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# Olfactory Preferences of *Popillia japonica*, *Vanessa cardui*, and *Aphis glycines* for *Glycine max* Grown Under Elevated CO<sub>2</sub>

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**ABSTRACT** Levels of atmospheric CO<sub>2</sub> have been increasing steadily over the last century and are projected to increase even more dramatically in the future. Soybeans (*Glycine max* L.) grown under elevated levels of CO<sub>2</sub> have larger herbivore populations than soybeans grown under ambient levels of CO<sub>2</sub>. Increased abundance could reflect the fact that these herbivores are drawn in by increased amounts of volatiles or changes in the composition of volatiles released by plants grown under elevated CO<sub>2</sub> conditions. To determine impacts of elevated CO<sub>2</sub> on olfactory preferences, Japanese beetles (*Popillia japonica* Newman) and soybean aphids (*Aphis glycines* Matsumura) were placed in Y-tube olfactometers with a choice between ambient levels of CO<sub>2</sub> gas versus elevated levels of CO<sub>2</sub> gas or damaged and undamaged leaves and plants grown under ambient levels of CO<sub>2</sub> versus damaged and undamaged plants grown under elevated levels of CO<sub>2</sub>. All plants had been grown from seeds under ambient or elevated levels of CO<sub>2</sub>. Painted lady butterflies (*Vanessa cardui* L.) were placed in an oviposition chamber with a choice between plants grown under ambient and elevated levels of CO<sub>2</sub>. *A. glycines* and *V. cardui* showed no significant preference for plants in either treatment. *P. japonica* showed no significant preference between ambient levels and elevated levels of CO<sub>2</sub> gas. There was a significant *P. japonica* preference for damaged plants grown under ambient CO<sub>2</sub> versus undamaged plants but no preference for damaged plants grown under elevated CO<sub>2</sub> versus undamaged plants. *P. japonica* also preferred damaged plants grown under elevated levels of CO<sub>2</sub> versus damaged plants grown under ambient levels of CO<sub>2</sub>. This lack of preference for damaged plants grown under elevated CO<sub>2</sub> versus undamaged plants could be the result of the identical elevated levels of a green leaf volatile (2-hexenal) present in all foliage grown under elevated CO<sub>2</sub> regardless of damage status. Green leaf volatiles are typically released from damaged leaves and are used as kairomones by many herbivorous insects for host plant location. An increase in production of volatiles in soybeans grown under elevated CO<sub>2</sub> conditions may lead to larger herbivore outbreaks in the future.

**KEY WORDS** herbivorous insects, climate change, green leaf volatiles, Y-tube olfactometers

Multiple insect herbivores use individual volatile chemicals or blends of volatiles specific to their host plants to locate their food by olfaction. Changes in the composition of the volatile blend released from damaged plants over time have been found to be attractive to Japanese beetles (*Popillia japonica* Newman) (Loughrin et al. 1996). *P. japonica* Newman feed on >300 species of plants and are considered an economic pest in all feeding life stages (Fleming 1972). This olfactory preference for general volatiles released from many different plants after they have been damaged may be an evolved trait, allowing the insect to locate a suitable plant that another organism has successfully consumed.

There are many factors that can affect a plant's chemical composition, including changing concentrations of atmospheric gases and pollutants. These atmospheric changes can lead to increased or decreased volatile production and can further lead to modifications in the behavior of the herbivores that depend on these plants. Multiple atmospheric change studies have shown that insect herbivores have distinct gustatory and olfactory preferences in different atmospheric conditions. *Malacosoma disstria* (forest tent caterpillar) larvae prefer feeding on *Populus tremuloides* Michaux (quaking aspen) foliage over *Betula papyrifera* Marshall (paper birch) foliage in an ambient CO<sub>2</sub> atmosphere, but in an elevated CO<sub>2</sub> atmosphere they prefer the opposite (Agrell et al. 2005); Kopper and Lindroth (2003) found that *Phyllonorycter tremuloidiella* Braun (aspen blotch leafminer) colonization rates were decreased on *P. tremuloides* grown under elevated CO<sub>2</sub> and O<sub>3</sub> conditions.

How elevated CO<sub>2</sub> affects the composition and quantity of volatile chemicals released from damaged

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plants remains an open question. Olfaction is important for host location for most herbivores, especially ones that feed on a transient resource such as an agricultural crop (Carroll et al. 2006, Couty et al. 2006, Gouinguene and Staedler 2006). A change in production of volatiles could lead to an increased preference for plants grown under elevated CO<sub>2</sub> conditions. Previous research conducted at the soybean free air gas concentration enrichment (SoyFACE) site at the University of Illinois, Urbana-Champaign, found that herbivory was increased in the elevated CO<sub>2</sub> treatment. This increase was associated with higher numbers of *P. japonica* on those plants (Hamilton et al. 2005). *P. japonica* were accidentally introduced from Japan to the east coast of the United States in the early 1900s, and the species has been spreading steadily west ever since, reaching Illinois in the 1990s (Gray 1997). The adults are leaf skeletonizers, feeding on the tissue in between the leaf veins giving the leaves a lacy, skeletal appearance (Potter and Held 2002). Populations of soybean aphids, *Aphis glycines* Matsumura, are also increased on soybeans (*Glycine max* L.) grown under elevated CO<sub>2</sub> conditions (Dermody et al. 2008). *A. glycines* were accidentally introduced from Asia to the midwest United States in the early 2000s (Ragsdale et al. 2004). All life stages are phloem feeders and can have an economic impact on *G. max* yield (Ragsdale et al. 2007). Although these increases were associated with improved insect performance on these plants (O'Neill et al. 2008, O'Neill 2008), the role of insect preference for plants grown under this treatment was undetermined and potentially could also be contributing to increased populations of these herbivores.

We examined the preference for undamaged and insect-damaged foliage of *G. max* grown under ambient and elevated CO<sub>2</sub> conditions exhibited by three important soybean herbivores: *P. japonica*, *A. glycines*, and the painted lady butterfly (*Vanessa cardui* L.). *V. cardui* is native to Illinois, with larvae that are foliage chewers on various species of legumes and population outbreaks every few years (Poston et al. 1977). Although these outbreaks do not currently result in economic levels of damage to soybeans, it is unknown how populations of this species may respond to effects of elevated levels of CO<sub>2</sub> on their host plants. We also measured leaf volatile compounds produced and released by undamaged and insect-damaged foliage grown under ambient and elevated levels of CO<sub>2</sub> that might account for potential differences in insect preference. We hypothesized that the higher numbers of herbivores found on *G. max* growing under elevated levels of CO<sub>2</sub> result from increased olfactory preference for *G. max* caused by increased volatile chemical production and release from plants.

### Materials and Methods

**Plant Material.** All assays with freshly cut plant material were conducted during July 2004. All whole-plant assays were conducted during July and August 2007. All plant material was grown at the soybean free air gas concentration enrichment (SoyFACE) site in

SoyFACE, IL. FACE systems allow researchers to fumigate a large area with elevated levels of atmospheric gases. This site covers 32.4 ha and is a part of the South Farms area at the University of Illinois, Urbana-Champaign. There are three experiment treatments and a control treatment at this site: ambient air (387 μmol/mol CO<sub>2</sub>), elevated CO<sub>2</sub> with a target of 550 μmol/mol CO<sub>2</sub>, addition of 1.2 times ambient levels O<sub>3</sub>, and combination of the elevated CO<sub>2</sub> and O<sub>3</sub> treatments. The plots are fumigated during daylight hours, every day, throughout the growing season. This farm has been in continuous crop production for 100 yr, mostly using the crop rotation system with corn and soybean. The cultivar used in all assays was Pioneer 93B15. The field is divided into four sections, with each treatment replicated four times in a randomized block design, once per section, for a total of 16 plots. Treatment plots have a diameter of 20 m, cover 350 m<sup>2</sup>, and are at least 100 m from any other plots. This buffer zone between experimental plots ensures that there is minimal drift of elevated atmospheric gases from one plot to another. Further details of the SoyFACE experimental set up can be found in Long et al. (2004).

***Glycine max* Leaf Extracts.** Undamaged and beetle-damaged leaves were picked from all ambient and elevated CO<sub>2</sub> plots at the SoyFACE site on 19 July 2005 and placed immediately in liquid nitrogen. Leaves were cut at the petiole with scissors and taken back to the laboratory for extracting. Six 1-cm leaf disks from each plot and each treatment were ground, and 500 μl hexane was added to each sample. Samples were centrifuged at 10,000/min for 2 min, supernatant was separated, and a 3-μl subsample was analyzed on a gas chromatograph (GC) (5890; Hewlett-Packard, Palo Alto, CA). The GC had a DB-5 capillary column (30 m by 0.32 mm by 0.25 μm film; J&W Scientific, Folsom, CA). The GC program was started at 40°C for 3 min, 5°/min increase to 150°, 40°/min increase to 290°C, and held for 5 min. Six leaf disks from beetle-damaged plants grown under elevated CO<sub>2</sub> levels were also analyzed on a separate GC/mass spectrophotometer (MS) using the same program as described (GC 6890, MS 5970; Hewlett-Packard) to identify sample peaks with the help of the NIST library (National Institute of Standards and Technology, Gaithersburg, MD). The GC/MS had a DB-5MS capillary column (30 m by 0.25 mm by 0.25-μm film; J&W Scientific). Resulting peaks were compared across treatments and identity of peaks that differed in area significantly in the foliage grown under elevated levels of CO<sub>2</sub> from those found in the foliage grown under ambient levels of CO<sub>2</sub> was confirmed by commercial standards when available. Peak identities were confirmed by adding commercial standard to samples of undamaged foliage grown under ambient levels of CO<sub>2</sub>, analyzing extracts on the GC, and identifying the resulting peaks. Peak areas were compared by a repeated-measures analysis of variance (ANOVA) with a between-subject effect of CO<sub>2</sub> treatment and damage repeated (16 statistical software; SPSS, Chicago, IL). Damage was considered a repeated measure because matched insect-damaged and control samples were taken from the same plots.

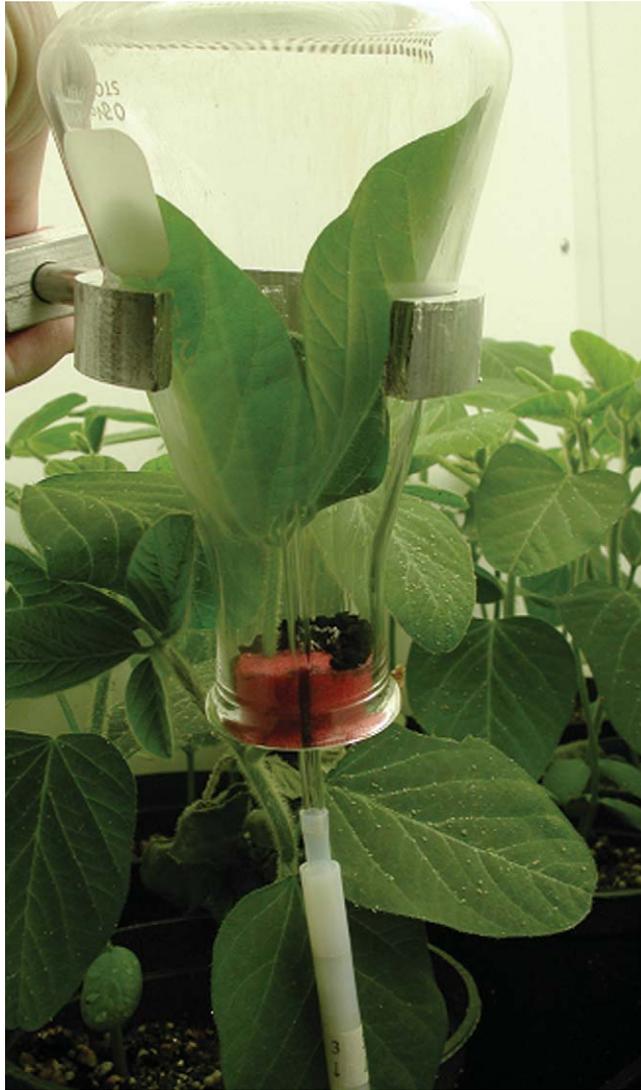


Fig. 1. *Glycine max* volatile collection apparatus. The sealed flask was used to help create a head-space around the leaf tissue where volatiles could be contained. A vacuum pump attached to the glass outlet tube was used to collect the accumulated plant volatile chemicals. (Online figure in color.)

***Glycine max* Volatile Collection and Analysis.** Volatile compounds were collected in situ from undamaged and mechanically damaged plants in all ambient and elevated CO<sub>2</sub> plots at the SoyFACE site twice during July 2008 by head space collection (Fig. 1). In the field, living *G. max* leaves were clamped inside a flask that was sealed with a foam cork to create a headspace. The cork was sealed around the leaf petiole and charcoal was placed around the air inlet hole to scrub the incoming air. Air was pulled through the flask by an attached vacuum pump at a rate of 500 ml/min. Volatiles were collected on Porapak type Q absorbent (80–100 Mesh; Waters, Milford, MA). A glass outlet tube, connected to the column containing the Porapak, was inserted well up into the flask next to the trifoliolate leaf for volatile collection. Collections

lasted for 16 h. Back in the laboratory, volatiles were eluted from the Porapak type Q absorbent with hexane and analyzed on the GC/MS used previously. The GC was programmed from 35°C for 30 s, 5°/min increase to 150°, 40°/min increase to 200°C, and held for 2 min. Samples were run through a mass spectrophotometer to identify sample peaks with the help of a NSB75K library. Peaks were matched across samples, and concentrations were compared by repeated-measures ANOVA with a between-subject effect of CO<sub>2</sub> treatment. Damage was considered as repeated measures because matched insect-damaged and control samples were taken from the same ambient and elevated CO<sub>2</sub> treatment plots (16 statistical software; SPSS). Date was not used in this analysis because very few volatile compounds were found in the headspace

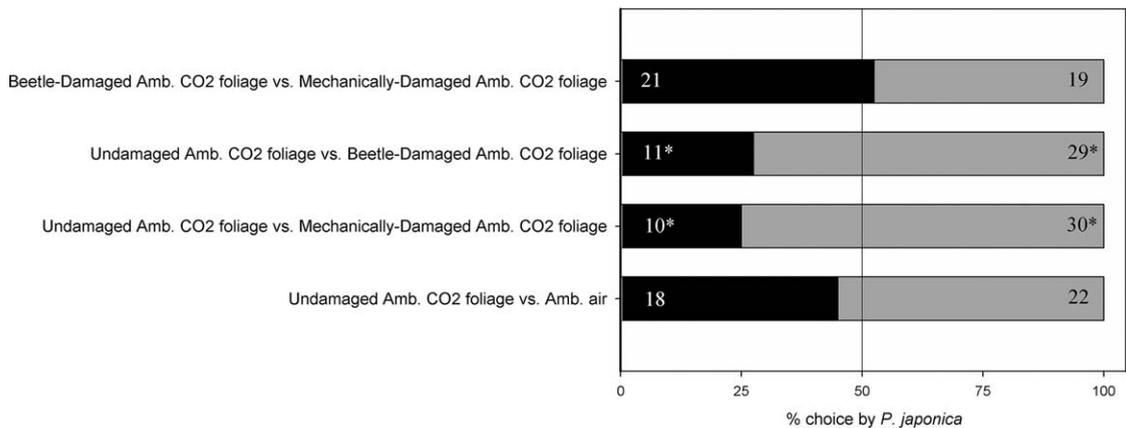


Fig. 2. *Popillia japonica* olfactory preference for *G. max* cut foliage grown under ambient levels of CO<sub>2</sub>. Each row represents a different olfactory trial with unique odor sources. The bars show the percentage of beetles that chose each of the two options. The numbers in the bars are the absolute numbers of beetles that chose each of the two options. Choices between odor sources were analyzed by  $\chi^2$  analysis (stars indicate significant *P* values at the 0.001 level). Beetle-damaged ambient CO<sub>2</sub> foliage versus mechanically damaged ambient CO<sub>2</sub> foliage ( $\chi^2 = 0.1$ , *P* = 0.752). Undamaged ambient CO<sub>2</sub> foliage versus beetle-damaged ambient CO<sub>2</sub> foliage ( $\chi^2 = 8.1$ , *P* = 0.004). Undamaged ambient CO<sub>2</sub> foliage versus mechanically damaged ambient CO<sub>2</sub> foliage ( $\chi^2 = 10$ , *P* = 0.002). Undamaged ambient CO<sub>2</sub> foliage versus ambient air ( $\chi^2 = 0.4$ , *P* = 0.527).

collected on the first date so we were unable to compare them to ones collected on the second date.

**Insects.** *Popillia japonica* were collected for the olfactory choice tests with freshly cut foliage by hand from soybean plants at the SoyFACE site in the last week of July 2004 during the mornings before the beetles had warmed up enough to fly away. Beetles were again collected by hand from soybean plants at the SoyFACE site during the mornings in the first week of July 2007 for the olfactory choice tests with whole plants. *A. glycines* were obtained from a campus laboratory colony in the second week of July 2007. The aphids had been reared for multiple generations on *G. max* maintained in growth chambers. *V. cardui* were reared from eggs purchased from Carolina Biological Supply Company (Burlington, NC) in July 2007. The newly hatched larvae were reared on *G. max* at the SoyFACE research site from first instars to pupation.

**Description of Y-Tube Choice Test.** Insect olfactory preference was tested for undamaged and mechanically/insect-damaged *G. max* foliage or whole plants grown at the SoyFACE research site under ambient and elevated levels of CO<sub>2</sub>. Olfactory preference was measured for *P. japonica* and *A. glycines* with a Y-tube olfactometer made of Pyrex with a stem 10 cm long and each arm also 10 cm long. The tube had a diameter of 1 cm, and the Y arms were at an angle of 140° to each other. Trials were performed in the laboratory under 1,500-W fluorescent bulbs. Odor sources were placed in Plexiglas boxes at the ends of each arm of the Y-tube with charcoal-filtered air flowing over the choices through the Y arms of the tube toward the insects in the stem of the Y. For the trials with *P. japonica*, air flowed through each Y arm at a flow rate of 4 ml/min. For the trials with *A. glycines*, air flowed through each Y arm at a flow rate of 2 ml/min. Undamaged plants were deliberately damaged, by insects or mechani-

cally, for the olfactory trials. *P. japonica* or *A. glycines* were added to plants/foliage and contained on them with fine mesh bags for 24 h. Plants/foliage that had 20–40% feeding damage were chosen as insect-damaged foliage, and only plants damaged by the species being tested were used in each trial. Plants/foliage given 20–40% tears, rips, or holes by a razor blade were chosen as mechanically damaged foliage. Plants/foliage were covered with fine mesh bags for 24 h to prevent any additional damage from occurring to the plants/foliage. Percent foliar damage was visually estimated by plant/foliage collectors, with the aid of a reference collection of photographs. Equal numbers of male and female beetles were introduced individually into the Y-tube. Forty beetles in total were used in the assays with freshly cut foliage; 50 beetles in total were used in the assays with whole intact plants. Ten individuals of one sex were introduced individually into the olfactometer; the olfactometer was rinsed with hexane, and 10 individuals of the other sex were introduced individually into the olfactometer. This procedure ensured that all traces of pheromones or frass would be removed from the olfactometer before the opposite sex was introduced to it. Each individual insect was considered a replicate. Aphid alates were not sexed but were also introduced individually into the Y-tube, for a total of 50 aphids for each test. A preference trial was considered complete once an insect had walked from the base of the Y to one of the arms. For both the beetles and the aphids, the odor source was switched to the opposite Y arm after every 10th insect to ensure there was no directional preference being recorded instead of odor preference, and the olfactometer was rinsed with hexane at this time to clean it. All Y-tube results were analyzed by  $\chi^2$  analysis (16.0 statistical software; SPSS).

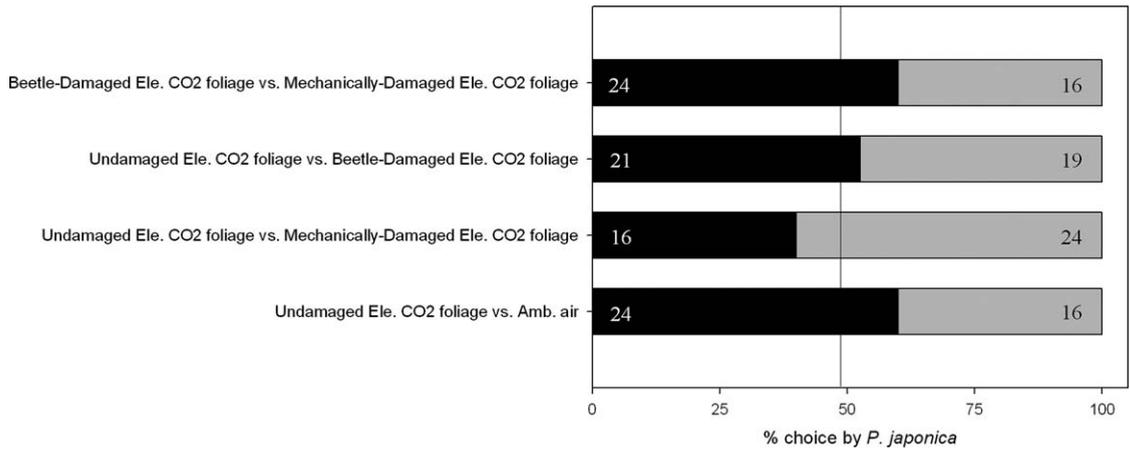


Fig. 3. *Popillia japonica* olfactory preference for *G. max* cut foliage grown under elevated levels of CO<sub>2</sub>. Each row represents a different olfactory trial with unique odor sources. The bars show the percentage of beetles that chose each of the two options. The numbers in the bars are the absolute numbers of beetles that chose each of the two options. Choices between odor sources were analyzed by  $\chi^2$  analysis. Beetle-damaged elevated CO<sub>2</sub> foliage versus mechanically damaged elevated CO<sub>2</sub> foliage ( $\chi^2 = 1.6, P = 0.206$ ). Undamaged elevated CO<sub>2</sub> foliage versus beetle-damaged elevated CO<sub>2</sub> foliage ( $\chi^2 = 0.1, P = 0.752$ ). Undamaged elevated CO<sub>2</sub> foliage versus mechanically damaged elevated CO<sub>2</sub> foliage ( $\chi^2 = 1.6, P = 0.206$ ). Undamaged elevated CO<sub>2</sub> foliage versus ambient air ( $\chi^2 = 1.6, P = 0.206$ ).

**Popillia japonica Olfactory Choice Tests.** Beetle olfactory preferences were tested for elevated and ambient levels of CO<sub>2</sub> gas with pure elevated (700 ppm with nitrogen as a carrier gas) CO<sub>2</sub> flowing through one arm of the Y-tube and pure ambient (370 ppm with nitrogen as a carrier gas) CO<sub>2</sub> flowing through the second arm of the Y-tube. Beetle olfactory preferences were tested for various combinations of freshly cut foliage with and without beetle damage. Foliage was cut from soybean plants at the SoyFACE

research site, placed in water, and immediately brought to the laboratory for use in olfactory testing. Cutting the foliage may have increased the release of volatiles, but because all foliage in all treatments was treated exactly the same way, this potential increase was equivalent across treatments. Various combinations of undamaged and beetle-damaged freshly cut foliage grown under ambient and elevated levels of CO<sub>2</sub> were presented to beetles in olfactory choice tests (Figs. 2–5). Beetle olfactory preferences were

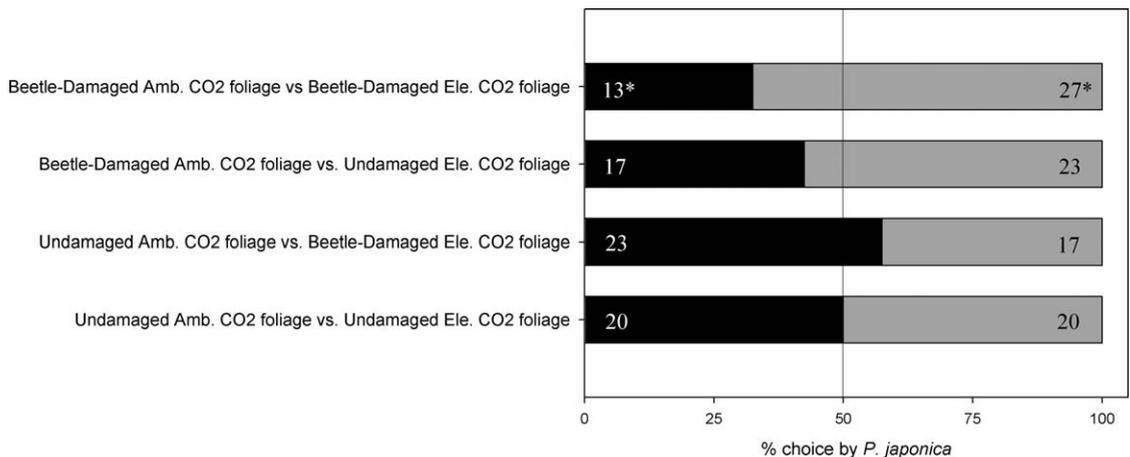


Fig. 4. *Popillia japonica* olfactory preference for *G. max* cut foliage grown under ambient or elevated levels of CO<sub>2</sub>. Each row represents a different olfactory trial with unique odor sources. The bars show the percentage of beetles that chose each of the two options. The numbers in the bars are the absolute numbers of beetles that chose each of the two options. Choices between odor sources were analyzed by  $\chi^2$  analysis (stars indicate significant *P* values at the 0.01 level). Beetle-damaged ambient CO<sub>2</sub> foliage versus beetle-damaged elevated CO<sub>2</sub> foliage ( $\chi^2 = 4.9, P = 0.027$ ). Beetle-damaged ambient CO<sub>2</sub> foliage versus undamaged elevated CO<sub>2</sub> foliage ( $\chi^2 = 0.9, P = 0.343$ ). Undamaged ambient CO<sub>2</sub> foliage versus beetle-damaged elevated CO<sub>2</sub> foliage ( $\chi^2 = 0.9, P = 0.343$ ). Undamaged ambient CO<sub>2</sub> foliage versus undamaged elevated CO<sub>2</sub> foliage ( $\chi^2 = 0.0, P = 1.0$ ).

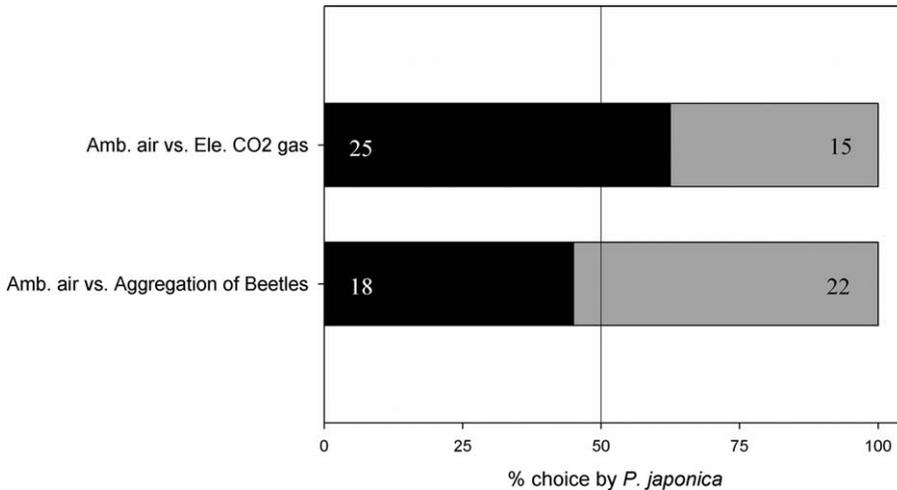


Fig. 5. *Popillia japonica* olfactory preference for ambient or elevated levels of CO<sub>2</sub> gas. Each row represents a different olfactory trial with unique odor sources. The bars show the percentage of beetles that chose each of the two options. The numbers in the bars are the absolute numbers of beetles that chose each of the two options. Choices between odor sources were analyzed by  $\chi^2$  analysis. Ambient air versus elevated CO<sub>2</sub> gas ( $\chi^2 = 2.5$ ,  $P = 0.114$ ). Ambient air versus beetle aggregation ( $\chi^2 = 0.4$ ,  $P = 0.527$ ).

also tested for various combinations of undamaged and beetle-damaged entire soybean plants grown in pots at the SoyFACE research site under ambient and elevated levels of CO<sub>2</sub> (Fig. 6).

***Aphis glycines* Olfactory Choice Tests.** Aphid olfactory preference was tested for various combinations of entire soybean plants with and without aphid damage. Alate aphids were used, as this is the only life stage that can fly and find new soybean plants. Various combi-

nations of undamaged and aphid-damaged plants grown in pots at the SoyFACE research site under ambient or elevated levels of CO<sub>2</sub> were presented to aphids and olfactory preference was recorded (Fig. 7).

***Vanessa cardui* Oviposition Choice Tests.** Adult butterflies were not used in Y-tube olfactory choice tests, because they did not respond to any stimuli other than sunlight when in the olfactometer. Butterflies were placed in an oviposition choice chamber. The ovipo-

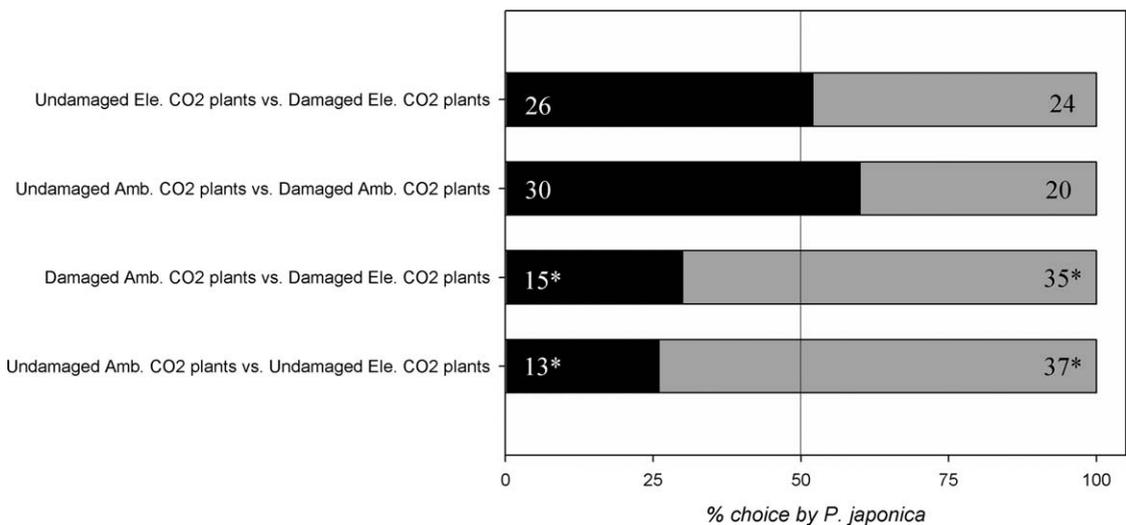


Fig. 6. *Popillia japonica* olfactory preference for *G. max* whole plants grown under ambient or elevated levels of CO<sub>2</sub>. Each row represents a different olfactory trial with unique odor sources. The bars show the percentage of beetles that chose each of the two options. The numbers in the bars are the absolute numbers of beetles that chose each of the two options. Choices between odor sources were analyzed by  $\chi^2$  analysis (stars indicate significant  $P$  values at the 0.001 level). Undamaged elevated CO<sub>2</sub> plants versus damaged elevated CO<sub>2</sub> plants ( $\chi^2 = 0.08$ ,  $P = 0.777$ ). Undamaged ambient CO<sub>2</sub> plants versus damaged ambient CO<sub>2</sub> plants ( $\chi^2 = 2.0$ ,  $P = 0.157$ ). Damaged ambient CO<sub>2</sub> plants versus damaged elevated CO<sub>2</sub> plants ( $\chi^2 = 4$ ,  $P = 0.004$ ). Undamaged ambient CO<sub>2</sub> plants versus undamaged elevated CO<sub>2</sub> plants ( $\chi^2 = 11.5$ ,  $P = 0.001$ ).

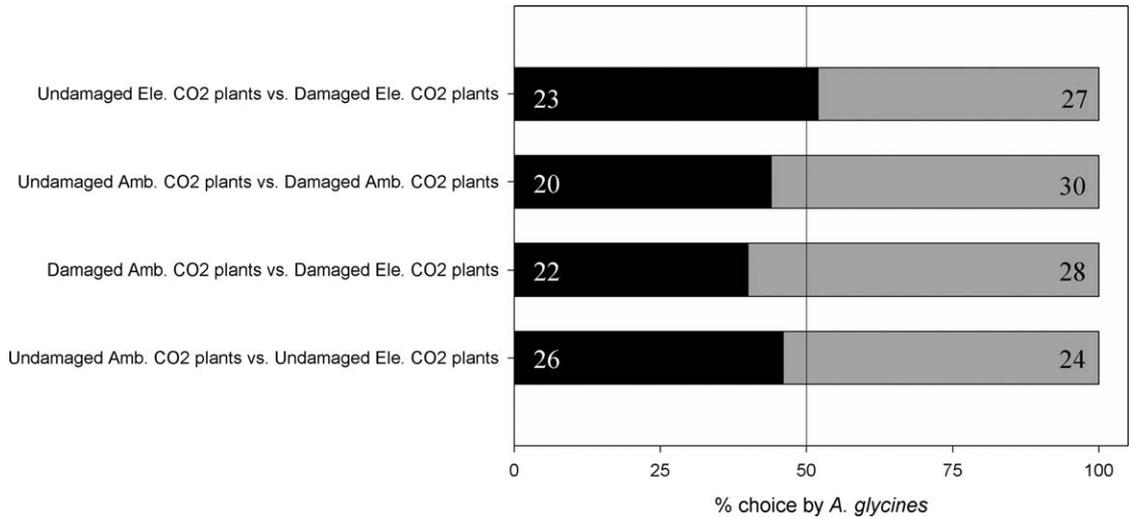


Fig. 7. *Aphis glycines* olfactory preference for *G. max* grown under ambient or elevated levels of CO<sub>2</sub>. Each row represents a different olfactory trial with unique odor sources. The bars show the percentage of aphids that chose each of the two options. The numbers in the bars are the absolute numbers of aphids that chose each of the two options. Choices between odor sources were analyzed by  $\chi^2$  analysis. Undamaged elevated CO<sub>2</sub> plants versus damaged elevated CO<sub>2</sub> plants ( $\chi^2 = 0.1, P = 0.777$ ). Undamaged ambient CO<sub>2</sub> plants versus damaged ambient CO<sub>2</sub> plants ( $\chi^2 = 0.7, P = 0.396$ ). Damaged ambient CO<sub>2</sub> plants versus damaged elevated CO<sub>2</sub> plants ( $\chi^2 = 2.0, P = 0.157$ ). Undamaged ambient CO<sub>2</sub> plants versus undamaged elevated CO<sub>2</sub> plants ( $\chi^2 = 0.3, P = 0.572$ ).

sition chamber was a mesh cage 1.219 m long, 76.2 cm wide, and 1.022 m tall. One soybean plant was placed at each of the opposite long ends of the cage: a plant grown under ambient levels of CO<sub>2</sub> on one side and a plant grown under elevated levels of CO<sub>2</sub> on the other side. Soybean plants were grown in pots at the SoyFACE research site and moved into the laboratory when needed. Plants were replaced every 48 h, and treatment sides were switched to the opposite end of the cage at this time as well. Because of size restrictions in the cage and the limited availability of butterflies, only undamaged soybean plants were used. All eggs were counted on the foliage of plants after removal and choices were recorded. One female butterfly was placed in the chamber with one male butterfly for 24 h. Mating occurred readily, and this time frame was found to be sufficient. Once mated, the male butterfly was removed from the cage and the female butterfly was kept in the chamber by herself for another 24 h. Only 12 individuals were tested because

of high mortality in the laboratory colony. Each individual insect was considered a replicate. Oviposition choice test results were analyzed by  $\chi^2$  analysis (16 statistical software; SPSS).

Results

**Glycine max Leaf Extraction of Potential Volatile Compounds.** Two compounds were significantly increased in plants grown under elevated CO<sub>2</sub> levels over the amounts measured in plants grown under ambient CO<sub>2</sub> levels. These compounds were identified by the NIST library accessed by the GC/MS as 2-hexenal ( $F = 14.04, P = 0.010$ ) and 7-octen-4-ol ( $F = 9.68, P = 0.021$ ; Table 1). There were no significant interactions between CO<sub>2</sub> level and damage level.

**Glycine max Volatile Collection and Analysis.** One compound was significantly decreased in mechanically damaged foliage from both the ambient and el-

Table 1. Effects of elevated CO<sub>2</sub> (between-subject effects) on extract component relative amounts in undamaged and beetle-damaged soybean foliage

Compound	CO <sub>2</sub> P value	CO <sub>2</sub> F value	Ambient/elevated CO <sub>2</sub> mean relative abundance	Ambient/elevated CO <sub>2</sub> SE	Damage P value	Damage F value	Undamaged/damaged mean relative abundance	Undamaged/damaged SE
3-Hexanal	0.988	0.000	352.17/351.00	52.52/52.52	0.681	0.195	334.00/369.17	40.56/65.41
2-Hexenal	<b>0.010</b>	<b>14.04</b>	<b>7,912.00/13,575.13</b>	<b>1,068.72/1,068.72</b>	0.081	4.396	11,834.88/9,652.25	910.78/924.37
7-Octen-4-ol	<b>0.021</b>	<b>9.679</b>	<b>2,714.25/4,670.25</b>	<b>444.56/444.56</b>	0.422	0.741	3,890.75/3,493.75	311.23/455.09

Changes in extract component relative amounts across the control and beetle-damaged treatments were compared in foliage grown under ambient and elevated levels of CO<sub>2</sub> by repeated-measures ANOVA. Significant P values at the 0.05 level are in bold text. Relative abundance values represent peak areas.

Table 2. Effects of elevated CO<sub>2</sub> (between-subject effects) on relative amounts of volatile head-space components in undamaged and mechanically damaged soybean foliage

Compound	CO <sub>2</sub> P value	CO <sub>2</sub> F value	Ambient/elevated CO <sub>2</sub> mean relative abundance	Ambient/elevated CO <sub>2</sub> SE	Damage P value	Damage F value	Undamaged/damaged mean relative abundance	Undamaged/damaged SE
2-penten-1-ol	0.376	0.992	112,200,963.33/10,335,630.17	72,309,735.36/72,309,735.36	0.017	15.558	63,938,332/58,598,261	50,614,270/75/51,650,845.65
Hexane, 2,2,3-trimethyl-	0.690	0.193	29,099,475.00/33,733,260.83	8,171,515.17/6,672,015.01	0.728	0.146	35,175,055.08/27,657,680.75	14,086,737.03/7,108,025.78
Cyclopentanone, 3-methyl-	0.774	0.095	299,595.38/283,167.00	30,817.88/162,161.03	0.671	0.210	283,747.63/299,017.75	25,979.20/36,129.67
Cyclopentanol, 3-methyl-	0.892	0.024	384,466.30/377,177.00	31,622.21/35,354.71	0.777	0.086	387,083.55/374,559.75	39,853.65/21,061.65
1,2,6-hexanetriol	0.276	1.766	515,087.50/808,734.25	139,760.55/171,171.01	0.373	1.093	600,509.67/723,312.08	140,824.24/107,166.58

Changes in relative amounts of volatile head-space components across the control and mechanically damaged treatments were compared in foliage grown under ambient and elevated levels of CO<sub>2</sub> by double repeated-measures ANOVA. Significant P values at the 0.05 level are in bold text. Relative abundance values represent peak areas.

evated CO<sub>2</sub> treatments in comparison with the amounts seen in undamaged foliage from both the ambient and elevated CO<sub>2</sub> treatments. This compound was identified by the library accessed by the GC/MS as an alcohol, 2-penten-1-ol ( $F = 15.58, P = 0.017$ ). No compounds differed in plants grown under elevated levels of CO<sub>2</sub> versus plants grown under ambient levels of CO<sub>2</sub> (Table 2). There was a significant interaction between CO<sub>2</sub> level and damage level for 2-penten-1-ol ( $F = 9.08, P = 0.039$ ), with levels decreasing more after damage in elevated CO<sub>2</sub> than in ambient CO<sub>2</sub> (undamaged ambient CO<sub>2</sub> mean  $\pm$  SE, 116,910,924.00  $\pm$  71,579,388.14; damaged ambient CO<sub>2</sub> mean  $\pm$  SE, 107,491,002.67  $\pm$  73,045,326.42; undamaged elevated CO<sub>2</sub> mean  $\pm$  SE, 10,965,740.33  $\pm$  71,579,388.14; damaged elevated CO<sub>2</sub> mean  $\pm$  SE, 9,705,520.00  $\pm$  73,045,326.42).

**Popillia japonica Olfactory Preferences for Cut Foliage Grown Under Ambient Levels of CO<sub>2</sub>.** *Popillia japonica* displayed significant preferences for beetle-damaged leaves over undamaged leaves ( $\chi^2 = 8.1, P = 0.004$ ) and mechanically damaged leaves over undamaged leaves ( $\chi^2 = 10, P = 0.002$ ; Fig. 2).

**Popillia japonica Olfactory Preferences for Cut Foliage Grown Under Elevated Levels of CO<sub>2</sub>.** The beetles did not differentiate between beetle-damaged leaves and undamaged leaves ( $\chi^2 = 0.1, P = 0.752$ ) or between mechanically damaged leaves and undamaged leaves ( $\chi^2 = 1.6, P = 0.206$ ). Instead, beetles went readily to both choices presented (Fig. 3).

**Popillia japonica Olfactory Preferences for Cut Foliage Grown Under Ambient or Elevated Levels of CO<sub>2</sub>.** There was a significant preference for beetle-damaged leaves grown under elevated levels of CO<sub>2</sub> over beetle-damaged leaves grown under ambient levels of CO<sub>2</sub> ( $\chi^2 = 4.9, P = 0.027$ ; Fig. 4). Significant preferences were not observed for any of the other olfactory combinations presented (Figs. 2–5).

**Popillia japonica Olfactory Preferences for Whole Plants Grown Under Ambient or Elevated Levels of CO<sub>2</sub>.** There were significant beetle preferences for undamaged plants grown under elevated levels of CO<sub>2</sub> versus undamaged plants grown under ambient levels of CO<sub>2</sub> ( $\chi^2 = 11.5, P = 0.001$ ) and for beetle-damaged plants grown under elevated levels of CO<sub>2</sub> versus beetle-damaged plants grown under ambient levels of CO<sub>2</sub> ( $\chi^2 = 4, P = 0.004$ ; Fig. 6). Significant preferences were not observed for any of the other olfactory combinations presented (Fig. 6).

**Aphis glycines Olfactory Preferences.** There were no significant aphid preferences shown for any of the olfactory combinations presented (Fig. 7).

**Vanessa cardui Oviposition Preferences.** Butterflies displayed no significant preference in oviposition choice between plants grown under ambient levels of CO<sub>2</sub> and plants grown under elevated levels of CO<sub>2</sub> ( $\chi^2 = 0.7, P = 0.403$ ; Fig. 8). There was a significant butterfly preference in oviposition choice related to the side of the chamber in which the plant was positioned, with the butterflies preferring to oviposit on the plant on the right side of the cage, which received more sunlight ( $\chi^2 = 0.03, P = 0.000$ ; Fig. 8).

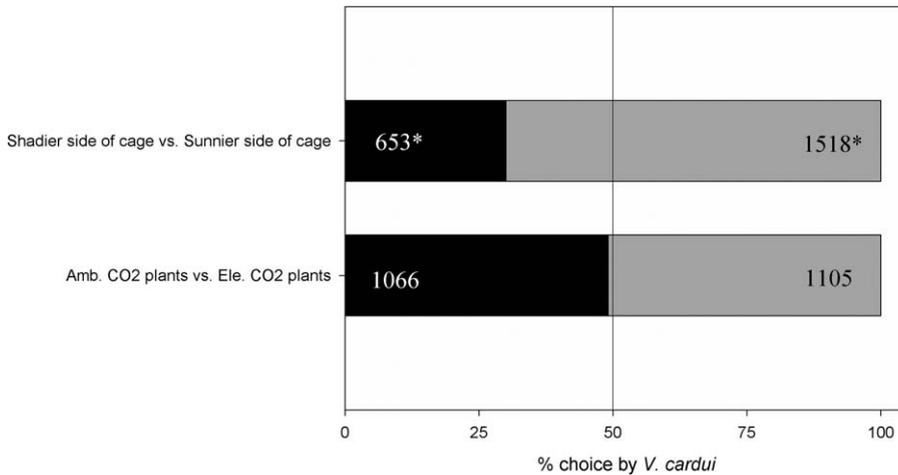


Fig. 8. *Vanessa cardui* oviposition preference for *G. max* grown under ambient or elevated levels of CO<sub>2</sub>. Each row represents a oviposition trial with unique odor sources/locations in the oviposition cage. The bars show the percentage of butterflies that chose each of the two options. The numbers in the bars are the absolute numbers of butterflies that chose each of the two options. Choices between odor sources were analyzed by  $\chi^2$  analysis (stars indicate  $P$  values significant at the 0.001 level). Shadier side of the oviposition cage versus sunnier side of the oviposition cage ( $\chi^2 = 0.03$ ,  $P = 0.000$ ). Ambient CO<sub>2</sub> plants versus elevated CO<sub>2</sub> plants ( $\chi^2 = 0.7$ ,  $P = 0.403$ ).

### Discussion

Our finding that increased levels of compounds produced by *G. max* under elevated CO<sub>2</sub> conditions may be attractive as kairomones for herbivores is consistent with our hypothesis that larger herbivore populations on *G. max* grown under elevated levels of CO<sub>2</sub> are the result of increased olfactory preference. This increased production of kairomones in all plants, regardless of level of herbivore damage, is likely to act as a superstimulus, potentially eliciting an exaggerated feeding response in herbivores. If all leaves are equally attractive, especially to *P. japonica*, the beetles may not congregate on preexisting damage sites and instead feed readily on all foliage available to them. This behavioral response may account in part for the increased numbers of *P. japonica* found on soybeans grown under elevated levels of CO<sub>2</sub> at the SoyFACE site (Hamilton et al. 2005).

The principal green leaf volatile that was increased in elevated CO<sub>2</sub> foliage in this experiment was the aldehyde 2-hexenal. The aldehyde 3-hexenal is produced by the plant and stored in tissue until damage occurs (Turlings et al. 1995). It rapidly converts to 2-hexenal and is released to facilitate a swift wound response (Hatanaka et al. 1987). Another volatile compound that was increased under elevated CO<sub>2</sub>, 7-octen-4-ol, is a "fruity" volatile (Guedes et al. 2004). *P. japonica* are attracted to more complex blends of volatiles, because volatile complexity increases at a later stage of plant damage (Loughrin et al. 1998). The combination of the green leaf volatile and this fruity volatile would make up a more complex volatile blend and may be more attractive to *P. japonica* (Loughrin et al. 1998).

Comparisons between plants and herbivores grown in ambient and elevated levels of atmospheric gases

are quite common, and we ourselves performed some of these comparisons with elevated CO<sub>2</sub> during this study (Veteli et al. 2002, Goverde and Erhardt 2003, Chen et al. 2005). Although they are extremely useful for recognizing the potential for adaptation that may occur between current and future conditions, it is also important to examine changes between plants and herbivores grown solely in elevated atmospheres. Thus, we also compared preference for damaged and undamaged foliage grown under only one treatment. *P. japonica* preferred the odors released from damaged foliage rather than those released from undamaged foliage when both sets of foliage were grown under ambient levels of CO<sub>2</sub>. This preference was expected as *P. japonica* orient toward plants that have been previously damaged (Loughrin et al. 1996). As stated earlier, these beetles feed on multiple plant families, and evidence of previous damage may be an indicator of palatability (Fleming 1972). However, when the choice was between undamaged and damaged foliage grown under elevated CO<sub>2</sub>, the beetles had no clear olfactory preference. The increased amounts of the hexenal/floral blend released from all plants grown under elevated CO<sub>2</sub>, regardless of damage-level to the foliage, may have confused the beetles. *P. japonica* has evolved to feed on plants releasing a complex blend of volatiles that, until now, has usually indicated a plant with herbivore damage at least a day old (Loughrin et al. 1995). If all soybean plants in a field growing under elevated levels of CO<sub>2</sub> produce large amounts of complex volatile blends, beetles may settle on any of these plants to feed, causing more widespread damage than is currently seen in soybean fields.

Only one volatile compound, 2-penten-1-ol, was significantly different between undamaged and mechanically damaged soybeans when we measured the

volatiles actively being released from these plants. The lack of significant changes in volatiles released, particularly 2-hexanal, may have been a function of weather and the type of damage inflicted on the plants. The summer of 2008, when this experiment was conducted, was atypical, in that flooding caused a large delay in plant and insect growth. Mechanically damaging the plants may have resulted in a different complement of volatile compounds being released than would be if the plants had been damaged by feeding herbivores (Beauchamp et al. 2005, Loreto et al. 2006).

The lack of discrimination shown by *A. glycines* and *V. cardui* may reflect their host-finding behavior. Previous studies have remarked on *V. cardui* females' illogical oviposition choices, because they tend to oviposit on plants with available nectar for the adults, even if larval feeding on these plants results in high mortality (Janz and Nylin 1997, Janz 2005). This behavior suggests that volatile chemicals released from potential host plants do not play a major role in oviposition choice for this species. Alate *A. glycines* locate their host plants by a combination of visual cues (Favret and Voegtlin 2001) and olfactory ones, particularly benzaldehyde (Zhu and Park 2005). Benzaldehyde is released in large amounts from *G. max* during the reproductive growth stages (Boue et al. 2003). The plants used during our trials were still in the vegetative growth stages and were not releasing benzaldehyde in detectable amounts. This response is more typical of monophagous species, such as *A. glycines*, which tend to locate their host plants using only one or two volatiles (Smart and Blight 1997, Hori 2007). Polyphagous species that use volatile chemicals in their host location, such as *P. japonica*, tend to recognize several compounds that can be used to locate their host plants (Smart and Blight 2000, Bruce and Cork 2001).

If *P. japonica* and other herbivores change their feeding patterns in a future elevated CO<sub>2</sub> atmosphere and feed on all host foliage available regardless of previous damage, economic impacts associated with them may increase. The combination of reduced foliage specificity and increased levels of feeding stimulants in foliage may lead to an increase in the severity of damage inflicted by *P. japonica* in the future.

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