May, 2009

Leaf Herbivory Increases Floral Fragrance in Male But Not Female *Cucurbita pepo* subsp. *texana* (Cucurbitaceae) Flowers

Nina Theis

Karen Kesler

Lynn Adler, *University of Massachusetts - Amherst*

LEAF HERBIVORY INCREASES FLORAL FRAGRANCE IN MALE BUT NOT FEMALE *CUCURBITA PEPO* SUBSP. *TEXANA* (CUCURBITACEAE) FLOWERS¹

NINA THEIS,² KAREN KESLER, AND LYNN S. ADLER

Department of Plant, Soil and Insect Sciences, University of Massachusetts, Amherst, Massachusetts 01003 USA

Mutualisms are key interactions that affect population dynamics and structure communities, but the extent to which mutualists can attract potential partners may depend on community context. Many studies have shown that leaf herbivory reduces pollinator visitation and have focused on reduced floral visual display and rewards as potential mechanisms. However, olfactory display plays a critical role in mediating interactions between plants, herbivores, and pollinators. We simulated leaf damage in *Cucurbita pepo* subsp. *texana* and measured fragrance emission and other floral characters of both male and female flowers. Contrary to our expectations, damage increased fragrance production, but only in male flowers. Female flowers, which were bigger and produced more fragrance than males, were unaffected by leaf damage. The greatest increase in floral fragrance compounds was in the terpenoids, which we hypothesize could be byproducts of defensively induced cucurbitacins, or they may function defensively themselves. In summary, this study is the first to demonstrate changes in floral fragrance due to leaf damage. Such changes in floral fragrance following herbivory may be a critical and overlooked mechanism mediating interactions between plants, herbivores, and pollinators.

Key words: *Acalymma vittatum*; *Apis mellifera*; *Cucurbita pepo* subsp. *texana*; Cucurbitaceae; diabroticite beetles; floral volatiles; generalist; honeybees; *Peponapis pruinosa*; plant–insect interactions; specialist; squash bees.

Mutualisms play a critical role in affecting population dynamics and structuring communities (Bronstein, 1994). However, the ability to promote beneficial mutualist interactions may depend on community context. For many plants, attracting pollinators is partially dependent on the herbivore community. Studies in numerous systems have shown that leaf damage triggers the transcriptional activation of genes, which results in the induction of defense compounds, the emission of wound volatiles, the repair of damaged tissue, and the reassessment of internal nutritional needs (Leon et al., 2001). Consequently, leaf damage can affect plant reproduction not only via direct effects on the quality and number of pollen grains and seeds (Marquis, 1984; Quesada et al., 1995; Mutikainen and Delph, 1996; Strauss et al., 1996; Lehtila and Strauss, 1999; Avila-Sakar et al., 2003), but also by reducing a plant's ability to attract pollinators, with subsequent reduction in female and sometimes male reproduction (reviewed in Strauss and Whittall, 2006; Adler, 2007). Leaf herbivory reduces the visual display or rewards in many systems, leading to the hypothesis that these changes are the underlying mechanisms reducing pollinator attraction (Lehtila and Strauss, 1999; Mothershead and Marquis, 2000). However, chemical changes in flowers due to leaf damage have been rarely studied and may be of equal or greater importance in mediating plant interactions with pollinators (Kessler et al., 2008).

Floral fragrance affects a pollinator's decision to forage at a flower (Goyret et al., 2007), but the effect of leaf damage on floral fragrance has rarely been considered (but see Euler and

Baldwin, 1996; Effmert et al., 2008). The olfactory display advertises reward, attracting pollinators from a distance, and manipulating pollinator movements within the confines of the flower (Dobson and Bergström, 2000). Fragrance can be the key factor for pollinator attraction even in small, weakly scented flowers (Ashman et al., 2005). Furthermore, even minor chemical changes in fragrance can affect visitation (Okamoto et al., 2007). Therefore, even a small change to the fragrance blend could affect reproductive fitness.

Leaf herbivory could change the olfactory display of a plant by altering bouquet composition of individual flowers or by affecting sex ratio or flower number. Folivory has been shown to bias the sex ratio in plants with unisexual flowers, favoring male flowers, which are relatively cheap to produce (Schlichting and Delesalle, 1997; Krupnick et al., 2000). Furthermore, according to Bateman's principle, in sexually dimorphic species, males are likely to be the more fragrant sex (Bateman, 1948; Charnov, 1979; Bell, 1985; Stanton et al., 1986). If herbivory increases the sex ratio toward a male bias, the result could be a more fragrant plant. However, fragrance from individual flowers of either sex may decline following damage because herbivory reduces resource availability for fragrance production. In addition, fragrance-emitting floral parts such as nectar and pollen (Dobson and Bergström, 2000; Raguso, 2004) may decline. Therefore, leaf herbivory could arguably reduce or increase fragrance production at the whole-plant level via a variety of mechanisms, resulting in very different consequences for subsequent pollinator attraction.

We chose a wild cucurbit for this research, *Cucurbita pepo* subsp. *texana* (Texas gourd) because fragrance is an important signal in this system for pollinators and specialist herbivores, and effects of leaf herbivory on other floral traits have been demonstrated. The Texas gourd is an annual vine native to Texas and Mexico. It is closely related to the domesticated *C. pepo* and is possibly the wild progenitor of cultivated squashes and pumpkins (Paris et al., 2003). Pollinator attraction is critical for reproduction of this monoecious species, which has

¹ Manuscript received 4 September 2008; revision accepted 9 January 2009.

The authors thank J. Normanly for use of her GC-MS and S. T. Petsch for use of his evaporator. J. Hugh, J. Bellino, A. Filshtinsky, and B. Chaney-Ryan helped with plant care and pollen counts. This work was funded by NSF-DEB-0542819 to N.T.

² Author for correspondence (e-mail: ntheis@nsm.umass.edu)

sexually dimorphic floral fragrance production (Ferrari et al., 2006). Specialist pollinators, squash bees, *Peponapis* spp. (Anthophoridae) (Hurd et al., 1971) pollinate at or just before sunrise, when squash flowers open (Hurd et al., 1971). Generalists such as bumble bees (*Bombus* spp.; Apidae) and the introduced honeybee *Apis mellifera* (Apidae) can also pollinate squash flowers (Shuler et al., 2005). Species in the genus *Cucurbita* are attacked at all stages of development by diabroticite beetles (squash and cucumber beetles; Metcalf and Lampman, 1989, 1991). Defensive cucurbitacins are induced after beetle feeding but also by mechanical damage (Tallamy, 1985). Diabroticite beetles have overcome the defenses of cucurbits and feed compulsively in the presence of these bitter tetracyclic terpenoids (Metcalf et al., 1980). In addition, they are attracted to *Cucurbita* floral volatiles (Andersen and Metcalf, 1987; Metcalf et al., 1998; Andrews et al., 2007).

The effect of leaf herbivory on floral traits in *C. pepo* subsp. *texana* has been studied extensively. Leaf herbivory resulted in a more male-biased sex ratio (Krupnick et al., 2000). However, variable effects have been measured in studies on the effect of leaf herbivory on pollen production. Depending on timing and intensity, herbivory can reduce (Quesada et al., 1995; Avila-Sakar et al., 2003), have no effect on (Avila-Sakar et al., 2003), or increase pollen production (Avila-Sakar and Stephenson, 2006). If leaf feeding by cucurbit beetles affects floral traits, beetles are ostensibly changing their own food resource (Quesada et al., 1995; Krupnick et al., 2000; Avila-Sakar et al., 2003) and potentially the fragrance signals used to locate it. Thus, changes to floral fragrance have the potential for large effects on plant fitness via attracting both pollinators and herbivores.

We damaged leaves of *C. pepo* subsp. *texana* in the greenhouse and subsequently measured floral characters to ask how leaf damage affects floral fragrance emitted per flower, floral visual display, rewards, sex ratio, and the number of flowers produced per plant. Although data from leaf damage studies have been equivocal, the majority of studies have shown a reduction in floral traits following early damage (Quesada et al., 1995; Avila-Sakar et al., 2003; Thomson et al., 2004), so we predict that fragrance per flower will decline similarly. Moreover, damage reduced the number of flowers produced in the cucurbit *Cucumis sativus* (Thomson et al., 2004) and increased male bias in the sex ratio of *C. pepo* subsp. *texana* (Krupnick et al., 2000). Given that males in *C. pepo* subsp. *texana* are less fragrant than females (Ferrari et al., 2006), any of these mechanisms could reduce fragrance at the whole-plant level.

MATERIALS AND METHODS

Herbivory simulation experimental design—Ninety *Cucurbita pepo* subsp. *texana* plants were germinated on 31 March 2006 from seeds acquired through the USDA North Central Regional Plant Introduction Station (NCRPIS) in Ames, Iowa, USA. On 11 April they were transplanted to 10 cm pots and into 19 L pots on 2 May when they were matched for size and bud number in groups of three, and then randomly assigned to one of three treatments. We have measured herbivory in the field and found a peak in damage (averaged 10% of leaf area but reached 95% in some plants) during the second two weeks after transplanting, which then rapidly declined to an average of less than 1% per plant (N. Theis, unpublished data). Leaf damage in the greenhouse was accomplished in a manner that mimicked beetle damage in the field. Damage occurred from 3 May until 14 May on expanding leaves whose axillary flower bud was ~8–15 mm long. Leaf damage was therefore gradual, and plants at different stages of development lost different amounts of leaf tissue. To mimic high field levels of herbivory (Quesada et al., 1995), we removed an estimated 15% of each leaf using a hole punch. An extreme level of simulated herbivory (50%) was

accomplished with scissors, removing half the leaf while avoiding the midvein (as in Avila-Sakar et al., 2003). For the control, we touched but did not damage the leaf.

Measuring floral traits—We counted and sexed every flower from the start of flowering on 10 May until 23 June. From each plant, we sampled three early male flowers and, because female flowers are fewer and flower later, just one early female flower so that male and female flowers were measured at roughly equivalent times in the season. For each flower, we measured the floral length (calyx to apex), diameter at the top of floral tube, nectar volume using micro-capillary tubes (Drummond Scientific, Broomall, Pennsylvania, USA), nectar sugar concentration with a refractometer (Fisherbrand, ThermoFisher Scientific, Waltham, Massachusetts, USA), and pollen production. Flowers are open for only one day, so nectar volume approximates nectar production. To count pollen, anthers were dried in the oven at 40°C, crushed and suspended in 1.5 ml of distilled water, and sonicated. Pollen grains were counted in eight 10- μ L drops from each sample.

Fragrance collection—We collected fragrance using dynamic headspace sampling from one male and one female flower per plant for 3 h beginning between 0700–0900 hours. The temperature was recorded throughout. Individual intact flowers were enclosed in polyethylene bags (Toppits, Cofresco Frischhalteprodukte GmbH & Co. Kg, Minden, Germany) with a hole cut in the bag to allow air flow. Ambient air was pulled by vacuum pump at a flow rate of ca. 200 mL/min (Air Check 52 or Air Check 2000 diaphragm pump, SKC, Eighty Four, Pennsylvania) through the hole over the flower and onto a cartridge packed with 100 mg Super Q (Alltech Associates, Deerfield, Illinois, USA). Fragrance was also measured in ambient air throughout the collection period for subtraction purposes. Cartridges were frozen at –20°C until they were eluted with 3 mL of hexane (Honeywell, Burdick & Jackson, Morristown, New Jersey, USA). An internal standard of 3 μ L of anisole was added to the elutions, which were then dried under a nitrogen stream to 75 μ L.

Fragrance analysis—Fragrance analysis was performed by combined capillary gas chromatography–mass spectrometry (GC-MS), with an Agilent GC 6890 equipped with a Mass Selective Detector 5973 (Agilent Technologies, Santa Clara, California, USA). A 1- μ L sample was injected splitless onto a nonpolar column (DB5, 30 m $\times$ 0.25 mm; J&W Scientific, Agilent Technologies) at an initial temperature of 50°C. The oven temperature was held there for the first 2 min and was then increased 10°C per min until 275°C, where it was held for 3.5 min. Compounds were identified by matching the retention time to previously injected standards and to the Wiley Mass Spectral Library. Quantification was achieved by dividing the mass ion of each scent compound by the mass ion of the internal standard and multiplying by both the mass of the internal standard and a coefficient that corrected for the response of the GC-MS to the specific scent compound.

Statistical analysis—All nonparametric analyses were conducted using the program SYSTAT version 7.0 (Systat Software, Chicago, Illinois, USA), and parametric data were analyzed using the program SAS version 9.1 (SAS Institute, Cary, North Carolina, USA) unless stated otherwise. All responses were analyzed separately for male and female flowers. We asked whether herbivory affected the date of the first flower produced (Kruskal–Wallis test) and the cumulative number of male and female flowers (in 5-d increments) using multiple one-way ANOVAs. Female flower number was log transformed. Using a MANOVA, we tested for an herbivory effect on attractive floral traits including corolla size (length $\times$ diameter; log transformed in males only), nectar volume (log transformed), sugar concentration, and pollen production. Paired *t* tests were also used to contrast all floral traits between sexes (except pollen production).

Because fragrance is a complex trait, we analyzed it separately. *Cucurbita pepo* subsp. *texana* flowers produced more than 50 compounds in the fragrance blend. We reduced the dimensionality of the data using principal component analysis (PCA) in the program R version 2.3.1 (R Development Core Team, 2006), using the 10 most abundant compounds, which on average represented more than 95% of the fragrance. The PCA identified three principal components (PCs) that explained 68% of the variation in the fragrance data (Table 1).

The reduced variables from the PCA were then analyzed in a MANOVA to determine the effect of treatment on fragrance per flower. One high outlier (male flower with 15% damage) was deleted from the analysis to better approximate normality. Univariate ANOVAs identified the significant responses in the MANOVA, with PCs 2 and 3 log transformed to improve fit to normality.

TABLE 1. Loadings for fragrance compounds on to principal components (PCs) based on fragrance flux per flower.

Compound	PC1	PC2	PC3
6-Methyl-5-hepten-2-one	-0.22	0.31	-0.57
(E)-4,8-dimethyl-1,3,7-nonatriene	-0.37	0.31	0.30
1,4-Dimethoxybenzene	-0.35	0.19	0.40
<i>p</i> -Anisaldehyde	-0.25	0.44	0.17
1,2,4-Trimethoxybenzene	-0.41	-0.07	0.22
α -Gurjunene	-0.36	-0.47	-0.07
Geranyl acetone	-0.33	0.22	-0.57
Germacrene D	-0.33	-0.35	-0.10
Sesquiterpenoid unknown	-0.31	-0.43	-0.02
Unidentified terpenoid	-0.15	0.08	-0.02
Eigen values	3.1	2.4	1.3
Cumulative percentage	31	55	68

Because the scent data were not normally distributed, we also tested for differences using the Kruskal–Wallis test on individual compounds. We analyzed data separately for male and female flowers. To detect differences between the sexes of *C. pepo* subsp. *texana*, total scent emissions and the same PCs, determined above, were used in paired *t* tests. These tests were also conducted on PCs calculated on data analyzed by gram dry mass to account for mass differences between sexes.

RESULTS

Herbivory simulation experiment—Herbivory significantly delayed the date that the first male flower bloomed on a plant ($H = 8.08$, $P = 0.018$) on average by 9 ± 2 ($\pm$ SE) d (for both 15% and 50%). This delay was at least partially a result of male bud abortion on treated plants (N. Theis, personal observation). As a result, control plants initially produced more male flowers than did either the 15% or 50% defoliated plants, but they did not produce more female flowers. The flowering date for the first female flower was not affected by herbivory ($H = 0.465$, $P = 0.8$). When analyzing the cumulative number of open flowers per plant in 5-d increments, this difference in male flower production was significant up to 80 d after sowing (male $F_{2,85} = 5.43$, $P = 0.006$, female $F_{2,85} = 0.03$, $P = 0.97$). By 85 d, there were no significant differences in cumulative flower number (male $F_{2,85} = 2.44$, $P = 0.09$, female $F_{2,85} = 0.3$, $P = 0.8$, Fig. 1), indicating that damaged plants had compensatory male flower production.

Leaf damage had no significant effect on floral traits in male or female flowers, including corolla size, nectar sugar concentration, nectar volume, and the number of pollen grains (male: Wilks' $\lambda = 0.90$, $F_{8,162} = 1.1$, $P = 0.39$; female: Wilks' $\lambda = 0.93$, $F_{6,132} = 0.82$, $P = 0.56$).

Simulated herbivory did not affect fragrance production in female flowers (Wilks' $\lambda = 0.91$, $F_{6,100} = 0.82$, $P = 0.56$). In male flowers, however, herbivory significantly affected fragrance (Wilks' $\lambda = 0.83$, $F_{6,148} = 2.4$, $P = 0.03$, Fig. 2). Univariate tests determined that this result was due to PC1, which was represented by high negative loadings for all 10 compounds except the unidentified terpenoid (Table 1). PC1 was significantly higher in control plants ($F_{2,76} = 5.0$, $P < 0.009$), indicating lower fragrance production in controls compared to damaged plants. These results are corroborated by nonparametric tests, which demonstrated that while the dominant compound 1,4-dimethoxybenzene was not affected by damage treatment ($H = 1.8$, $P = 0.4$, Table 2, Fig. 2) all other compounds summed together were significantly higher in plants with 15% damage than con-

Fig. 1. Cumulative number of male and female flowers in plants with 0%, 15%, or 50% leaf damage. Error bars represent standard errors.

trol plants ($H = 6.2$, $P = 0.05$, Fig. 2). Individual compounds, such as the terpenoids (E)-4,8-dimethyl-1,3,7-nonatriene (DMNT) ($H = 7.4$, $P = 0.03$, Fig. 2), geranyl acetone ($H = 7.3$, $P = 0.03$), germacrene D ($H = 7.7$, $P = 0.02$), and an unknown sesquiterpenoid ($H = 7.8$, $P = 0.02$) and the aromatic *p*-anisaldehyde ($H = 7.2$, $P = 0.03$, Fig. 2), were all significantly higher in plants with 15% damage compared to controls (Table 2). However, only DMNT and *p*-anisaldehyde were also significantly higher in plants with 50% damage compared to controls (Table 2, Fig. 2).

We used the same previously calculated PCs in a paired *t* test to ask whether fragrance differed between the sexes, regardless of treatment. For control plants, total fragrance ($df = 24$, $t = 4.1$, $P = 0.0004$) and PC1 ($t = -3.2$, $P = 0.004$) were both significantly higher in females than males (results were qualitatively similar with all treatments combined; data not shown). We also generated PCs for fragrance produced per gram of dry floral mass to determine if differences between sexes were due solely to differences in floral mass. In this case, the three PCs explained 66% of the data, and all compounds loaded highly onto PC1 (data not shown). The sexes differed significantly for PC1 ($t = -4.2$, $P = 0.003$), PC2 ($t = -2.7$, $P = 0.013$) and total fragrance per gram dry mass ($t = 5.2$, $P < 0.0001$), indicating that females were more fragrant than males on a per gram basis as well as overall.

Other floral characters were also contrasted by sex, without regard to treatment (only reported for control plants, but results similar for all plants). Nectar volume was higher in female flowers (mean $\pm$ SE; female: 66 ± 6 μ L, male: 29 ± 3 μ L; $t = 6.74$, $P < 0.0001$). Corolla size did not significantly differ between sexes (female: 187.5 ± 16.1 cm, male: 135.1 ± 8.9 cm; $t = 1.4$, $P = 0.16$), nor did sugar concentration (female: $26 \pm 1\%$ brix, male: $24 \pm 1\%$ brix; $t = -0.15$, $P = 0.88$).

DISCUSSION

Does leaf damage affect floral fragrance in *C. pepo* subsp. *texana*?—Leaf herbivory significantly increased floral fragrance emissions of male *C. pepo* subsp. *texana* flowers. In this study, there were no significant changes detected in any component of the floral display other than fragrance. An increase in fragrance was contrary to our expectations because other components of cucurbit floral display, such as flower size and pollen production, either did not significantly change (Avila-Sakar et al., 2003) or decline with herbivory (Quesada et al., 1995;

Fig. 2. Mean flux rate of 1,4-dimethoxybenzene, total fragrance except 1,4-dimethoxybenzene, *p*-anisaldehyde, and (*E*)-4,8-dimethyl-1,3,7-nonatriene (DMNT) from male and female flowers with different levels of leaf damage. Different letters represent significantly different means in Kruskal–Wallis tests at the $P = 0.05$ level. Error bars represent standard errors.

Avila-Sakar et al., 2003; Thomson et al., 2004; but see Avila-Sakar and Stephenson, 2006, where herbivory increased pollen production). Given that we found effects of damage on floral fragrance but not visual cues or rewards, studies that examine the effect of herbivory only on visual cues may miss important mechanisms by which leaf herbivores could influence pollinator preference and plant reproduction.

There are a number of nonmutually exclusive explanations for the increased floral fragrance in response to leaf damage. One possibility is that resources rescued from aborted flowers could be channeled to increase fragrance production. However, there was not a general increase in all fragrance components with damage. Instead, the principal change was in the terpenoid compounds, which are biosynthetically related to cucurbitacins, the triterpenoid defense compounds produced by cucurbits (Newman, 1972). Thus, an alternate hypothesis is that the increased floral volatiles are a consequence of cucurbitacin in-

duction. It is interesting to note that no compound had greater production following 50% leaf damage compared to the 15% treatment, and one compound had significantly higher emissions with 15% damage compared to 50% damage (Table 2). The hole punch damage is a better mimic of beetle feeding than the scissor damage and is therefore likely to induce more cucurbitacins in leaves (Tallamy, 1985; Mithofer et al., 2005). A third possibility is that leaf damage induces other changes, such as the release of leaf volatiles that attract natural enemies, incidentally increasing related floral volatiles. The defense response causes an immediate increase in signaling molecules including jasmonates, salicylic acid, and ethylene, leading to the upregulation of genes that trigger the accumulation and release of secondary metabolites, including volatile terpenoids (Ryan, 2000; Arimura et al., 2005; Dudareva et al., 2006). It is not known whether this upregulation could extend to genes that encode for floral volatiles. Our understanding of the regulation of floral

TABLE 2. Average emission rates of the ten most abundant compounds $\pm$ SE in ng-flower⁻¹·h⁻¹ for control and damaged plants. Boldface type indicates a significant effect of herbivory on fragrance in Kruskal–Wallis tests at the $P = 0.05$ level; different letters indicate differences in posthoc tests with no adjusted α -value.

Compounds	Male flowers			Female flowers		
	0% Damage	15% Damage	50% Damage	0% Damage	15% Damage	50% Damage
6-Methyl-5-hepten-2-one	10.1 $\pm$ 1.2	13.7 $\pm$ 1.9	13.5 $\pm$ 1.7	19.0 $\pm$ 4.3	11.8 $\pm$ 1.9	16.4 $\pm$ 2.1
(<i>E</i>)-4,8-Dimethyl-1,3,7-nonatriene	1.8 $\pm$ 0.7 a	6.2 $\pm$ 2.8 b	4.7 $\pm$ 1.3 b	5.8 $\pm$ 1.7	5.7 $\pm$ 1.5	7.8 $\pm$ 2.1
1,4-Dimethoxybenzene	385 $\pm$ 79	553 $\pm$ 165	543 $\pm$ 101	1195 $\pm$ 176	1158 $\pm$ 142	1541 $\pm$ 298
<i>p</i>-Anisaldehyde	1.6 $\pm$ 1.1 a	7.2 $\pm$ 4.5 b	2.2 $\pm$ 0.7 b	3.1 $\pm$ 1.0	2.2 $\pm$ 1.1	4.8 $\pm$ 1.9
1,2,4-Trimethoxybenzene	5.7 $\pm$ 1.0	8.2 $\pm$ 1.5	6.5 $\pm$ 1.2	21.5 $\pm$ 3.6	14.8 $\pm$ 2.6	20.3 $\pm$ 3.5
α -Gurjunene	7.6 $\pm$ 1.4	12.8 $\pm$ 2.0	7.0 $\pm$ 1.6	10.3 $\pm$ 2.3	9.6 $\pm$ 2.8	8.8 $\pm$ 2.1
Geranyl acetone	3.0 $\pm$ 0.5 a	6.4 $\pm$ 1.1 b	4.5 $\pm$ 0.8 ab	4.0 $\pm$ 1.2	2.5 $\pm$ 0.7	4.5 $\pm$ 0.9
Germacrene D	1.8 $\pm$ 0.8 a	5.7 $\pm$ 1.4 b	4.7 $\pm$ 2.1 ab	3.1 $\pm$ 1.0	4.7 $\pm$ 2.3	3.4 $\pm$ 1.8
Sesquiterpenoid unknown	3.9 $\pm$ 0.8 a	8.9 $\pm$ 1.4 b	4.1 $\pm$ 0.9 a	6.8 $\pm$ 1.5	4.6 $\pm$ 1.0	5.5 $\pm$ 1.1
Unidentified terpenoid	2.2 $\pm$ 0.9	7.5 $\pm$ 4.0	14.7 $\pm$ 5.6	3.6 $\pm$ 2.5	3.0 $\pm$ 1.7	4.5 $\pm$ 1.6
Total	449 $\pm$ 82	660 $\pm$ 175	626 $\pm$ 108	1298 $\pm$ 188	1246 $\pm$ 151	1645 $\pm$ 301

volatiles is still incomplete. For example, enzyme production appears to be controlled at the pretranslational level, but emission levels do not always correlate with enzyme levels, and controls are likely to also exist further down the line (Dudareva et al., 2000; Schuurink et al., 2006). Before we can draw a link between leaf damage and the induction of floral volatiles, a better understanding of the regulation of floral fragrance emission is necessary.

Empirical data suggest possible connections between the induction of defensive and volatile compounds in leaves and floral tissue. For example, in *Raphanus sativus*, glucosinolates are induced in both leaves and petals following leaf herbivory (Strauss et al., 2004). Conversely, herbivory on flower buds of *Gossypium hirsutum* induced the production of volatiles from the leaves as well as from the buds themselves (Bezemer et al., 2004). In *Nicotiana tabacum* and *N. sylvestris*, leaf herbivory of bolting plants induced alkaloids in nectar or other floral parts rather than in leaves (Ohnmeiss and Baldwin, 2000; Adler et al., 2006). However, leaf but not floral volatiles increased in *N. suaveolens* when herbivory occurred just before anthesis (Effmert et al., 2008). This timing of damage is much later phenologically than the one chosen for our experiment. The authors hypothesize that at this late stage in floral development, the flower is no longer a sink, and instead, floral volatile production may rely on stored carbohydrates and be unaffected by leaf herbivory (Effmert et al., 2008). Thus, there is some evidence for interrelationships between induction in flowers and leaves, suggesting that an induced response to damage in leaves of *C. pepo* subsp. *texana* could result in the release of related compounds from the flowers.

Leaf damage increased individual male floral fragrance but delayed blooming of male flowers, potentially affecting fragrance production at the whole plant level. Moreover, female flower production and fragrance were unaffected, leading to a change in the sex ratio in damaged plants. Although damaged plants had a combination of more fragrant male flowers and a sex ratio biased (early on) toward females (which are more fragrant than males), control plants were probably more fragrant at this stage because they had produced more flowers. The early reduction in male flowers in damaged plants was followed by a compensatory increase, ultimately leading to an equal sex ratio and equal flower number in control and treated plants. Thus, for an initial period after damage, control plants may have been more fragrant, but if male flowers continue to produce higher emission levels in damaged plants, then they may have been more fragrant ultimately.

Theory and empirical data suggest that male flowers in sexually dimorphic species will be larger, more fragrant, and receive a greater number of pollinator visits (reviewed in Agren et al., 1999; Mena Granero et al., 2004; Theis et al., 2007). The fragrance dimorphism between the sexes in *C. pepo* subsp. *texana*, in which females are more fragrant (our study and Ferrari et al., 2006), is therefore contrary to theoretical predictions. Selection is expected to act on male function to attract pollinators, driving the evolution of a showy display (Bateman, 1948; Charnov, 1979; Bell, 1985; Stanton et al., 1986), while female flowers are expected to be limited by resources. However, in *C. pepo* subsp. *texana*, female flowers are bigger and more fragrant, both overall and on a per gram basis. Together these differences suggest that pollinator visits rather than resources limit seed production in female *C. pepo* subsp. *texana*. However, no study has yet examined natural populations of *C. pepo* subsp. *texana* to determine whether pollen receipt limits seed set. Pollinator limita-

tion may be high because flowers are short lived, lasting only a few hours. Moreover, because this species is monoecious, a foraging pollinator needs to visit the female flower (to deposit allogamous pollen) before the male flowers on a plant for optimal cross fertilization (Johannsson et al., 1998; Hayes et al., 2004; Ferrari et al., 2006). The architecture of monoecious and/or dichogamous plants has been shown to promote pollinator visitation first to the female sex (Harder et al., 2000), and perhaps a showy display by the female sex is an extension of this phenomenon. The hypothesis that female flowers should be more fragrant than males in monoecious species should be tested by comparing floral fragrance dimorphism in monoecious and dioecious species.

Are pollinators and herbivores affected by the compounds that increase with leaf damage?—Previous research on the functional role of fragrance components indicate some potentially interesting effects of the compounds that increased with damage in this study. Numerous studies using scent-baited trapping have informed our understanding of fragrance attraction for specialist pollinators and herbivores of cucurbits. However, none of the fragrance components significantly affected by leaf damage are known attractants (Lewis et al., 1990; Metcalf et al., 1995). Of the floral volatiles that increased in damaged plants, the only one that has been identified as a generalist pollinator attractant was *p*-anisaldehyde, which attracts honeybees (Theis, 2006). Increased floral fragrance could be an adaptation to increase pollinator visitation despite fewer resources and ensure pollination of flowers from damaged plants. However, honeybees are not specialized pollinators of this plant, and *p*-anisaldehyde is a minor component of the fragrance blend of *C. pepo* subsp. *texana*. The predominant group of compounds that increased following hole punch damage, including sesquiterpenoids (e.g., germacrene D) and the homoterpene DMNT, are difficult to attain commercially and thus unlikely to be tested in field experiments using scent-baited traps; however, they are known to play important roles in insect attraction in other systems. For example, DMNT is induced in leaves of a number of plant species following herbivory, attracting herbivores' natural enemies (Arimura et al., 2005). Similarly, sesquiterpenoids can act as attractants of natural enemies in tritrophic interactions or as deterrents of herbivores in plant defense (Dey and Harborne, 1997; Arimura et al., 2005). Therefore, it is not known how the overall increase in fragrance from flowers following leaf damage will affect pollinator or herbivore attraction. To fully understand the ramifications of the fragrance increase, we need to conduct field experiments that directly measure pollinator and herbivore interactions with flowers on plants with beetle damage.

Conclusion—In spite of the reduction in resources that followed leaf damage, we found a significant increase induced in the floral volatiles of *C. pepo* subsp. *texana*. This effect was limited to male flowers; female flowers were unaffected by damage both in fragrance production and also in initial flowering time. It may be that production of female flowers, fragrance, and seeds are under tighter genetic control than is production of male flowers and pollen. Alternatively, it is possible that female flowers were unaffected by damage because the bout of herbivory ended considerably before females bloomed. Thus, damage later in the season, although unlikely in this system, could have different effects on whole plant fragrance. In male flowers,

damage increased floral terpenoids, which we hypothesize could be byproducts of induced cucurbitacins or function defensively themselves. We therefore do not expect this increase in fragrance to increase herbivore attraction, although it may increase honeybee attraction due to higher levels of *p*-anisaldehyde, a honeybee attractant (Theis, 2006). Alternatively, if heightened terpenoids repel pollinators, this result may explain pollinator deterrence from damaged plants. Our results indicate that changes in floral fragrance following damage could be a potential mechanism mediating plant interactions with both mutualists and antagonists.

LITERATURE CITED

- ADLER, L. S. 2007. Selection by pollinators and herbivores on attraction and defense. In K. J. Tilmon [ed.], *Specialization, speciation, and radiation: The evolutionary biology of herbivorous insects*, 162–173. University of California Press, Berkeley, California, USA.
- ADLER, L. S., M. WINK, M. DISTL, AND A. J. LENTZ. 2006. Leaf herbivory and nutrients increase nectar alkaloids. *Ecology Letters* 9: 960–967.
- AGREN, J., K. DANELL, E. ELMQVIST, L. ERICSON, AND J. HJALTEN. 1999. Sexual dimorphism and biotic interactions. In M. A. Geber, T. E. Dawson, and L. F. Delph [eds.], *Gender and sexual dimorphism in flowering plants*, 217–246. Springer, Berlin, Germany.
- ANDERSEN, J. F., AND R. L. METCALF. 1987. Factors influencing distribution of *Diabrotica* spp. (Coleoptera, Chrysomelidae) in blossoms of cultivated *Cucurbita* spp. *Journal of Chemical Ecology* 13: 681–699.
- ANDREWS, E. S., N. THEIS, AND L. S. ADLER. 2007. Pollinator and herbivore attraction to *Cucurbita* floral volatiles. *Journal of Chemical Ecology* 33: 1682–1691.
- ARIMURA, G., C. KOST, AND W. BOLAND. 2005. Herbivore-induced, indirect plant defences. *Biochimica et Biophysica Acta* 1734: 91–111.
- ASHMAN, T. L., M. BRADBURN, D. H. COLE, B. H. BLANEY, AND R. A. RAGUSO. 2005. The scent of a male: The role of floral volatiles in pollination of a gender dimorphic plant. *Ecology* 86: 2099–2105.
- AVILA-SAKAR, G., S. M. SIMMERS, AND A. G. STEPHENSON. 2003. The interrelationships among leaf damage, anther development, and pollen production in *Cucurbita pepo* ssp. *texana* (Cucurbitaceae). *International Journal of Plant Sciences* 164: 395–404.
- AVILA-SAKAR, G., AND A. G. STEPHENSON. 2006. Effects of the spatial pattern of leaf damage on growth and reproduction: Whole plants. *International Journal of Plant Sciences* 167: 1021–1028.
- BATEMAN, A. J. 1948. Intra-sexual selection in *Drosophila*. *Heredity* 2: 349–368.
- BELL, G. 1985. On the function of flowers. *Proceedings of the Royal Society of London, B, Biological Sciences* 224: 223–265.
- BEZEMER, T. M., R. WAGENAAR, N. M. VAN DAM, W. H. VAN DER PUTTEN, AND F. L. WÄCKERS. 2004. Above- and below-ground terpenoid aldehyde induction in cotton, *Gossypium herbaceum*, following root and leaf injury. *Journal of Chemical Ecology* 30: 53–67.
- BRONSTEIN, J. L. 1994. Our current understanding of mutualism. *Quarterly Review of Biology* 69: 31–51.
- CHARNOV, E. L. 1979. Simultaneous hermaphroditism and sexual selection. *Proceedings of the National Academy of Sciences, USA* 76: 2480–2484.
- DEY, P. M., AND J. B. HARBORNE. 1997. *Plant biochemistry*. Academic Press, San Diego, California, USA.
- DOBSON, H. E. M., AND G. BERGSTRÖM. 2000. The ecology and evolution of pollen odors. *Plant Systematics and Evolution* 222: 63–87.
- DUDAREVA, N., L. M. MURFITT, C. J. MANN, N. GORENSTEIN, N. KOLOSOVA, C. M. KISH, C. BONHAM, AND K. WOOD. 2000. Developmental regulation of methyl benzoate biosynthesis and emission in snapdragon flowers. *Plant Cell* 12: 949–961.
- DUDAREVA, N., F. NEGRE, D. A. NAGEGOWDA, AND I. ORLOVA. 2006. Plant volatiles: Recent advances and future perspectives. *Critical Reviews in Plant Sciences* 25: 417–440.
- EFFMERT, U., C. DINSE, AND B. PIECHULLA. 2008. Influence of green leaf herbivory by *Manduca sexta* on floral volatile emission by *Nicotiana suaveolens*. *Plant Physiology* 146: 1996–2007.
- EULER, M., AND I. T. BALDWIN. 1996. The chemistry of defense and apparency in the corollas of *Nicotiana attenuata*. *Oecologia* 107: 102–112.
- FERRARI, M. J., A. G. STEPHENSON, M. C. MESCHER, AND C. M. DE MORAES. 2006. Inbreeding effects on blossom volatiles in *Cucurbita pepo* subsp. *texana* (Cucurbitaceae). *American Journal of Botany* 93: 1768–1774.
- GOYRET, J., P. M. MARKWELL, AND R. A. RAGUSO. 2007. The effect of decoupling olfactory and visual stimuli on the foraging behavior of *Manduca sexta*. *Journal of Experimental Biology* 210: 1398–1405.
- HARDER, L. D., S. C. H. BARRETT, AND W. W. COLE. 2000. The mating consequences of sexual segregation within inflorescences of flowering plants. *Proceedings of the Royal Society of London, B, Biological Sciences* 267: 315–320.
- HAYES, C. N., J. A. WINSOR, AND A. G. STEPHENSON. 2004. Inbreeding influences herbivory in *Cucurbita pepo* ssp. *texana* (Cucurbitaceae). *Oecologia* 140: 601–608.
- HURD, P. D., E. G. LINSLEY, AND T. W. WHITAKER. 1971. Squash and gourd gees (*Peponapis*, *Xenoglossa*) and the origin of the cultivated *Cucurbita*. *Evolution* 25: 218–234.
- JOHANSSON, M. H., M. J. GATES, AND A. G. STEPHENSON. 1998. Inbreeding depression affects pollen performance in *Cucurbita texana*. *Journal of Evolutionary Biology* 11: 579–588.
- KESSLER, D., K. GASE, AND I. T. BALDWIN. 2008. Field experiments with transformed plants reveal the sense of floral scents. *Science* 321: 1200–1202.
- KRUPNICK, G. A., G. AVILA, K. M. BROWN, AND A. G. STEPHENSON. 2000. Effects of herbivory on internal ethylene production and sex expression in *Cucurbita texana*. *Functional Ecology* 14: 215–225.
- LEHTILA, K., AND S. Y. STRAUSS. 1999. Effects of foliar herbivory on male and female reproductive traits of wild radish, *Raphanus raphanistrum*. *Ecology* 80: 116–124.
- LEON, J., E. ROJO, AND J. J. SÁNCHEZ-SERRANO. 2001. Wound signalling in plants. *Journal of Experimental Botany* 52: 1–9.
- LEWIS, P. A., R. L. LAMPMAN, AND R. L. METCALF. 1990. Kairomonal attractants for *Acalymma vittatum* (Coleoptera, Chrysomelidae). *Environmental Entomology* 19: 8–14.
- MARQUIS, R. J. 1984. Leaf herbivores decrease fitness of a tropical plant. *Science* 226: 537–539.
- MENA GRANERO, A., F. J. EGEE GONZÁLEZ, A. GARRIDO FRENICH, J. M. GUERRA SANZ, AND J. L. MARTÍNEZ VIDAL. 2004. Single step determination of fragrances in *Cucurbita* flowers by coupling headspace solid-phase microextraction low-pressure gas chromatography–tandem mass spectrometry. *Journal of Chromatography, A* 1045: 173–179.
- METCALF, R. L., AND R. L. LAMPMAN. 1989. The chemical ecology of diabroticites and Cucurbitaceae. *Experientia* 45: 240–247.
- METCALF, R. L., AND R. L. LAMPMAN. 1991. Evolution of diabroticite rootworm beetle (Chrysomelidae) receptors for *Cucurbita* blossom volatiles. *Proceedings of the National Academy of Sciences, USA* 88: 1869–1872.
- METCALF, R. L., R. L. LAMPMAN, AND L. DEEM-DICKSON. 1995. Indole as an olfactory synergist for volatile kairomones for diabroticite beetles. *Journal of Chemical Ecology* 21: 1149–1162.
- METCALF, R. L., R. L. LAMPMAN, AND P. A. LEWIS. 1998. Comparative kairomonal chemical ecology of diabroticite beetles (Coleoptera: Chrysomelidae: Galerucinae: Luperini: Diabroticina) in a reconstituted tallgrass prairie ecosystem. *Journal of Economic Entomology* 91: 881–890.
- METCALF, R. L., R. A. METCALF, AND A. M. RHODES. 1980. Cucurbitacins as kairomones for diabroticite beetles. *Proceedings of the National Academy of Sciences, USA* 77: 3769–3772.
- MITHOFER, A., G. WANNER, AND W. BOLAND. 2005. Effects of feeding *Spodoptera littoralis* on lima bean leaves. II. Continuous mechanical wounding resembling insect feeding is sufficient to elicit herbivory-related volatile emission. *Plant Physiology* 137: 1160–1168.
- MOTHERSHEAD, K., AND R. J. MARQUIS. 2000. Fitness impacts of herbivory through indirect effects on plant–pollinator interactions in *Oenothera macrocarpa*. *Ecology* 81: 30–40.
- MUTIKAINEN, P., AND L. F. DELPH. 1996. Effects of herbivory on male reproductive success in plants. *Oikos* 75: 353–358.

- NEWMAN, A. A. 1972. Chemistry of terpenes and terpenoids. Academic Press, New York, New York, USA.
- OHNMEISS, T. E., AND I. T. BALDWIN. 2000. Optimal defense theory predicts the ontogeny of an induced nicotine defense. *Ecology* 81: 1765–1783.
- OKAMOTO, T., A. KAWAKITA, AND M. KATO. 2007. Interspecific variation of floral scent composition in *Glochidion* and its association with host-specific pollinating seed parasite (*Epicephala*). *Journal of Chemical Ecology* 33: 1065–1081.
- PARIS, H. S., N. YONASH, V. PORTNOY, N. MOZEES-DAUBE, G. TZURI, AND N. KATZIR. 2003. Assessment of genetic relationships in *Cucurbita pepo* (Cucurbitaceae) using DNA markers. *Theoretical and Applied Genetics* 106: 971–978.
- QUESADA, M., K. BOLLMAN, AND A. G. STEPHENSON. 1995. Leaf damage decreases pollen production and hinders pollen performance in *Cucurbita texana*. *Ecology* 76: 437–443.
- R DEVELOPMENT CORE TEAM. 2006. R: A Language and Environment for Statistical Computing. R Foundation for Statistical Computing, Vienna, Austria.
- RAGUSO, R. A. 2004. Why are some floral nectars scented? *Ecology* 85: 1486–1494.
- RYAN, C. A. 2000. The systemin signaling pathway: Differential activation of plant defensive genes. *Biochimica et Biophysica Acta* 1477: 112–121.
- SCHLICHTING, C. D., AND V. A. DELESALLE. 1997. Stressing the differences between male and female functions in hermaphroditic plants. *Trends in Ecology & Evolution* 12: 51–52.
- SCHUURINK, R. C., M. A. HARING, AND D. G. CLARK. 2006. Regulation of volatile benzenoid biosynthesis in petunia flowers. *Trends in Plant Science* 11: 20–25.
- SHULER, R. E., T. H. ROULSTON, AND G. E. FARRIS. 2005. Farming practices influence wild pollinator populations on squash and pumpkin. *Journal of Economic Entomology* 98: 790–795.
- STANTON, M. L., A. A. SNOW, AND S. N. HANDEL. 1986. Floral evolution: Attractiveness to pollinators increases male fitness. *Science* 232: 1625–1627.
- STRAUSS, S. Y., J. K. CONNER, AND S. L. RUSH. 1996. Foliar herbivory affects floral characters and plant attractiveness to pollinators: Implications for male and female plant fitness. *American Naturalist* 147: 1098–1107.
- STRAUSS, S. Y., R. E. IRWIN, AND V. M. LAMBRIX. 2004. Optimal defence theory and flower petal colour predict variation in the secondary chemistry of wild radish. *Journal of Ecology* 92: 132–141.
- STRAUSS, S. Y., AND J. B. WHITTALL. 2006. Non-pollinator agents of selection on floral traits. In L. D. Harder and S. C. H. Barrett [eds.], *Ecology and evolution of flowers*, 120–138. Oxford University Press, Oxford, UK.
- TALLAMY, D. W. 1985. Squash beetle feeding behavior: An adaptation against induced cucurbit defenses. *Ecology* 66: 1574–1579.
- THEIS, N. 2006. Fragrance of Canada thistle (*Cirsium arvense*) attracts both floral herbivores and pollinators. *Journal of Chemical Ecology* 32: 917–927.
- THEIS, N., M. LERDAU, AND R. A. RAGUSO. 2007. The challenge of attracting pollinators while evading floral herbivores: Patterns of fragrance emission in *Cirsium arvense* and *Cirsium repandum* (Asteraceae). *International Journal of Plant Sciences* 168: 587–601.
- THOMSON, V. P., A. B. NICOTRA, AND S. A. CUNNINGHAM. 2004. Herbivory differentially affects male and female reproductive traits of *Cucumis sativus*. *Plant Biology* 6: 621–628.