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Elevated CO_2 reduces the response of *Sitobion avenae* (Homoptera: Aphididae) to alarm pheromone

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ABSTRACT

The aphid alarm pheromone (*E*)-β-farnesene (EβF) is an efficient signal that warns aphids of attack by natural enemies. In this field study, eight open-top chambers (OTCs) located in Beijing, China (40°11′N, 116°24′E) with spring wheat *Triticum aestivum* were used to examine the response of the grain aphid *Sitobion avenae* to CO_2 (ambient vs. double ambient) and EβF (applied zero, two, or five times each day). We experimentally tested the hypotheses that, depending on frequency of EβF release, elevated CO_2 reduces the response (in terms of population density) of *S. avenae* to EβF, and that lower activity of acetylcholinesterase (AChE) in *S. avenae* may be involved in its reduced sensitivity to EβF under elevated CO_2. Numbers of *S. avenae* declined with increased frequency of EβF application under ambient CO_2 but were unaffected by EβF application under elevated CO_2. Additionally, the mean relative growth rate (MRGR) and the dry material and amino acid content of *S. avenae* increased with elevated CO_2 but declined when with EβF application. Activities of superoxide dismutase and catalase were higher in *S. avenae* under elevated vs. ambient CO_2. Under elevated CO_2, however, AChE activity remained low when *S. avenae* was exposed to the lower EβF frequency, while the highest AChE activity occurred in aphids exposed to the higher EβF frequency. These results indicate that aphids become insensitive to EβF under elevated CO_2, perhaps because of decreased AChE activity.

1. Introduction

Atmospheric carbon dioxide (CO_2) concentration has increased from a pre-industrial value of about 280–379 ppm in 2005, and the level of CO_2 is anticipated to double by the end of this century (IPCC, 2007). Plant responses to CO_2 have been well documented, with the consensus emerging that elevated CO_2 stimulates plant growth (Jablonski et al., 2002; Barbehenn et al., 2004). In addition, elevated CO_2 changes the C:N ratio of plant tissues, and accordingly, alters the nutritional and defensive metabolites in those tissues. These changes sometimes cascade through ecosystems and impact higher trophic levels, including insect herbivores and their natural enemies (Agrell et al., 2000; Harley et al., 2000). For example, when consuming plants grown under elevated CO_2, the fitness of chewing insects (e.g., lepidopteran larvae) was generally reduced because of reduced fecundity, survivorship, and developmental rates (Chen et al., 2005b, 2007; Wu et al., 2006). It was considered that, however, the population of aphid has been found to be the only feeding guild to respond positively to elevated CO_2 (Bezemer and Jones, 1998; Lesley and Fakhri, 2001).

(*E*)-β-Farnesene (EβF), the major component of alarm pheromone of most aphid species, is secreted from the cornicles upon predator attack, resulting in various behavioral reactions, such as increased alertness, non-feeding, and moving away from or dropping off the host plant (Bowers et al., 1972; Edwards et al., 1973; Montgomery and Nault, 1977). Apparently, EβF is an altruistic chemical signal released by attacked aphids to protect other aphids from natural enemies (Nault et al., 1973; Pickett and Griffiths, 1980). Furthermore, several cases have confirmed that, when aphids perceive EβF, the pheromone can greatly affect their behavior, life history, physiology, and morphology (Kunert et al., 2005; Su et al., 2006). In the ambient CO_2 environment, aphids perceiving the EβF signal would increase production of alate offspring and reduce their foraging rate, which would increase the ability to disperse to enemy-free space and reduce exposure to predators.

Pheromone-mediated responses of herbivores to predators are recognized as important for understanding predator–prey systems (Hassell, 1978; Mangel and Roitberg, 1992). Previous studies suggest that, under elevated CO_2, parasitoids and predators are more abundant or effective (Stilling et al., 1999; Percy et al., 2002;

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Chen et al., 2005a) whereas aphids seem less sensitive to alarm pheromones. Awmack et al. (1997) reported that the potato aphid *Aulacorthum solani* (Kalt.) was less sensitive to disturbance under elevated CO_2 than under ambient CO_2. Mondor et al. (2004) found that the aphid *Chaitophorus stevensis* on trembling aspen trees exhibited diminished escape responses under elevated CO_2. Additionally, aphids react more strongly to the frequency of pheromone release than to the amount of pheromone released (Kunert et al., 2005). Thus, how aphids reared under elevated CO_2 respond to the quantity of EβF released and frequency of EβF release is not well understood but should be studied because such information could increase our ability to manage aphid pests in crop plants in elevated CO_2 environments.

Although exposure to alarm pheromone greatly affects aphid behavior and physiology, elevated CO_2 is also considered to affect aphids via changes in plant defenses and plant nutrients supplied to the aphids (Sudderth et al., 2005; Pritchard et al., 2007). Part of the plant defense against herbivory are exogenous reaction oxygen species (ROS). The response of aphids to ROS may involve two of the antioxidant enzymes in herbivorous insects, superoxide dismutase (SOD) and catalase (CAT), enzymes which may also be involved in aphid response to EβF (Orozco-cardenas and Ryan, 1999). Plant defense includes a variety of secondary metabolites such as phenolic compounds and tannins, which have been reported to increase under elevated CO_2 (Harborne, 1997; Chen et al., 2005b). Furthermore, Dawson et al. (1983) reported that insecticide resistance in the peach aphid *Myzus persicae* (Sulz.) decreased the response to EβF. Therefore, acetylcholinesterase (AChE), a key enzyme in neurotransmission, has been studied as an important factor affecting insecticide resistance (Matés, 2000; Li and Han, 2004) and may be involved in aphid response to elevated CO_2 and EβF. In this study, these three enzymes (SOD, CAT, and AChE) were used to evaluate the aphid defense as affected by CO_2 level and EβF frequency of release.

The current study explored how elevated CO_2 affects wheat plants *Triticum aestivum* and modifies the responses of the grain aphid *Sitobion avenae* to the frequency of EβF release. We test the hypotheses that, depending on EβF frequency, elevated CO_2 reduces the response of *S. avenae* to EβF. Three specific objectives were to determine: (1) the effects of EβF application (zero, two, or five times daily) on population abundance of *S. avenae* under elevated CO_2, (2) whether the growth and chemical components of individual aphids change with EβF application under elevated CO_2, and (3) whether SOD, CAT, and AChE were involved in the response of *S. avenae* to elevated CO_2 and EβF application.

2. Materials and methods

2.1. Open top chambers

The experiment was carried out in eight octagonal, open-top chambers (OTCs) (1.6 m wide, 4.2 m diameter, and 2.4 m high) at the Observation Station on Global Chang Biology of the Institute of Zoology, CAS in Xiaotangshan County, Beijing, China (40°11′N, 116°24′E). The current ambient level of CO_2 (375 ppm) and double the current ambient level (750 ppm, the predicted level in about 100 years) (IPCC, 2007) were applied continuously in the OTCs. Four OTCs were used for each CO_2 treatment. The OTCs were arranged in four blocks, with one ambient CO_2 OTC and one double-ambient CO_2 OTC in each block. Double-ambient CO_2 concentrations were monitored and controlled by an infrared CO_2 transmitter (Ventostat 8102, Telaire Company, USA) and were maintained throughout the experiments. Details of the automatic control system for CO_2 levels and OTCs were provided in Chen et al. (2005a,b). The tops of the OTCs were covered with nylon netting to exclude insects.

2.2. Host plants

Seeds of wheat (cv. Longfu 174379) were sown on 23 April 2007 in plastic pots (14 cm diameter and 13 cm high) filled with 8:3:1 (by volume) loam:cow dung:earthworm castings. There were 20 seeds per pot, and 64 pots per OTC. Pot placement was re-randomized within each OTC once every week. On 8 May 2007, wheat plants were thinned to 10 stems per pot. No chemical fertilizers or insecticides were applied. Water was added to each pot once every 2 days.

2.3. Effects of CO_2 on plants in the absence of aphids

Twenty stems from four pots per OTC (160 stems in total) were randomly selected on 22 May for plant height measurement. After plant height (from base to terminal of stem) was measured, the stems were then dried at 80 °C for 72 h to measure the above-ground dry biomass. Fifty stems from five pots per OTC (400 stems in total) were randomly selected on 22 May, weighed, and stored at −20 °C until subjected to chemical analysis. Foliar water content, as a proportion of fresh weight, was calculated after these stems were dried at 80 °C for 72 h. Total non-structural carbohydrates (TNC), mainly starch and sugar, were assayed by acid hydrolysis following the method of Tissue and Wright (1995). Nitrogen content was assayed using Kjeltec nitrogen analysis (Foss automated Kjeltec™ instruments, Model 2100).

2.4. Aphid infestation and EβF treatments

The apterous grain aphid, *S. avenae*, was obtained from the Institute of Crop Protection, Chinese Agricultural Academy of Science, and was reared on wheat seedlings in photoclimatic chambers (HPG280H; Orient Electronic, Haerbin, China) for use as stock cultures. The chambers were maintained at 24 ± 1 °C, 60–70% RH, and 16:8 (L:D)-h photoperiod.

Aphid alarm pheromone, (*E*)-β-farnesene (EβF), was provided by Professor Zhang Z.N. of the Institute of Zoology, Chinese Academy of Sciences (Zhang et al., 1989, 1997; Xiangyu et al., 2002; Su et al., 2006); EβF had been synthesized according to the methods of Dawson and Pickett (1982). The standard solution of EβF (containing 40% of the active isomer) was diluted to the most active concentration of 0.1 μl EβF per ml *n*-hexane (*n*-hex) (Zhang et al., 1997; Su et al., 2006), packed and sealed in glass ampoules, and stored at 4 °C until use.

Each OTC contained four combinations of treatments, including two levels of EβF (added or not added, with *n*-hexane as the carrier and control) and there were two frequencies (two and five times per day) of EβF application: (1) 20 μl *n*-hexane twice daily (8:00, 18:00); (2) 20 μl EβF twice daily (8:00 and 18:00); (3) 20 μl *n*-hexane five times daily (8:00, 10:30, 13:00, 15:30 and 18:00); (4) 20 μl EβF five times daily (8:00, 10:30, 13:00, 15:30 and 18:00).

2.5. Experiment 1: exposure of aphids to four treatments

Each CO_2 level was represented by four OTCs, with three replicate pots for each of the four treatments within each OTC. Each pot was covered with an air-permeable cellophane bag (18.8 cm × 39.0 cm) to prevent aphid escape. On 22 May, each pot was inoculated with 10 apterous adults of *S. avenae*. Starting on 23 May, the four treatments were applied daily through 9 June. To avoid contamination among the four treatments, pots were moved out of OTC during treatment application, and the solutions (EβF/*n*-hexane) were added by a micropipette to a piece of filter paper (3 cm × 3 cm) at the base and center of each pot. After 10 min, the plants were transferred back to the OTC. Because EβF is easily oxidized and highly volatile (Dawson et al., 1982), we considered it

very unlikely that volatiles from one pot would affect another pot once the pots had been returned to the OTCs. After aphids had been added to the pots, aphid numbers per pot (10 stems) were recorded by developmental stage (1st and 2nd instars, 3rd and 4th instars, and adults) and morph type (apterous and alate) every 3 days from 25 May to 9 June 2007, to give six sampling dates. Pots were rotated in each OTC after each count.

2.6. Experiment 2: growth and chemical components of S. avenae exposed to four treatments

On 22 May, 10 randomly selected pots in each OTC (40 pots total) for each treatment were collected (these pots were not part of experiment 1), and the aphids were added and four treatments were applied as described for experiment 1. On 3 June, after 13 days exposure to the four treatments, two kinds of nymphs (4th instar and 1st/2nd instar) were collected and were transferred to a −20 °C refrigerator for later chemical composition assays and enzyme activity quantification. Simultaneously, 10 1st instar nymphs were randomly collected from 3 of 10 pots described above, weighed (W_1), and placed on plants in a new pot. The nymphs were re-weighed (W_2) after 4 days of the inoculation. The mean relative growth rate (MRGR) of *S. avenae* was calculated based on the method of Viskari et al. (2000): $\text{MRGR} = (\ln W_2 - \ln W_1)/t$, where W_1 is the weight of 1st instar nymphs, W_2 the final weight of aphid nymphs and t is the larval duration (day). Ten adults from previous 10 pots were also weighed, dried in an oven, and re-weighed with a Sartorius R200D automatic electro balance (Sartorius, Gottingen, Germany).

Two hundred 4th instar nymphs from each treatment stored at −20 °C were homogenized for 1.5 min at 4 °C in 1:10 (fresh weight/buffer volume ratio) 100 mM phosphate buffer, pH 7.4, containing 100 mM KCl and 1 mM EDTA. Homogenates were centrifuged at 10,000 × *g* for 10 min, and the supernatants were subjected to chemical component analysis. Protein concentration was determined by the Bradford (1976) assay. Total amino acids and free fatty acids were analyzed with reagent kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, Jiangsu Province, China) (Wu et al., 2007).

2.7. Experiment 3: quantification of enzyme activity in aphids exposed to four treatments

All sampled 1st/2nd instar nymphs (≈300) from experiment 2 stored at −20 °C were homogenized and centrifuged by the same protocol used in experiment 2, and collected supernatants were stored at −20 °C. The activities of superoxide dismutase (SOD), catalase (CAT), and acetylcholinesterase (AChE) were measured with reagent kits (Nanjing Jiancheng Bioengineering Institute). As indicated by kit protocol, SOD activity was assayed spectrophotometrically at 550 nm by use of the xanthine and xanthine oxidase system. One unit (U) of SOD activity was defined as the amount of SOD required for 50% inhibition of the xanthine and xanthine oxidase system reaction per minute and per milligram of total protein in the homogenate. CAT activity was based on the decomposition rate of H_2O_2 by the enzyme, which can be measured as the decrease in absorbance per minute at 405 nm. Enzyme activity values were also expressed in CAT units, where one unit (U) is the amount of enzyme needed to hydrolyze 1 μmol H_2O_2 per minute and per milligram of total proteins present in the homogenate. AChE activity was measured by the hydrolysis of acetylthiocholine, which was assayed by the release of sulfhydrylic groups to react with bis-(3-carboxy-4-nitrophenyl) disulfide at 412 nm (Ellman's reagent) (Ellman et al., 1961). One unit (U) of AChE activity was defined as 1 μmol acetylthiocholine decomposed at 37 °C one milligram of protein per six minutes. Finally, activity of the three enzymes was standardized by total protein in the supernatant (U/mg protein).

2.8. Statistical analyses

Analyses of variance (ANOVA, SAS Institute, 1996) were used to analyze the effects of CO_2 levels on plant height, aboveground biomass, and foliar chemical components in the absence of aphids. A split-split plot design was used to analyze the univariate responses of the measured variables of aphid (MRGR, protein, amino acid, fatty acid, SOD, CAT and AChE). CO_2 and block (a pair of ambient and elevated OTCs) were the main effects, EβF level (added or not added) was the subplot effect, and EβF frequency (low or high) was the sub-subplot effect. Effects were considered significant if $p < 0.05$. The effect of block was not significant ($p > 0.30$), and the effect of block and its interaction with other factors are not presented to facilitate data presentation in tables and in text.

Repeated-measures ANOVAs were used to demonstrate the effects of CO_2 levels, EβF level, and EβF frequency on aphid densities (numbers per 10 stems) at different developmental stages. If variables or interactions were significant, least significant difference (LSD) tests were used to separate the different levels. Data from aphid densities were transformed using $\ln(x + 1)$, and proportional data were transformed using the arcsine square root to satisfy assumptions of normality. Data from plant trait and aphid chemical components were $\ln(x + 10)$ transformed if necessary. In addition, Pearson's correlations were calculated to analyze the relationships between the population abundance of 1st and 2nd instar of *S. avenae* and its AChE activity when exposed to different frequencies of EβF under ambient and elevated CO_2.

3. Results

3.1. Plant height, biomass, and foliar chemical components in the absence of aphids

Elevated CO_2 significantly increased plant height ($F_{1,38} = 7.36$, $p = 0.010$), aboveground biomass per plant ($F_{1,38} = 10.758$, $p = 0.002$), TNC ($F_{1,6} = 13.0$, $p = 0.023$), and TNC:N ratio ($F_{1,6} = 125.2$, $p < 0.001$). Elevated CO_2 significantly decreased the nitrogen content in leaves ($F_{1,6} = 464.4$, $p < 0.001$; Table 1). There were no significant differences in foliar water content between ambient and elevated CO_2.

3.2. Experiment 1: effects of CO_2 and EβF on S. avenae population abundance

CO_2 level and EβF level (added or not added) significantly affected the abundance of all aphid developmental stages, with the

Table 1
Traits and foliar chemical components of wheat plants grown under ambient (370 ppm) and elevated CO_2 (750 ppm).

Plant traits	CO_2 level	
	Ambient	Elevated
Plant height (cm)	30.9 ± 2.36 b	33.0 ± 2.44 a
Biomass[a] (g)	0.319 ± 0.083 b	0.397 ± 0.067 a
Nitrogen (mg/g)	29.0 ± 0.64 a	21.0 ± 0.04 b
TNC[b] (mg/g)	227.6 ± 18.7 b	267.6 ± 4.48 a
TNC:N	7.85 ± 0.73 b	12.7 ± 0.21 a
Water content (%)	81.6 ± 1.66 a	80.5 ± 1.21 a

Each value represents the average (±SD) of four replicates (one replicate = one open top chamber), with 10 stems assayed per replicate. Different lowercase letters indicate significant differences between CO_2 treatments by LSD test at $p < 0.05$.
[a] Above-ground biomass per wheat stem.
[b] Total non-structural carbohydrates.

Table 2
p-Values from repeated-measures ANOVAs for the effect of CO_2 level, (*E*)-β-farnesene, and (*E*)-β-farnesene frequency on aphid numbers.

Dependent variable (numbers of aphids)	Main effects and interactions						
	CO_2 [a]	EβF[b]	Frequency[c]	$CO_2 \times$ EβF	$CO_2 \times$ Frequency	EβF × Frequency	$CO_2 \times$ EβF × Frequency
1st and 2nd instars	<0.001	<0.001	0.013	0.144	0.100	0.001	0.848
3rd and 4th instars	<0.001	<0.001	0.584	0.495	0.857	0.022	0.164
Apterous adults	<0.001	<0.001	0.748	0.329	0.915	0.164	0.049
Alate aphids	0.06	0.215	0.281	0.628	0.519	0.481	0.129
Total number	<0.001	<0.001	0.446	0.401	0.945	0.106	0.746

[a] Ambient CO_2 vs. elevated CO_2.
[b] *n*-hexane vs. (*E*)-β-farnesene.
[c] Exposure to EβF (*n*-hex) twice a day vs. five times a day.

exception of alate morphs (Table 2). EβF frequency (low vs. high) was only significant for the abundance of 1st and 2nd instars ($F_{1,88} = 6.470$, $p = 0.013$). The interaction between EβF level and EβF frequency affected the abundance of 1st and 2nd instars ($F_{1,88} = 11.170$, $p = 0.001$) and 3rd and 4th instars ($F_{1,88} = 5.415$, $p = 0.022$). The interaction among CO_2 level, EβF level and EβF frequency was significant for the abundance of apterous adults ($F_{1,88} = 3.972$, $p = 0.049$). Moreover, none of the interactions between/among CO_2 level, EβF level, and EβF frequency was significant for total numbers of aphids (Table 2).

Regardless of EβF frequency, EβF significantly reduced the abundance of all developmental stages of apterous aphid under ambient CO_2. Under elevated CO_2, numbers of 1st and 2nd instars as well as 3rd and 4th instars declined when exposed to a high frequency of EβF (1st and 2nd instars: $F_{1,22} = 18.794$, $p < 0.001$; 3rd and 4th instars: $F_{1,22} = 21.254$, $p < 0.001$) but did not change when exposed to a low frequency of EβF (1st and 2nd instars: $F_{1,22} = 2.063$, $p = 0.165$; 3rd and 4th instars: $F_{1,22} = 2.657$, $p = 0.117$). Furthermore, regardless of EβF frequency, the abundance of apterous adults did not change when exposed to EβF under elevated CO_2 (Figs. 1 and 2). Regardless of CO_2 level, the abundance of 1st and 2nd instar decreased in response to higher EβF frequency vs. lower EβF frequency (ambient CO_2: $F_{1,22} = 7.408$, $p = 0.038$; elevated CO_2: $F_{1,22} = 21.385$, $p < 0.001$).

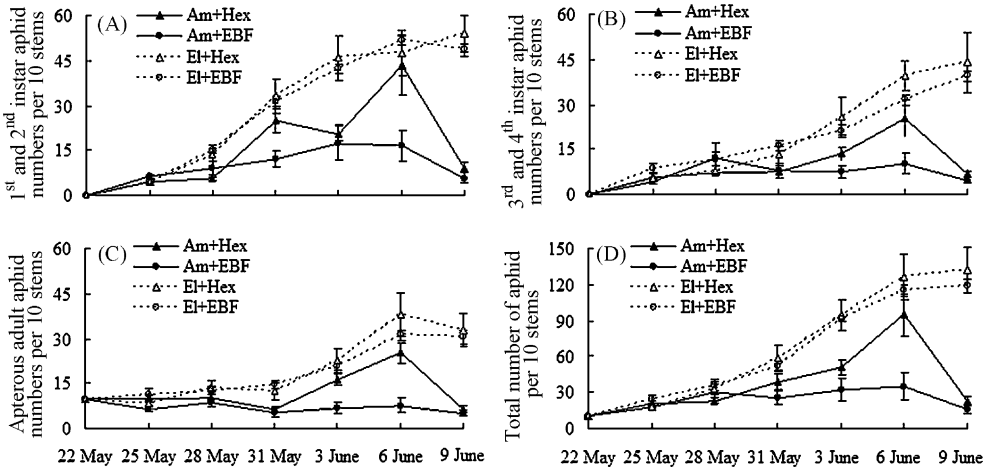

Fig. 1. Abundance (mean ±SD) of *S. avenae* exposed to (*E*)-β-farnesene (EβF) or *n*-hexane (Hex) twice a day under ambient (Am) and elevated (El) CO_2. (A) 1st and 2nd instar, (B) 3rd and 4th instar, (C) apterous adult and (D) total number.

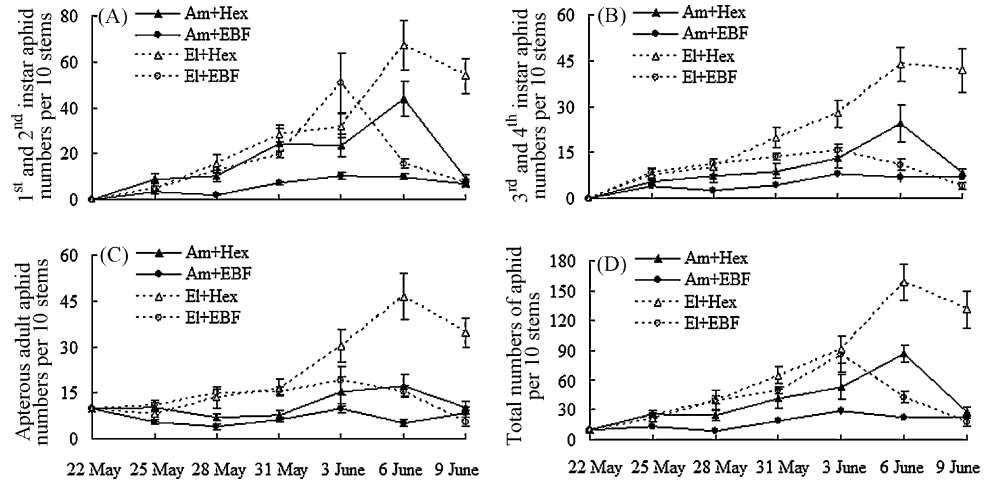

Fig. 2. Abundance (mean ±SD) of *S. avenae* exposed to (*E*)-β-farnesene (EβF) or *n*-hexane (Hex) five times a day under ambient (Am) and elevated (El) CO_2. (A) 1st and 2nd instar, (B) 3rd and 4th instar, (C) apterous adult and (D) total number.

Table 3
p-Values from ANOVAs for the effect of CO_2 level, (*E*)-β-farnesene, frequency on growth, chemical components, and enzyme activity of the aphid *S. avenae*.

Dependent variable	Main effects and interactions						
	CO_2 [a]	EβF[b]	Frequency[c]	CO_2 × EβF	CO_2 × Frequency	EβF × Frequency	CO_2 × EβF × Frequency
MRGR[d]	<0.001	<0.001	0.011	0.062	0.319	0.09	0.058
Dry material (%)	<0.001	<0.001	<0.001	<0.001	0.456	<0.001	0.767
Protein (mg/ml)	<0.001	<0.001	0.006	<0.001	0.027	0.005	0.016
TAA (μmol/mg) [e]	<0.001	<0.001	0.314	<0.001	0.715	0.568	0.314
FFA (μmol/mg) [f]	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
SOD (U)[g]	<0.001	<0.001	0.46	<0.001	0.669	0.416	0.645
CAT (U)[h]	<0.001	<0.001	<0.001	0.425	0.004	<0.001	0.003
AChE (U)[i]	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001

[a] Ambient CO_2 vs. elevated CO_2.
[b] *n*-hexane vs. (*E*)-β-farnesene.
[c] Exposure to EβF (*n*-hex) twice a day vs. five times a day.
[d] Mean relative growth rate.
[e] Total amino acids (μmol/mg protein).
[f] Free fatty acids (μmol/mg protein).
[g] Superoxide dismutase.
[h] Catalase.
[i] Acetylcholinesterase.

3.3. Experiment 2: growth and chemical components of S. avenae

CO_2 level and EβF level (added or not added) significantly influenced MRGR, dry material, protein content, amino acid content, and fatty acid content of *S. avenae* (Table 3). With the exception of MRGR, the interaction between CO_2 level and EβF was significant for all measured variables. Moreover, EβF frequency significantly affected all variables except for amino acids (Table 3).

Regardless of EβF frequency, elevated CO_2 caused higher MRGR, protein content, and amino acid content, and lower fatty acid content of *S. avenae* exposed to *n*-hexane (the carrier for EβF) (Table 4). Furthermore, regardless of CO_2 level and EβF frequency, the MRGR, dry material percentage, and amino acid content of *S. avenae* was significantly lower with EβF than *n*-hexane. Under both CO_2 levels, higher dry material percentage was found in *S. avenae* when EβF was applied at high rather than low frequency. Moreover, aphids exposed to EβF under elevated CO_2 had a higher protein content ($F_{1,6} = 14.7$, $p = 0.010$) when exposed to higher frequency rather than lower frequency EβF (Table 4).

3.4. Experiment 3: SOD, CAT, and AChE of S. avenae

CO_2 level, EβF level (added or not added), and their interactions significantly affected SOD activity. All factors, with the exception of the interaction between CO_2 level and EβF, significantly influenced CAT activity. All factors significantly affected AChE activity (Table 3).

EβF caused higher activity of aphid SOD and AChE under ambient CO_2 (Table 4). For aphids exposed to *n*-hexane, the activity of SOD, CAT, and AChE increased in response to elevated CO_2. Elevated CO_2 decreased SOD activity when aphids were exposed to EβF. Activity of AChE decreased in response to elevated CO_2 when aphids were exposed to lower frequency EβF ($F_{1,6} = 1286.9$, $p < 0.001$) but increased in response to elevated CO_2 when aphids

Table 4
Growth, chemical components, and enzyme activity of the aphid *S. avenae* as exposed to different frequencies of EβF and reared on wheat under ambient (370 ppm) and elevated CO_2 (750 ppm).

EβF frequency	Measured indices	370 ppm		750 ppm	
		n-Hexane	EβF	*n*-Hexane	EβF
Twice a day	MRGR[a]	0.250 ± 0.014b,A	0.198 ± 0.011d,A	0.275 ± 0.008a,A	0.223 ± 0.026c,A
	Dry material (%)	27.1 ± 0.290b,A	24.0 ± 0.493d,B	28.0 ± 0.170a,A	25.8 ± 0.157c,B
	Protein (mg/ml)	0.956 ± 0.011c,A	1.13 ± 0.025a,A	1.14 ± 0.025a,A	1.03 ± 0.050b,B
	TAA (μmol/mg)[b]	1.25 ± 0.690b,A	0.470 ± 0.204c,A	2.78 ± 0.591a,A	0.453 ± 0.222c,B
	FFA (μmol/mg)[c]	228.3 ± 9.23b,A	228.6 ± 8.40b,A	175.7 ± 7.24c,A	373.8 ± 9.00a,A
	SOD (U)[d]	6.01 ± 0.163c,A	16.3 ± 1.33a,A	14.4 ± 0.803b,A	14.6 ± 1.25b,A
	CAT (U)[e]	3.48 ± 0.380c,A	3.80 ± 0.383c,B	5.51 ± 0.527b,A	7.09 ± 0.646a,B
	AChE (U)[f]	0.922 ± 0.019d,A	3.38 ± 0.165a,A	2.35 ± 0.101b,A	1.58 ± 0.121c,B
Five times a day	MRGR	0.250 ± 0.009a,A	0.160 ± 0.018c,B	0.265 ± 0.008a,A	0.215 ± 0.013b,A
	Dry material (%)	27.0 ± 0.193b,A	25.0 ± 0.261c,A	28.1 ± 0.144a,A	26.8 ± 0.178b,A
	Protein (mg/ml)	0.958 ± 0.010b,A	1.14 ± 0.032a,A	1.14 ± 0.033a,A	1.15 ± 0.038a,A
	TAA (μmol/mg)	1.41 ± 0.227b,A	0.500 ± 0.182c,A	2.75 ± 0.558a,A	0.887 ± 0.135c,A
	FFA (μmol/mg)	234.4 ± 7.33a,A	239.6 ± 9.98a,A	167.9 ± 6.42b,A	130.3 ± 6.09c,B
	SOD (U)	6.03 ± 0.517c,A	16.1 ± 0.846a,A	14.4 ± 1.13b,A	13.9 ± 1.08b,A
	CAT (U)	3.48 ± 0.347d,A	7.23 ± 0.422b,A	5.57 ± 0.401c,A	8.54 ± 0.171a,A
	AChE (U)	0.914 ± 0.018d,A	3.97 ± 0.080b,B	2.35 ± 0.155c,A	4.69 ± 0.243a,A

Each value represents the average (±SD) of four replicates. Different lowercase letters within a row indicate significant differences (LSD test: d.f. = 3, 12; $p < 0.05$). Different uppercase letters indicate significant differences between EβF frequency within the same CO_2 and EβF treatment (*n*-hexane vs. EβF) (LSD test: d.f. = 1, 6; $p < 0.05$).
[a] Mean relative growth rate.
[b] Total amino acids (μmol/mg protein).
[c] Free fatty acids (μmol/mg protein).
[d] Superoxide dismutase.
[e] Catalase.
[f] Acetylcholinesterase.

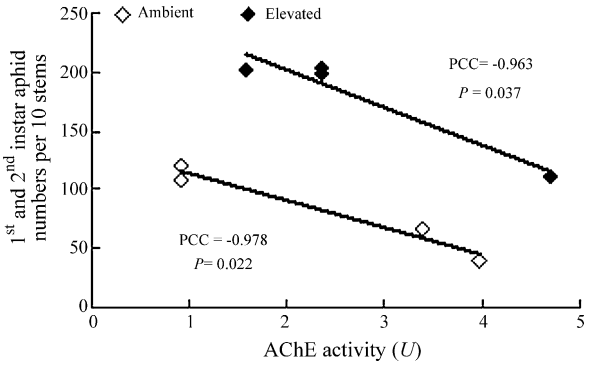

Fig. 3. Pearson correlation between the population abundance of 1st and 2nd instar of *S. avenae* and its AChE activity when exposed to different frequencies of EβF under ambient (370 ppm) and elevated CO_2 (750 ppm) (PCC: Pearson correlation coefficient).

were exposed to higher frequency EβF ($F_{1,6} = 78.8$, $p < 0.001$). Moreover, for aphids exposed to EβF under ambient CO_2, higher frequency EβF increased CAT activity ($F_{1,6} = 187.7$, $p < 0.001$) and decreased AChE activity ($F_{1,6} = 883.6$, $p < 0.001$). In contrast, for aphids exposed to EβF under elevated CO_2, higher frequency EβF increased the activities of CAT ($F_{1,6} = 186.3$, $p < 0.001$) and AChE ($F_{1,6} = 263.3$, $p < 0.001$) (Table 4). Population abundance of 1st and 2nd instar of *S. avenae* was negatively correlated with its AChE activity when exposed to different frequencies of EβF under both ambient and elevated CO_2 (Fig. 3).

4. Discussion

Our study clearly showed that addition of EβF substantially suppressed aphid abundance under ambient CO_2 (Table 2). The reduced abundance could have resulted from the combined effects of low survival rate, long developmental times, and lower fecundity. Conversely, elevated CO_2 increased *S. avenae* abundance when the aphid was exposed to *n*-hexane without added EβF, and the interaction between EβF and CO_2 level was not significant for aphid abundance. Thus, at the population level, the negative effects of EβF on aphid abundance were counteracted by elevated CO_2.

EβF generally increases the proportion of alate progeny in pea aphid, and the "pseudo-crowding" hypothesis proposed by Kunert et al. (2005) is well documented in ambient environments. In our study, however, CO_2 level, EβF, and EβF frequency did not affect the production of *S. avenae* alate morphs; perhaps EβF alone cannot trigger the alate morph or perhaps production of alates was inhibited by the presence of other natural molecules (Dawson et al., 1984). The pseudo-crowding hypothesis proposes that perception of alarm pheromone increases walking behavior in aphids, which increases the number of physical contacts between individuals, as happens when aphids are crowded (Kunert et al., 2005). This hypothesis could explain why elevated CO_2 counteracted the EβF effect on aphid abundance. The larger or higher plants grown under elevated CO_2 might reduce the physical contacts between aphids, which in turn might decrease the perception of EβF and therefore decrease EβF-mediated walking behavior.

The consequences of EβF for population dynamics will depend on the complex relationship between host density, rate of predator attack (EβF frequency), and trait modification (Kunert et al., 2003). Under ambient CO_2, the abundance of 1st and 2nd instar decreased in response to higher EβF frequency vs. lower EβF frequency. Kunert et al. (2005) also found that the pea aphid reacts more strongly to the frequency of EβF release than to the amount of EβF released. EβF frequency, however, was not significant for the abundance of the 3rd and 4th as well as apterous adults, suggesting that aphid abundance exhibits a stage-specific response to higher EβF frequency under ambient CO_2. Su et al. (2006) found that different instars of the cotton aphid *Aphis gossypii* responded differently to EβF, and that the 1st instar was the most sensitive to EβF in terms of development time and fecundity. Frazer et al. (1981) reported that juvenile aphids are more susceptible to predators than later instars and adult aphids. This could explain why, under ambient CO_2 in the current study, 1st and 2nd instars of *S. avenae* were more sensitive than 3rd and 4th instars to higher EβF frequency.

As a chemical signal, EβF affects interactions among plant, aphid, and natural enemy (Beale et al., 2006), and these interactions could be modified by elevated CO_2 (Gao et al., 2008). Clearly, elevated CO_2 changes the quality and quantity of the plant, and may further influence the performance of the herbivore and its natural enemies. Chen et al. (2005a) indicated that elevated CO_2 increased the MRGR of the lady bird beetle *Leis axyridis*, increased the preference of the beetle for *A. gossypii*, and enhanced the biological control of the aphid by the beetle. Stacey and Fellowes (2002) found that the changes in plant quality under elevated CO_2 did not seem to alter aphid quality as a prey species. In this study, elevated CO_2 enhanced the MRGR of the grain aphid *S. avenae* when the aphid was exposed to *n*-hexane without EβF and increased the nutrient content (dry material percentage, amino acids, and protein but not free fatty acids) of the aphid. This indicates that elevated CO_2 enhanced the quality of the aphid as a food resource for its predator. In contrast, aphid MRGR, dry material content, and amino acid content decreased in response to EβF. At the level of individuals, EβF has been considered an alarm or a stress perceived by aphids to indicate the presence of predators, and has been widely acknowledged for its non-lethal and non-consumptive interactions within the perceiving individual. Thus, EβF-induced changes in the perceiving individual can be costly because the altered behaviors that help reduce the risk of predation or parasitism often reduce aphid growth rates. Furthermore, EβF is also acting as a foraging cue that attracts the predators of aphid (Francis et al., 2004). Thus, EβF production entails significant ecological cost which may reflect the population information of aphid to its natural enemies (Verheggen et al., 2009). It is seems that, under elevated CO_2 environment, the benefit of EβF to aphids would be reduced while the cost would be increased because of insensitivity of aphid to EβF and increasing exposure to natural enemies.

Elevated CO_2 enhanced the activities of antioxidant enzymes (SOD and CAT) in aphids exposed to higher EβF frequency. Phenolics and other plant allelochemicals altered by elevated CO_2 can stimulate or deter aphid settling and feeding (Montllor, 1991). Dreyer and Jones (1981) found that some phenolics such as flavonoid aglycones are feeding deterrents for aphids. Thus, the activities of antioxidant enzymes were up-regulated when aphids perceived stress. Moreover, Dawson et al. (1983) proposed that peach aphids resistant to insecticide might be insensitive to EβF. AChE is one of the most important enzymes involved in nerve transmission, and perhaps is the key enzyme in EβF perception and signal transduction. In this study, AChE activity of *S. avenae* was negatively correlated with aphid population abundance when exposed to different frequencies of EβF (Fig. 3). This suggests that AChE activity may be involved in aphid insensitivity to EβF under elevated CO_2. We proposed that higher AChE activity of *S. avenae* may indicate more sensitive to EβF, while lower AChE activity implied less sensitive to EβF. In this study, when lower frequency of EβF was applied, elevated CO_2 reduced the activity of AChE in aphids, which may decrease the sensitive of aphids to EβF, thereby increased the numbers of 1st and 2nd instars under elevated CO_2. Conversely, when exposed to higher frequency of EβF, elevated CO_2 increased the activity of AChE, which increased the sensitive of aphids to EβF, and in turn decreased the numbers of 1st and 2nd instars. Although the underlying mechanisms leading to lack of

aphid response to EβF under elevated CO_2 are not well understood, our study shows that higher activities of SOD and CAT are involved in how aphids respond to changes in various volatile and non-volatile plant compounds induced by elevated CO_2, and that lower activity of AChE may contribute to aphid insensitivity to EβF.

Although alarm pheromone is the principal anti-predator defense for aphids, the pheromone could be used in new pest control methods to repel aphids or attract natural enemies (Micha and Wyss, 1996; Al abassi et al., 2000; Beale et al., 2006). This use of the pheromone for pest control, however, requires that we understand how aphids respond to the pheromone in various environments, including those with elevated CO_2. According to our study, however, a plant that constantly releases EβF under elevated CO_2 would neither limit aphid abundance nor increase the proportion of alates produced. However, more efficient predation (Chen et al., 2005a), more plant secondary metabolites (Peltonen et al., 2006), and reduced response to alarm pheromone could limit aphid abundance under elevated CO_2 in the future. Unfortunately, how elevated CO_2 reduces the response of aphids to EβF is still unclear. We offer the following speculations about the mechanism underlying this phenomenon. First, EβF and its inhibitors, β-caryophyllene and (−)-germacrene D, are naturally emitted from many plants (Dawson et al., 1984). Direct effects of elevated CO_2 on plant secondary metabolism are expected to increase the emission of volatile organic compounds because of allocation of excess carbon to secondary metabolites. This increase in production of secondary metabolites, many of which are volatile, could alter the emission ratio between EβF and its inhibitor. On the other hand, given that plants produce more EβF under elevated CO_2, the aphid may acclimate and become less sensitive to EβF. Second, elevated CO_2 increases the quality and quantity of food available to the aphids, and perhaps these increases in food quality and quantity outweigh the perceived danger of predator indicated by increases in alarm pheromone (Dill et al., 1990; Losey and Denno, 1998). This is the first study to report that elevated CO_2 alleviates the response of *S. avenae* population to EβF, and the possible mechanisms underlying this phenomenon remain to be elucidated.

5. Conclusion

Overall, our results showed that numbers of *S. avenae* declined with increased frequency of EβF application under ambient CO_2 but were unaffected by EβF application under elevated CO_2, and suggested that elevated CO_2 reduces the response (in terms of population density) of *S. avenae* to EβF. Although the underlying mechanisms are still unknown, lower activity of acetylcholinesterase in *S. avenae* may be involved in its reduced sensitivity to EβF under elevated CO_2.

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