decrease in secretion mass production under elevated CO<sub>2</sub>. Consequently, eggs are less protected against unsuitable environmental conditions and mechanical damage, which might reduce hatching success in the following year. In addition, the prolonged nymphal development time at elevated CO<sub>2</sub> retards the first egg deposition until later in the season, which could also lead to a smaller total egg production or lower hatching success in the next season. It is generally assumed that only eggs deposited early in the summer have the chance to fully develop by the next year. In some recent studies investigating the biology of the genus *Miramella* it was found that only up to 50% of all eggs actually developed by the next growing season (Köhler et al. 1999; Asshoff and Köhler 2003). Both less egg deposition and less protection of the remaining eggs might negatively influence the life cycle and population dynamics of *M. alpina*.

## Food choice

*M. alpina* females and males showed a different food plant preference, which was additionally influenced by CO<sub>2</sub> concentration. Diet selection for specific nutrients can vary between female and male grasshoppers. For example, females of two acridoid species displayed a higher preference for diets with a high proline content compared to males (Behmer and Joern 1994). Furthermore, it is known that elevated CO<sub>2</sub> can alter herbivore food plant preferences (Peters et al. 2000; Goverde and Erhardt 2002). In our experiment, a preference for *G. punctata* increased in males of *M. alpina*, whereas it decreased in females at elevated CO<sub>2</sub>, leading to a substantially higher proportion of *G. punctata* consumption at elevated CO<sub>2</sub> in males than females. A shift in food plant species preference may have the greatest impact in response to high CO<sub>2</sub>-induced leaf quality changes in generalist insect herbivores (Hättenschwiler and Schafellner 2004). In our field experiment where we focused on the two supposedly most suitable host species of *M. alpina*, the grasshoppers could not choose freely among different alternative food plant species, which might have influenced their response to elevated CO<sub>2</sub>. For example, caterpillar growth of the butterfly *Coenonympha pamphilus* was not significantly affected by elevated CO<sub>2</sub> in a calcareous grassland (Goverde et al. 2003). This lack of effect was interpreted as being due to the possibility of insects being able to maintain an optimal nutritional status in the field by altering the proportions of different plant species consumed. A second reason discussed was the harsher climatic conditions in the field compared to the greenhouse, which is most often used for the assessment of herbivore responses to elevated CO<sub>2</sub>, which could limit growth to such an extent that even reduced leaf nitrogen concentration will not limit growth any further. The grasshoppers in our experiment showed a somewhat lower adult biomass and a longer development time than those inferred from grasshoppers observed in the field. This might indicate that a single food plant is not optimal for *M. alpina*, but it is not at all clear to what extent polyphagous grasshoppers really depend on a mixed diet (Joern 1979). In greenhouse experiments it was observed that a single food plant species is as suitable as a mixture of different species for *M. alpina* (Hägele and Rowell-Rahier 1999).

In summary, we have demonstrated that elevated CO<sub>2</sub> reduces leaf quality in two dominant dwarf shrub species of the alpine treeline ecotone, and that this has consequences for grasshopper performance. Growth of early instar nymphs was particularly strongly affected by elevated CO<sub>2</sub>, but adult reproduction was also impaired. The observed plant species-specific CO<sub>2</sub> effects on grasshopper performance suggest changes in feeding behavior and host plant-grasshopper interactions with the continuing rise in atmospheric CO<sub>2</sub> concentrations, which in turn has important implications for the structure and function of the treeline ecotone.

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