

ATTRACTING MUTUALISTS AND ANTAGONISTS: PLANT TRAIT VARIATION EXPLAINS THE DISTRIBUTION OF SPECIALIST FLORAL HERBIVORES AND POLLINATORS ON CROPS AND WILD GOURDS¹

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- *Premise of the study:* Floral traits play important roles in pollinator attraction and defense against floral herbivory. However, plants may experience trade-offs between conspicuousness to pollinators and herbivore attraction. Comparative studies provide an excellent framework to examine the role of multiple traits shaping mutualist and antagonist interactions.
- *Methods:* To assess whether putative defensive and attractive traits predict species interactions, we grew 20 different Cucurbitaceae species and varieties in the field to measure interactions with pollinators and herbivores and in the greenhouse to assess trait variation. Cucurbits are characterized by the production of cucurbitacins, bitter nonvolatile terpenoids that are effective against generalist herbivores but can attract specialist beetles. We determined whether plant traits such as cucurbitacins predict herbivore resistance and pollinator attraction using an information-theoretic approach.
- *Key results:* Mutualists and floral antagonists were attracted to the same cucurbit varieties once they flowered. However, rather than cucurbitacin concentration, we found that the size of the flower and volatile emissions of floral sesquiterpenoids explained both pollinator and floral herbivore visitation preference across cucurbit taxa. This pattern held across cucurbit taxa and within the *Cucurbita* genus.
- *Conclusions:* Surprisingly, floral sesquiterpenoid volatiles, which are associated with direct defense, indirect defense, and attraction, rather than defense traits such as cucurbitacins, appeared to drive interactions with both pollinators and floral herbivores across cucurbit taxa. Identifying the relevant plant traits for attraction and deterrence is important in this economically valuable crop, particularly if pollinators and floral herbivores use the same plant traits as cues.

Key words: cucumber beetles; *Cucurbita*; cucurbitacins; Cucurbitoideae; defense; fragrance; herbivore; squash bees; terpenoids; volatile organic compounds.

For plants engaged in a mutualism with insects to facilitate cross pollination, the evolution of floral cues may be constrained by costs of attracting antagonists such as herbivores (e.g., Theis et al., 2007). Understanding the constraints imposed on the evolution of plant traits is of increasing interest for both basic ecological and agricultural research. Since the 1700s, floral biologists have focused their efforts on understanding the functional importance of floral traits for interactions with pollinators (Lloyd and Barrett, 1996). In contrast, agricultural research has focused to a large extent on pest control, even on crops that require cross pollination because pollination has not been limiting when farmers can rely on hired bee colonies

(James and Pitts-Singer, 2008). However, the two research approaches have begun to converge. On the one hand, floral biologists have a growing interest in interpreting the evolution and functional significance of floral traits in a wider perspective, encompassing multispecies interactions and abiotic factors (Strauss and Whittall, 2006; Adler, 2007). On the other hand, with the recent crises in managed honey bee colonies and reductions in native bee populations, agricultural strategies are beginning to emphasize the importance of attracting pollinators as well as deterring pests (Allen-Wardell et al., 1998; Garibaldi et al., 2013). Thus, research from both basic and agricultural perspectives is converging to focus on how plant traits attract both mutualists and antagonists.

Floral herbivores and pollinators may use the same traits to find flowers, resulting in conflicting selection pressure on those traits. This predicament has been found for flower number (Lay et al., 2011), size (Kudoh and Whigham, 1998), shape (Galen and Cuba, 2001), scape length (Ehrlén et al., 2002), fragrance emission (Theis and Adler, 2012), nectar production (Adler and Bronstein, 2004), and phenology (Ehrlén and Munzbergova, 2009). In a few cases, traits can be optimized for pollinator visitation while repelling floral herbivores. For example, defensive compounds like nicotine repel floral herbivores while increasing the efficiency of pollinator visits (Kessler and Baldwin, 2007), fragrance components such as terpenoids attract pollinators

¹Manuscript received 11 April 2014; revision accepted 21 July 2014.

The authors thank N. Woodard and A. Brown for field cultivation, M. Messer for project management, F. Halaweish (South Dakota State University) for cucurbitacin analysis, M. Doall (Stony Brook University) for C and N analyses, J. Normanly for the use of the GC-MS, T. Beauchesne and C. Joyner for greenhouse support, and J. Hazzard-Watkins, K. Bouley, S. Brindle, N. Freeman, and S. Subramony for field assistance. This research was funded by a USDA-CSREES Northeastern Integrated Pest Management Grant awarded April 2005.

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while repelling herbivores (Junker and Bluethgen, 2010), and an aposematic extrafloral display increases pollinator attraction but repels herbivores (Gerchman et al., 2012).

Understanding the role of traits in attraction of mutualists vs. antagonists is particularly important in crop plants that require cross pollination. Rather than natural selection driving trait evolution, artificial selection is the primary force. Crops could be bred to be more attractive to pollinators to increase pollinator services. However, traits attractive to pollinators can also attract pests (Adler and Bronstein, 2004; Theis and Adler, 2012). There can also be trade-offs in resource allocation to different functions, such as attractive and defensive traits. Therefore, to maximize yield in crop plants, we need to identify traits important for pollinator attraction and to assess potential allocation trade-offs and ecological costs in terms of increased herbivore damage.

We examined the functional significance of plant traits for attraction of both pollinators and herbivores to the Cucurbitoideae subfamily of the squash family, Cucurbitaceae. This is a large family of plants with ca. 740 species in 111 genera, which includes many economically important crops such as *Cucurbita* (summer and winter squash, zucchini, and pumpkin), *Cucumis* (cantaloupe and cucumber), and *Citrullus* (watermelon) (Kocyan et al., 2007). Trait variation across the subfamily is large, and there are both specialist pollinators and herbivores that have co-evolved with squash. The squash bees (*Peponapis* and *Xenoglossa*; Hymenoptera: Apidae) are obligately oligolectic (Hurd and Linsley, 1970). Squash flowers open at dawn, and squash bees fly at low light and temperature (Tepedino, 1981). Generalists such as honeybees and bumblebees can also be effective pollinators (Artz and Nault, 2011). Cucumber beetles (*Diabrotica* spp., *Acalymma vittatum*) can reduce yield in cucurbits through direct damage (Barber et al., 2011; Barber et al., 2012) and indirectly by reducing pollinator attraction (Theis and Adler, 2012) and acting as a vector of pathogens (Sasu et al., 2010). Cucumber beetle adults damage leaves and flowers early in the season and lay eggs in the soil. In the northeastern United States where our study was conducted, the primary cucumber beetle is *Acalymma vittatum*, whose larvae can extensively damage cucurbit root systems and reduce yield (Barber et al., 2011). Second-generation adults emerge and continue to feed, damaging leaves, flowers, and fruit before leaving the fields for overwintering sites. The semiochemicals of cucurbits have been of much interest (Metcalf and Metcalf, 1992). Cucurbitacins, oxygenated tetracyclic triterpenoids, are among the bitterest compounds known. However, cucumber beetles feed compulsively in their presence (Chambliss and Jones, 1966), and the consumption of these compounds does not appear to reduce beetle fitness (Tallamy et al., 1997). In fact, it has been proposed that cucumber beetles choose plants based on floral cucurbitacin content, with a preference for higher concentrations (Andersen and Metcalf, 1987). Another lure for the beetles are the floral volatiles emitted by squash. In a study on eight varieties of squash, the more fragrant varieties were more likely to be attacked (Andersen and Metcalf, 1987). That study did not consider how floral fragrance affects pollinating squash bees, although bioassays have demonstrated that fragrance is an important lure for both beetles (Metcalf and Metcalf, 1992) and squash bees (Andrews et al., 2007; Theis and Adler, 2012). Specifically, the compound 1,4-dimethoxybenzene has been shown to be a critical attractant (Theis and Adler, 2012).

To determine the relative importance of floral and defensive traits in attracting mutualists and antagonists, we examined 20 cucurbit varieties, including agricultural and wild species. Of these, 14 were in the genus *Cucurbita*. We included non-*Cucurbita*

species because they are also attacked by cucumber beetles and provide a broader taxonomic base for examining how trait variation affects interactions. To assess floral and defensive traits, we grew plants in the greenhouse, thereby avoiding interactions with insects in the field that could induce changes in defense or floral traits (Tallamy, 1985; Theis et al., 2009). The approach of assessing traits in a controlled environment that are then used to interpret ecological interactions in the field has been used previously (Agrawal et al., 2012). We assessed pollinator and herbivore visitation over 2 yr in field-grown plants. We then correlated greenhouse trait levels with field measures of interactions to ask the following questions: (1) How do leaf defenses and floral traits vary across taxa, and is there evidence of allocation trade-offs? (2) What leaf and floral traits are associated with herbivore and pollinator attraction? (3) Are any traits associated with attracting both pollinators and herbivores creating a cost associated with pollinator attraction?

MATERIALS AND METHODS

Greenhouse study to assess traits—Plant material—Twenty taxa (Table 1) were sown from seed between 15 August and 14 September 2005 using ProMix HP (Premiere Horticulture, Quakertown, Pennsylvania, USA). Because some of our taxa are varieties of one species and some are separate wild or cultivated species, we will use “variety” hereafter to refer to each separate taxonomic variety or species in the study. Seedlings were transplanted ($n = 10$ per variety) into 3.7-L pots in the greenhouse in a randomized block design. They were fertilized on 31 October with 20-20-20 General Purpose Peters Professional at 300 ppm and again on 4 November with slow-release Osmocote Classic 14-14-14 at 5 g per pot. Both are Sierra Horticultural Products (Marysville, Ohio, USA). The greenhouse was sprayed with fungicide regularly to control the spread of powdery mildew. In the sample collections described later, fully expanded leaves were used. Leaf number was noted and kept constant for each trait measurement (e.g., leaf 13 for trichome density). If mildew was obvious on a leaf, the next oldest leaf was chosen for that measurement.

Leaf and root traits—To assess the percentage water content in each variety, leaves were cut and weighed with the petiole, then dried at 55°C to a constant mass and weighed again. The upper and lower trichome density was measured on two discs (6 mm diameter) per leaf and averaged to produce a single count. The nitrogen and carbon content of leaves and roots were measured at the Functional Ecology Research and Training Laboratory at Stony Brook University on a Carlo Erba CHN analyzer. Samples were analyzed in triplicate on two young but fully formed leaves and on roots from each plant to calculate percentage nitrogen and carbon.

Floral traits—Not all of the varieties produced female flowers, so traits are only reported for male flowers. We did not collect floral trait data for two species (*C. ecuadoriensis* and Buffalo gourd) that did not flower and one (Speckled Swan) that flowers nocturnally. Fragrance was collected using dynamic headspace sampling (as described in Theis et al., 2009) from a single male flower per plant starting between 07:00 and 09:00 hours and continuing for 50 min to 3 h based on the fragrance intensity per variety. Fragrance concentrations are reported in nanograms per flower per hour. For subtraction purposes, fragrance was also measured from a vegetative sample per species. Fragrance analysis was performed by combined capillary gas chromatography–mass spectrometry (GC-MS) with an Agilent 6890 GC equipped with Mass Selective Detector 5973 (Agilent Technologies, Santa Clara, California, USA) as described by Theis et al., (2009). Other floral traits were measured on one male flower per plant, including corolla length and width at top of floral tube, nectar volume using microcapillary tubes (Drummond Scientific, Broomall, Pennsylvania, USA) and nectar sugar concentration with a refractometer (Fisherbrand, ThermoFisher Scientific, Waltham, Massachusetts, USA). Pollen production was assessed by suspending crushed, dried anthers in ethanol, sonicating them and then counting the pollen grains using a hemacytometer. Six replicate counts were conducted per anther to estimate pollen grains per flower. We do not report pollen grains per anther since *Cucurbita* species have a fused anther structure.

TABLE 1. Species and varieties included in greenhouse and field experiments.

Species	Variety	Common name	Study code	Seed source
<i>C. maxima</i> subsp. <i>andreaana</i>	wild		CxA	USDA PI 458659 90GI
<i>C. maxima</i>	Burgess	Buttercup	CxB	Rupp, Wauseon, Ohio, USA
<i>C. maxima</i>	Cinderella	Cinderella pumpkin	CxC	Johnny's Selected Seeds, Waterville, Maine, USA
<i>C. maxima</i>	Giant Pumpkin	Prizewinner	CxG	Johnny's
<i>C. maxima</i>	Blue Hubbard	Blue Hubbard	CxH	Johnny's
<i>C. pepo</i>	Baby Pam	Sugar pumpkin	CpB	Rupp
<i>C. pepo</i>	Rocket	Standard pumpkin	CpR	Johnny's
<i>C. pepo</i> subsp. <i>Texana</i>	wild	Texas gourd	CpT	USDA PI 540907 LOT 97NCAB01
<i>C. pepo</i>	Embassy	Zucchini	CpZ	Rupp
<i>C. moschata</i>	Waltham	Butternut	CmB	Johnny's
<i>C. moschata</i>	La Estrella	Calabaza	CmC	Rupp
<i>C. argyrosperma</i>	Cushaw pumpkin	Navajo Cushaw	Ca	Native Seeds, Tucson, Arizona, USA
<i>C. ecuadoriensis</i>	wild		Ce	USDA PI 432441
<i>C. foetidissima</i>	wild	Buffalo gourd	Cf	USDA PI 442192 FROM MEXICO
<i>Cucumis melo</i>	Athena	Canteloupe	CA	Johnny's
<i>C. sativus</i>	Marketmore	Cucumber	CU	Johnny's
<i>Momordica charantia</i>		Bittergourd	BG	Rupp
<i>Lagenaria siceraria</i>		Speckled Swan	SS	Johnny's
<i>Citrullus lanatus</i>	Red Festival	Watermelon	WA	Johnny's
<i>Benincasa hispida</i>		Winter melon	WI	Rupp

Cucurbitacins—Cucurbitacins were measured using HPLC by Dr. Fathi Halaweish (South Dakota State University) from flowers, leaves, and roots from three individuals from each variety, except that all floral samples from butternut and Texas gourd were lost during analysis. For detailed methods, see Appendix S1, Supplemental Data with the online version of this article.

Field study to measure pollination and herbivory—Seeds were sown from the 20 varieties on 20 May 2005 and 23 May 2006 in Metromix 360 soil in the greenhouse with natural light and planted at the University of Massachusetts Center for Agriculture (South Deerfield, Massachusetts, USA) on 11 June 2005 and on 9 June 2006. Six individuals from each variety were planted in pairs (pairs were considered the unit of replication, $n = 3$) in a randomized block design in the field and fertilized with 19-19-19 granular fertilizer (Crop Production Services, Loveland, Colorado, USA). Sample sizes, in terms of number of varieties for which we could collect herbivore and pollinator data, differed between years. In 2005, *C. ecuadoriensis* did not flower. In 2006, *C. ecuadoriensis* and Buffalo gourd did not survive, and pollinator data were not collected for *C. maxima* subsp. *andreaana*, Texas gourd, and Speckled Swan.

To determine preferences of herbivorous cucumber beetles and leaf damage, we censused plants on a weekly basis throughout June for the number of live and dead beetles and the degree of damage to the four most recently expanded leaves. In 2005, leaf damage was scored from 0 to 4 (based on 25% damage increments), and in 2006, the percentage damage was used to provide a finer scale measurement of damage.

Pollinator observations began at the onset of flowering for each plant. Up to two male flowers and two female flowers were observed per plant, first recording the number of beetles in the flower and then recording all pollinator visits, time per visit and all rejections (pollinators approaching the floral headspace but not alighting) in a 5-min period. Pollinator and floral herbivore visitation was positively correlated for male and female flowers within taxa (Spearman rank correlations: number of squash bee visits $r_s = 0.567$, $P < 0.001$; number of beetle visits: $r_s = 0.353$, $P = 0.025$; number of squash bee time: $r_s = 0.521$, $P = 0.049$; number of rejections: $r_s = 0.444$, $P = 0.004$), so visitation was averaged within sexes for each taxa. We did not record flower age because the flowers bloom at dawn and only last a few hours for most of these varieties. Pollinators were identified as *Peponapis pruinosa*, *Apis mellifera*, *Bombus* sp., or other. Visits from squash bees (*Peponapis pruinosa*) made up 80% of total visits; other taxa were not common enough to analyze separately. Therefore, only squash bee pollination is considered below. However, rejections were often difficult to identify on the wing, and so all rejections are analyzed as one category without identifying the visitor.

Statistical analyses—Our main goal was to determine which putative defensive and attractive traits, measured in greenhouse plants, predicted interactions with pollinators and herbivores in the field. We did not measure traits in the field-grown plants since interactions such as leaf herbivory can alter both

defensive and floral traits (Tallamy, 1985; Theis et al., 2009), resulting in spurious correlations between traits and interactions where cause and effect cannot be dissected. Because this is a common problem, traits are often estimated on plants other than those used to assess interactions in field studies (e.g., Lankau and Strauss, 2007; Agrawal et al., 2012).

To determine the plant traits that influence pollinators and herbivores, we calculated trait values for each variety from greenhouse plants using a generalized linear model with variety and block as fixed factors. We used the effects package (Fox, 2003) in the program R (R Development Core Team, 2012) to calculate mean trait values after accounting for variation due to block. These analyses were conducted for percentage water content, trichome number, fragrance classes (ng-flower⁻¹·h⁻¹), corolla length (mm), pollen production (grains/flower), and nectar volume (μL). Leaf % N, % C, and leaf and root cucurbitacin concentration (μg/g dry mass) were determined from a subset of 1–3 plants per variety, so values for these traits are raw means that do not account for block variation. Separate analyses of cucurbitacin B and D produced very similar results (data not shown), so for simplicity the compounds were added, and only the results for total cucurbitacins are presented. Volatile emissions were not normally distributed and were log-transformed prior to calculating variety mean values.

To summarize herbivore and pollinator preference for each variety, we calculated average leaf damage, number of beetles on leaves and flowers, squash bee visits, squash bee probe time, and total pollinator rejections for each variety in the same way, treating block as a fixed factor and calculating response values using the R effects package. For consistency, leaf damage in 2006 was converted into the damage scale used in 2005 by converting individual leaf damage estimates. Because of the variation in yearly data, z-scores were computed for graphing purposes, so that data across years can be visually compared. We also calculated Pearson correlations to analyze whether leaf damage was correlated with cucumber beetle abundance and whether cucumber beetle abundance was correlated with pollinator abundance or pollinator rejections.

To assess whether there were trade-offs across varieties in allocation to defensive vs. attractive traits, we determined Pearson correlations between total leaf and floral cucurbitacins and corolla length, pollen production, nectar volume, and four classes of floral volatiles. We also correlated floral traits to determine whether floral traits such as nectar and pollen, which act as rewards to pollinators, were correlated with characters more easily perceived by pollinators on the wing, such as visual characters such as corolla size or chemical characters such as fragrance. To control the number of variables in our models, we analyzed fragrance traits at the class level, rather than analyzing individual compounds, except for the compound 1,4-dimethoxybenzene, an important insect attractant in squash (Theis and Adler, 2012). Because recent work has demonstrated that defense concentrations are correlated across tissues in *Nicotiana* species (Adler et al., 2012), we also analyzed whether root, leaf, and floral cucurbitacins were correlated across varieties.

To determine which plant traits correlated most strongly with herbivory, beetle abundance, and pollinator behavior, we constructed model sets using

a subset of plant traits hypothesized to be important to the responses (see below). For each response variable, we evaluated the full set of nested models using AICc (corrected Akaike information criterion) and used model averaging (Burnham and Anderson, 2002) to estimate parameter coefficients, unconditional standard errors, and unconditional 95% confidence intervals. We used the full set of nested models, rather than a subset of models, as advocated by Burnham and Anderson (2002). We considered parameters where the unconditional 95% confidence intervals did not overlap zero to significantly predict responses. Field data from 2005 and 2006 were treated as independent observations with year included as a fixed factor in all models. Analyses were carried out in R using the AICcmodavg package (Mazerolle, 2013).

To analyze traits that predict leaf herbivory, models used leaf damage and number of live beetles on leaves as responses, and included as predictors leaf percentage N, C, water content, total leaf and root cucurbitacin concentration, and trichome density of both top and bottom surfaces of leaves. To analyze floral interactions, models included squash bee visits per 5 min, number of rejections, squash bee probe time per flower, and beetle abundance in flowers as responses, and nectar volume, pollen production, corolla length, and concentrations of sesquiterpenoids, monoterpenoids, and aromatics as predictors. To reduce the number of variables, we chose corolla length as a representative measurement of floral size based on a correlation analysis of the various measurements (data not shown). A previous study identified 1,4-dimethoxybenzene as a potentially important attractor of squash bees (Theis and Adler, 2012), but because production of this compound is positively correlated with corolla length, we used mediation analysis with the R package mediation (Tingley et al., 2012) to determine if the relationship between 1,4-dimethoxybenzene and squash bee visitation is mediated by corolla length. Mediation analysis allows the identification of causal mechanisms by examining intermediate variables (“mediators”) in a causal pathway (MacKinnon, 2008). We found that corolla length was a significant mediator of the relationship between the production of this compound and squash bee visits (average causal mediation effect, 95%CI: 0.13, 0.06–0.22; direct effect, 95% CI: 0.07, –0.01 to 0.16), indicating that the response of squash bees is better explained by flower size than production of 1,4-dimethylbenzene. For this reason, we did not specify this compound as an independent variable in our model, but included it in total aromatic compounds.

Squash bee visitation and squash bee probe time were log-transformed to normalize global model residuals and reduce heteroscedasticity. *Cucurbita foetidissima* and *C. ecuadorensis* did not flower in the greenhouse, and *Lagenaria siceraria* blooms nocturnally; so all three were excluded from this analysis. In addition, because *Cucurbita* flowers are consistently larger than the non-*Cucurbita* varieties, there was the potential to find correlations between traits and interactions that appeared to be driven by flower size, but were instead due to other underlying differences between *Cucurbita* and other varieties. Thus, we reanalyzed data including only the *Cucurbita* varieties to ascertain whether patterns found across all varieties were consistent within the *Cucurbita* alone and were therefore not an artifact of phylogenetic relatedness.

RESULTS

Leaf traits—All wild cucurbits contained cucurbitacins, but not all cultivated cucurbits did. For example, one *C. pepo* variety, Rocket, had the highest concentration of total cucurbitacins in leaves, while another, Baby Pam, had zero (Appendix S2; see online Supplemental Data). Neither variety of *C. moschata* contained leaf cucurbitacins, nor did watermelon. Interestingly, though, watermelon had the second highest concentration of root cucurbitacins, indicating that defense concentrations are not necessarily correlated across tissues. Water content, leaf carbon, and leaf nitrogen are reported in Appendix S3 (see online Supplemental Data).

Floral traits—Non-*Cucurbita* species had the shortest petals, while crop varieties of *C. pepo* and *C. maxima* had the longest (online Appendix S4). Giant pumpkin had the highest nectar volume, whereas watermelon had no nectar but the highest number of pollen grains per flower. Floral cucurbitacins were highest in *C. andreana* and were undetectable in four varieties (Cinderella, cushaw, rocket, and watermelon, Appendix S2). The most fragrant flowers were from varieties of *C. maxima*. In these varieties, sesquiterpenoids dominated the blend. Cinderella had the highest emission rates, while watermelon emitted the lowest level of sesquiterpenoids (Appendix S4). Bittergourd had the greatest emission rates of monoterpenoids and aromatics, with two aromatics, indole and methyl anthranilate, dominating the blend.

Correlations between traits—There were no correlations between leaf or floral cucurbitacins and any floral attractive or reward trait ($|r| < 0.25$, $P > 0.05$ for all), providing no support for the hypothesis of trade-offs between attractive and defensive functions. Within floral traits, aromatics were positively correlated with sesquiterpenoids and monoterpenoids (Table 2). Interestingly, 1,4-dimethoxybenzene was highly correlated with sesquiterpenoids, corolla length, and nectar volume (Table 2). Corolla length was also positively correlated with nectar volume and sesquiterpenoids, but negatively correlated with pollen production. Within defensive traits, leaf and root total cucurbitacins were positively correlated, but there were no significant correlations between floral cucurbitacins and leaf or root cucurbitacins.

TABLE 2. Pearson correlation matrix showing R values for (A) floral traits (n = 17) and (B) field measurements from 2005 and 2006 (n = 25).

(A) Floral traits	Corolla length	Nectar	Pollen	Sesquiterpenoids	Monoterpenoids	Aromatics
Corolla length	1					
Nectar	0.52*	1				
Pollen	-0.50*	-0.13	1			
Sesquiterpenoids	0.62**	0.54*	-0.27	1		
Monoterpenoids	-0.34	-0.24	-0.03	0.17	1	
Aromatics	0.14	0.19	-0.13	0.52*	0.59**	1
1,4-Dimethoxybenzene	0.76**	0.51*	-0.43	0.86**	-0.1	0.30

(B) Field measurements	Damage	Beetles (Flowers)	Squash bees	Beetles (leaves)	Squash bee time
Damage	1				
Beetles (on flowers)	-0.21	1			
Squash bees	0.13	0.62**	1		
Beetles (on leaves)	0.77	0.13	0.29	1	
Squash bee time	0.24	0.35	0.39	-0.18	1
Rejections	0.12	0.66**	0.63**	-0.04	0.48*

Notes: *P < 0.05, ** P < 0.01, without Bonferroni corrections.

Insect visitors—Per-variety visitation patterns for the number of beetles on leaves and flowers, squash bee visits and pollinator rejections were all consistent across years ($|r| > 0.66$, $P < 0.01$ for all), although leaf damage was not ($n = 18$, $r = -0.09$, $P = 0.73$ data not shown; $n = 18$, excluding *C. ecuadoriensis*, which did not flower/survive each year and Buffalo gourd which did not survive in 2006). Leaf beetle damage was also not correlated with leaf beetle abundance in either year ($n = 18$, $r < 0.36$, $P > 0.14$, Table 2). Cinderella had the most beetles on leaves both years (Fig. 1). The fewest beetles were observed on leaves of winter melon and butternut in 2005 and on Texas gourd in 2006. We only report insect visitation to flowers of varieties for which we measured floral traits ($n = 17$ varieties, excluding *C. ecuadoriensis*, Buffalo gourd, and Speckled Swan; see *Floral Traits* in Materials and Methods). Beetles were most likely to be found on flowers of calabaza in 2005 and Cinderella in 2006. The fewest beetles in flowers were observed in non-*Cucurbita* species in both years. Beetles in flowers and squash bee visits were highly correlated (Table 2, Fig. 2). Squash bees were most frequently observed at Cinderella in 2005 and Rocket in 2006, although *C. maxima* species had generally high visitation in both years. Rejections were also highly correlated with the presence of flower beetles (Table 2). Pollinators were most likely to reject zucchini flowers in both years. Rejections were unlikely in both years at non-*Cucurbita* species.

Models—Mean leaf damage was not significantly correlated with any plant traits, but cucumber beetle abundance on leaves increased with leaf water content and trichome density on the bottom surface of leaves, and declined with trichome density on the top surface of leaves (Appendix S5).

The analysis of floral interactions showed that cucumber beetle abundance in flowers and squash bee visitation was greatest in varieties with large flowers that produced greater amounts of sesquiterpenoids (Fig. 3; Appendix S6). Because the *Cucurbita* taxa have relatively large flowers, the model was also tested with non-*Cucurbita* taxa excluded from the analysis (model not shown). Within the *Cucurbita*, sesquiterpenoids and corolla length explained time spent at flowers by squash bees and pollinator rejections (Fig. 3, Appendix S6). However, sesquiterpenoids but not corolla length explained beetle and squash bee floral visitation (squash bees: sesquiterpenoids: $\beta = 0.096 \pm 0.034$, CI: 0.029–0.163; corolla: $\beta = 0.010 \pm 0.011$, CI: -0.011 to 0.030; beetles: sesquiterpenoids: $\beta = 2.91 \pm 1.28$, CI: 0.40–5.4; corolla: $\beta = 0.48 \pm 0.25$, CI: -0.022 to 0.98).

DISCUSSION

Corolla length and sesquiterpenoids were the best predictors of flower preference for both the specialist pollinator squash bees and the specialist cucumber beetle (Fig. 3, Appendix S6). Mutualists and antagonists chose the same flowering varieties to visit, and this preference was consistent across the 2 yr of the study. Cucumber beetles may reduce maternal fitness by directly damaging female reproductive parts but, consistent with results from another study (Theis and Adler, 2012), specialist pollinators were not deterred by the beetles or their damage (Table 2). In contrast to corolla length, sesquiterpenoid emission rates explained visitation regardless of whether non-*Cucurbita* species were excluded from the analysis. Thus, the relationship between sesquiterpenoids and visitation by specialist

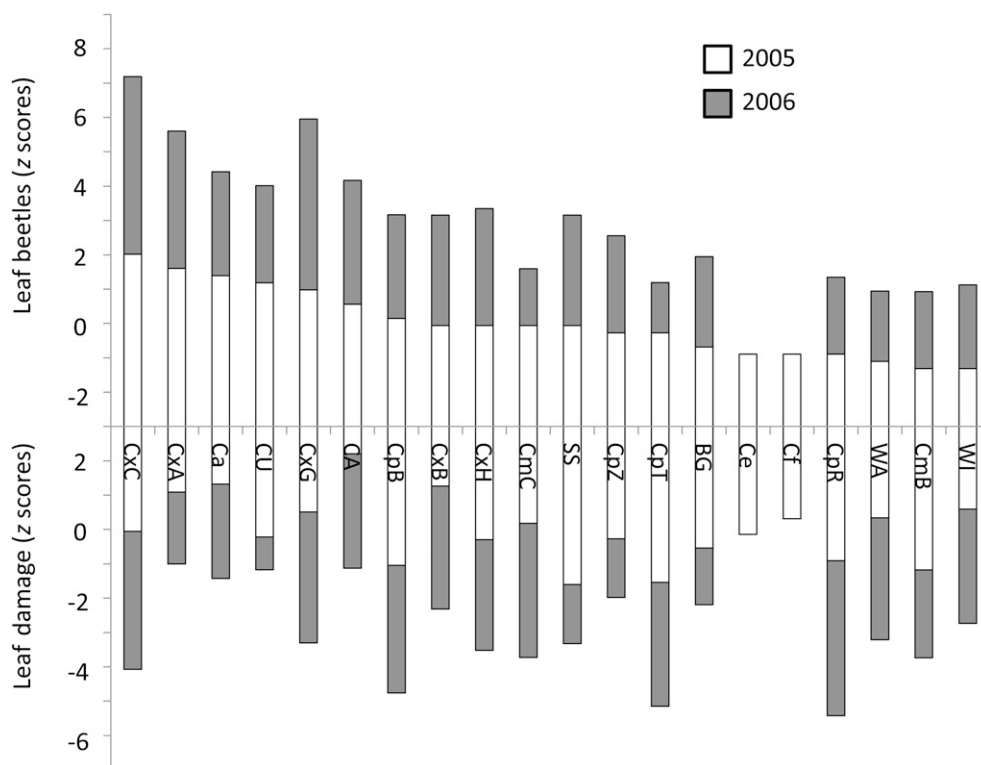


Fig. 1. Presence of leaf beetles and distribution of leaf damage on Cucurbitaceae in 2 yr. Data graphed as z -scores. In 2005, $n = 20$. In 2006, $n = 18$ due to exclusion of *C. ecuadoriensis* and Buffalo gourd which did not survive. Variety abbreviations are as shown in Table 1.

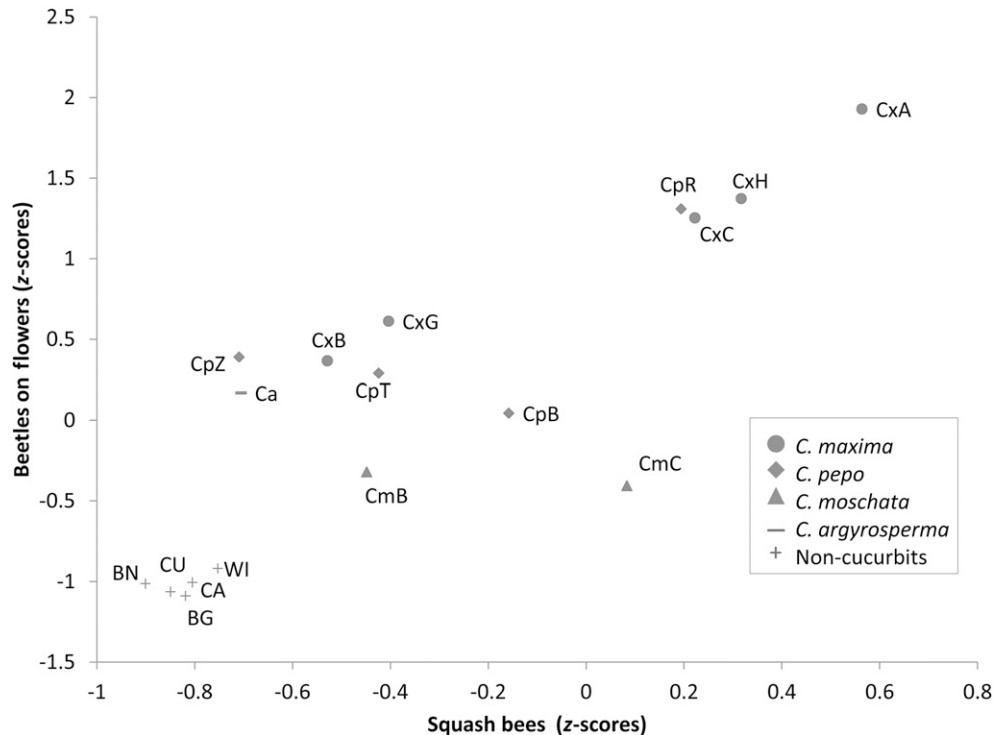


Fig. 2. Cucumber beetles in flowers and squash bee preference is correlated across taxa. Key to the abbreviations can be found in Table 1. Briefly, symbols indicate species affiliation. Plus sign indicates noncucurbit species. “C” followed by a lowercase letter indicates *Cucurbita*, and in most cases, is followed by a capital letter indicating variety. Non-*Cucurbita* taxa are indicated with two capital letters.

herbivores and pollinators holds for the subfamily Cucurbitoidae as well as within the *Cucurbita* alone and is not an artifact of differences between *Cucurbita* species and other cucurbits.

Mutualists provide pollination services in exchange for rewards such as nectar to fuel metabolism and pollen to provision bee larvae. Cucumber beetles are also pollen feeders, but may be detrimental to plant reproduction (Theis and Adler, 2012). However, neither nectar volume nor the number of pollen grains predicted pollinator or beetle flower preference, possibly because these traits may be difficult to assess on the wing. Correlated characters, such as floral size, are known to be used by pollinators to predict reward (e.g., Armbruster et al., 2005). At least for the Cucurbitaceae in our study, corolla length and sesquiterpenoids were highly correlated with nectar and pollen (Table 2), suggesting that these traits could be used by pollinators as reliable indicators of rewarding plants (Armbruster et al., 2005). However, while nectar was positively correlated with corolla length, pollen was negatively correlated with corolla length (Table 2), preventing insects from maximally foraging for both types of rewards simultaneously. Interestingly, both squash bees and beetles chose traits that indicated high quantities of nectar rather than pollen.

We found no evidence for trade-offs between allocation to defensive and attractive or reward functions. Even in wild systems, evidence for resource allocations can be difficult to detect (Strauss et al., 2002). In agricultural systems, where there are often high levels of nutrient input, irrigation, and release from interspecific competition (as well as artificial selection for reduced defensive compounds for palatability and large flowers for higher yield; reviewed in Mayrose et al., 2011), there may be little pressure for plants to allocate resources between these

functions. Leaf and root cucurbitacins were correlated across varieties, indicating that varieties with highly defended roots tended to have highly defended leaves as well. Such correlated defense concentrations may be adaptive since cucurbitacins are highly effective against an array of generalist herbivores (Tallamy et al., 1997) and cucurbits frequently suffer both above and belowground herbivory (Barber et al., 2011). Surprisingly, there were no correlations between floral cucurbitacins and either leaf or root levels, suggesting independent production. This is in contrast with *Nicotiana*, where floral and leaf alkaloid levels were correlated across species (Adler et al., 2012). Thus, in squash, selection for cucurbitacin levels in leaves should be unlikely to influence levels in flowers or vice versa.

The attraction of both beetles and bees to flowers with high sesquiterpenoid levels was unexpected. In previous trapping studies, the sesquiterpenoid β -caryophyllene was tested but did not attract squash bees. Instead, the aromatic compound 1,4-dimethoxybenzene was an important attractant for both squash bees and cucumber beetles (Theis and Adler, 2012). Because 1,4-dimethoxybenzene was highly correlated with sesquiterpenoid concentration, we wondered if this compound was driving the flower choice of mutualists and antagonists. Mediation analysis demonstrated that corolla length, which was correlated with 1,4-dimethoxybenzene, explained more variation in squash bee and cucumber beetle preference than 1,4-dimethoxybenzene. Sesquiterpenoids are rarely tested in bioassays, perhaps because few are commercially available. However, they are ubiquitous components of plant volatile profiles (Knudsen et al., 2006) and at least one compound (β -caryophyllene) has been identified as an important attractant to the related *Diabrotica* beetles (Hammack, 2001). In a

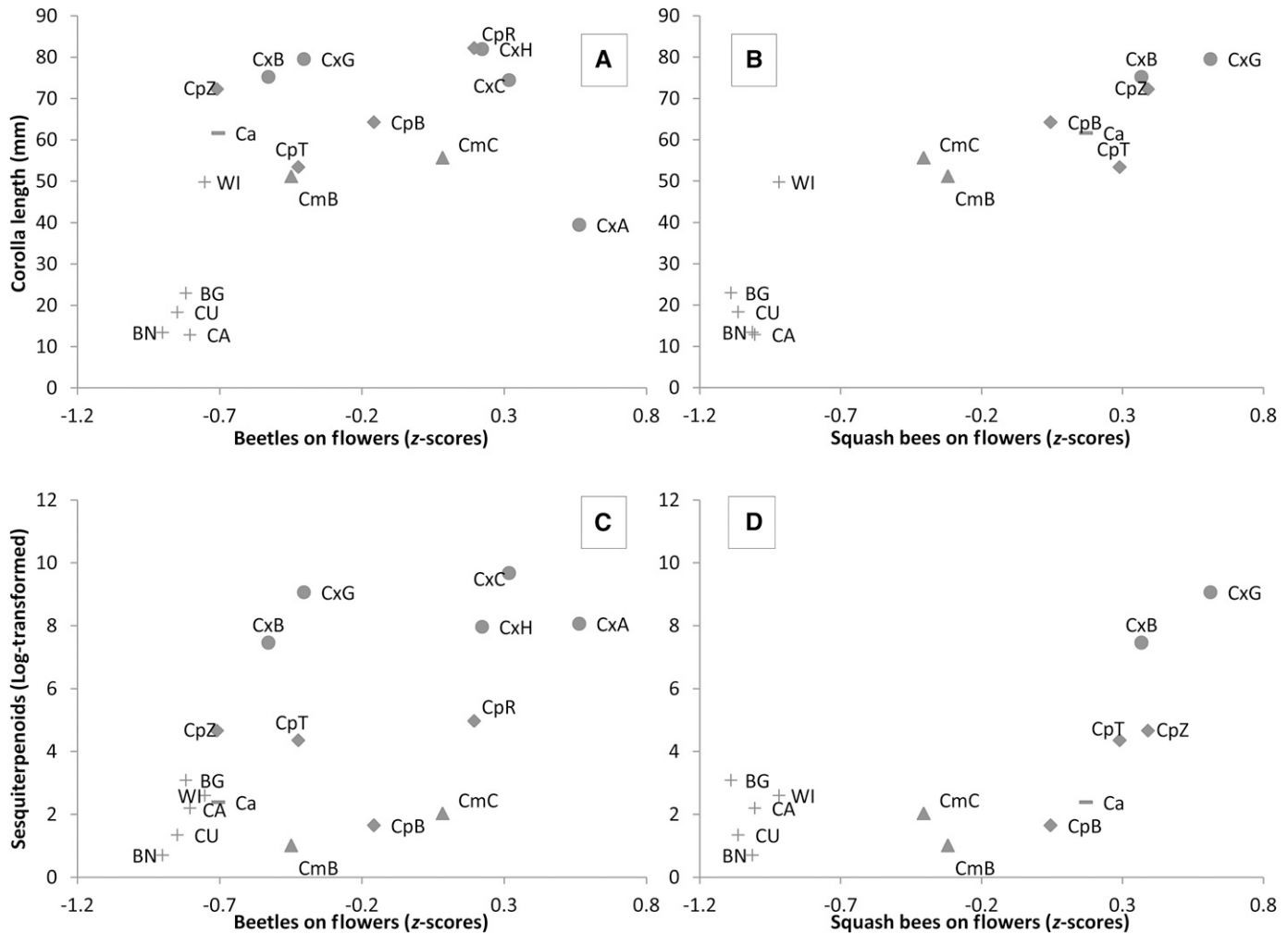


Fig. 3. (A, B) Corolla length and (C, D) sesquiterpenoids explain the (A, C) presence of cucumber beetles and (B, D) squash bees on flowers across taxa. Symbols indicate species affiliation as shown in the key in Fig. 2. Variety abbreviations are as shown in Table 1.

tritrophic system, β -caryophyllene is induced in corn roots following insect damage, luring entomopathogenic nematodes to prey on the detrimental beetle larvae (Turlings et al., 2012). Thus, a currently unidentified sesquiterpenoid may drive the patterns of attraction seen in cucurbits.

The cucumber beetle *Acalymma vittatum* attacks cucurbit plants at all stages of development, including leaves and flowers as adults and the roots as larvae. Before flowering, we found beetles on varieties whose leaves have high water content, fewer trichomes on the leaf top and more trichomes on the leaf bottom (Fig. 1, Appendix S3). Although water content is known to affect herbivores (e.g., Schadler et al., 2003), we were surprised that nitrogen, a nutritional necessity (e.g., Meloni et al., 2012), and cucurbitacins, a feeding stimulant (Metcalf et al., 1982) were not related to beetle abundance. Moreover, while leaf traits could explain the distribution of beetles across varieties of the Cucurbitaceae, it could not explain leaf damage. Beetle distribution was not correlated with leaf damage, suggesting that damage may be predicted more by plant resistance than by beetle presence across varieties. Furthermore, no trait we measured explained leaf damage. It is possible that leaf damage, which was not consistent across years, was related to

environmental factors such as leaf height (Marquis et al., 2001), which we did not measure, or a biotic factor such as the size of the leaf, specific leaf area, or a nutrient that we did not include in the model (Joern et al., 2012).

Understanding the significance of trait variation in the attraction of mutualists and antagonists has important implications for crop management, particularly in crops like squash that require pollination to set fruit. Our results have potential applications for integrated pest management strategies including trapping and monitoring of pest species using plant-based semiochemicals, which is already being used with some success in this system (e.g., Tóth et al., 2007), and for perimeter trap cropping (PTC). In PTC, the crop layout is designed to take advantage of pest colonization behavior and host preference. Border defenses are established by planting a more attractive trap crop to completely encircle the main crop, resulting in reduced infestation and reduced need for insecticides in the main crop (e.g., Aluja et al., 1997). Blue Hubbard (*C. maxima*) is the most common PTC crop for squash fields (Cavanagh et al., 2009). However, because it is not a highly prized crop, farmers have sought an alternative (Adler and Hazzard, 2009) and found success using buttercup squash (also *C. maxima*; Cavanagh et al., 2010). Our

visitation data suggest that Cinderella (*C. maxima*) or Giant pumpkin (*C. pepo*) might also be good alternatives because they are highly attractive to beetles, but only in regions with abundant squash bees since these species may compete with the main crop for pollinators (but see Adler and Hazzard, 2009; Cavanagh et al., 2010). Although some species, like cucumber and cantaloupe, were attractive at the leaf but not the flowering stage, we believe it is important to have a PTC that is attractive to beetles at the flowering stage because of the potential for cucumber beetles to spread microbial diseases during pollen feeding as well as leaf feeding (Sasu et al., 2010).

Overall, we found that pollinators and floral herbivores are attracted to the same flowers and use the same cues to choose those flowers. Therefore, breeding efforts cannot be focused on a trait that would be attractive to pollinators but not to floral herbivores. We found a good deal of variation in crop varieties, but in spite of thousands of years of breeding, traits such as corolla length and sesquiterpenoid concentrations grouped by species (Fig. 3). We also found that visual and olfactory signals that could be used as cues by pollinators honestly conveyed nectar and pollen reward status of the flower. Cucumber beetles were not steadfast in their plant preference through development. Cucumber beetles were commonly found on noncucurbit Cucurbitaceae in the vegetative state, but not once they flowered. Surprisingly, we found that sesquiterpenoids, which are associated with direct defense, indirect defense and attraction, rather than the nonvolatile cucurbitacins that define this group, were the chemical signal driving the distribution of both mutualist and antagonistic insect interactions.

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