Insect science

Insect Science (2011) 18, 409–418, DOI 10.1111/j.1744-7917.2011.01419.x

ORIGINAL ARTICLE

Growth responses of gypsy moth larvae to elevated CO_2: the influence of methods of insect rearing

Lan-Zhu Ji, Lin-Li An and Xiao-Wei Wang

Biodiversity Group, Institute of Applied Ecology, Chinese Academy of Sciences, Shenyang, China

Abstract The effects of elevated CO_2 on foliar chemistry of two tree species (*Populus pseudo-simonii* Kitag. and *Betula platyphylla*) and on growth of gypsy moth (*Lymantria dispar* L.) larvae were examined. Furthermore, we focused on the comparison of results on the growth responses of larvae obtained from two methods of insect rearing, the no-choice feeding trial performed in the laboratory or *in situ* in open-top chambers. On the whole, both primary and secondary metabolites in the leaves of the two tree species were significantly affected by main effects of time (sampling date), CO_2 and species. Elevated CO_2 significantly increased the C: N ratio and concentrations of the soluble sugar, starch, total nonstructural carbohydrates, total phenolics and condensed tannins, but significantly decreased the concentration of nitrogen. Higher contents of total phenolics and condensed tannins were detected in the frass of larvae reared in elevated CO_2 treatments. Overall, the growth of gypsy moth larvae were significantly inhibited by elevated CO_2 and CO_2-induced changes in leaf quality. Our study did not indicate the two methods of insect rearing could influence the direction of effects of elevated CO_2 on the growth of individual insects; however, the magnitude of negative effects of elevated CO_2 on larval growth did differ between the two insect rearing methods, and it seems that the response magnitude was also mediated by larval age and host plant species.

Key words elevated CO_2, leaf quality, *Lymantria dispar*, no-choice feeding, open-top chamber

Introduction

Exploring the biological and ecological consequences of climate change (e.g. atmospheric CO_2 enrichment) has been one of the most important subjects of modern ecology (Coviella & Trumble, 1999). Of these studies, the response of plant–insect interactions to elevated CO_2 has been given more and more attention because of its great theoretical and practical significance (Wang *et al.*, 2008). Accumulated studies indicated that the altered foliar chemistry in elevated CO_2 had negative effects on the performance of phytophagous insects, and some insects consumed more leaves under high CO_2 treatment (Lindroth *et al.*, 1993; Roth & Lindroth, 1994; Traw *et al.*, 1996; Lindroth *et al.*, 1997; Hättenschwiler & Schafellner, 1999; Percy *et al.*, 2002; Hättenschwiler & Schafellner, 2004; Wang *et al.*, 2009); however, in some other studies, the individual insects were only slightly affected or unaffected, although the leaf palatability was also reduced under elevated CO_2 (Williams *et al.*, 1994; Roth *et al.*, 1998; Buse *et al.*, 1998; Veteli *et al.*, 2002; Saxon *et al.*, 2004). In other words, the responses of leaf-feeding insects in different studies were inconsistent. In our analysis of the responses of 36 tree–insect systems to elevated CO_2 (Wang *et al.*, 2008), only approximately three-fifths showed that the total consumption or consumption rate increased. Approximately half of the tree-insect systems

Correspondence: Lan-Zhu Ji, Biodiversity Group, Institute of Applied Ecology, Chinese Academy of Sciences, P.O. Box 417, No. 72 Wenhua Road, Shenhe District, Shenyang 110016, China. Tel: +86 24 83970302; fax: +86 24 83970300; email: ji.lanzhu@iae.ac.cn

showed that the performance of individual insects became poorer under high CO_2 treatment.

The variability in insect responses to elevated CO_2 can be attributed to many factors. Different experimental studies use different plant and insect species, CO_2 exposure facilities, plant growth conditions, insect rearing methods, CO_2 concentration levels and experimental durations, and so on. However, how these factors affect the responses of insects to elevated CO_2 or affect the validity of extrapolation of results have not been thoroughly discussed and explained so far, although the idiosyncratic, species-specific responses to elevated CO_2 have been repeatedly mentioned (Lindroth, 1996a; Hunter, 2001; Williams *et al.*, 2003; Sanders *et al.*, 2004). Second, the interactive effects of other abiotic factors, such as temperature (Buse *et al.*, 1998; Veteli *et al.*, 2002; Williams *et al.*, 2003), soil nutrient contents (Hättenschwiler & Schafellner, 1999; Saxon *et al.*, 2004) and light availability (Agrell *et al.*, 2000) are believed to affect the response of phytochemistry to elevated CO_2, and in turn influence insect performance. Third, previous studies showed that the responses of different instar larvae to CO_2-induced changes in phytochemistry were different (Lindroth *et al.*, 1997; Brooks & Whittaker, 1998; Wang *et al.*, 2006), and the CO_2 effect did differ slightly between three generations of the insect *Gastrophysa viridula* (Brooks & Whittaker, 1998). Both cases implied that insect performances would tend to be different in long-term feeding trials compared with that in short-term ones. In addition, we assumed that the difference in insect rearing methods (and related insect growth conditions) is probably another reason that led to the variability of insect responses in different studies, which in turn influences the validity of extrapolated results. For example, because the physiological responses of host plants to elevated CO_2 is highly species-specific, the effects of elevated CO_2 on generalist insects may differ between feeding conditions with single or multiple diets.

So far, methods of insect rearing used to examine the responses of individual insects to elevated CO_2 can be divided into two categories. (i) Bioassays in constant conditions (controlled environment rooms or controlled environment chambers), including no-choice bioassays (Lindroth *et al.*, 1993; Buse *et al.*, 1998) and choice feeding bioassays (Goverde & Erhardt, 2003; Agrell *et al.*, 2005; Agrell *et al.*, 2006; Wang *et al.*, 2009), in which larvae are reared with detached leaves in Petri dishes or similar containers. In these bioassays, the direct effects of high-CO_2 concentration are often excluded. (ii) *In situ* feeding trials performed in CO_2 exposure facilities, in which both plants and insects are exposed to elevated CO_2 at the same time, including the no-choice feeding trial in which the test larvae are reared on caged branches or entire plants (Williams *et al.*, 2000; Percy *et al.*, 2002; Kopper & Lindroth, 2003; Holton *et al.*, 2003; Hättenschwiler & Schafellner, 2004) and the experiments in which the larvae are allowed to feed freely in natural or semi-natural plant communities where the CO_2 enrichment facilities established (Arnone *et al.*, 1995; Stiling *et al.*, 2002; Stiling *et al.*, 2003; Hall *et al.*, 2005).

In this study, we examined the effects of elevated CO_2 on foliar chemical contents of two tree species (*Populus pseudo-simonii* Kitag. and *Betula platyphylla*) and on growth of gypsy moth (*Lymantria dispar* L.) larvae. Furthermore, we focused on the comparison of results of larval growth obtained from two methods of insect rearing, viz. the no-choice feeding trial where larvae were fed with detached leaves in rearing containers in the laboratory and the no-choice feeding trial in which larvae were reared on caged branches of trees grown in CO_2-enrichment chambers.

Materials and methods

Plant growth conditions

Open-top chambers (OTCs) for plants were set up in the field at the Changbai Mountain Forest Ecosystem Research Station, Chinese Academy of Sciences (E128°28′, N42°24′; elevation 736 m, annual average temperature 3.5°C and precipitation 600–1000 mm). Two chambers were maintained at ambient CO_2 concentration and two were supplied with elevated CO_2 (650 ± 80 ppm) during daytime (7:00–19:00 hours) over the growing season (May to October). The construction of OTCs and the control of CO_2 concentrations have been described in detail in Wang *et al.* (2006) and Wang *et al.* (2009). Briefly, the octagonal OTCs (2.4 m diameter by 2.8 m height) were constructed of square steel and covered with clear, 0.02-cm polyvinyl chloride film. Four interconnected vertical PVC tubes served as the CO_2 injection system with well-distributed holes (2 mm diameter) on each tube. CO_2 was supplied from a gas cylinder, and levels of CO_2 were controlled by regulating the flow meter of the CO_2 pressure regulator and monitoring with an infrared gas analyzer (CI-301, CID Inc., Vancouver, WA, USA). An axial fan installed at 1.5 m height above ground in the centre of each OTC was used to mix CO_2 with air.

The top of each OTC was covered with 1-mm mesh nylon netting to avoid any unwanted insects and other animals. The topsoil (0–25 cm) of each chamber was removed and replaced with well-mixed topsoil collected from climax forest (mixed Korean pine and broadleaf forest) of

Changbai Mountain region, in order to reduce the soil heterogeneity among the chambers (Wang *et al.*, 2009).

Plant and insect materials

Saplings of 1-year-old *Populus pseudo-simonii* Kitag. and 2-year-old *Betula platyphylla* were randomly transplanted into each OTC (n = 8–10 for each species) on May 12, 2005. Egg masses of gypsy moth were collected from deciduous trees (mostly *Salix babylonica*) at Qingnian Park, Shenyang, and refrigerated at 4°C before use in the insect feeding experiments.

Insect experiments

On-tree feeding experiment Groups of 15 newly hatched larvae were randomly selected from approximately 20 egg masses on July 19, 2006. Five saplings of each tree species were randomly selected from each chamber and used for the on-tree feeding experiment. For each of the five saplings, a south-facing branch within the middle third of the tree crown was selected and enclosed in a fine nylon mesh bag (20 cm wide × 35 cm long; 0.25 mm mesh size). The group of 15 newly hatched larvae then was transferred into each bag (15 larvae per bag per tree). Each rearing bag was considered as a statistical replicate; hence, 10 replicates were set for each of the CO_2 × tree species treatments.

Larger nylon bags (30 × 50 cm, 0.25 mm mesh size) that could contain longer branches and more green leaves were used when the test larvae grew bigger. Once more than half of the leaves in a bag were consumed, the bag and the caterpillars were moved to a new branch on the same tree. The average body mass of larvae was successively measured on days 8, 14, 20 and 29 of larval development (July 27, August 2, August 8, and August 17, respectively). At a similar time of each measurement date, the larvae in each bag were weighed as a group; meanwhile, their frass were collected. Soon afterwards, the larvae were returned to the rearing bag for further growing. The larval growth was expressed as the average fresh mass per individual larva.

Feeding experiment in the laboratory environment At the beginning of this experiment on July 22 (3 days later than the on-tree feeding experiment), 15 newly hatched larvae were placed in a 15 cm diameter Petri dish and were maintained in an insect-rearing room at 25 ± 3°C, 80% ± 5% RH and a 16: 8 h L: D photoperiod. When the caterpillars grew bigger, they were transferred into 500 mL volume plastic containers. The leaves used for larvae rearing in the laboratory were collected from south-facing branches within the middle third of the tree crowns, and as much as possible, the leaves were collected from the saplings used for on-tree feeding experiments. Each rearing container was considered as a statistical replicate (n = 10 for each of the tree species × CO_2 treatments).

Leaves were cut at the base of the petioles and taken to the laboratory within 15 min. The petioles were inserted into a moistened cotton-wool ball and wrapped with plastic film to keep the leaves hydrated. Once more than half the leaves in a rearing container were consumed (visual inspection), they were replaced by freshly collected leaves.

The average fresh mass of larvae in each rearing container was successively measured on days 7, 14, 19, 23 and 27 of larval development (July 29, August 5, August 10, August 14 and August 18, respectively). At a similar time as each measurement date, the larvae in each container were weighed as a group, and were placed back into the container immediately. The average fresh mass per individual larva was calculated by dividing the pooled weight by the number of larvae in the container.

The feeding experiments were terminated when most larvae were still in the fourth instar stage. By that time, the total consumption by larvae per tree was considerable, and we estimated that the undamaged leaves (especially the birch leaves) on the south-facing branches within a third of the tree crowns could no longer meet the needs of the feeding experiments performed in the laboratory and OTCs. We did not use the leaves on other positions on the trees for the further experiments, because our earlier study showed that the palatability of leaves from the upper or lower crowns were different (Wang *et al.*, 2009). Because our experiments did not last until pupation, the development time from eggs to pupae, the pupal mass and other parameters of insect performance are not provided in this study.

Chemical analyses

Leaves for chemical analyses were also collected from the saplings used for on-tree feeding experiments (one leaf sample per sapling and n = 10 for each of the CO_2 × tree species treatments). All the leaf samples were collected from south-facing branches within the middle third of the tree crowns. Leaf sampling was performed four times on July 6, July 24, August 16 and September 6, 2006 (not just one time in the insect feeding period), in order to investigate the responses of leaf quality (food palatability) to elevated CO_2 over longer periods of time.

Leaves (≈ 2–3 g fresh weigh) for the phytochemistry analysis were cut at the base of the petiole, sealed in plastic bags and delivered to the laboratory within 10 min.

Total nitrogen and carbon content were measured by dry combustion using an elemental analyzer (Elementar, Vario EL III, Elementar Analysensysteme GmbH, Hanau Germany). Soluble sugar and starch were extracted using the procedures described by Tissue and Wright (1995). Sugar concentrations in the supernatant were determined by the phenol-sulphuric acid method (Dubois *et al.*, 1956), as modified by Chow and Landhäusser (2004), using glucose as a standard. Total non-structural carbohydrate was calculated as the sum of soluble sugar and starch.

The concentrations of total phenolics (TP) and condensed tannins (CT) were quantified by extraction with 50% methanol and assayed colorimetrically by the Folin-Ciocalteu and butanol-HCl-Fe^{III} methods (Waterman & Mole, 1994), against tannic acid (T0200; Sigma-Aldrich Co., St Louis, MO, USA) and purified aspen tannin standards, respectively. Aspen CTs were isolated and purified from bark of *P. pseudosimonii* according to the Ann E. Hagerman laboratory method (Tannin handbook, downloaded from http://www.users.muohio.edu/hagermae/tannin.pdf), and used as a standard for the two species.

We also measured the contents of foliar total phenolics and condensed tannins deposited in larval frass. Frass samples were collected from the fine nylon mesh bags in the on-tree feeding experiments as described above. Each frass sample corresponding to a nylon mesh bag was collected when the bag was moved from a branch to another and at the same date when fresh mass of larvae in the bag was measured. However, the dry mass (DM) of larval frass in each bag was not always enough for chemical analyses (especially for the early instar larvae). So, the frass samples from each treatment (time × species × CO_2) were mixed together and analyzed. Therefore, only the mean value of each treatment was given in the results (other statistical parameters, e.g. *P*-value and standard error, were not calculated); and the present study should be considered as a preliminary study. TP and CT deposited in larval frass were measured using the aforementioned methods that were used for phytochemical analyses.

Samples of leaves and larval frass were oven-dried to constant weight at 50°C. The samples used for further chemical analyses were ground into fine powders and stored in −20°C. The chemical contents were all expressed as mg/g DM.

Statistical analyses

Statistical analyses were performed using SPSS software (version 16.0 for Windows, SPSS Inc., Chicago, IL, US). Three-way analysis of variance (ANOVA) with repeated measures (CO_2 treatment and tree species as between-subjects factors and sampling date as within-subjects factor of variation) were used to analyze the concentrations of phytochemicals over time. The assumption of Type H covariance was tested by applying Mauchly's test of sphericity. If the assumption was not satisfied, the Huynh–Feldt Epsilon was used to correct the within-subjects effects (Peltonen *et al.*, 2005).

Data of larval growth were also analyzed using repeated measures ANOVA. Time (larval age) was used as a within-subjects factor, and the CO_2 treatment and tree species were used as between-subjects factors. The observation dates for the feeding trials performed in the laboratory and OTCs were not totally consistent (also see Fig. 2); therefore, we could not examine the effects of rearing method on larval growth by treating method as a within-subjects factor in the repeated measures ANOVA. The CO_2 effect on the growth of gypsy moth larvae was also expressed as Index of Response (IR), according to the formula: (average fresh mass of larvae at elevated CO_2 – average fresh mass of larvae at ambient CO_2)/average fresh mass of larvae at ambient CO_2.

Data were all expressed as mean ± SE and the level of significance was set at 0.05.

Results

Foliar chemistry

On the whole, both major primary and secondary metabolites in leaves of poplar and birch were significantly affected by main effects of time (sampling date), CO_2 and species (Table 1).

Main effect of CO_2 On average, elevated CO_2 significantly increased the concentration of soluble sugar by 12.4%, starch by 14.7%, total nonstructural carbohydrates by 12.9%, total phenolics by 9.5% and condensed tannins by 45.1%. The C: N ratio was also significantly increased by 16.6% under elevated CO_2 treatment. Elevated CO_2 significantly decreased the concentration of the nitrogen by 15.1% and carbon by 1.0% (Fig. 1 and Table 1).

Main effect of species On average, the concentration of nitrogen, carbon, starch and condensed tannins in birch leaves were 7%, 10%, 16% and 102% greater than that in poplar leaves, respectively (Table 1). In contrast, the concentration of soluble sugar and total nonstructural carbohydrates in poplar leaves were 46% and 30% higher than that in birch leaves, respectively. But the C: N ratio and the concentration of total phenolics in leaves of birch and poplar had no significant difference.

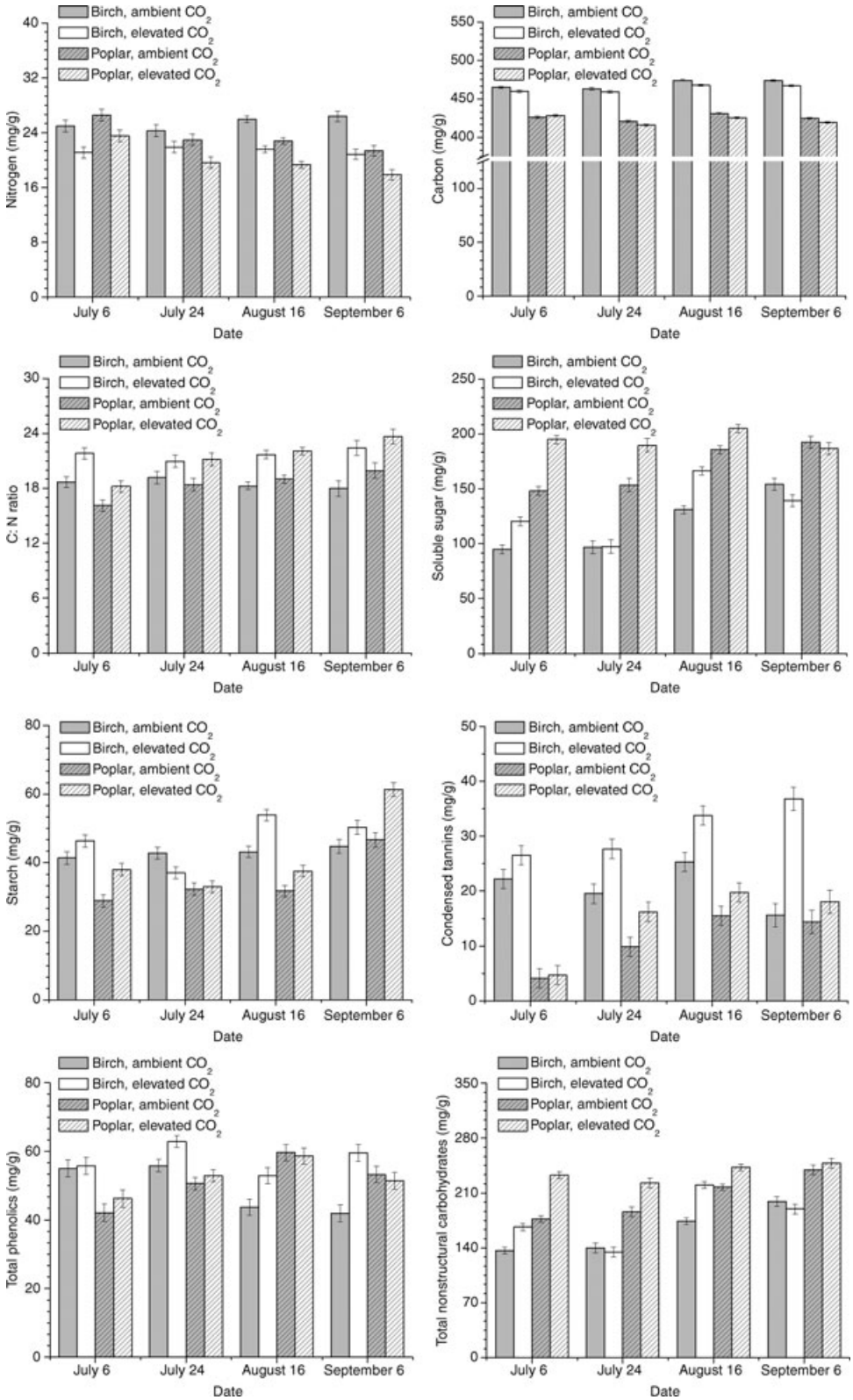

Fig. 1 Phytochemical concentrations (expressed as mg/g dry mass) in leaves of poplar (*Populus pseudo-simonii* Kitag.) and birch (*Betula platyphylla*) grown in ambient or elevated CO_2 conditions.

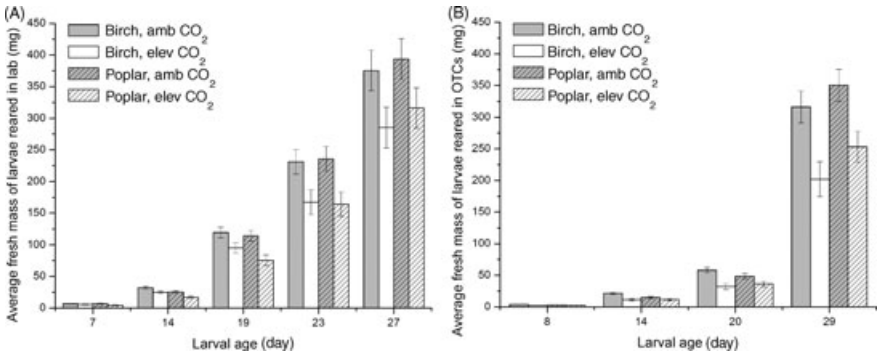

Fig. 2 Growth of gypsy moth larvae reared in containers in the laboratory (A) or on branches of trees in open-top chambers (B). Larval growth was expressed as mg fresh mass per larva. Amb CO_2, ambient CO_2; Elev CO_2, elevated CO_2.

Interactive effects of CO_2 and species The effects of elevated CO_2 on foliar chemistry were mediated by species, especially for the soluble sugar, total nonstructural carbohydrate (TNC), total phenolics and condensed tannins; but there were no interactive effects of CO_2 and species for nitrogen concentration and C: N ratio (Table 1).

Growth responses of larvae reared with two different methods

The growth of larvae (expressed as the average fresh mass per individual larva) reared either in the laboratory or in OTCs was significantly affected by the main effects of time (larval age) and CO_2, but unaffected by the main effect of host species and any one of the interaction effects (Table 2).

CO_2 effects The growth of larvae was significantly reduced in high CO_2 treatments (Table 2). The columns in Fig. 2 clearly showed that the average fresh mass of larvae in elevated CO_2 treatment was significantly lower than that in ambient CO_2 treatment on each measurement date.

Effect of host plant species The growth of larvae fed on birch or poplar leaves did not differ significantly in this study (Table 2), although most of the measured phytochemicals did differ significantly in the concentrations between the two tree species.

Effect of insect-rearing methods The magnitude of negative effects of CO_2 on larval growth were mediated by host tree species, larval age and insect-rearing method (Table 3), with index of response (IR) ranging from −6.5% to −46.7%. For larvae fed poplar, the magnitude of

Table 1 Summary of *P*-values of repeated measures ANOVA for the main effects of time, CO_2, species and their interactions on the concentrations of phytochemicals.

		Within-subjects effects			Between-subjects main effects		
	Time	Time × species	Time × CO_2	Time × species × CO_2	Species	CO_2	Species × CO_2
Nitrogen	< 0.001	< 0.001	0.151	0.257	0.029, poplar< birch	< 0.001, ↓	0.575
Carbon	< 0.001	< 0.001	0.075	0.156	< 0.001, poplar< birch	< 0.001, ↓	0.057
C: N ratio	< 0.001	< 0.001	0.126	0.508	0.541	< 0.001, ↑	0.768
Soluble sugar	< 0.001	< 0.001	< 0.001	0.003	< 0.001, poplar>birch	< 0.001, ↑	0.019
Starch	< 0.001	< 0.001	< 0.001	0.045	< 0.001, poplar< birch	< 0.001, ↑	0.055
TNC	< 0.001	< 0.001	< 0.001	0.001	< 0.001, poplar>birch	< 0.001, ↑	0.005
Total phenolics	< 0.001	< 0.001	0.261	0.001	0.311	0.004, ↑	0.017
Condensed tannins	< 0.001	< 0.001	0.001	0.005	< 0.001, poplar< birch	< 0.001, ↑	0.004

Time, sampling date; ↑, increase; ↓, decrease; <, smaller than; >, greater than; TNC, total nonstructural carbohydrates.

Table 2 Summary of *P*-values of repeated measures ANOVA for the main effects of time, CO_2, species and their interactions on the growth of gypsy moth larvae reared in OTCs or the laboratory environment.

		Within-subjects effects			Between-subjects main effects		
	Time	Time × species	Time × CO_2	Time × species × CO_2	Species	O_2	Species × CO_2
Rearing in laboratory	< 0.001	0.403	0.007	0.834	0.933	0.001	0.922
Rearing in OTCs	< 0.001	0.085	< 0.001	0.773	0.197	< 0.001	0.494

Time, larval age.

their growth response was smaller in feeding experiments performed *in situ* in OTCs than that in bioassays performed in the laboratory. In contrast, for larvae fed on birch leaves, stronger negative effects on larval growth were identified in feeding trials in OTCs than that indicated in bioassays in the laboratory.

Plant secondary metabolites detected in larval frass

Under elevated CO_2 treatment, higher concentrations of total phenolics and condensed tannins were detected in frass of larvae reared on caged branches of trees in OTCs, with 0.4%–28.5% increase in total phenolics and 25.0%–86.3% increase in condensed tannins (Table 4).

Table 3 The effects of elevated CO_2 (expressed as Index of Response [IR]) on the growth of gypsy moth larvae reared in open-top chambers (OTCs) or in the laboratory environment.

Rearing in laboratory			Rearing on tree in OTCs		
Larval age (days)	Tree species		Larval age (days)	Tree species	
	Poplar	Birch		Poplar	Birch
7	−36.0%	−15.7%	8	−6.5%	−28.6%
14	−33.0%	−22.6%	14	−22.3%	−46.7%
19	−33.7%	−20.5%	20	−25.3%	−44.4%
23	−30.3%	−27.7%	NA	NA	NA
27	−19.6%	−24.0%	29	−27.8%	−36.1%

NA, not available. IR was calculated by the formula: (average fresh mass of larvae at elevated CO_2 – average fresh mass of larvae at ambient CO_2)/ average fresh mass of larvae at ambient CO_2. A negative IR value indicates that the growth of larvae was inhibited by elevated CO_2.

Discussion

Responses of foliar chemistry and larval growth to elevated CO_2

In this study, the growth of gypsy moth larvae were significantly inhibited by elevated CO_2 under both insect rearing methods (the no-choice feeding trials performed in the laboratory or OTCs). On the whole, both primary and secondary metabolites in the leaves of two tree species (*Populus pseudo-simonii* Kitag. and *Betula platyphylla*) were significantly affected by elevated CO_2, with a decrease in nitrogen concentration, as well as an increase in the concentrations of soluble sugar, starch, total nonstructural carbohydrates, total phenolics, condensed tannins and C : N ratio.

We consider that the effects of elevated CO_2 on phytophagous insects can be attributed to four approaches: (i) the direct effect of high CO_2 concentration *per se*; (ii) the indirect effect of the CO_2-induced changes in plant quality (food palatability); (iii) the top-down effect of species at the third trophic level, such as parasitoids, predators and pathogens; and (iv) the more complex ecosystem approach, as well as the interactive effects of multiple factors.

The CO_2-induced changes in plant quality or foliar chemical compositions were commonly used to explain the responses of insects to elevated CO_2, and the decrease in tissue nitrogen concentrations have been widely recognized as a key influencing factor (Lincoln, 1993; Williams *et al*., 1994; Lindroth, 1996b; Bezemer & Jones, 1998; Hunter, 2001; Veteli *et al*., 2002). However, in a specific study, it is hard to determine which nutritional components, secondary metabolites (allelochemicals) or combination of physicochemical properties correlated positively or negatively with the responses of insects, and it is even harder to determine the relative magnitude of influence of these parameters.

Table 4 Concentrations of foliar total phenolics and condensed tannins in larval frass. The frass samples were collected from the on-tree feeding experiments performed in open-top chambers.

Tree species	Larval age (days)	Total phenolics (mg/g)			Condensed tannins (mg/g)		
		Ambient CO_2	Elevated CO_2	IR	Ambient CO_2	Elevated CO_2	IR
Birch	8	34.30	37.14	8.3%	5.26	7.25	37.9%
	20	31.59	37.58	19.0%	4.47	6.90	54.6%
	29	27.44	31.68	15.5%	4.41	6.68	51.6%
Poplar	8	29.14	32.75	12.4%	4.22	7.87	86.3%
	20	49.20	49.40	0.4%	8.38	10.48	25.0%
	29	27.01	34.70	28.5%	4.07	5.74	40.8%

IR, index of response, calculated by the formula: (metabolite concentration at elevated CO_2 – metabolite concentration at ambient CO_2)/metabolite concentration at ambient CO_2.

The direct effect of increased CO_2 concentration on performance of insects can be examined by rearing insects on artificial diets under different CO_2 treatments. Several experiments performed using this method consistently indicated that the direct effects of increased CO_2 concentration (corresponding to the doubled CO_2 scenario) on herbivore insects were weak or negligible (Fajer *et al.*, 1991; Agrell *et al.*, 2000; Yin *et al.*, 2009).

Therefore, in this study, the negative effects of elevated CO_2 on the growth of gypsy moth larvae can mainly be attributed to the overall changes in leaf physicochemical properties; furthermore, the decreased nitrogen and increased carbon-based secondary metabolites probably reduced leaf quality or food palatability, but higher nonstructural carbohydrate contents might be beneficial for the test insects.

The more accumulation of total phenolics and condensed tannin in the frass of larvae reared under elevated CO_2 treatment, as found in this study, meant that there was more intake of these secondary metabolites by larvae, which could also be used to explain the response of larvae to elevated CO_2. Because these secondary chemicals, especially the condensed tannins, have protein-binding characteristics and can cause decreases in food conversion rates.

Do the methods of insect rearing affect the magnitude of responses of larvae to elevated CO_2?

In addition to the methods used to investigate the responses of individual insects to elevated CO_2, namely the bioassays in constant conditions and the *in situ* feeding trials performed in CO_2 exposure facilities (see the Introduction for details), there have been studies examining the responses of herbivory, population dynamics or community composition of insects in natural plant communities where CO_2 exposure facilities (OTC or free-air CO_2 enrichment [FACE]) were established (e.g. Stiling *et al.*, 2002; Stiling *et al.*, 2003; Sanders *et al.*, 2004; Hamilton *et al.*, 2004; Hall *et al.*, 2005; Knepp *et al.*, 2005). Because many complex interacting factors influence insect performance and population dynamics in a high-CO_2 world (see the review by Lindroth, 1996a) and different research methods are corresponding to different influencing factors and approaches, we assume that the experimental methods and related plant/insect growth conditions probably affect the magnitude of responses of herbivorous insects to elevated CO_2 (as we discussed in the Introduction section). In this study, two methods of insect rearing, the no-choice feeding trials performed in the laboratory or in OTCs were used to examine the effects of elevated CO_2 on growth responses of gypsy moth larvae. The study did not indicate the two methods of insect rearing could influence the direction of effects of elevated CO_2 on the growth of individual insects; however, the magnitude of negative effects of elevated CO_2 on larval growth (expressed as Index of Response) did differ for the two methods, and it seems that the response magnitude was also mediated by larval age and host plant species.

The reasons may be as follows. There were some differences in larval diets between the two rearing methods. The diets for larvae reared in OTCs were the leaves *in situ* on branches of trees, thus including young and mature leaves, and tender and tough leaves. However, the diets for larvae reared in containers in the laboratory were abscised leaves from trees grown in OTCs and totally supplied by an experimental technician. Although the south-facing branches within a third of the tree crowns were chosen for larvae reared either in OTCs or laboratory conditions, only mature leaves in the middle part of a branch were chosen for the larvae reared in the laboratory. Therefore,

we believe, the differences in larval diets (*in situ* leaves and abscised leaves) probably could explain why the magnitude of negative effects of elevated CO_2 on larval growth differed for the two insect-rearing methods. In addition, the interval of 3days between the starting times of the two feeding experiments was not believed to significantly influence the leaf chemical contents and in turn the magnitude of growth responses of larvae to elevated CO_2.

The method of insect rearing on detached leaves in a constant environment was often criticized because of its methodological limitations (e.g. Hunter, 2001), but our study did not indicate it could influence the direction of effects of elevated CO_2 on individual insects. In fact, short-term bioassays are necessary for examining the physiological responses of individual insects; in contrast, field-based facilities and long-term experiments are especially required for exploring potential changes in herbivory, population dynamics and community composition of insects in a high CO_2 atmosphere.

Acknowledgments

This study was financially supported by the National Natural Science Foundation of China (30670306 & 30970515) and Special Research Funds for Public Welfare Sectors (Forestry), State Forestry Administration of China (200804023).

References

Agrell, J., Anderson, P., Oleszek, W., Stochmal, A. and Agrell, C. (2006) Elevated CO_2 levels and herbivore damage alter host plant preferences. *Oikos*, 112, 63–72.

Agrell, J., Kopper, B., McDonald, E.P. and Lindroth, R.L. (2005) CO_2 and O_3 effects on host plant preferences of the forest tent caterpillar (*Malacosoma disstria*). *Global Change Biology*, 11, 588–599.

Agrell, J., McDonald, E.P. and Lindroth, R.L. (2000) Effects of CO_2 and light on tree phytochemistry and insect performance. *Oikos*, 88, 259–272.

Arnone, J.A., Zaller, J.G., Körner, C., Ziegler, C. and Zandt, H. (1995) Leaf quality and insect herbivory in model tropical plant communities after long-term exposure to elevated atmospheric CO_2. *Oecologia*, 104, 72–78.

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

Brooks, G.L. and Whittaker, J.B. (1998) Responses of multiple generations of *Gastrophysa viridula*, feeding on *Rumex obtusifolius*, to elevated CO_2. *Global Change Biology*, 4, 63–75.

Buse, A., Good, J.E.G., Dury, S. and Perrins, C.M. (1998) Effects of elevated temperature and carbon dioxide on the nutritional quality of leaves of oak (*Quercus robur* L.) as food for the Winter Moth (*Operophtera brumata* L.). *Functional Ecology*, 12, 742–749.

Chow, P.S. and Landhäusser, S.M. (2004) A method for routine measurements of total sugar and starch content in woody plant tissues. *Tree Physiology*, 24, 1129–1136.

Coviella, C.E. and Trumble, J.T. (1999) Effects of elevated atmospheric carbon dioxide on insect-plant interactions. *Conservation Biology*, 13, 700–712.

Dubois, M., Gilles, K.A., Hamilton, J.K., Rebers, P.A., Smith, F. (1956) Colorimetric method for determination of sugars and related substances. *Analytical Chemistry*, 28, 350–356.

Fajer, E.D., Browers, M.D. and Bazzaz, F.A. (1991) The effect of enriched carbon dioxide atmospheres on the Buckeye butterfly, *Junonia coenia*. *Ecology*, 72, 751–754.

Goverde, M. and Erhardt, A. (2003) Effects of elevated CO_2 on development and larval food-plant preference in the butterfly *Coenonympha pamphilus* (Lepidoptera, Satyridae). *Global Change Biology*, 9, 74–83.

Hall, M.C., Stiling, P., Moon, D.C., Drake, B.G. and Hunter, M.D. (2005) Effects of elevated CO_2 on foliar quality and herbivore damage in a scrub oak ecosystem. *Journal of Chemical Ecology*, 31, 267–286.

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

Hättenschwiler, S. and Schafellner, C. (1999) Opposing effects of elevated CO_2 and N deposition on *Lymantria monacha* larvae feeding on spruce trees. *Oecologia*, 118, 210–217.

Hättenschwiler, S. and Schafellner, C. (2004) Gypsy moth feeding in the canopy of a CO_2-enriched mature forest. *Global Change Biology*, 10, 1899–1908.

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

Hunter, M.D. (2001) Effects of elevated atmospheric carbon dioxide on insect-plant interactions. *Agricultural and Forest Entomology*, 3, 153–159.

Knepp, R.G., Hamilton, J.G., Mohan, J.E., Zangerl, A.R., Berenbaum, M.R. and DeLucia, E.H. (2005) Elevated CO_2 reduces leaf damage by insect herbivores in a forest community. *New Phytologist*, 167, 207–218.

Kopper, B.J. and Lindroth, R.L. (2003) Responses of trembling aspen (*Populus tremuloides*) phytochemistry and aspen blotch leafminer (*Phyllonorycter tremuloidiella*) performance to elevated levels of atmospheric CO_2 and O_3. *Agricultural and Forest Entomology*, 5, 17–26.

Lincoln, D.E. (1993) The influence of plant carbon dioxide and nutrient supply on susceptibility to insect herbivores. *Vegetatio*, 104, 273–280.

Lindroth, R.L. (1996a) Consequences of elevated atmospheric CO_2 for forest insects. *Carbon Dioxide, Populations, and Communities* (eds. C. Körner & F.A. Bazzaz), pp. 347– 361. Academic Press, San Diego.

Lindroth, R.L. (1996b) CO_2-mediated changes in tree chemistry and tree-Lepidoptera interactions. *Carbon Dioxide and Terrestrial Ecosystems* (eds. G.W. Koch & H.A. Mooney), pp. 105–120. Academic Press, San Diego.

Lindroth, R.L., Kinney, K.K. and Platz, C.L. (1993) Responses of deciduous trees to elevated atmospheric CO_2: Productivity, phytochemistry, and insect performance. *Ecology*, 74, 763–777.

Lindroth, R.L., Roth, S., Kruger, E.L., Volin, J.C. and 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.

Peltonen, P.A., Vapaavuori, E. and Julkunen-Tiitto, R. (2005) Accumulation of phenolic compounds in birch leaves is changed by elevated carbon dioxide and ozone. *Global Change Biology*, 11, 1305–1324.

Percy, K.E., Awmack, C.S., Lindroth, R.L., Kubiske, M.E., Kopper, B.J., Isebrands, J.G., Pregitzer, K.S., Hendrey, G.R., Dickson, R.E., Zak, D.R., Oksanen, E., Sober, J., Harrington, R. and Karnosky, D.F. (2002) Altered performance of forest pests under atmospheres enriched by CO_2 and O_3. *Nature*, 420, 403–407.

Roth, S., Lindroth, R., Volin, J.C. and Kruger, E. (1998) Enriched atmospheric CO_2 and defoliation: effects on tree chemistry and insect performance. *Global Change Biology*, 4, 419–430.

Roth, S.K. and Lindroth, R.L. (1994) Effects of CO_2-mediated changes in paper birch and white-pine chemistry on gypsy moth performance. *Oecologia*, 98, 133–138.

Sanders, N.J., Belote, R.T. and Weltzin, J.F. (2004) Multitrophic effects of elevated atmospheric CO_2 on understory plant and arthropod communities. *Environmental Entomology*, 33, 1609–1616.

Saxon, M.E., Davis, M.A., Pritchard, S.G., Runion, G.B., Prior, S.A., Stelzer, H.E., Rogers, H.H. and Dute, R.R. (2004) Influence of elevated CO_2, nitrogen, and *Pinus elliottii* genotypes on performance of the redheaded pine sawfly, *Neodiprion lecontei*. *Canadian Journal of Forest Research*, 34, 1007–1017.

Stiling, P., Cattell, M., Moon, D.C., Rossi, A., Hungate, B.A., Hymus, G. and Drake, B. (2002) Elevated atmospheric CO_2 lowers herbivore abundance, but increases leaf abscission rates. *Global Change Biology*, 8, 658–667.

Stiling, P., Moon, D.C., Hunter, M.D., Colson, J., Rossi, A.M., Hymus, G.J. and Drake, B.G. (2003) Elevated CO_2 lowers relative and absolute herbivore density across all species of a scrub-oak forest. *Oecologia*, 134, 82–87.

Tissue, D.T. and Wright, S.J. (1995) Effect of seasonal water availability on phenology and the annual shoot carbohydrate cycle of tropical forest shrubs. *Functional Ecology*, 9, 518–527.

Traw, M.B., Lindroth, R.L. and Bazzaz, F.A. (1996) Decline in gypsy moth (*Lymantria dispar*) performance in an elevated CO_2 atmosphere depends upon host plant species. *Oecologia*, 108, 113–120.

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

Wang, X.W., Ji, L.Z. and Liu, Y. (2006) Effects of increased atmospheric CO_2 on nutritional contents in poplar (*Populus pseudo-simonii* [Kitag.]) tissues and larval growth of gypsy moth (*Lymantria dispar*). *Acta Ecologica Sinica*, 26, 3166–3173.

Wang, X.W., Ji, L.Z., Wang, G.Q. and Liu, Y. (2008) Potential effects of elevated carbon dioxide on leaf-feeding forest insects. *Frontiers of Biology in China*, 3, 68–77.

Wang, X.W., Ji, L.Z., Zhang, Q.H., Liu, Y. and Wang, G.Q. (2009) Effects of elevated CO_2 on feeding preference and performance of the gypsy moth (*Lymantria dispar*) larvae. *Journal of Applied Entomology*, 133, 47–57.

Waterman, P.G. and Mole, S. (1994) *Analysis of Phenolic Plant Metabolites*. Blackwell Scientific, Boston. pp. 80–85, 92–95.

Williams, R.S., Lincoln, D.E. and Norby, R.J. (2003) Development of gypsy moth larvae feeding on red maple saplings at elevated CO_2 and temperature. *Oecologia*, 137, 114–122.

Williams, R.S., Lincoln, D.E. and Thomas, R.B. (1994) Loblolly pine grown under elevated CO_2 affects early instar pine sawfly performance. *Oecologia*, 98, 64–71.

Williams, R.S., Norby, R.J. and Lincoln, D.E. (2000) Effects of elevated CO_2 and temperature-grown red and sugar maple on gypsy moth performance. *Global Change Biology*, 6, 685–695.

Yin, J., Sun, Y.C., Wu, G., Parajulee, M.N. and Ge, F. (2009) No effects of elevated CO_2 on the population relationship between cotton bollworm, *Helicoverpa armigera* Hübner (Lepidoptera: Noctuidae), and its parasitoid, *Microplitis mediator* Haliday (Hymenoptera: Braconidae). *Agriculture, Ecosystems and Environment*, 132, 267–275.

Accepted February 24, 2010