Acclimation to Elevated CO_2 Increases Constitutive Glucosinolate Levels of *Brassica* Plants and Affects the Performance of Specialized Herbivores from Contrasting Feeding Guilds

J. Klaiber · S. Dorn · A. J. Najar-Rodriguez

Abstract Plants growing under elevated CO_2 concentration may acclimate by modifying chemical traits. Most studies have focused on the effects of environmental change on plant growth and productivity. Potential effects on chemical traits involved in resistance, and the consequences of such effects on plant-insect interactions, have been largely neglected. Here, we evaluated the performance of two *Brassica* specialist herbivores from contrasting feeding guilds, the leaf-feeding *Pieris brassicae* and the phloem-feeding *Brevicoryne brassicae,* in response to potential CO_2-mediated changes in primary and major secondary metabolites (glucosinolates) in *Brassica oleracea*. Plants were exposed to either ambient (400 ppm) or elevated (800 ppm) CO_2 concentrations for 2, 6, or 10 weeks. Elevated CO_2 did not affect primary metabolites, but significantly increased glucosinolate content. The performance of both herbivores was significantly reduced under elevated CO_2 suggesting that CO_2-mediated increases in constitutive defense chemistry could benefit plants. However, plants with up-regulated defenses could also be subjected to intensified herbivory by some specialized herbivores, due to a chemically-mediated phagostimulatory effect, as documented here for *P. brassicae* larvae. Our results highlight the importance of understanding acclimation and responses of plants to the predicted increases in atmospheric CO_2 concentrations and the concomitant effects of these responses on the chemically-mediated interactions between plants and specialized herbivores.

Introduction

Plant chemistry can be strongly affected by environmental changes, including elevated carbon dioxide (CO_2) concentrations, predicted under some climate scenarios (Houghton et al., 2001; Bidart-Bouzat and Imeh-Nathaniel, 2008). Atmospheric CO_2 concentrations have increased since the pre-industrial era and are predicted to double by the end of this century (Houghton et al., 2001). Elevated CO_2 concentrations are expected to increase rates of photosynthesis, which in turn could enhance plant growth, biomass accumulation, plant size (Frenck et al., 2011; Klaiber et al., 2013b), and allocation to plant primary and secondary metabolites (Coley et al., 2002).

Both carbon-based and nitrogen-based secondary metabolites play important roles in plant defense against insect herbivores (Awmack and Leather, 2002). These metabolites can modify insect behavior and performance by acting as repellents or toxins (Bennett and Wallsgrove, 1994), or by reducing plant digestibility (Ahuja et al., 2010). Thus, changes in allocation to secondary metabolites could directly impact plant-insect herbivore interactions (Agrell et al., 2006).

Allocation to carbon-based defense compounds such as phenolics is reported to increase in response to CO_2 enrichment (e.g. Coviella et al., 2002; Ballhorn et al., 2011). In contrast, changes in allocation to nitrogen-based compounds

J. Klaiber · S. Dorn · A. J. Najar-Rodriguez (✉)
ETH Zurich, Institute of Agricultural Sciences/Applied Entomology, Schmelzbergstrasse 9/LFO,
8092 Zurich, Switzerland
e-mail: adriana.najar-rodriguez@ipw.agrl.ethz.ch

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due to CO_2 enrichment have received less attention, and the available evidence is contradictory. For instance, the cyanogenic glycoside content of *Eucalyptus calocalyx* (Gleadow et al., 1998) and *Trifolium repens* (Frehner et al., 1997), and the glucosinolate content of radish *Raphanus sativus*, turnip *Brassica rapa* var. *rapa* (Karowe et al., 1997), oilseed rape *Brassica rapa* var. *oleifera*, cabbage *Brassica oleracea* var. *capitata* (Reddy et al., 2004), and *Arabidopsis thaliana* (Bidart-Bouzat et al., 2005) did not differ between plants gown under ambient and elevated CO_2 concentrations. In contrast, growth under elevated CO_2 led to a decrease in cyanogenic glycoside content of lima bean *Phaseolus lunatus* (Ballhorn et al., 2011) and *Lotus corniculatus* (Goverde and Erhardt, 2003), to a general decrease in the glucosinolate content of rapeseed *Brassica napus* (Himanen et al., 2008) and mustard *Brassica juncea* (Karowe et al., 1997), and to an increase in the glucosinolate content of broccoli *Brassica oleracea* var. *italica* (Schonhof et al., 2007) and Chinese kale *Brassica alboglabra* (La et al., 2009). Thus, it seems that the investment in nitrogen-containing secondary metabolites is species-specific (Karowe et al., 1997).

Most studies, particularly those on crop plants, have tested short periods of exposure to elevated CO_2, neglecting the potential effects that prolonged periods may have on plant allocation to both carbon- and nitrogen-containing secondary metabolites. Acclimation of plants to elevated CO_2 over several weeks may change chemical composition (Coley et al., 2002). In contrast to short periods of exposure, which often led to marginal or negligible effects (Karowe et al., 1997; Bidart-Bouzat et al., 2005), prolonged periods of exposure may lead to pronounced effects, as shown for the allocation of the carbon-containing compounds saponin and cardenolide (Agrell et al., 2004; Vannette and Hunter, 2011). However, to date, no one has comprehensively assessed how a prolonged period of exposure to elevated CO_2 could alter nutrient allocation, particularly to nitrogen-containing compounds, and the effects that such alteration could have on insect herbivores.

Plants from the genus *Brassica* are important annual crops grown worldwide (http://www.faostat.fao.org). They also are important model plants used to study chemically mediated plant-insect interactions (Gutbrodt et al., 2011a), in particular those involving their characteristic nitrogen-containing defense compounds, the glucosinolates (Gols et al., 2008; Gutbrodt et al., 2011a). Glucosinolates themselves have little biological activity (Winde and Wittstock, 2011). However, upon hydrolysis by myrosinases, they are transformed to bioactive products responsible for toxicity and deterrence, such as isothiocyanates, thiocyanates, nitriles, and epithionitriles (Hopkins et al., 2009). Several specialized insect herbivores use *Brassica* species as preferred host plants (Ahuja et al., 2010), and these insects share the ability to tolerate or detoxify glucosinolates and/or their by-products (Hopkins et al., 2009).

CO_2-mediated changes in secondary metabolites could affect the performance of even specialized insects if the threshold levels of tolerance for such metabolites are exceeded in the host plant (Ballhorn et al., 2007). The feeding guild to which an insect belongs might also affect its responses to secondary metabolites (Awmack and Leather, 2002). Whereas several studies have simultaneously looked at the CO_2-mediated effects on specialist and generalist herbivores belonging to the same feeding guild (e.g., Reddy et al., 2004), studies that compare the effects on specialist insect herbivores from contrasting feeding guilds are lacking. Furthermore, few studies have simultaneously assessed the effects of elevated CO_2 on glucosinolate content and insect performance. The studies to date have been limited to a single time interval of plant exposure to elevated CO_2 and to measurements of single insect performance parameters (Reddy et al., 2004; Bidart-Bouzat et al., 2005).

Here, we evaluated the performance of two *Brassica* specialist herbivores from contrasting feeding guilds on plants that might be subject to CO_2-mediated changes in leaf glucosinolate content. *Pieris brassicae* (L.) (Lepidoptera: Pieridae) exclusively feeds on leaves from plants of a limited number of families within the order Brassicales such as Brassicaceae, and represents a major pest of *Brassica* crops (Chew, 1995). *Brevicoryne brassicae* (L.) (Hemiptera: Aphididae) exclusively feeds on phloem sap of brassicacean plants, causing considerable economic damage in *Brassica* crops (Blackman and Eastop, 2000). The annual crop Brussels sprout *Brassica oleracea* L. var. *gemmifera* (Brassicaceae) is regularly subjected to herbivory by these two insect species, but the likely effects of elevated CO_2 on the plant chemical defenses and the concomitant effects on insects are not well understood. Thus, in this study, we measured the effects of exposure of Brussels sprout plants to elevated CO_2 for 2, 6, or 10 weeks on the primary metabolites carbon and nitrogen, and on glucosinolates as secondary metabolites, as well as on the performance of *P. brassicae* and *B. brassicae*.

Methods and Materials

Plant Material and CO_2 Concentrations Four-week-old Brussels sprout plants (*Brassica oleracea* var. *gemmifera*) (Sativa Rheinau AG, Rheinau, Switzerland) were placed in groups of 80 into one of 2 walk-in climate chambers (Conviron PGV36 - Controlled Environments Limited, Winnipeg, MB, Canada) that maintained two different CO_2 concentrations: (1) ambient CO_2 (corresponding to the background concentration of air entering the climate chamber facility; 400 ± 10 ppm) or (2) elevated CO_2 (double the ambient concentration; 800 ± 10 ppm). Plants were grown in these CO_2 concentrations for 2, 6, or 10 weeks at day/night temperatures of 24/20 °C, with a photoperiod of

16:8 h L:D, light intensity of 250 μmol m^{-2} s^{-1} (based on Himanen et al., 2009), and 50 % relative humidity in 16× 16×16 cm pots on 'Klasman Substrat 2' soil (a peat substitute with pH-value 5.5, NPK: 280, 320, 360 mg l^{-1}, 100 mg l^{-1} magnesium, trace elements and chelates; Klasmann-Deilmann GmbH, Geeste, Germany). Both elevated CO_2 concentration and temperature level were chosen based on the moderate climate change scenario values predicted for the year 2060 (Houghton et al., 2001). Plants in each climate chamber were randomized weekly to avoid any positional effects. During exposure to CO_2, plants were watered twice a week and fertilized weekly (Wuxal liquid fertilizer, 5 ml per pot, concentration 0.5 ml/l, N:P:K 10:10:7.5, Maag Syngenta Agro, Dielsdorf, Switzerland), to prevent changes in plant metabolism due to nutritional deficiency (Reddy et al., 2004). All experiments were conducted twice under these fully controlled conditions (similar to Klaiber et al. 2013a, b). Between replications, the CO_2 concentration in each chamber was switched to the other concentration to control for potential chamber effects and exclude pseudoreplication (Agrell et al., 2006) (see also "Statistical Analysis" below). Growth chamber conditions were monitored at 30 min intervals throughout the experiments with a 'Telaire 7001 CO_2 and temperature monitor' (Ge Measurement & Control, Fremont, CA, USA) connected to an HOBO data logger (Onset Computer Corporation, Bourne, MA, USA). The CO_2 concentrations in the chambers were: ambient CO_2: 400±10 ppm day/430± 10 ppm night; elevated CO_2: 800±10 ppm day/830±10 ppm night.

Insects Cabbage white (*P. brassicae*) stock colonies were maintained on Brussels sprout plants at a temperature of 21±2 °C, with a photoperiod of 16:8 h L:D and 50–70 % relative humidity as described in Gutbrodt et al. (2012). The stock colony was first randomly split into two separate lines, and each line was kept separately throughout the experimental phase.

Cabbage aphid (*B. brassicae*) stock colonies originated from parthenogenetic females that were collected in Wädenswil (Zurich region, Switzerland). Stock colonies were kept on Brussels sprout plants that were placed inside insect rearing cages (30×30×30 cm) (BugDorm, Megaview Science CO., Ltd., Taichung, Taiwan) and maintained under controlled conditions (day/night temperatures of 22/18 °C with a photoperiod of 16:8 h L:D and 50 % relative humidity) (Klaiber et al., 2013a, b).

Plant Acclimation to CO_2: Changes in Plant Chemical Parameters To assess the effects of plant exposure to elevated CO_2 on carbon and nitrogen as primary metabolites, and on glucosinolates as secondary metabolites, a group of 10 Brussels sprout plants that had been

exposed to either the ambient or the elevated CO_2 concentration for 2, 6, or 10 weeks were randomly collected for destructive leaf-sampling.

Total leaf carbon and nitrogen content was assessed by C-N elemental analysis. The 7th leaf from each plant was oven-dried at 40 °C to achieve constant mass and milled to a fine powder using a Retsch ball mill MM200 (*Retsch* GmbH, Haan, Germany). A subsample (2.5 mg) was used for analysis using a Flash EA 1112 Series elemental analyzer (Thermo Italy, Rhodano, Italy) coupled to a Finnigan MAT Delta plus XP isotope ratio mass spectrometer (Finnigan MAT, Bremen, Germany) via a six-port valve and a ConFlo III, as described in Plath et al. (2011).

Leaf glucosinolate content was assessed by high-performance liquid chromatography (HPLC). The 6th and 10th leaves from each plant were freeze-dried and milled to a fine powder using a Retsch ball mill MM200 (Retsch GmbH, Haan, Germany). The purification technique used followed the basic Sephadex/sulphatase *Arabidopsis* protocol as per Kliebenstein et al. (2001), with minor modifications. Freeze-dried leaf material (10–12 mg) was placed into deep-well microtiter tubes (Qiagen, Basel, Switzerland) and methanol (400 μl), 0.3 M lead acetate (10 μl), and water (120 μl) were added to the tubes. Samples were centrifuged, and supernatants were added onto Sephadex columns (Sigma-Aldrich, Buchs, Switzerland) together with glucotropaeolin (12 μl) (Phytoplan, Heidelberg, Germany) as internal standard. To desulfate glucosinolates, water (10 μl) and sulfatase (10 μl) (Sigma-Aldrich, Buchs, Switzerland) were added to each column, followed by overnight incubation at room temperature. Desulfoglucosinolates were eluted twice with 60 % (v/v) methanol (100 μl) and finally with water (100 μl). The chromatographic analyses were conducted by HPLC (Agilent 1,200 Series, Santa Clara, CA, USA) equipped with a diode-array detector and a SymmetryShield RP18 column (4.6×150 mm, 5 μm particle size, Waters Corporation, Milford, MA, USA) with a flow rate of 1 ml min^{-1} and constant temperature of 30 °C. A gradient program consisting of 3 min at 100 % H_2O, 23 min from 0 % to 25 % (v/v) acetonitrile, 1 min at 25 % acetonitrile, 9 min from 25 % to 0 % acetonitrile, and a final 4 min at 100 % H_2O was used. Chromatograms were recorded at 229 nm. The concentrations of glucosinolate compounds were calculated by means of the internal standard and based on certified glucosinolate reference materials (Phytoplan, Heidelberg, Germany). Total glucosinolate content per leaf sample was calculated as the sum of all the individual glucosinolates in that sample.

Effect of Plant Acclimation to CO_2 on the Performance of the Leaf-feeding Specialist Pieris brassicae We tested whether plant exposure to elevated CO_2 for 2, 6, or 10 weeks affected the performance of the leaf-feeding *P. brassicae*.

Prior to bioassays, one generation of pre-breeding was conducted in order to obtain adults that had developed entirely under one of the two CO_2 concentrations tested. This also ensured that the insects were not directly related, i.e., were not siblings. We established 20 families at each CO_2 concentration and at each tested length of exposure by pairing one virgin adult female to one virgin adult male, each 1–2 day-old. Both females and males were randomly selected from the two different stock colony lines maintained in our laboratory. Each pair was kept in a separate BugDorm cage (30×30×30 cm) (BugDorm, Megaview Science CO., Ltd., Taichung, Taiwan) and provided with a 4-week-old Brussels sprout plant grown under controlled conditions (21±2 °C with a photoperiod of 16:8 h L:D and 50 % relative humidity) as substrate for egg deposition. All eggs laid by each female during the first 5 days after mating were equally divided into two groups. Each group then was transferred to one of the two experimental CO_2 concentrations. The resulting larvae per family, herein referred to as F1 families, were reared separately until adulthood.

From these F1 adults, 20 virgin females, one per family, were mated to a virgin male each from a different F1 family, thereby avoiding sibling mating, i.e., inbreeding. After mating, females were kept separately in custom-made mesh cages (30×30×70 cm) and were allowed to lay eggs on Brussels sprout plants grown for 2, 6, or 10 week under the same CO_2 concentration under which the females were reared. After 7 days, all females were removed. Eggs were left to hatch and from the resulting larvae per female, herein referred to as F2 families, 8 randomly selected larvae per family were allowed to complete development. Development time, measured as the time period from egg hatching to adult emergence, as well as pupal and adult mass of these eight individuals per family were recorded. To ensure that all individuals were at the same developmental stage at the time of quantification, pupal mass was determined as soon as the black wing marks became visible through the cuticle (AB204-S/FACT Analytical Lab Balance Scale, Mettler Toledo, Switzerland, accuracy: 0.1 mg). Adult mass was measured 1 day after emergence, using the same precision scale. From the resulting F2 adult individuals, one virgin female per family ($N=20$) was randomly selected and mated to a virgin male from a different F2 family. The females were offered 4-week-old Brussels sprout plants grown under controlled conditions (21±2 °C with a photoperiod of 16:8 h L:D and 50 % relative humidity) as a substrate for egg deposition. All females were provided with sugar solution *ad libitum* and allowed to lay eggs until death. Total fecundity, i.e., total number of eggs laid until death, fertility, i.e., percentage of eggs hatching, and longevity of each of these females were recorded.

To quantify the effect of plant acclimation to elevated CO_2 on larval leaf consumption, 7 first instar F2 larvae per CO_2 concentration and tested period of exposure were randomly selected. Each larva was placed onto a leaf of a Brussels sprout plant that had been grown under the corresponding CO_2 concentrations for 2, 6, or 10 weeks. The leaf was covered with a custom-made cage (30×40×7 cm, Sefar Nitex, SEFAR AG, Heiden, Switzerland) to prevent escape. As each larva developed, it was transferred onto a new leaf. This was done at regular intervals until pupation, as to prevent consumption of entire leaves. Each time a larva was removed from a leaf, that leaf was detached from the plant and scanned. The leaf area consumed by each larva was determined by using a reference area of 1 cm^2 and the software Adobe Photoshop CS5 Extended (Adobe Systems Incorporated, CA, USA) (Mody et al., 2007). All areas consumed by each larva were summed to obtain the total consumed leaf area per larva during its development.

To test whether plant nutritional quality has an effect on *P. brassicae* performance, nutritional indices were calculated. Since plant acclimation response to elevated CO_2 was previously found to be particularly pronounced after the 10 week exposure period (Klaiber et al., 2013b), and this period of plant exposure also yielded the highest total leaf glucosinolate content and the most pronounced effect on *P. brassicae* adult mass (this study), nutritional indices were estimated for this period of plant exposure only. Twenty first instar F2 larvae per CO_2 concentration were randomly selected and each was placed onto a leaf of a Brussels sprout plant that had been grown under the corresponding CO_2 concentration for 10 weeks. The leaf was covered with a custom-made cage to prevent escape. All larvae were monitored until pupation and were moved onto new leaves at regular intervals to prevent consumption of whole leaves. Before being placed onto a new leaf, each larva was weighed on the microbalance, and the feces were collected, air-dried, and weighed. The leaf material not consumed by the larva was weighed and scanned, and the unconsumed leaf area (NCA) and the consumed leaf area (CA) per larva were quantified as above. The consumed fresh leaf mass (CM) was calculated based on the NCA, CA, and the fresh mass of the unconsumed leaf area (NCM): CM = (NCM/NCA) x CA (Gutbrodt et al., 2011b). Nutritional indices for each larva were calculated using standard formulas for approximate digestibility AD = [(CM – dry mass of feces)/CM] and efficiency of conversion of ingested food ECI = [larval mass gain/CM] (Waldbauer, 1968).

Effect of Plant Acclimation to CO_2 on the Performance of the Phloem-feeding Specialist Brevicoryne brassicae We also tested whether plant exposure to elevated CO_2 for 2, 6, or 10 weeks has any effect on the performance of colonies and individuals of the specialist aphid *B. brassicae*. Aphid colony performance was assessed as colony growth rate, taken as the difference in aphid numbers on a given day

compared to the aphid numbers recorded on the previous day. Aphid individual performance was characterized using the following traits: (1) the pre-reproductive time of individual aphids from the first (F1) and the second generation (F2) feeding on a plant; (2) the effective fecundity; and (3) the intrinsic rate of increase of F1 aphid individuals. Furthermore, we quantified the relative honeydew excretion rate of F2 individuals. All aphids used in the bioassays originated from colonies exposed to one of the two CO_2 concentrations for 20 days (i.e., 2–3 generations) prior to the bioassays. Thus, at the time of experiment onset, aphids were already exposed to their respective CO_2 concentration.

To assess aphid colony performance, 10 Brussels sprout plants per combination of CO_2 concentration and period of exposure tested were placed into separate custom-made cylindrical mesh cages (70 cm×60 cm diam), and groups of 15 young winged adult aphids (F0 generation) were released onto the cages. They were allowed to colonize the plants and to lay a total of 30 nymphs (F1 generation) per plant. Winged adult aphids were removed, and the F1 nymphs were allowed to develop into adults and to reproduce. The resulting F2 individuals and their progeny were counted for eight consecutive days and a final count was conducted on day 16 after initial infestation. The aphid colony growth rates on each plant, taken as the difference between aphid numbers on a given day and the aphid numbers on the previous day, were calculated as in Najar-Rodriguez et al. (2007).

To assess individual aphid performance, a group of 10 young winged adult aphids (F0 generation) per combination of CO_2 concentration and period of exposure colonized a Brussels sprout plant that had been grown under either CO_2 concentration for 2, 6, or 10 weeks. Each winged aphid was allowed to produce two nymphs, herein referred to as F1 generation, within 24 h. If a single nymph only was produced during this period, the winged aphid was discarded. If, in exceptional cases, more than two nymphs were produced, only the two oldest (largest) were kept. Each F1 nymph was confined alone to a leaf with a custom-made mesh cage. When an F1 nymph turned into an adult, this adult and one of its nymphal progeny (F2 generation) were monitored daily for offspring production (F1) and development until adulthood (F2). This procedure allowed determination of (1a) the pre-reproductive time of each F1 aphid, taken as the number of days from the birth of a F1 nymph to the onset of its reproduction, (1b) the pre-reproductive time of the F2 individuals produced by each F1 aphid (d), (2) the effective fecundity (M_d) of each F1 aphid, taken as the number of nymphs produced by each F1 aphid during a period equivalent to the pre-reproductive time of its F2 nymph, and (3) the intrinsic rate of increase (r_m) using Wyatt and White's (1977) equation $r_m = 0.738$ (ln M_d)/d.

To better understand how aphids deal with a glucosinolate-enriched diet, the excretion rate of honeydew (Mittler and Meikle, 1991) by adult aphids was assessed. We used 20 F2 individuals originating from winged adult aphids (F0) and their progeny (F1) feeding on Brussels sprout plants exposed to the corresponding CO_2 concentration for 2, 6, or 10 weeks, and quantified their honeydew excretion as described in Warrington and Whittaker (1985). Twenty aphids were singly confined for 48 h to the abaxial surface of leaves provided with a film of aluminum foil underneath. The foil containing the honeydew droplets produced by each single aphid was oven-dried to constant mass at 40 °C, weighed on the microbalance, cleaned of honeydew with hot distilled water, dried and reweighed in order to calculate honeydew production by mass difference. To relate honeydew production to the fresh body mass of the aphids reared under the same CO_2 conditions, we also weighed adult F2 aphids ($N=30$ per CO_2 concentration and duration of exposure). The ratio of mean amount of honeydew excreted by either group of aphids to individual aphid body mass yielded the relative honeydew excretion rate.

Statistical Analysis Potential differences between climate chambers were examined by including 'chamber' as a random effect in all analyses. As no significant chamber effects were detected ($P\geq0.05$ in all cases), this variable was excluded from the analyses described below, and data from the two replications were pooled. Plant primary metabolites and glucosinolate contents were analyzed with separate Wilcoxon rank sum tests for each period of exposure to the different CO_2 concentrations. The effects of plant acclimation to elevated CO_2 on *Pieris brassicae* F2 developmental time, total consumed leaf area, approximate digestibility (AD), efficiency of conversion of ingested food (ECI), pupal and adult mass, and adult female fertility and longevity at the different periods of exposure were analyzed with separate Wilcoxon rank sum tests. The effects of plant acclimation to elevated CO_2 on total female fecundity were analyzed with a separate general linear model (GLM), with CO_2 concentration and period of exposure included in the analysis as fixed factors. Interpretation of the GLM was carried out by a first examination of interactions followed by an examination of main effects. When not significant, interactions were excluded from further analysis. Whenever effects were detected, means were subsequently compared by the *Tukey's HSD* test. Prior to analysis, if necessary, data were $\log_{10}(X+1)$ transformed to meet the assumptions of normality and heteroscedasticity for parametric tests. The effects of plant acclimation to elevated CO_2 on aphid colony growth rates from day 1 to 8 and at day 16 of the reproductive period were analyzed with one-way repeated measures ANOVAs (SPSS 20.0 for Mac OS X, Chicago, IL, USA),

after a $\log_{10}$ $(X+1)$ transformation. Whenever effects were detected, means were subsequently compared by the *Tukey's HSD* test. The pre-reproductive time of F1 and F2 aphids, and the effective fecundity and intrinsic rate of increase of F1 individuals at the different periods of exposure tested were analyzed with separate Wilcoxon rank sum tests and one-way ANOVAs, respectively. Aphid honeydew excretion rate was analyzed separately for each period of exposure tested with Wilcoxon rank sum tests. Unless otherwise stated, Software package R, version 2.14.0 (R Development Core Team, 2009) was used for the statistical analysis.

Results

Plant Acclimation to CO_2: Changes in Plant Primary and Secondary Metabolite Content Growing plants under elevated CO_2 for a total of 10 weeks did not significantly affect total plant carbon (C) and nitrogen (N) level or C/N ratios, regardless of the length of exposure tested (Online Resource 1).

The elevated CO_2 concentration, in contrast, affected the total leaf glucosinolate levels as well as the level of individual glucosinolates. Mean values of total glucosinolate levels were consistently higher in plants grown under elevated compared to ambient CO_2 concentrations, with significant differences after 2 or 10 weeks (Fig. 1 and Online Resource 2). Among individual glucosinolates, glucoiberin levels were always significantly higher in plants grown under elevated CO_2 (Fig. 1 and Online Resource 2). Progoitrin and glucoraphanin levels were significantly higher in plants grown under elevated CO_2 after 2 and 6 weeks, while sinigrin and gluconasturtiin levels were significantly higher in plants grown under elevated CO_2 after 2 and 10 weeks (Fig. 1 and Online Resource 2). Gluconapin, 4-hydroxyglucobrassicin and glucobrassicin levels were all significantly higher in plants grown under elevated CO_2 after 2 weeks but not thereafter (Fig. 1 and Online Resource 2). Neoglucobrassicin, detected in low amounts, was found in significantly lower levels in plants grown under elevated compared to ambient CO_2 after 6 weeks of exposure (Fig. 1 and Online Resource 2).

Effects of Plant Acclimation to CO_2 on Pieris brassicae Performance Feeding on plants grown under elevated CO_2 for 2, 6 and 10 weeks markedly affected the performance, i.e., the development time, pupal and adult mass, and adult female longevity, of F2 *P. brassicae* individuals. The development time of F2 individuals was always significantly shorter under elevated CO_2 (Fig. 2a and Online Resource 3). F2 larvae consumed significantly larger amounts of leaf material under elevated CO_2 concentrations, regardless of the length of exposure to CO_2 (Table 1). Despite consuming more

leaf material, the AD, ECI, and pupal mass gained were significantly lower when larvae fed on plants grown under elevated CO_2 for 10 weeks (Table 2). The pupal mass attained was always lower when larvae had fed on plants grown under elevated CO_2 (Fig. 2b and Online Resource 3). Adult mass also was consistently lower when larvae fed on plants grown under elevated CO_2, with differences becoming significant after 6 weeks (Fig. 2c and Online Resource 3). Adult females developed from larvae fed on plants exposed to the elevated CO_2 for 2 weeks had a significantly longer lifespan compared to those fed on plants exposed to the ambient CO_2 concentration, whereas adult females derived from individuals fed on plants exposed to elevated CO_2 for 6 weeks had a significantly shorter adult lifespan when reared under elevated compared to ambient CO_2. Although mean values suggested a similar relationship after 10 weeks of exposure, differences were not significant (Fig. 2d and Online Resource 3). Total F2 female fecundity (Online Resource 5) as well as fertility (Online Resource 6) were the only parameters not significantly affected by any of the CO_2 treatments.

Effect of Plant Acclimation to CO_2 on Brevicoryne brassicae Colony and Individual Performance Feeding on plants grown under elevated CO_2 for a total of 10 weeks affected both the colony and the individual performance of *B. brassicae* aphids. Aphid colony growth rates differed significantly between CO_2 concentrations. Colonies grew more slowly and contained fewer individuals when they developed under elevated compared to ambient CO_2 concentrations, with significant differences noted after 6 and 10 weeks of plant exposure (Fig. 3 and Online Resource 4).

The number of days from birth until onset of reproduction for F1 and F2 individuals was significantly shorter under elevated CO_2 after the 6 week plant exposure (Figs. 4a, d, and Online Resource 3), but significantly longer (approximately 1 day) after the 10 week exposure (Figs. 4a, d, and Online Resource 3). The effective fecundity of the F1 generation aphids was reduced under elevated compared to ambient CO_2 after 6 or 10 weeks of plant exposure to CO_2, with values diminished nearly 20 % and 40 %, respectively (Fig. 4b and Online Resource 3). There was no difference after only 2 weeks of plant exposure to the elevated CO_2 (Fig. 4b and Online Resource 3). The intrinsic rate of increase (r_m) of F1 aphid individuals was significantly reduced by about 20 % under elevated compared to ambient CO_2 after 10 weeks of plant exposure to controlled CO_2 (Fig. 4c and Online Resource 3).

Relative honeydew excretion rate was significantly higher in aphids feeding on plants grown under elevated CO_2 compared to those feeding on plants grown under ambient CO_2 (Fig. 4e and Online Resource 3).

Fig. 1 Glucosinolate content (μmol/g $\pm$ SE) of *Brassica oleracea* var. *gemmifera* plants exposed to either ambient (400$\pm$10 ppm) or elevated CO_2 concentration (800$\pm$10 ppm) for **a** 2, **b** 6, or **c** 10 weeks. $N=10$ plants per CO_2 concentration and period of exposure to CO_2. *P*-values based on Wilcoxon rank sum tests: *=$P\le0.05$, ns = not significant. *GNA* = gluconapin, *GRA* = glucoraphanin, *PRO* = progoitrin, *NEO* = neoglucobrassicin, *SIN* = sinigrin, *4OH-GBS*=4-hydroxyglucobrassicin, *GNT* = gluconasturtiin, *GBS* = glucobrassicin, *GIB* = glucoiberin, *TOTAL* = total sum of all glucosinolate compounds. To account for the considerably higher content, GBS, GIB and TOTAL are displayed on the secondary *y*-axis to the right of the figure
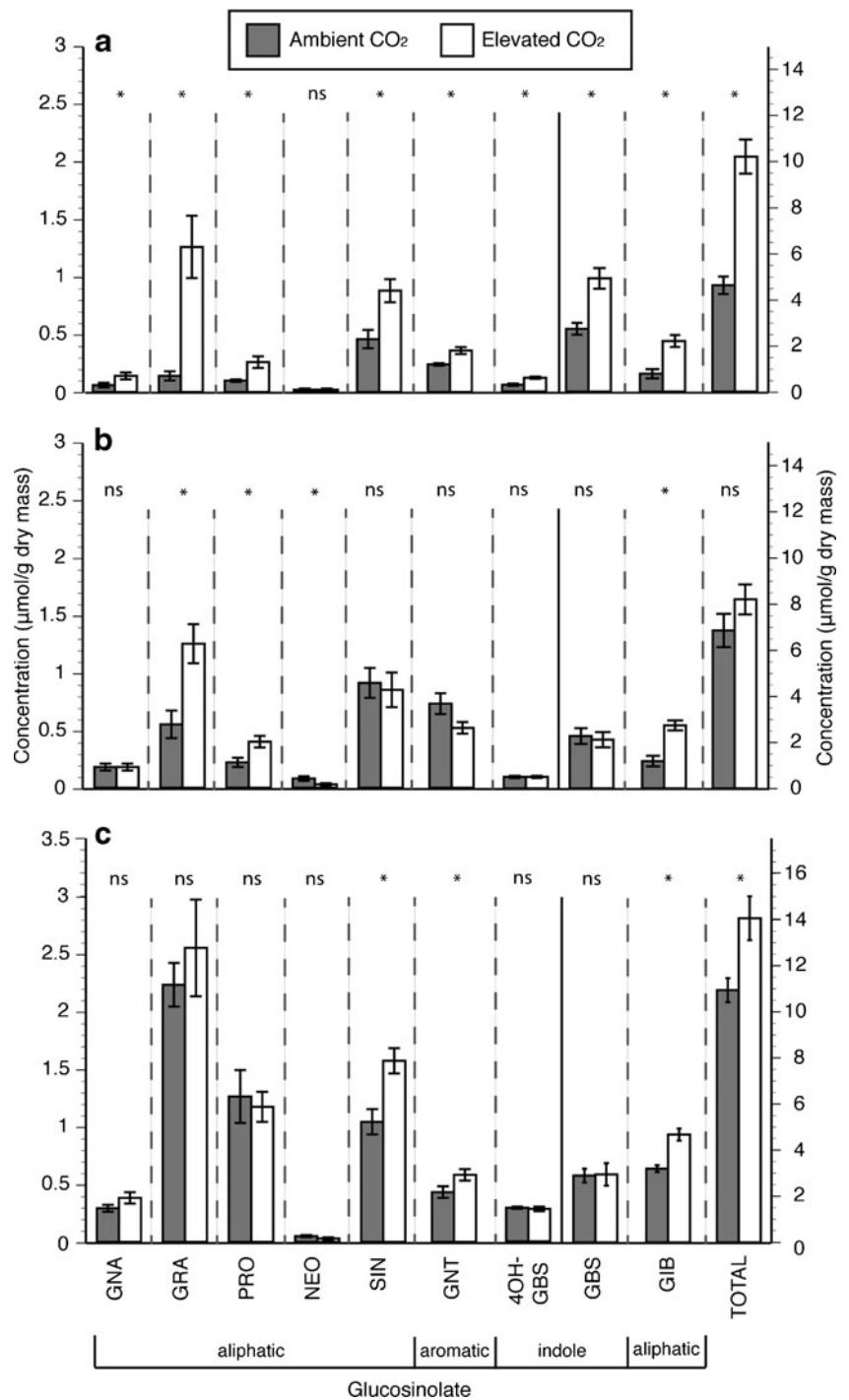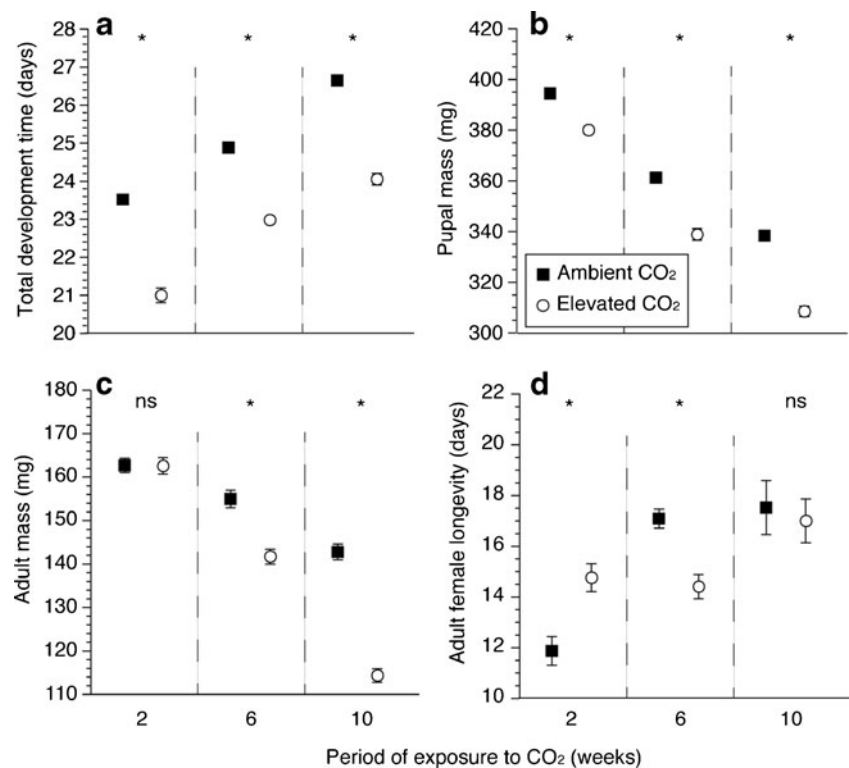

Discussion

CO_2-mediated changes in plant chemical composition, often attributed to plant acclimation or adaptation and occurring within several weeks or months of treatment with elevated CO_2 (Coley et al., 2002; Frenck et al., 2011), could significantly affect interactions between plants and their associated specialized insect herbivores. In this study, exposure of

Brassica plants to elevated CO_2 for up to 10 weeks did not lead to significant changes in primary metabolites, i.e., carbon and nitrogen content. However, it led to a significant increase in constitutive secondary metabolites, specifically the nitrogen-containing, defense-related glucosinolates, with changes being most striking after a 10-week exposure to elevated CO_2. Concomitantly with changes in leaf glucosinolates, two specialized herbivores from contrasting

feeding guilds performed more poorly when feeding on plants grown under elevated CO_2. This suggests that CO_2-mediated changes in constitutive plant defense compounds increase plant resistance to these specialized insects.

Plant acclimation to elevated CO_2 significantly increased the total content of glucosinolates as well as the content of several individual aliphatic glucosinolates after 2, 6, and 10 weeks of plant exposure. Plants may allocate photosynthate and nutrients to growth, storage, reproduction, or defense, depending on the needs of the plant and the availability of the resources (Ryan et al., 2010 and references therein). In the current study, plants were fertilized both under ambient and elevated CO_2 conditions on a weekly basis with low levels of nitrogen, to prevent changes in plant metabolism due to nutrient deficiency (Reddy et al., 2004). This fertilizer treatment was chosen to resemble agricultural settings, which characteristically have enriched soil nitrogen levels (Coviella et al., 2002).

In a previous study of Chinese kale (*Brassica alboglabra*), elevated CO_2 decreased plant carbon and nitrogen levels regardless of nitrogen fertilization levels (La et al., 2009). In kale, the driving factor for variation in glucosinolate content was not the CO_2 concentration, but the nitrogen fertilization level (La et al., 2009). In contrast, nitrogen fertilization, even at a very low concentration, negatively affected the glucosinolate content of broccoli *Brassica oleracea* var. *italica* (Aires et al., 2006). Here, we found no differences in plant carbon and nitrogen content, irrespective of CO_2 concentration, but an increase in glucosinolate content under elevated CO_2. Hence, the relationship between nitrogen supply, plant nitrogen, and glucosinolate content seems to be species-specific, at least in *Brassica* plants.

Table 1 Leaf area consumed ($cm^2 \pm$ SE) by *Pieris brassicae* larvae feeding on *Brassica* plants exposed to either an ambient (400±10 ppm) or an elevated (800±10 ppm) CO_2 concentration for 2, 6, or 10 weeks

| Period of plant exposure to CO_2	Ambient CO_2	Elevated CO_2	*P*
2 weeks	154.13±5.24	176.18±6.95	0.034
6 weeks	88.64±11.37	140.09±14.12	0.026
10 weeks	125.44±8.00	166.21±13.36	0.017

N=7 individuals per CO_2 concentration and period of exposure. *P*-values based on Wilcoxon rank sum tests: $P>0.05$=not significant

Table 2 Approximate digestibility (mean ± SE) and efficiency of conversion of ingested food (mean ± SE) of *Pieris brassicae* individuals developing on *Brassica* plants exposed to either an ambient (400± 10 ppm) or an elevated (800±10 ppm) CO_2 concentration for 10 weeks

Nutritional index	Ambient CO_2	Elevated CO_2	*P*
Approximate digestibility	72.29±1.39	68.78±1.00	0.006
Efficiency of conversion of ingested food	14.94±1.99	11.10±0.94	0.043

N=20 individuals per CO_2 concentration. *P*-values based on Wilcoxon rank sum tests: $P>0.05$=not significant
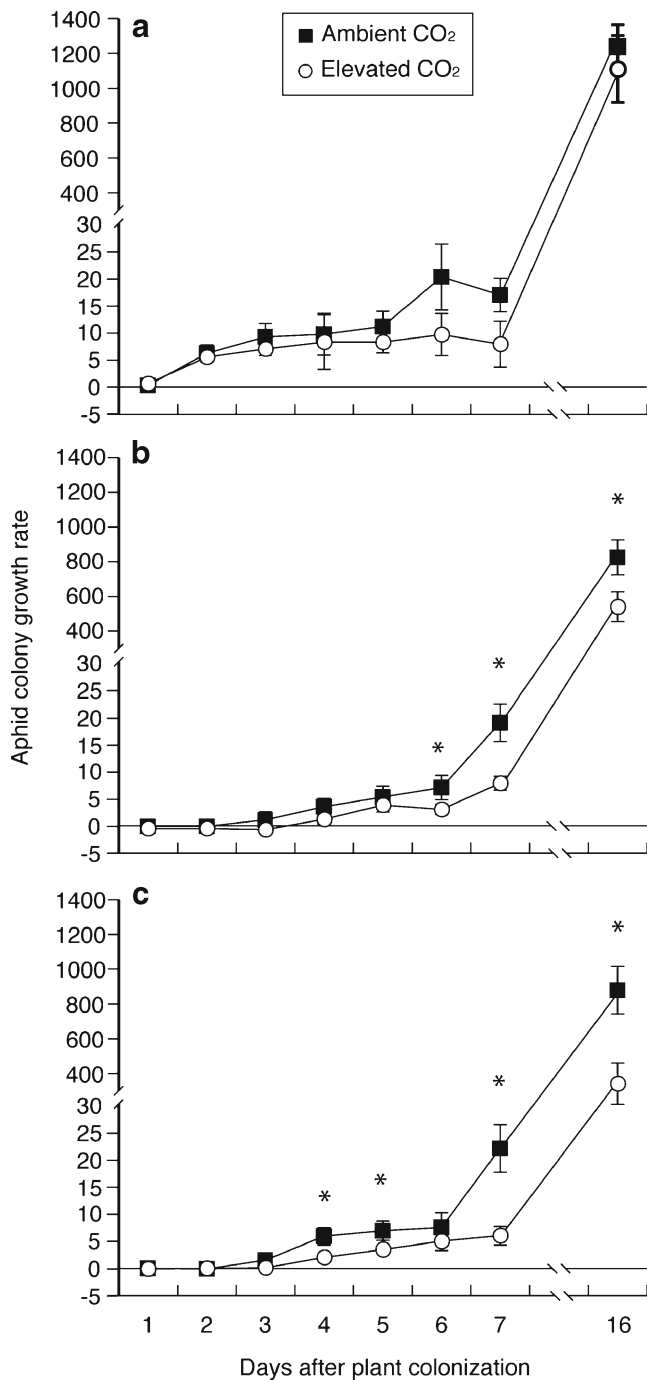
Fig. 3 Colony growth rates of *Brevicoryne brassicae* aphids developing on *Brassica oleracea* var. *gemmifera* plants exposed to either ambient (400±10 ppm) or elevated (800±10 ppm) CO_2 concentration for **a** 2, **b** 6, or **c** 10 weeks. Colony growth rates over the first 7 days were calculated as the difference in the number of aphids on a given day compared to the previous day (with negative values indicating aphid mortality). Colony growth rate at day 16 was calculated as the difference in the number of aphids on day 16 compared with day 8. $N=$ 10 colonies per CO_2 concentration and period of exposure to CO_2. *P*-values based on one-way ANOVAs': *$=P≤0.05$

Leaf nitrogen is regarded as the most important and limiting nutrient for insect herbivores, and a decrease in

nitrogen has been suggested as one of the main reasons for a decrease in insect performance under elevated CO_2 (Awmack and Leather, 2002; Douglas, 2003). However, in our study, nitrogen content was not affected by plant exposure to elevated CO_2. Thus, the increase in larval leaf consumption and the decrease in pupal and adult mass and in food conversion of *P. brassicae*, and the reduction in colony and individual performance of *B. brassicae* aphids, under elevated CO_2 cannot be explained based on plant primary metabolites. In accordance with our results, nitrogen did not play a role in the decrease in pupal mass of *Spodoptera exigua* larvae reared on CO_2-exposed cotton seedlings (Akey and Kimball, 1989), or on the increased consumption and decreased efficiency of conversion of food by *Colias philodice* larvae feeding on legume plants (*Medicago sativa*, *Trifolium repens*, and *Lotus corniculatus*) grown under elevated CO_2 (Karowe, 2007). Overall, these findings suggest that CO_2-mediated effects on plant metabolites other than nitrogen were responsible for the decrease in performance of the two specialized insect herbivores observed here.

Glucosinolates have been suggested to play an important role in determining the nutritional quality of *Brassica* plants for *Brassica*-specialized insect herbivores (Gols et al., 2008). Thus, the CO_2-induced increase in total leaf glucosinolate content, and in the content of some aliphatic glucosinolates, particularly glucoiberin and sinigrin, could have altered the nutritional quality of our *Brassica* plants for both *P. brassicae* and *B. brassicae*, thus affecting their performance. High levels of glucoiberin in white cabbage, *Brassica oleracea* var. *alba*, and in black mustard, *Brassica nigra*, negatively affected larval mass gain in the white butterfly *Pieris rapae* (Poelman et al., 2009). High levels of either sinigrin or glucobrassicin led to a decrease in adult mass of the specialist herbivores *P. rapae* and diamondback moth *Plutella xylostella* (Gols et al., 2008). Similarly, *B. brassicae* colonies were found less frequently on plants containing high foliar levels of glucoiberin and sinigrin compared to plants containing high foliar levels of other aliphatic glucosinolates (Newton et al., 2009; Poelman et al., 2009).

For most *Brassica*-specialized insect herbivores, glucosinolates are cues mediating plant consumption due to a positive phagostimulatory effect (Gabrys et al., 1997; Renwick and Lopez, 1999). For instance, high foliar aliphatic glucosinolate content in *Arabidopsis thaliana* lines stimulated feeding by *P. rapae in vivo* (Müller et al., 2010), and high levels of sinigrin were sufficient to increase leaf consumption by both *P. rapae* (Renwick and Lopez, 1999) and *P. xylostella* larvae *in vitro* (van Loon et al., 2002). In addition, high foliar contents of glucoiberin increased leaf consumption by *Pieris* spp. and *P. xylostella* larvae in the field (Olsson and Jonasson, 1994). In our study, *P. brassicae* larvae consumed significantly more leaf material when

F1 generation
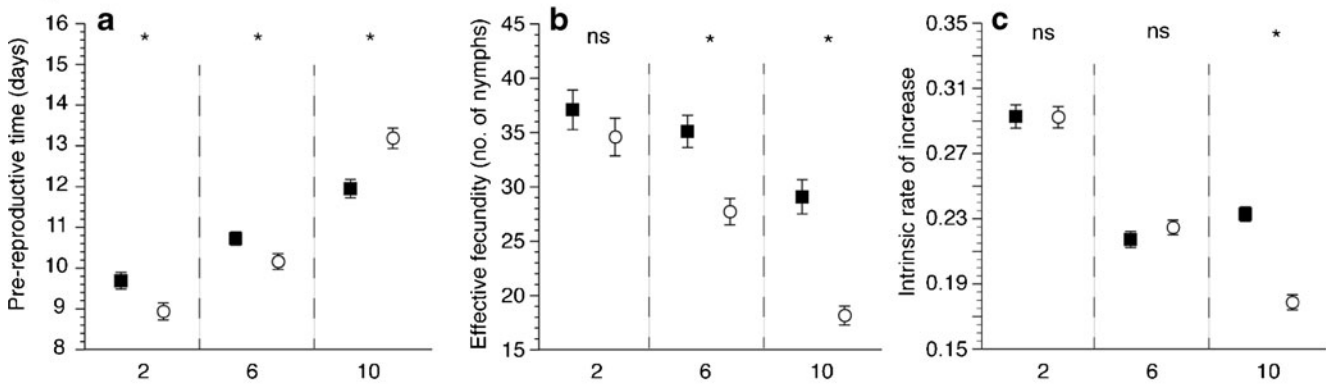
F2 generation
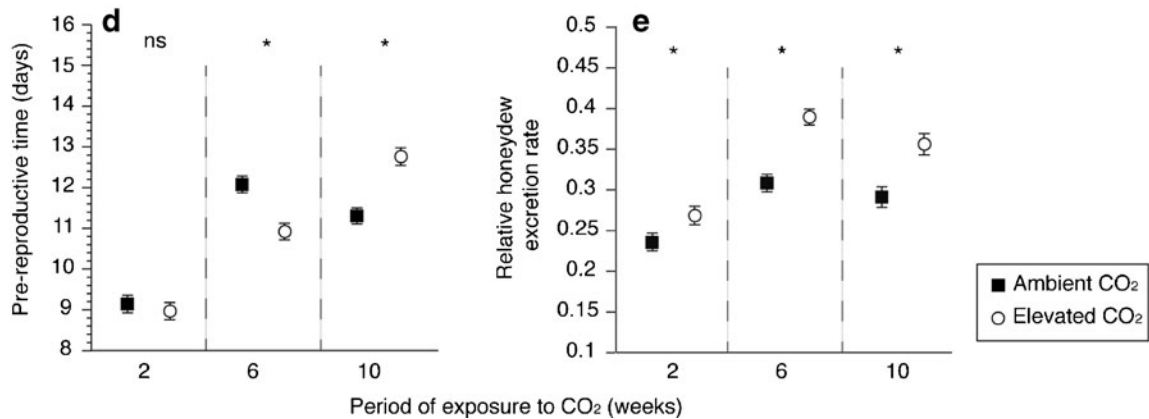
Fig. 4 Performance of *Brevicoryne brassicae* individuals developing on *Brassica oleracea* var. *gemmifera* plants exposed to either ambient (400±10 ppm) or elevated (800±10 ppm) CO_2 concentration for 2, 6, or 10 weeks. **a** pre-reproductive time, **b** effective fecundity and **c** intrinsic rate of increase of first (F1) generation individuals, and **d** pre-reproductive time and **e** relative honeydew excretion rate of second (F2) generation individuals. $N=20$ individuals each per CO_2 concentration and period of exposure to CO_2, respectively. *P*-values based on Wilcoxon rank sum tests: *=$P \leq 0.05$, ns = not significant

plants were grown under elevated compared to ambient CO_2, and these plants contained more glucosinolates, particularly more sinigrin and glucoiberin. Thus, this increased glucosinolate content might have stimulated *P. brassicae* larval feeding.

Glucosinolates can be phagostimulants, but also can inhibit growth and development of insects. For instance, when given the choice between plants containing high or standard glucosinolate content, *P. brassicae* larvae preferred to feed on plants containing high glucosinolate content, although this food source prolonged its pupal period (Mattiacci et al., 2001), and led to a reduced pupal mass (Gutbrodt et al., 2011a). *Pieris brassicae* blocks the formation of the toxic glucosinolate hydrolysis products by means of a nitrile specifier protein in its gut (Hopkins et al., 2009; Winde and Wittstock, 2011). However, feeding on plants containing elevated glucosinolate levels might require biosynthesis of more nitrile specifier protein for efficient detoxification, which could incur energetic costs leading to a reduction in insect performance, as suggested for the related species *P. rapae* (Kos et al., 2012). Thus, feeding on *Brassica* plants under elevated CO_2 could have incurred a similar energetic cost for *P. brassicae*. The AD, which measures the proportion of ingested food that is actually digested by an herbivore, and the ECI, which indicates how efficiently food is converted into biomass, were significantly diminished in *P. brassicae* larvae that were feeding on plants grown under elevated CO_2. Increased leaf consumption might reduce ECI due to an additional energetic cost associated with the metabolism of larger amounts of food (Brooks and Whittaker, 1998), even in the absence of additional demand for detoxification. Both the energetic costs of processing the diet and of detoxifying glucosinolates could contribute to the overall decrease in the performance of the specialized leaf-feeding insect *P. brassicae* under elevated CO_2.

Glucosinolates have phagostimulatory effects on aphids (Newton et al., 2009 and references therein), and sinigrin in particular promoted feeding by *B. brassicae* aphids (Gabrys et al., 1997). Here, we found that *B. brassicae* aphids feeding on plants grown under elevated CO_2 had a significantly higher

relative honeydew excretion rate compared to aphids feeding on plants grown under ambient CO_2. For most aphid species, the rate of phloem intake is equivalent to the rate of excretion (Klingler et al., 1998). Thus, the increased aphid honeydew excretion under elevated CO_2 suggests an increase in the rate of feeding. *Brevicoryne brassicae* aphids do not detoxify glucosinolates but sequester them as a means to circumvent their toxicity (Winde and Wittstock, 2011). However, the ability of aphids to sequester glucosinolates may be impaired under elevated CO_2 (Klaiber et al., 2013a). This physiological constraint might force the aphids to increase their rates of excretion through increased honeydew production. An excretory mechanism has been reported for *B. brassicae* winged aphids (Winde and Wittstock, 2011), which unlike wingless aphids are not able to sequester glucosinolates from their diets. A similar mechanism has been proposed for non-specialist aphids like the green peach aphid *Myzus persicae* (Douglas, 2003). Rapid excretion is an herbivore adaptation that enables the insect to overcome toxic secondary metabolites in its diet (Winde and Wittstock, 2011). However, increased honeydew excretion implies an additional energetic cost for the aphids (Opitz and Müller, 2009), reducing their overall performance and even leading to increased water loss, and lower body mass (Daniels et al., 2009). In accordance with previous work, aphids under elevated CO_2 in our study system excreted more honeydew, had lower body weights (Klaiber et al., 2013a), and performed poorly. Hence, the phloem-feeding aphid *B. brassicae,* similar to the leaf-feeding *P. brassicae,* was negatively affected by the energetic costs required to cope with the higher plant glucosinolate content of plants under elevated compared to ambient CO_2.

Enhanced constitutive glucosinolate content in plants growing under elevated CO_2 might leave them better protected against insect herbivores, as suggested by the results presented here. However, increased glucosinolate content of *Brassica* plants could be disadvantageous for organisms from higher trophic levels. Furthermore, humans are sensitive to many bitter compounds (des Gachons et al., 2009), including glucosinolates (Cartea et al., 2008), and generally reject foods that are perceived as excessively bitter (des Gachons et al., 2009). Sinigrin, which is one of two compounds responsible for the bitterness of *Brassica* plants (van Doorn et al., 1998), increased under elevated CO_2 concentration in our study. Thus, human acceptance of *Brassica* crops containing higher glucosinolate contents could be negatively affected under the expected future CO_2 scenario.

In summary, our results provide evidence for glucosinolate-mediated, bottom-up regulation of specialized herbivores under elevated CO_2. We showed a significant impairment in the performance of two *Brassica* specialized insect herbivores from two contrasting feeding guilds likely due to CO_2-mediated increases in leaf glucosinolate content. Thus, even

Brassica-specialized insects might have threshold levels of tolerance to the glucosinolate content in their diets, as already suggested for a Fabaceae-specialized herbivore under ambient CO_2 on plants with cyanogenic precursors (Ballhorn et al., 2007). In our study, plant acclimation to CO_2 seemed to have increased plant resistance to insect herbivory by up-regulating constitutive chemical defense. This defense up-regulation is expected to be beneficial for plants, but due to chemically-mediated feeding stimulatory effects as shown here for the leaf-feeding specialist *P. brassicae*, negative effects of such up-regulation on the plants could also occur.

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