Ecological Entomology

Ecological Entomology (2011), **36**, 1–13 DOI: 10.1111/j.1365-2311.2010.01240.x

CO_2-mediated changes of plant traits and their effects on herbivores are determined by leaf age

DANIEL J. BALLHORN,[1] IMKE SCHMITT,[1] JONATHON D. FANKHAUSER,[1] FUMIAKI KATAGIRI[1] and HARDY PFANZ[2] [1]Department of Plant Biology, University of Minnesota, St Paul, Minnesota, U.S.A. and [2]Institute of Applied Botany, University of Duisburg-Essen, Essen, Germany

Abstract. 1. Concentration of atmospheric CO_2 is predicted to double during the 21st century. However, quantitative effects of increased CO_2 levels on natural herbivore–plant interactions are still little understood.

2. In this study, we assess whether increased CO_2 quantitatively affects multiple defensive and nutritive traits in different leaf stages of cyanogenic wildtype lima bean plants (*Phaseolus lunatus*), and whether plant responses influence performance and choice behaviour of a natural insect herbivore, the Mexican bean beetle (*Epilachna varivestis*).

3. We cultivated lima bean plants in climate chambers at ambient, 500, 700, and 1000 ppm CO_2 and analysed cyanogenic precursor concentration (nitrogen-based defence), total phenolics (carbon-based defence), leaf mass per area (LMA; physical defence), and soluble proteins (nutritive parameter) of three defined leaf age groups.

4. In young leaves, cyanide concentration was the only parameter that quantitatively decreased in response to CO_2 treatments. In intermediate and mature leaves, cyanide and protein concentrations decreased while total phenolics and LMA increased.

5. Depending on leaf stage, CO_2-mediated changes in leaf traits significantly affected larval performance and choice behaviour of adult beetles. We observed a complete shift from highest herbivore damage in mature leaves under natural CO_2 to highest damage of young leaves under elevated CO_2. Our study shows that leaf stage is an essential factor when considering CO_2-mediated changes of plant defences against herbivores. Since in the long run preferred consumption of young leaves can strongly affect plant fitness, variable effects of elevated CO_2 on different leaf stages should receive highlighted attention in future research.

Key words. Cyanogenesis, global climate change, herbivory, insect performance, leaf stage, lima bean (*Phaseolus lunatus*), Mexican bean beetle (*Epilachna varivestis*), plant defence.

Introduction

Carbon dioxide (CO_2) concentration in the atmosphere is expected to rise continuously in the foreseeable future and to reach levels of 540–970 ppm by the end of this century (Houghton *et al*., 1996; Prather & Ehhalt, 2001). Plants growing under elevated CO_2 commonly show alteration of leaf chemical composition that can affect the palatability and nutritional quality of foliage for leaf-feeding arthropods (Lincoln *et al*., 1993; Peñuelas & Estiarte, 1998; Hamilton *et al*., 2004; Zvereva & Kozlov, 2006; Valkama *et al*., 2007). For example, plants grown under enhanced CO_2 often exhibit lower nitrogen and soluble protein content in leaves (Mulchi *et al*., 1992; Cotrufo *et al*., 1998) and, thus, reduced nutritional value to herbivores. Furthermore, in response to increased CO_2, plants commonly accumulate mono- or disaccharides and starch in their foliage, affecting palatability by altering the C : N ratio (Cotrufo *et al*., 1998; Long *et al*., 2004). Low nutritional quality of tissues, however, can have different effects on insect herbivores, depending on the feeding guilds

Correspondence: Daniel J. Ballhorn, Department of Plant Biology, University of Minnesota, St Paul, MN 55108, U.S.A. E-mail: ballhorn@umn.edu

© 2010 The Authors
Ecological Entomology © 2010 The Royal Entomological Society

(Bezemer & Jones, 1998). It has repeatedly been reported that some leaf-chewing herbivores exhibit 'compensatory feeding' by increased consumption of foliage with a lower nitrogen content to meet their nutritional requirements (Bezemer & Jones, 1998; Whittaker *et al*., 1999). In addition to digestible sugars, special structures can be alternative sinks for carbon, such as thick cell walls or trichomes. These structures could make the leaves more difficult to consume for herbivores, and additionally dilute essential nutrients (reviewed by Bezemer & Jones, 1998). When an insect cannot compensate for the dilution of nutrients by increased feeding its growth will be retarded and it will be subject to predation for a longer period of time (slow growth–high mortality hypothesis) (Lill & Marquis, 2001).

Besides shifts in nutritional component composition and physical traits, enhanced CO_2 may substantially affect chemical plant defences against herbivores (Coviella *et al*., 2002; Hamilton *et al*., 2005; Bidart-Bouzat *et al*., 2008). Carbon-based phenolic compounds have been frequently reported to increase in response to CO_2 enrichment (Lambers, 1993; Mansfield *et al*., 1999; Coley *et al*., 2002). In contrast to carbon-based defences, it remains elusive whether nitrogen-based defensive compounds are quantitatively affected by CO_2 availability (but see Rufty *et al*., 1989; Gleadow *et al*., 1998, 2009). Until now there is only sparse information on quantitative effects of CO_2 on multiple plant defences (but see Lindroth *et al*., 1997; Bazin *et al*., 2002; Holton *et al*., 2003; Donaldson & Lindroth, 2007), and even less is known about the effects of changing CO_2 levels on different leaf developmental stages (Milligan *et al*., 2008; Zavala *et al*., 2009). Allocation of resources into synthesis of secondary compounds depends on type and age of the respective plant organ (Reichardt *et al*., 1984; Bryant & Julkunen-Tiitto, 1995; Jones & Hartley, 1999; Bidart-Bouzat *et al*., 2005). Within an individual plant, variability of CO_2-mediated changes depending on leaf developmental stage might be of great ecological relevance, because younger and older leaves have a vastly different importance for plant fitness. According to the optimal defence hypothesis (ODH) three main factors – cost of defence, risk of attack, and value of the respective plant organ – determine the allocation of defensive secondary metabolites (McKey, 1974, 1979; Rhoades, 1979; Stamp, 2003). The higher the risk of a given plant tissue to be consumed by herbivores and the higher its value for the plant fitness, the more energy should be allocated to its defence (Zangerl & Bazzaz, 1992; Rostás & Eggert, 2008). Following the assumptions of the ODH, within the total foliage of a plant, young leaves make a larger contribution to plant fitness than old leaves as they have a higher potential photosynthetic value resulting from a longer expected lifetime (Rhoades, 1979; Coley, 1980, 1988; Coley *et al*., 1985; Stamp, 2003). In addition, younger leaves are often more nutritious and thus more attractive to herbivores (Calvo & Molina, 2005) and should be better defended (Anderson & Agrell, 2005).

The aim of the present study is to contribute to our understanding of functional associations between ontogenetic variations of plant traits, CO_2-mediated changes in defence-associated and nutritive parameters, and plant–herbivore interaction. In our experiments, we used wildtype lima bean plants (Fabaceae: *Phaseolus lunatus* L.) derived from a natural population in southern Mexico (Oaxaca) and the Mexican bean beetle (Coccinellidae: *Epilachna varivestis* Muls.) as a natural insect herbivore of lima bean.

Lima bean represents a prominent experimental plant for studies on inducible indirect plant defences against herbivores, such as the release of herbivore-induced volatile organic compounds (VOCs) and the secretion of extrafloral nectar (Arimura *et al*., 2002; Ballhorn *et al*., 2008a; Mumm *et al*., 2008; Radhika *et al*., 2008). In contrast to indirect defences, the contribution of direct chemical defences as well as of physical leaf traits and nutritive leaf parameters to the overall resistance of lima bean to herbivores have received less attention. Only recently, in our own studies the functional ecology of lima beans' cyanogenesis in herbivore–plant interaction was analysed under laboratory (Ballhorn *et al*., 2006, 2007, 2010a,b) and field conditions (Ballhorn *et al*., 2009). Cyanogenesis was demonstrated to act as an efficient defence against both generalist and specialist insect herbivores. In the present study, we focus on quantitative effects of enhanced CO_2 on two direct chemical defences (cyanogenesis and total phenolics), a physical leaf trait potentially affecting herbivory (leaf mass per area, LMA), and on soluble protein concentration as a selected nutritive parameter crucially determining leaf quality (Mattson, 1980; Ganzhorn, 1992). Specifically, we address the following questions: (i) how will increased CO_2 quantitatively affect multiple defensive and nutritive traits of cyanogenic wildtype lima bean, (ii) how are different ontogenetic leaf stages affected by CO_2 treatments, and (iii) how will plant responses influence performance and choice behaviour of a natural insect herbivore, the Mexican bean beetle?

Materials and methods

Experimental design

In this study we used closed-top climatic chambers in which we applied four different CO_2 treatment: (i) ambient (= control) CO_2 (mean 360 ppm), (ii) 500 ppm CO_2, (iii) 700 ppm CO_2, and (iv) 1000 ppm CO_2. In each chamber we placed 10 lima bean plants. Climatic chambers were adjusted to 29 : 23 °C in a LD 13 : 11 h period (light: photon flux density 450–500 μmol photons s^{-1} m^{-2} at table height, Son-T Agro 400, Philips®, Hamburg, Germany) and an air humidity of 70–80%. During the experimental period, the plants were moved between the four chambers at regular intervals (one time per week) and the respective CO_2 atmospheres were re-adjusted to avoid any effects of physical chamber environment (i.e. the factor 'chamber') on plant development.

Lima bean plants were grown from seeds collected in a natural population at a coastal site 10 km west of Puerto Escondido, Oaxaca, Mexico (~ 15°55′446″N, 97°09′107″W, elevation 11 m). The testa of the seeds was scratched to facilitate water absorption and to ensure homogeneous germination of seeds. Plants were cultivated separately in black plant containers with 8 cm in diameter in a 1 : 1 ratio of standard substrate (TKS®-1-Instant, Floragard®, Oldenburg, Germany) and sand (grain size 0.5–2.0 mm), and were fertilised with 50 ml of a 0.1% aqueous solution of Flory-3® (NPK-Fertilizer, EUFLOR

© 2010 The Authors

GmbH, Munich, Germany) twice a week and watered daily. Plants were grown in the climatic chambers for 31 days and were allowed to climb up wooden sticks of 60 cm (from substrate surface). When reaching the top of these sticks (after approx. 2 weeks), developing tendrils were wrapped around the sticks by hand to avoid burning of shoot tips at the lamps.

Mexican bean beetles (Coccinellidae: *Epilachna varivestis* Muls.) used as herbivores in this study were maintained on non-cyanogenic snap bean (Fabaceae: *Phaseolus vulgaris* var. Saxa) under the same climatic chamber conditions as the lima bean plants (control treatment without experimentally elevated CO_2). Freshly hatched larvae were used in feeding experiments on herbivore performance (i.e. biomass accumulation per time), whereas adult beetles in random sex ratios were used in choice experiments.

Plant growth measurements and leaf characterisation

The length of the shoots was measured daily until lima bean plants reached the top of the sticks. Fresh and dry weight of total plants was determined at the end of the experiment after 31 days. For dry weight determination, plants were dried at 45 °C for 96 h until constancy of weight. In addition, leaf mass per area (LMA) of three defined leaf developmental stages (young, intermediate, and mature) was determined for each plant individual by dividing the biomass of dried leaves by the area of the leaves. Young leaves inserted three leaf positions down from the apex and did not completely reach the final leaf size at time of analysis. Leaves inserted seven positions down from the apex were defined as 'intermediate'. They were completely expanded, however still showed thin and delicate leaf tissue. Mature leaves were located at the stem 11 insertion positions below the apex. These leaves were characterised by a dark green colour and a tougher leaf structure. For measurement of leaf area, leaves were photographed on a scale and leaf area was measured using analySIS® software (Olympus Soft Imaging Solutions GmbH, Münster, Germany). For determination of total biomass accumulation of the plants the calculated dry weight of the defined leaves that were removed for biochemical analyses and feeding trials was added to the total dry weight per plant individual.

Leaf sampling

For comparative analyses of chemical and physical plant traits eight plants per treatment were selected randomly. After 31 days growing under experimentally enhanced CO_2, young leaves were removed for quantification of cyanide, total phenolics, soluble protein concentration, and determination of LMA. Two leaflets of each trifoliate young leaf were used for bioassays (body mass accumulation of larvae and choice test), whereas the remaining leaflet was portioned and used for biochemical analyses. Since the same individual leaves were used in bioassays with Mexican bean beetles, leaf chemical parameters could be directly related to insect performance and choice behaviour. After excision of leaf discs for choice tests, the remaining leaf material of the specific leaflet was used for determination of LMA. The position of the leaflets used for the two different bioassays and the chemical analyses was set at random. Leaf chemical characteristics were assumed to be similar among all three leaflets (Ballhorn *et al*., 2007).

Larval body mass accumulation

For quantification of larval body mass accumulation, single leaflets were removed from the plant with their petiole intact. The petiole was immersed in a water-filled Eppendorf® tube with perforated lid. Each of these water supplied leaflets was placed in a Petri dish (diameter 90 mm) lined with moistened filter paper. Freshly hatched larvae were weighed (Sartorius Electronic Microbalance 4503 MP6) and placed individually on single leaflets. This procedure was conducted for leaves of all three different developmental stages ($n = 8$ bioassays per leaf stage). Larvae used in feeding trials were derived from different egg batches from different female beetles to reduce potential genetic effects on performance. Bioassays were conducted at the same ambient conditions as adjusted for plant cultivation (control treatment, i.e. ambient CO_2). After an experimental period of 48 h the larvae were re-weighed and biomass accumulation was calculated. After removing the larvae from the leaflets, we waited for 45–60 min before quantification of weight to excluded variation of weight due to ingested leaf material from the analysis of body mass. After this period of time, ingested leaf material in the gastrointestinal tract of larvae (which easily can be seen as a dark colouration in the translucent yellowish insect bodies) was excreted. Values given on larval biomass accumulation were corrected for initial larval weight. We chose the relatively short experimental period of 48 h to minimise potential changes in leaf biochemistry due to transfer of CO_2 treated leaves to ambient CO_2 concentrations. Furthermore, when leaves are detached from the plant, biochemical parameters are likely to change over extended time periods. Using individual leaves for both chemical analyses and bioassays enabled us to directly correlate chemical features of individual leaves with responses of herbivores.

Choice behaviour of adult beetles

To test for CO_2-mediated treatment effects on feeding choice of adult Mexican bean beetles, we offered leaves of the same developmental stage derived from plants treated with the different CO_2 concentrations (ambient, 500, 700, and 1000 ppm CO_2) simultaneously in a choice arena (Petri dishes with 14 cm in diameter, supplemented with slightly moist filter paper; $n = 8$ choice experiments per leaf stage). In an additional choice experiment, we simultaneously offered leaves of different developmental stages derived from control plants grown at natural CO_2 concentration to evaluate effects of leaf age (independent of CO_2 treatment) on the beetles' feeding preference ($n = 8$ bioassays). For all choice experiments, leaf discs (1.8 cm in diameter) were cut with a cork-borer from leaflets. We used leaf discs instead of intact leaflets to avoid potential

© 2010 The Authors

effects of leaf size on the beetles' choice behaviour. Leaf discs were placed upside down (because the beetles preferred to feed on the lower surface of leaves) and in equal distance to each other on the filter paper; position of leaflets was random. We waited for 30 min before starting the experiment, to ensure complete diffusion of gaseous HCN released from the cutting edge that might deter beetles before reaching the leaf disc. The release of HCN from fresh leaf discs was quantified in preliminary experiments using an air-flow system for cyanide detection according to Ballhorn *et al*. (2005). These experiments demonstrated that 10 min were sufficient for release of all cyanide from injured cells at the cutting edge (data not shown). Beetles were food deprived for 2 h before the experiment and then a single beetle was placed in the middle of the plate. Beetles were allowed to feed for 2 h at the same ambient conditions as adjusted for plants (control treatment). In choice experiments we used natural sex ratios of beetles. Leaf area consumption in experiments with larvae (body mass accumulation) and choice tests with adult Mexican bean beetles was quantified by digitally photographing leaflets and leaf discs on a scale (Canon, EOS 40D; 10 000 pixels) and computer-based determination of missing leaf area using analySIS software (Olympus, Hamburg, Germany).

Leaf cyanogenic potential (HCNp)

Quantitative analyses of the cyanogenic potential (HCNp; the total amount of cyanide that can be released by a given tissue) were conducted by complete enzymatic degradation of extracted cyanogenic glycosides and subsequent quantitative measurement of released cyanide according to Ballhorn *et al*. (2005). All steps of preparation were conducted at $<4\ ^{\circ}C$ to avoid any premature release of gaseous hydrogen cyanide. For hydrolysis of cyanogenic glycosides in leaf extracts we used β-glucosidase isolated from young leaves of rubber tree (Euphorbiaceae: *Hevea brasiliensis*) according to Ballhorn *et al*. (2006), which showed strong affinity to cyanogenic glycosides in lima bean. After 20 min of incubation at 30 °C in gas-tight glass vessels (Thunberg vessels) released cyanide was spectrophotometrically measured at 585 nm using the Spectroquant® cyanide test (Merck, Darmstadt, Germany). Analyses of HCNp in defined leaf developmental stages as well as total amount of cyanide per plant were conducted following the same procedure. Both single leaves and plants were harvested between 10.00 and 10.45 a.m. to exclude diurnal effects on chemical composition.

Total phenolics

Concentration of total phenolics in leaf material was analysed following Tikkanen and Julkunen-Tiitto (2003). Homogenates of dried leaves were extracted three times for 15 min in 5 ml acetone diluted with aqua dest. (60 : 40). After each extraction, samples were incubated in an ultrasonic bath (3 min) and were finally centrifuged for 10 min at $5000 \times \boldsymbol{g}$. The supernatant was transferred to 2 ml concentrated acetic acid (Merck KGaA, Darmstadt, Germany), acetone was removed under vacuum (60 mbar) at 40 °C, and the residue was quantitatively transferred by using deionised water. Samples were diluted with 2.5% acetic acid, and 1 ml of this solution was mixed with 0.5 ml Folin–Ciocalteu phenol reagent (Merck). After adding 2 ml 20% Na_2CO_3, the solution was made up to 10 ml with deionised water. Samples were incubated at 70 °C and, after cooling, spectrophotometrically quantified at 730 nm against blank containing water instead of sample. Epicathechine (Sigma, Deisenhofen, Germany) served as standard.

Soluble protein

Concentration of soluble proteins in defined leaves and total amount of protein per plant was quantified according to Bradford (1976) with modifications following Ballhorn *et al*. (2007). Bradford reagent (Biorad Laboratories, Munich, Germany) was diluted 1 : 5 with deionised water and 20 µl of each homogenised plant sample were added to 1 ml of diluted Bradford solution. Bovine serum albumine (BSA; Fluka Chemie AG, Buchs, Switzerland) was used as standard at different dilutions. After 5 min of incubation, concentration of protein was spectrophotometrically measured at 595 nm (Genesys 20, Thermo Spectronic, Madison, Wisconsin). We used the same plant extracts for protein measurements and HCNp analyses, thus being able to quantitatively relate the two parameters.

Leaf mass per area (LMA)

After excision of leaf discs for feeding trials, smaller leaf discs (1.2 cm in diameter) were cut from the leftover leaf material. Leaf discs were weighed to the nearest 0.001 g and dried at 45 °C to constancy of weight. Leaf samples were consecutively weighed for determination of dry matter and LMA was calculated.

Statistical analyses

For analysis of CO_2 effects on plant growth, the square root of the shoot length value was taken for the homogeneity of the variance. A polynomial linear model was fitted through the curves of the square-rooted shoot length value for individual plants, and a mixed-effect linear model was fitted on the coefficients of these curves: $\mathrm{sqrt}(\mathrm{Sl}_{ij}) \sim 0 + C_i + C : (\mathrm{Tm} + \mathrm{Tm}^2 + \mathrm{Tm}^3 + \mathrm{Tm}^4)_i + (1 + \mathrm{Tm} + \mathrm{Tm}^2 + \mathrm{Tm}^3 + \mathrm{Tm}^4)|P_{ij} + \varepsilon_{ij}$, where Sl, C, Tm, P, and ε are shoot length, CO_2 condition (fixed effect), time (fixed effect), individual plant (random effect), and residual, and i and j are the indices for the CO_2 condition and the individual plant. To avoid convergence problems, the coefficients of the random effect $(1 + \mathrm{Tm} + \mathrm{Tm}^2 + \mathrm{Tm}^3 + \mathrm{Tm}^4)|P_{ij}$ were assumed to be independent, and time was centred and scaled to range from -1 to 1. For the polynomial model, the third to fifth orders were tested, and the fourth order was chosen because it resulted in

© 2010 The Authors

the lowest AIC value. The nlme package in the R environment (version 2.10.0, www.r-project.org) was used for this analysis.

General linear model (GLM) was applied for analysing effects of 'Leaf stage' and 'Treatment' on chemical and physical plant parameters as well as on biomass accumulation and leaf area consumption of insects. We used *post hoc* analysis (LSD, $P < 0.05$) after one-way ANOVA to test for significant differences of treatment effects on specific leaf developmental stages (young, intermediate, mature) as well as on number of leaves and plant dry weight among treatment groups. In the same way, differences of leaf area consumption of beetles within leaf stages of different plants grown at ambient (experimentally unchanged) CO_2 concentrations were analysed by ANOVA and LSD ($P < 0.05$) *post hoc* analysis. GLM, ANOVA, and *post hoc* analyses were carried out using Statistical Package for Social Sciences (SPSS) 13.0 (SPSS for Windows, SPSS, Chicago, Illinois).

Results

Morphological plant parameters

Shoot length of lima bean plants quantitatively increased in response to elevated CO_2 conditions (Fig. 1). The difference in shoot length between controls and plants grown under elevated CO_2 concentrations of 700 and 1000 ppm was significant after 11 days of cultivation and remained significant until the end of the experiment [according to *post hoc* analysis (LSD, $P < 0.05$) after one-way ANOVA]. Plants grown at 500 ppm CO_2 showed significantly increased shoot length compared with the controls on the 13th day of cultivation, however at that time were significantly smaller than plants growing under 1000 ppm CO_2 treatments (Fig. 1). No significant differences in shoot length between plants grown at 500 and 700 ppm as well as between plants that had developed under 700 and 1000 ppm were observed (Fig. 1).

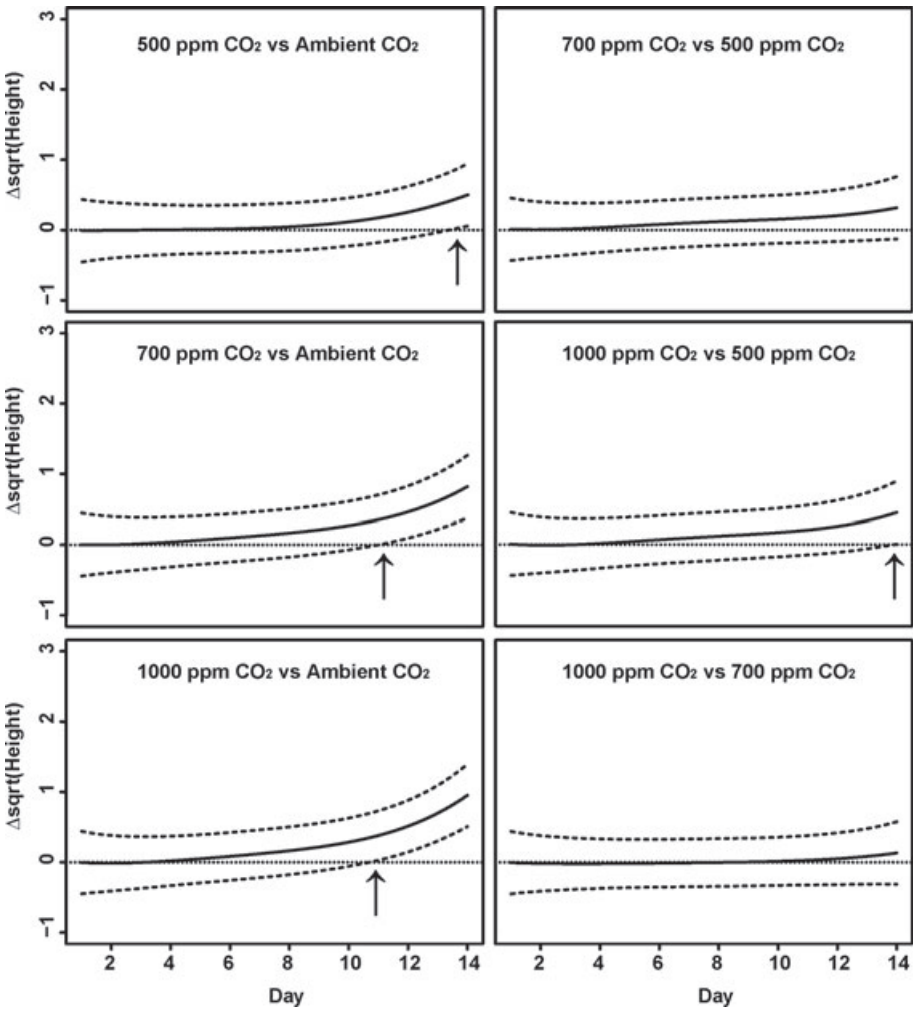

Fig. 1. Plant growth under different CO_2 regimes. Shoot length of lima bean plants was measured daily over an experimental period of 14 days. Response curves are plotted using square root transformed mean difference values for each solid curve, and 95% confidence intervals are represented by dotted curves in each comparison. Curves are based on mixed effects linear model estimating growth for each individual plant with fourth-order polynomial time function. Arrows indicate the time point at which plant heights show significant differences between treatments.

© 2010 The Authors

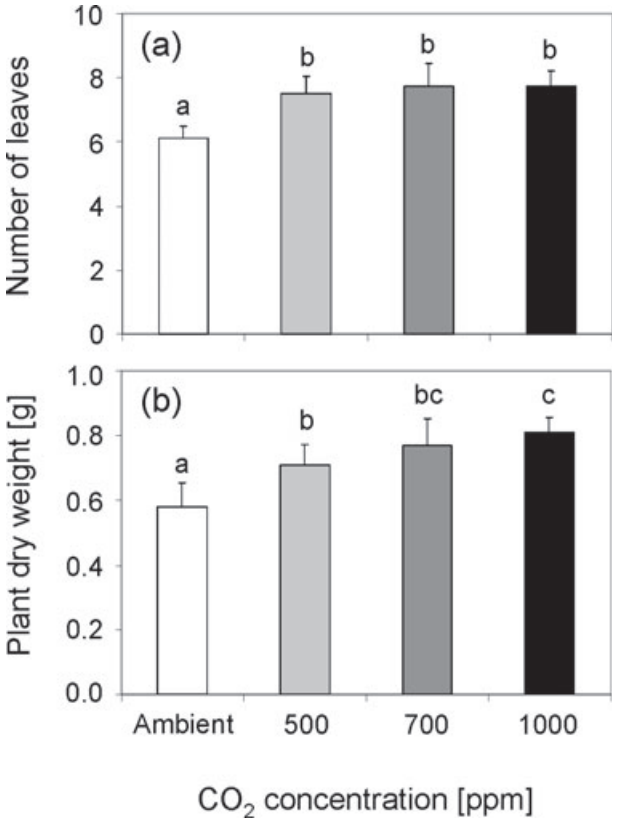

Fig. 2. Leaf number and plant biomass under enhanced CO_2 conditions. Number of leaves (a) and total plant dry weight of lima bean plants (b) growing under different CO_2 concentrations were determined. Letters at the columns represent significant differences among treatment groups [according to *post hoc* analysis (LSD, $P < 0.05$) after one-way ANOVA]. Values represent means ± SD.

By the time of harvest (after 31 days of cultivation), plants grown under all CO_2 treatments revealed significantly higher leaf numbers and total biomass than the controls [according to *post hoc* analysis (LSD, $P < 0.05$) after one-way ANOVA; Fig. 2a,b)]. Total dry weight of plants treated with 1000 ppm CO_2 was significantly higher compared with plants growing under a CO_2 atmosphere of 500 ppm. Plants treated with 700 ppm CO_2 took an intermediate position to both other treatment groups and differences in dry weight of these plants to the other groups were not significant (Fig. 2b).

Leaf cyanogenic potential (HCNp)

Both leaf age and treatment with different CO_2 concentrations significantly affected cyanogenic potential (HCNp) of lima bean plants (according to GLM, Table 1). Among all treatments, young leaves exhibited a significantly higher HCNp than intermediate leaves, while mature leaves consistently showed significantly lower values than intermediate leaves [according to *post hoc* analysis (LSD, $P < 0.05$) after one-way ANOVA; Fig. 3a]. Comparing leaves of the same ontogenetic stage revealed characteristic treatment effects (Fig. 3a). Cyanogenic potential of young and mature leaves was significantly reduced by all three treatments compared with corresponding leaf stages of the controls, whereas intermediate leaves showed significantly reduced HCNp when treated with CO_2 concentrations of 700 and 1000 ppm. Among all leaf developmental stages, we observed a distinct quantitative association between reduction of HCNp and intensities of CO_2 treatments.

Table 1. Effects of CO_2 treatment and leaf stage on chemical and physical leaf traits.

Source	Dependent variable	SS	d.f.	*F*	*P*
Model	HCNp	5395.089	11	68.690	<0.001
	Phenolics	1.738	11	58.435	<0.001
	Protein	498.487	11	48.989	<0.001
	LMA	26.453	11	78.902	<0.001
Leaf stage	HCNp	4590.845	2	321.476	<0.001
	Phenolics	1.456	2	269.247	<0.001
	Protein	458.609	2	247.885	<0.001
	LMA	20.523	2	336.673	<0.001
Treatment	HCNp	622.412	3	29.057	<0.001
	Phenolics	0.197	3	24.334	<0.001
	Protein	31.406	3	11.317	<0.001
	LMA	3.671	3	40.144	<0.001
Leaf stage × Treatment	HCNp	181.832	6	4.244	<0.01
	Phenolics	0.085	6	1.527	<0.001
	Protein	8.473	6	5.216	0.179
	LMA	2.260	6	12.357	<0.001
Error	HCNp	599.781	84	—	—
	Phenolics	0.227	84	—	—
	Protein	77.703	84	—	—
	LMA	2.560	84	—	—

Results obtained using the GLM for analysis of variance with cyanogenic potential (HCNp), total phenolics, soluble protein concentration, and leaf mass per area (LMA) as variables. The terms 'Leaf stage' and 'Treatment' were set as fixed factors.

Total phenolics

Among all CO_2 treatments and the controls, young leaves always showed the lowest and mature leaves the highest concentrations of total phenolics, while intermediate leaves had intermediate levels (Fig. 3b). Thus, phenolics and HCNp exhibited an inverse distribution across the three leaf age groups. The GLM revealed a significant effect of leaf age and treatment on total phenolics concentration (Table 1). In young leaves, concentration of total phenolics was not significantly affected by any of the CO_2 treatments, whereas in intermediate leaves CO_2 treatments with 700 and 1000 ppm resulted in a significantly higher accumulation of phenolics (Fig. 3b). Total phenolics concentration in mature leaves was significantly enhanced in response to all three CO_2 treatments [according to *post hoc* analysis (LSD, $P < 0.05$) after one-way ANOVA; Fig. 3b].

© 2010 The Authors

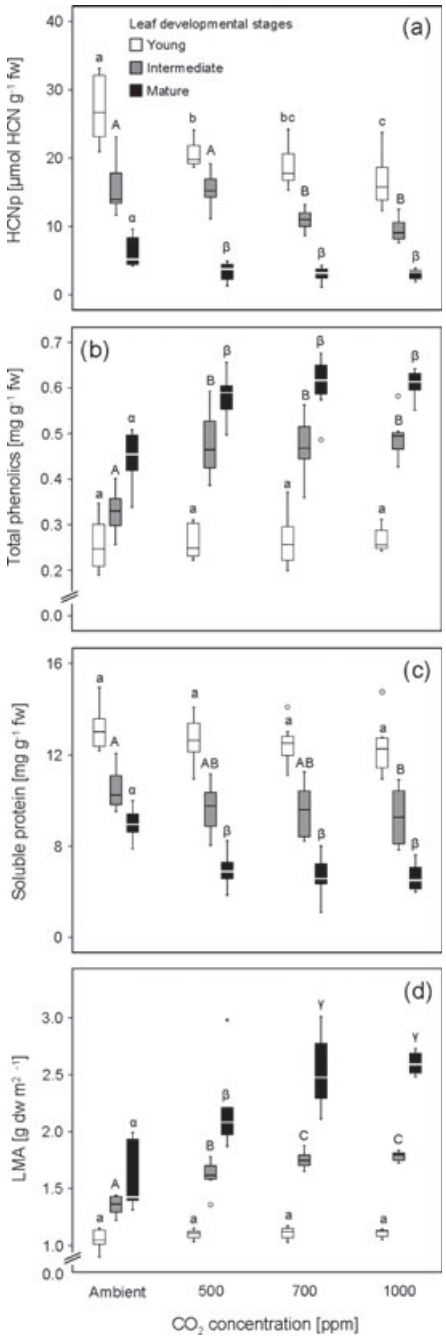

Fig. 3. Chemical and physical leaf parameters. Different leaf ages of plants growing under enhanced CO_2 atmospheres were analysed for cyanogenic potential (a), concentration of total phenolics (b), and soluble proteins (c). In addition to chemical leaf parameters, leaf mass per area (LMA) was quantified (d) as a physical trait affecting palatability for herbivores. Among treatment groups, differences in leaf parameters were analysed separately for each leaf age and significant differences are indicated by different letters (lower-case letters for young leaves, capital letters for intermediate leaves, and Greek letters for mature leaves) at the columns [according to *post hoc* analysis (LSD: $P < 0.05$) after one-way ANOVA]. Values represent means ± SD; open circles indicate outliers; asterisks indicate extremes. fw, fresh weight; dw, dry weight.

Soluble protein concentration

Concentration of soluble proteins was significantly affected by both leaf age and treatment (according to GLM, Table 1). However, the interaction of both factors revealed no significant effect on leaf protein concentration (Table 1). In all treatment groups and the controls, concentration of proteins was higher in young compared with intermediate and mature leaf developmental stages (Fig. 3c). Among all leaf developmental stages CO_2 treatments resulted in a reduction of protein concentration. However, treatment effects were relatively weak. No significant differences in protein concentration could be observed for young leaves and among intermediate leaves significant differences occurred only between control leaves and leaves grown under the highest CO_2 concentrations (Fig. 3). Among mature leaves, a significant reduction of soluble protein concentration was observed between control leaves and leaves developed under a CO_2 atmosphere of 500 ppm, whereas further enhanced CO_2 concentrations had no significant effects on protein concentration [according to *post hoc* analysis (LSD, $P < 0.05$) after one-way ANOVA; Fig. 3c].

Leaf mass per area (LMA)

Independent of CO_2 treatments, LMA increased with leaf age (Fig. 3d). However, the GLM revealed significant effects of both leaf age and treatment on LMA (Table 1). While treatment had no effect on LMA of young leaves, LMA of intermediate and mature leaf stages was significantly increased in response to all three CO_2 treatments [according to *post hoc* analysis (LSD, $P < 0.05$) after one-way ANOVA; Fig. 3d].

Leaf area consumption and body mass accumulation of larvae

The factor 'Leaf stage' and the interaction of 'Leaf stage × Treatment' had significant effects on leaf area consumption by Mexican bean beetle larvae whereas CO_2 'Treatment' alone did not significantly affect larval feeding (according to GLM, Table 2). However, comparing missing leaf area of young as well as intermediate leaves among the different treatments showed a continuous increase of leaf consumption with increasing CO_2 concentrations (Table S1). In contrast to young and intermediate leaves, consumption of mature leaves decreased with enhanced CO_2 concentrations (Table S1). Among CO_2 treatments, larval leaf consumption and body mass accumulation were significantly correlated for all three leaf stages [according to Pearson's correlation (young leaves): $r = 0.797$, $P < 0.001$; (intermediate): $r = 0.437$, $P < 0.05$; (mature): $r = 0.834$, $P < 0.001$].

The GLM predicted significant effects of leaf age and treatment on larval body mass (Table 2). Under natural CO_2 concentrations, biomass accumulation of Mexican bean beetle larvae was higher for intermediate and mature leaves compared with young leaves (Fig. 4). Carbon dioxide treatments resulted in substantial shifts in body mass accumulation of larvae depending on leaf age. Larval growth was significantly increased on young leaves treated with CO_2 at all

© 2010 The Authors

Table 2. Effects of CO_2 treatment and leaf stage on larval feeding and biomass accumulation.

Source	Dependent variable	SS	d.f.	*F*	*P*
Model	Leaf area consumption	1175.58	11	4.0847	<0.001
	Biomass accumulation	19.257	11	16.759	<0.001
Leaf stage	Leaf area consumption	645.02	2	12.3266	<0.001
	Biomass accumulation	10.374	2	49.656	<0.001
Treatment	Leaf area consumption	57.25	3	0.7294	0.538
	Biomass accumulation	1.057	3	3.372	<0.05
Leaf stage × Treatment	Leaf area consumption	473.31	6	3.0151	<0.01
	Biomass accumulation	7.826	6	12.487	<0.001
Error	Leaf area consumption	2197.75	84	—	—
	Biomass accumulation	8.775	84	—	—

Results obtained using the GLM for analysis of variance with leaf area consumption and biomass accumulation as variables. The terms 'Leaf stage' and 'Treatment' were set as fixed factors.

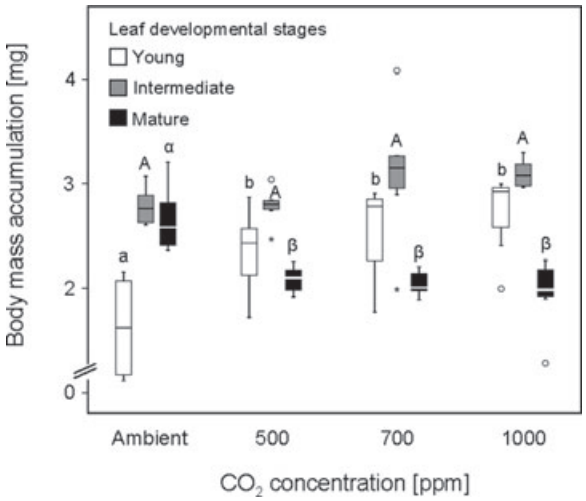

Fig. 4. Larval body mass accumulation. In no-choice feeding experiments leaves of different developmental stages derived from plants cultivated under different CO_2 regimes were offered to the insects and larval body mass accumulation was measured after an experimental period of 2 days. Among treatment groups, differences in larval body mass accumulation were analysed separately for each leaf age group. Significant differences are indicated by lower-case letters for young leaves, capital letters for intermediate leaves, and Greek letters for mature leaves [according to *post hoc* analysis (LSD: $P < 0.05$) after one-way ANOVA]. Values represent means ± SD; open circles indicate outliers; asterisks indicate extremes.

concentrations (500, 700, and 1000 ppm). Similar to young leaves, larval body mass was enhanced on intermediate leaves derived from plants treated with elevated CO_2. However, for all treatments and the controls, differences in larval body mass accumulation on intermediate leaves were not significant. On mature leaves larval body mass was significantly reduced in response to all CO_2 treatments compared with young and intermediate leaves, and compared with the controls (Fig. 4).

Considering plants of all treatment groups, larval body mass accumulation on young leaves was significantly negatively correlated to HCNp (according to Pearson's correlation: $r = -0.913$, $P < 0.001$; $n = 32$), whereas other leaf traits showed no correlation to larval body mass. On intermediate leaves, larval body mass showed no significant correlation to any of the leaf parameters measured, whereas biomass accumulation of larvae feeding on mature leaves was negatively correlated to total phenolic concentration (according to Pearson's correlation: $r = -0.689$, $P < 0.001$) and LMA ($r = -0.599$, $P < 0.001$) and positively correlated to soluble protein ($r = 0.623$, $P < 0.001$) and cyanide concentration ($r = 0.617$, $P < 0.001$).

Choice behaviour of adult beetles on leaves of CO_2 treated plants

Feeding behaviour of beetles on leaves of different developmental stages was affected by CO_2 treatment of the plants (Fig. 5). While 'Treatment' and the interaction of 'Treatment × Leaf stage' significantly affected feeding of beetles among CO_2 treatments 'Leaf stage' alone had no significant effects on feeding (according GLM; Table 3). With focus on young leaves, beetles significantly preferred leaves that had developed under high CO_2 concentration over leaves that had been treated with lower CO_2 concentrations (Fig. 5). However, the increase in leaf area consumption was significant only for leaves treated with the highest CO_2 concentrations (1000 ppm) [according to *post hoc* analysis (LSD, $P < 0.05$) after one-way ANOVA; Fig. 5]. In feeding trials with intermediate leaves, beetles showed significant preference for leaves derived from plants grown at 700 and 1000 ppm CO_2 compared with leaves that had developed under 500 ppm and ambient CO_2. While patterns of choice among differently treated young and intermediate leaves were similar, feeding choice of beetles among mature leaves showed an inverse pattern. Here, leaves that had developed under control conditions were significantly preferred compared with leaves from plants treated with CO_2.

Among all treatments, feeding damage of young leaves was significantly negatively correlated to HCNp (according to Pearson's correlation: $r = -0.538$, $P < 0.01$). In intermediate leaves both HCNp ($r = -0.558$, $P < 0.05$) and protein concentration ($r = -0.439$, $P < 0.05$) showed a negative correlation to consumed leaf area. In mature leaves we observed a negative correlation between leaf damage and total phenolics ($r = -0.728$, $P < 0.001$) as well as LMA ($r = -0.592$, $P < 0.001$). However, we observed a positive correlation between soluble protein concentration ($r = 0.684$, $P < 0.001$), HCNp ($r = 0.745$, $P < 0.001$) and leaf area consumed.

© 2010 The Authors

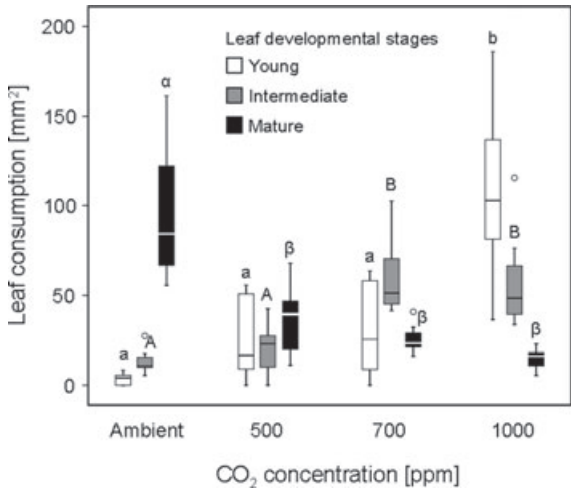

Fig. 5. Feeding choice of beetles. In free-choice feeding trials, leaves of the same age, but subjected to different CO_2 treatments were offered simultaneously to adult beetles. Leaf area consumption was quantified over an experimental period of 2 h. Among treatment groups, differences in leaf consumption were analysed separately for each leaf age group and significant differences are indicated by lower-case letters for young leaves, capital letters for intermediate leaves, and Greek letters for mature leaves [according to *post hoc* analysis (LSD: $P < 0.05$) after one-way ANOVA]. Values represent means ± SD; open circles indicate outliers.

Table 3. Effects of CO_2 treatment and leaf stage on feeding choice of adult beetles.

Source	Dependent variable	SS	d.f.	*F*	*P*
Model	Leaf area consumption	95801.953	11	15.833	<0.001
Leaf stage	Leaf area consumption	536.706	2	0.488	0.616
Treatment	Leaf area consumption	13343.449	3	8.086	<0.001
Leaf stage × Treatment	Leaf area consumption	81921.799	6	24.821	<0.001
Error	Leaf area consumption	46206.522	84	—	—

Results obtained using the GLM for analysis of variance with leaf area consumption as variable. The terms 'Leaf stage' and 'Treatment' were set as fixed factors.

When given the choice to select between different leaf stages from plants cultivated under natural CO_2 concentrations, adult Mexican bean beetles significantly preferred mature over intermediate and young leaves [according to *post hoc* analysis (LSD, $P < 0.05$) after one-way ANOVA; Fig. 6]. Comparing insect feeding on different leaf stages of individual plants revealed a significant negative correlation between feeding damage and both HCNp (according to Pearson's correlation: $r = -0.856$, $P < 0.001$) and soluble protein concentration ($r = -0.869$, $P < 0.001$) whereas total phenolics ($r = 0.756$, $P < 0.001$) and LMA ($r = 0.757$, $P < 0.001$) were positively correlated to leaf consumption.

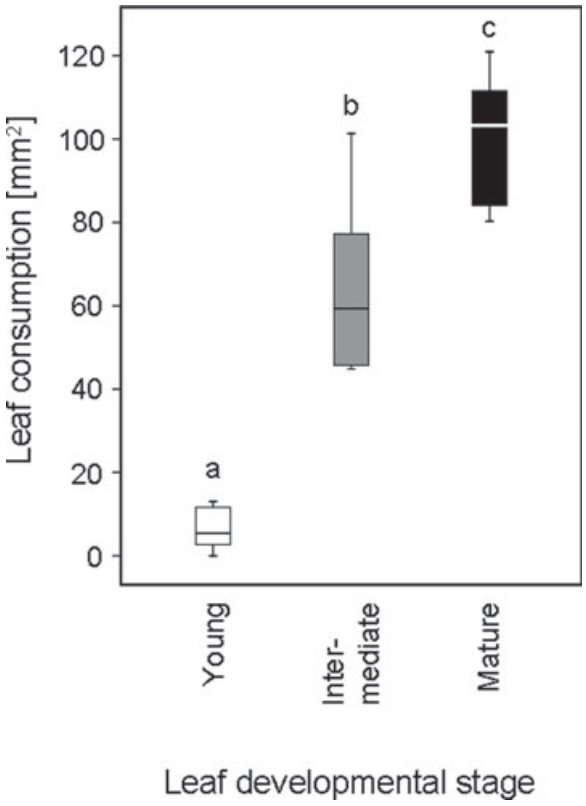

Fig. 6. Feeding on different leaf developmental stages grown at ambient CO_2. In free-choice feeding trials, young, intermediate, and mature leaves were offered simultaneously to adult beetles, and leaf area consumption was quantified. Leaves were derived from plants cultivated under ambient CO_2. Letters over the boxes represent significant differences of leaf area consumption [according to *post hoc* analysis (LSD, $P < 0.05$) after one-way ANOVA].

Discussion

During the last few years much attention has been paid to potential effects of CO_2-mediated changes in plant chemical and physical traits (Parry, 1992; Bazin *et al*., 2002). However, only in few cases have clear quantitative effects of enhanced CO_2 on multiple plant traits, variation of plant responses depending on developmental stage, and consequences of CO_2-mediated changes of plant traits on natural herbivores been demonstrated (e.g. Lindroth *et al*., 1997; Bazin *et al*., 2002; Holton *et al*., 2003; Donaldson & Lindroth, 2007; Gleadow *et al*., 1998, 2009; Zavala *et al*., 2008, 2009). Using a natural plant–herbivore system consisting of wildtype lima bean and Mexican bean beetles, we show here that CO_2-mediated changes in defensive and nutritive plant traits critically depend on leaf age, and that variation of traits in different leaf age groups significantly affects performance and choice behaviour of the insect herbivore.

In our study, lima bean plants showed distinct responses to enhanced ambient CO_2. As expected, plants revealed increased linear growth, leaf number, and biomass production under elevated CO_2 regimes (Fig. 1, Table 1). Generally, photosynthesis

© 2010 The Authors

is intensified under elevated CO_2 by stimulation of the carboxylation function of ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) and inhibition of the oxygenation function (Woodrow, 1994), frequently leading to an increase in plant biomass (Gleadow *et al*., 1998). Raised levels of CO_2 increase photosynthesis and the accumulation of carbohydrates beyond the amount required for growth, maintenance and storage (Bazzaz, 1990). A number of studies report an increase in total non-structural carbohydrates and a decrease in leaf nitrogen content that goes along with enhanced photosynthesis and promoted biomass production (Poorter *et al*., 1997; Peñuelas & Estiarte, 1998; Veteli *et al*., 2002).

In contrast to the numerous observations on effects of elevated CO_2 levels on total leaf nitrogen, quantitative effects of CO_2 on specific nitrogen-containing defensive plant compounds have rarely been reported (Rufty *et al*., 1989; Frehner *et al*., 1997; Gleadow *et al*., 1998, 2009; Goverde *et al*., 1999; Bazin *et al*., 2002). However, differentiation of compounds contributing to the overall leaf nitrogen pool is important, because these compounds may have completely different functions in interactions with higher trophic levels (Mattson, 1980; Baldwin, 1994; Ballhorn *et al*., 2009). In the present study, we demonstrate that enhanced CO_2 levels result in substantial changes of leaf cyanogenic potential (HCNp) that depended on both concentration of CO_2 applied and leaf age. In leaves of all developmental stages, HCNp showed a significant decrease in response to CO_2 treatment. In young and mature leaves, all CO_2 treatments (500, 700, and 1000 ppm CO_2) resulted in significantly reduced HCNp compared with plants grown at ambient CO_2, whereas in intermediate leaves a significant reduction was observed only under the highest CO_2 concentrations (700 and 1000 ppm). Our findings on lowered HCNp in CO_2-treated lima bean plants are in contrast to a recent study by Gleadow *et al*. (2009) who found quantitatively increased cyanide accumulation in another legume species (White clover, *Trifolium repens*) in response to approximately twice-ambient CO_2. In addition to increased cyanide levels, Gleadow *et al*. (2009) reported a CO_2-mediated decrease of total protein by 25%. This is also in contrast to the present study, where protein concentrations in young lima bean leaves were not significantly reduced under any of the CO_2 treatments, and in intermediate leaves were reduced only under highest CO_2 concentrations (1000 ppm). Only in mature leaves we observed a significant reduction of leaf soluble protein in response to all CO_2 treatments.

Our results on variation of nitrogen-based defensive (cyanogenic glycosides) and nutritive (soluble proteins) plant compounds among different leaf ages suggest that: (i) even relatively closely related plant species such as lima bean and clover (Fabaceae, Papilionoideae) show substantial differences in their responses to increased atmospheric CO_2 and (ii) considering total foliage for evaluating effects of enhanced CO_2 is not suitable to identify small-scale shifts in plant biochemistry. Such small-scale variation may have limited impact on plant interaction with large grazing mammals consuming entire plants, but might be of crucial importance for interaction of plants with herbivorous insects, which often consume specific plant organs and tissues or specific ontogenetic stages of plant parts. In most food webs, insect herbivores are one of the major conduits of energy flow between the primary producers (autotrophs) and the rest of the food web (Becerra, 1997; Farrell & Mitter, 1998). Thus, small-scale CO_2-mediated shifts in food plant quality, as we report here, may critically influence interaction of plants and their insect herbivores, ecosystem stability and, in the long-run co-evolution of plants and insects in a currently potentially underestimated way.

In this study we also focused on phenolics a widely occurring group of carbon-based defensive compounds (Nomura & Itioka, 2002; Matsuki *et al*., 2004). Phenolics inhibit the digestion of proteins in various herbivores and, thus commonly act as plant defences (Bryant *et al*., 1983; Jones & Hartley, 1999; Hartley *et al*., 2000). In lima bean, concentration of phenolics increased intrinsically with leaf age (Fig. 3b) and thus showed a converse quantitative pattern to the accumulation of cyanogenic glycosides in leaves. While in young leaves the naturally low concentrations of phenolics remained unaffected by CO_2 treatments, in intermediate and mature leaves they were significantly increased in response to all CO_2 treatments (Fig. 3b). Consequently, the dichotomy of investment in different defences, i.e. cyanide or phenolics depending on leaf age was further promoted by increased CO_2 levels.

In addition to allocation in carbon-based defensive plant compounds, excess carbon, fixed under elevated CO_2 regimes, can be allocated to the production of physical structures such as thick cell walls. These strengthened structures increase LMA and limit palatability of leaf tissues to herbivores (Bezemer & Jones, 1998). In our experimental system, we observed significantly increased levels of LMA for intermediate and mature leaf developmental stages (Fig. 3d), whereas young leaves showed no significant changes in LMA. This is reasonable, as young and actively growing cells commonly show constraints in investment in physical structures (Herms & Mattson, 1992).

Most importantly, we found distinct correlations between quantitative shifts in plant traits and insect responses such as larval feeding, larval body mass accumulation and feeding choice behaviour of adult Mexican bean beetles. At ambient CO_2, leaf area consumption and body mass accumulation of larvae feeding on young lima bean leaves – characterised by naturally high cyanide levels – was lower compared with intermediate and mature leaves accumulating smaller amounts of cyanide (Fig. 4; Table S1). Consumption of young leaves and body mass accumulation increased when plants were treated with CO_2. Thus, a functional association between enhanced body mass of larvae and decreasing HCNp is likely, because in young leaves HCNp was the only trait that was significantly affected by CO_2 treatments (Fig. 3). This interpretation is further supported by our own previous studies, in which the central role of cyanogenesis for performance of this herbivore species has been reported (Ballhorn & Lieberei, 2006; Ballhorn *et al*., 2007, 2008b).

On intermediate leaves, larval leaf consumption and body mass accumulation was increased corresponding to the CO_2-mediated reduction of HCNp – even though differences to larvae feeding on intermediate leaves of control plants were not significant (Fig. 4). Significantly lower protein concentration

© 2010 The Authors

in intermediate leaves of plants treated with CO_2 at high concentration (1000 ppm) had no limiting effects on larval body mass and we observed no compensatory feeding on leaves with reduced protein concentration. In contrast to young and intermediate leaves, in mature leaves CO_2 treatments lead to reduced consumption of leaf area and larval body mass accumulation (Fig. 4; Table 3). The decrease of consumed leaf area and larval growth on mature leaves was significant under all three CO_2 treatments. This finding can be explained by enhancement of defence-associated carbon-based traits in mature leaves of CO_2 treated plants. Compared with younger leaf stages, in mature leaves we observed a characteristic increase of total phenolics and LMA that quantitatively corresponded to reduced larval performance. However, we also observed a decrease in HCNp in response to CO_2 treatments in mature leaves that apparently contradicts data on increased larval performance (Fig. 3a, Fig. 4). This contradiction can be explained by variation in efficiency of cyanogenesis in different leaf developmental stages. Although efficiently limiting larval performance on younger leaves, cyanogenesis of mature leaves appears less important for plant resistance. Due to their naturally low HCNp, mature leaves are weakly defended against Mexican bean beetles (Ballhorn *et al*., 2008b). Thus, additionally decreased cyanide concentrations in mature leaves of CO_2 treated plants may not substantially affect leaf quality for herbivores. On the other hand, phenolics and LMA (tougher tissues) may be less important defence mechanisms under ambient CO_2 but contributed significantly to leaf defence when quantitatively enhanced under elevated CO_2 concentrations.

We found clear effects of CO_2 treatments on leaf traits and larval body mass accumulation. However, quantitative shifts of leaf traits can directly affect leaf consumption but also the conversion efficiency of ingested food (Scriber, 1977). In our study, CO_2-mediated changes in concentration of cyanide-containing precursors and total phenolics were quantitatively correlated to leaf area consumed by Mexican bean beetle larvae. Furthermore, among all leaf stages and all CO_2 treatments we found positive correlations between consumed leaf area and larval body mass. Our findings indicate that the observed CO_2-mediated effects on herbivore body mass accumulation were due to plant responses directly limiting feeding (such as increased levels of cyanide and phenolics), rather than to differences in conversion efficiency of ingested food.

In choice tests with leaf material grown under natural CO_2 concentration, adult beetles showed a preference of mature over intermediate and young leaves (Fig. 6) (see also Ballhorn *et al*., 2008b). Higher levels of soluble proteins in young leaves and, thus, a potentially higher nutritive value, obviously did not compensate for efficient defence by high HCNp in these leaves. When simultaneously offering young leaves grown under different CO_2 atmospheres, beetles preferred leaves from CO_2 treated plants over the respective controls. The same situation of decreased defence at elevated CO_2 concentrations was observed for intermediate leaves, whereas the insects rejected mature leaves from CO_2 treated plants compared with the controls (Fig. 5). These patterns correspond to the feeding experiments with larvae, and further support the suggestion that reduced HCNp is the crucial component determining enhanced feeding on young and intermediate leaves under elevated CO_2 concentrations, while quantitative changes of carbon-based defences were of higher importance in mature leaves. Thus, both experiments on feeding preference of adult beetles and larval performance revealed a complete shift from highest defence of young leaves under ambient CO_2 to lowest defence of young leaves under enhanced CO_2 levels (Fig. 4 and Fig. 5), whereas the reciprocal pattern was observed for mature leaves (Fig. 4). These findings demonstrate strongly interacting effects of CO_2 concentration and leaf age on plant–herbivore interaction (Table 2).

Our study provides new insights into ontogenetic variability of CO_2-mediated shifts of multiple plant traits and consequences of this variation on higher trophic levels (Tylianakis *et al*., 2008). These different within-plant shifts of defence-associated traits in response to rising CO_2 levels might have strong implications on a plant's overall fitness. Especially the lowered defence of young leaves may have significant effects on plant fitness, because young leaves with a longer life expectancy have a higher value for the plant than old leaves. The within-plant variation of multiple traits under enhanced CO_2 represents an underestimated source of variation that should be considered in future global change research.

Acknowledgements

We wish to thank Christa Kosch (Essen) for her technical support with the climate chambers. The University of Duisburg-Essen is acknowledged for financial support. DJB gratefully acknowledges funding through the Deutsche Forschungsgemeinschaft (DFG grant BA 3966/1-1). Martin Heil (CINVESTAV, Irapuato, Mexico) and Ralf Krüger are acknowledged for providing seed material of lima bean.

Supporting Information

Additional Supporting Information may be found in the online version of this article under the DOI reference: 10.1111/j.1365-2311.2010.01240.x

Table S1. Leaf area consumption of Mexican bean beetle larvae.

Please note: Neither the Editors nor Wiley-Blackwell are responsible for the content or functionality of any supplementary material supplied by the authors. Any queries (other than missing material) should be directed to the corresponding author for the article.

References

Anderson, P. & Agrell, J. (2005) Within-plant variation in induced defence in developing leaves of cotton plants. *Oecologia*, **144**, 427–434.

Arimura, G.I., Ozawa, R., Nishioka, T., Boland, W., Koch, T., Kuhnemann, F. *et al*. (2002) Herbivore-induced volatiles induce the emission of ethylene in neighboring lima bean plants. *Plant Journal*, **29**, 87–98.

© 2010 The Authors

Baldwin, I.T. (1994) Chemical changes rapidly induced by folivory. *Insect–Plant Interactions* (ed. by E. A. Bernays), pp. 1–23. CRC Press, Boca Raton, Florida.

Ballhorn, D.J. & Lieberei, R. (2006) Oviposition choice of Mexican bean beetle (*Epilachna varivestis*) depends on host plant cyanogenic capacity. *Journal of Chemical Ecology*, **32**, 1861–1865.

Ballhorn, D.J., Lieberei, R. & Ganzhorn, J.U. (2005) Plant cyanogenesis of *Phaseolus lunatus* and its relevance for herbivore–plant interaction: the importance of quantitative data. *Journal of Chemical Ecology*, **31**, 1445–1473.

Ballhorn, D.J., Heil, M. & Lieberei, R. (2006) Phenotypic plasticity of cyanogenesis in Lima bean *Phaseolus lunatus* – activity and activation of β-glucosidase. *Journal of Chemical Ecology*, **32**, 261–275.

Ballhorn, D.J., Heil, M., Pietrowski, A. & Lieberei, R. (2007) Quantitative effects of cyanogenesis on an adapted herbivore. *Journal of Chemical Ecology*, **33**, 2195–2208.

Ballhorn, D.J., Kautz, S., Lion, U. & Heil, M. (2008a) Trade-offs between direct and indirect defense of lima bean (*Phaseolus lunatus*). *Journal of Ecology*, **96**, 971–980.

Ballhorn, D.J., Schiwy, S., Jensen, M. & Heil, M. (2008b) Quantitative variability of direct chemical defense in primary and secondary leaves of lima bean (*Phaseolus lunatus*) and consequences for a natural herbivore. *Journal of Chemical Ecology*, **34**, 1298–1301.

Ballhorn, D.J., Kautz, S., Heil, M. & Hegeman, A.D. (2009) Cyanogenesis of wild lima bean (*Phaseolus lunatus* L.) is an efficient direct defence in nature. *PLoS ONE*, **4**, e5450, DOI: 10.1371/journal.pone.0005450

Ballhorn, D., Pietrowski, A. & Lieberei, R. (2010a) Direct trade-off between cyanogenesis and resistance to a fungal pathogen in lima bean (*Phaseolus lunatus* L.). *Journal of Ecology*, **98**, 226–236.

Ballhorn, D.J., Kautz, S. & Lieberei, R. (2010b) How generalist and specialist herbivores respond to various cyanogenic plant features. *Entomologia Experimentalis et Applicata*, **134**, 245–259.

Bazin, A., Goverde, M., Erhardt, A. & Shykoff, J.A. (2002) Influence of atmospheric carbon dioxide enrichment on induced response and growth compensation after herbivore damage in *Lotus corniculatus*. *Ecological Entomology*, **27**, 271–278.

Bazzaz, F.A. (1990) The response of natural ecosystems to rising global CO_2 levels. *Annual Review of Ecology, Evolution, and Systematics*, **21**, 167–196.

Becerra, J.X. (1997) Insects on plants: macroevolutionary chemical trends in host uses. *Science*, **276**, 253–256.

Bezemer, T.M. & Jones, T.H. (1998) Plant–insect herbivore interactions in elevated CO_2: quantitative analyses and guild effects. *Oikos*, **82**, 212–222.

Bidart-Bouzat, M.G. & Imeh-Nathaniel, A. (2008) Global change effects on plant chemical defenses against insect herbivores. *Journal of Integrative Biology*, **50**, 1339–1354.

Bidart-Bouzat, M.G., Mithen, R. & Berenbaum, M.R. (2005) Elevated CO_2 influences herbivory-induced defense responses of *Arabidopsis thaliana*. *Oecologia*, **145**, 415–424.

Bradford, M.M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein–dye binding. *Analytical Biochemistry*, **72**, 248–254.

Bryant, J.P. & Julkunen-Tiitto, R. (1995) Ontogenic development of chemical defense by seedling resin birch: energy cost of defense production. *Journal of Chemical Ecology*, **21**, 883–896.

Bryant, J.P., Chapin, S.F. & Klein, D.R. (1983) Carbon/nutrient balance of boreal plants in relation to vertebrate herbivory. *Oikos*, **40**, 357–368.

Calvo, D. & Molina, J.M. (2005) Effects of tangerine (*Citrus reticulata*) foliage age on *Streblote panda*. Larval development and performance. *Phytoparasitica*, **33**, 450–459.

Coley, P.D. (1980) Effects of leaf age and plant life history patterns on herbivory. *Nature*, **284**, 545–546.

Coley, P.D. (1988) Effects of plant growth rate and leaf lifetime on the amount and type of antiherbivore defense. *Oecologia*, **74**, 531–536.

Coley, P.D., Bryant, J.P. & Chapin, F.S. III (1985) Resource availability and plant antiherbivore defense. *Science*, **230**, 895–899.

Coley, P.D., Massa, M., Lovelock, C.E. & Winter, K. (2002) Effects of elevated CO_2 on foliar chemistry of saplings of nine species of tropical tree. *Oecologia*, **133**, 62–69.

Cotrufo, M., Ineson, P. & Scott, A. (1998) Elevated CO_2 reduces the nitrogen concentration of plant tissues. *Global Change Biology*, **4**, 43–54.

Coviella, C.E., Stipanovic, R.D. & Trumble, J.T. (2002) Plant allocation to defensive compounds: interaction between elevated CO_2 and nitrogen in transgenic cotton plants. *Journal of Experimental Botany*, **53**, 323–331.

Donaldson, J.R. & Lindroth, R.L. (2007) Genetics, environment, and their interaction determine efficacy of chemical defense in trembling aspen. *Ecology*, **88**, 729–739.

Farrell, B.D. & Mitter, C. (1998) The timing of insect–plant diversification: might *Tetraopes* (Coleoptera: Cerambycidae) and *Asclepias* (Asclepiaceae) have co-evolved? *Biological Journal of the Linnean Society*, **63**, 553–557.

Frehner, M., Lüscher, A., Hebeisen, T., Zanetti, S., Schubiger, F. & Scalet, M. (1997) Effects of elevated partial pressure of carbon dioxide and season of the year on forage quality and cyanide concentration of *Trifolium repens* L. from a FACE experiment. *Acta Oecologia*, **18**, 297–304.

Ganzhorn, J.U. (1992) Leaf chemistry and the biomass of folivorous primates in tropical forests: test of a hypothesis. *Oecologia*, **91**, 540–547.

Gleadow, R.M., Foley, W.J. & Woodrow, I.E. (1998) Enhanced CO_2 alters the relationship between photosynthesis and defence in cyanogenic *Eucalyptus cladocalyx* F. Muell. *Plant, Cell and Environment*, **21**, 12–22.

Gleadow, R.M., Edwards, E.J. & Evans, J.R. (2009) Changes in nutritional value of cyanogenic *Trifolium repens* grown at elevated atmospheric CO_2. *Journal of Chemical Ecology*, **35**, 476–478.

Goverde, M., Bazin, A., Shykoff, J.A. & Erhardt, A. (1999) Influence of leaf chemistry of *Lotus corniculatus* (Fabaceae) on larval development of *Polyommatus icarus* (Lepidoptera, Lycaenidae): effects of elevated CO_2 and plant genotype. *Functional Ecology*, **13**, 801–810.

Hamilton, J.G., Zangerl, A.R., Berenbaum, M.R., Pippen, J., Aldea, M. & DeLucia, E.H. (2004) Insect herbivory in an intact forest understory under experimental CO_2 enrichment. *Oecologia*, **138**, 566–573.

Hamilton, J.G., Dermody, O., Aldea, M., Zangerl, A.R., Rogers, A., Berenbaum, M.R. *et al*. (2005) Anthropogenic changes in tropospheric composition increase susceptibility of soybean to insect herbivory. *Environmental Entomology*, **34**, 479–485.

Hartley, S.E., Jones, C.G., Couper, G.C. & Jones, T.H. (2000) Biosynthesis of plant phenolic compounds in elevated atmospheric CO_2. *Global Change Biology*, **6**, 497–506.

Herms, D.A. & Mattson, W.J. (1992) The dilemma of plants: to grow or defend. *Quarterly Review of Biology*, **67**, 283–335.

Holton, M.K., Lindroth, R.L. & Nordheim, E.V. (2003) Foliar quality influences tree-herbivore-parasitoid interactions: effects of elevated CO_2, O_3, and genotype. *Oecologia*, **137**, 233–244.

Houghton, J.T., Meira Filho, L.G., Callander, B.A., Harris, N., Kattenburg, A. & Maskell, K. (eds) (1996) The science of climate change. *Intergovernmental Panel on Climate Change*, 572 pp. Cambridge University Press, Cambridge, U.K.

© 2010 The Authors

Jones, C.G. & Hartley, S.E. (1999) A protein competition model of phenolic allocation. *Oikos*, **86**, 27–44.

Lambers, H. (1993) Rising CO_2, secondary plant metabolism, plant–herbivore interactions and litter decomposition. Theoretical considerations. *Vegetatio*, **104**, 263–271.

Lill, J.T. & Marquis, R.J. (2001) The effects of leaf quality on herbivore performance and attack from natural enemies. *Oecologia*, **126**, 418–428.

Lincoln, D.E., Fajer, E.D. & Robert, J.H. (1993) Plant–insect herbivore interactions in elevated CO_2 environments. *Trends in Ecology & Evolution*, **8**, 64–68.

Lindroth, R.L., Roth, S., Kruger, E.L., Volin, J.C. & Koss, P.A. (1997) CO_2-mediated changes in aspen chemistry: effects on gypsy moth performance and susceptibility to virus. *Global Change Biology*, **3**, 279–289.

Long, S., Ainsworth, E., Rogers, A. & Ort, D.R. (2004) Rising atmospheric carbon dioxide: plants FACE the future. *Annual Review of Plant Biology*, **55**, 591–628.

Mansfield, J.L., Curtis, P.S., Zak, D.R. & Pregitzer, K.S. (1999) Genotypic variation for condensed tannin production in trembling aspen (*Populus tremuloides*, Salicaceae) under elevated CO_2 and in high- and low-fertility soil. *American Journal of Botany*, **86**, 1154–1159.

Matsuki, S., Sano, Y. & Koike, T. (2004) Chemical and physical defence in early and late leaves in three heterophyllous birch species native to northern Japan. *Annals of Botany*, **93**, 141–147.

Mattson, W.J. (1980) Herbivory in relation to plant nitrogen. *Annual Review of Ecology, Evolution and Systematics*, **11**, 119–161.

McKey, D. (1974) Adaptive patterns in alkaloid physiology. *American Naturalist*, **108**, 305–320.

McKey, D. (1979) The distribution of secondary compounds within plants. *Herbivores: Their Interactions with Secondary Plant Metabolites* (ed. by G. A. Rosenthal and D. H. Janzen), pp. 55–133. Academic Press, New York, New York.

Milligan, J.R., Krebs, R.A. & Mal, T.K. (2008) Separating developmental and environmental effects on fluctuating asymmetry in *Lythrum salicaria* and *Penthorum sedoides*. *International Journal of Plant Sciences*, **169**, 625–630.

Mulchi, C., Slaughter, L., Saleem, M., Lee, E.H., Pausch, R. & Rowland, R. (1992) Growth and physiological characteristics of soybean in open-top chambers in response to ozone and increased atmospheric CO_2. *Agriculture, Ecosystems and Environment*, **38**, 107–118.

Mumm, R., Posthumus, M.A. & Dicke, M. (2008) Significance of terpenoids in induced indirect plant defence against herbivorous arthropods. *Plant Cell and Environment*, **31**, 575–585.

Nomura, M. & Itioka, T. (2002) Effects of synthesized tannin on the growth and survival of a generalist herbivorous insect, the common cutworm, *Spodoptera litura* Fabricius (Lepidoptera: Noctuidae). *Applied Entomology and Zoology*, **37**, 285–289.

Parry, M. (1992) The potential effect of climate changes on agriculture and land use. *Advances in Ecological Research*, **22**, 63–91.

Peñuelas, J. & Estiarte, M. (1998) Can elevated CO_2 affect secondary metabolism and ecosystem function? *Trends in Ecology & Evolution*, **13**, 20–24.

Poorter, H., van Berkel, Y., Baxter, R., Den Hertog, J., Dijkstra, P., Gifford, R.M. *et al*. (1997) The effect of elevated CO_2 on the chemical composition and construction costs of leaves of 27 C3 species. *Plant, Cell and Environment*, **20**, 472–482.

Prather, M. & Ehhalt, D. (2001) Chapter 4. Atmospheric chemistry and greenhouse gases. *Climate Change 2001: The Scientific Basis* (ed. by J. T. Houghton, Y. Ding, D. S. Griggs, M. Nogver, P. J. van der Linden, X. Dai. *et al*.), pp. 239–287. Cambridge University Press, Cambridge, U.K.

Radhika, V., Kost, C., Bartram, S., Heil, M. & Boland, W. (2008) Testing the optimal defence hypothesis for two indirect defences: extrafloral nectar and volatile organic compounds. *Planta*, **228**, 449–457.

Reichardt, P.B., Bryant, J.P., Clausen, T.P. & Wieland, G.D. (1984) Defense of winter-dormant Alaska paper birch against snowshoe hare. *Oecologia*, **68**, 58–69.

Rhoades, D.F. (1979) Evolution of plant chemical defense against herbivores. *Herbivores: Their Interactions with Secondary Plant Metabolites* (ed. by G. A. Rosenthal and D. H. Janzen), pp. 4–53. Academic Press, New York, New York.

Rostás, M. & Eggert, K. (2008) Ontogenetic and spatio-temporal patterns of induced volatiles in *Glycine max* in the light of the optimal defence hypothesis. *Chemoecology*, **18**, 29–38.

Rufty, T.W. Jr, Jackson, D.M., Severson, R.F., Lam, J.J. & Snook, M.E. (1989) Alterations in growth and chemical constituents of tobacco in response to CO_2 enrichment. *Journal of Agricultural and Food Chemistry*, **37**, 552–555.

Scriber, J.M. (1977) Limiting effects of low leaf-water content on the nitrogen utilization, energy budget, and larval growth of *Hyalophora cecropia*. *Oecologia*, **28**, 269–287.

Stamp, N. (2003) Out of the quagmire of plant defense hypotheses. *Quarterly Review of Biology*, **78**, 23–55.

Tikkanen, O.P. & Julkunen-Tiitto, R. (2003) Phenological variation as protection against defoliating insects: the case study *Quercus robur* and *Operophtera brumata*. *Oecologia*, **136**, 244–251.

Tylianakis, J.M., Didham, R.K., Bascompte, J. & Wardle, D.A. (2008) Global change and species interactions in terrestrial ecosystems. *Ecology Letters*, **11**, 1–13.

Valkama, E., Koricheva, J. & Oksanen, E. (2007) Effects of elevated O_3, alone and in combination with elevated CO_2, on tree leaf chemistry and insect herbivore performance: a meta-analysis. *Global Change Biology*, **13**, 184–201.

Veteli, T.O., Kuokkanen, K., Julkunen-Tiitto, R., Roininen, H. & Tahvanainen, J. (2002) Effects of elevated CO_2 and temperature on plant growth and herbivore defensive chemistry. *Global Change Biology*, **8**, 1240–1252.

Whittaker, J.B., Roininen, H. & Tahvanainen, J. (1999) Impacts and responses at population level of herbivorous insects to elevated CO_2. *European Journal of Entomology*, **96**, 149–156.

Woodrow, I.E. (1994) Optimal acclimation of the C3 photosynthetic system under enhanced CO_2. *Photosynthesis Research*, **39**, 410–412.

Zangerl, A.R. & Bazzaz, F.A. (1992) Theory and pattern in plant defense allocation. *Plant Resistance to Herbivores and Pathogens, Ecology, Evolution and Genetics* (ed. by R. Fritz and E. Simms), pp. 363–391. University of Chicago Press, Chicago, Illinois.

Zavala, J.A., Casteel, C.L., DeLucia, E.H. & Berenbaum, M.R. (2008) Anthropogenic increase in carbon dioxide compromises plant defense against invasive insects. *Proceedings of the National Academy of Sciences of the United States of America*, **105**, 5129–5133.

Zavala, J.A., Casteel, C.L., Nabity, P.D., Berenbaum, M.R. & DeLucia, E.H. (2009) Role of cysteine proteinase inhibitors in preference of Japanese beetles (*Popillia japonica*) for soybean (*Glycine max*) leaves of different ages and grown under elevated CO_2. *Oecologia*, **161**, 35–41.

Zvereva, E.L. & Kozlov, M.V. (2006) Consequences of simultaneous elevation of carbon dioxide and temperature for plant–herbivore interactions: a meta-analysis. *Global Change Biology*, **12**, 27–41.

Accepted 6 September 2010
First published online 22 October 2010

© 2010 The Authors