

## Pollinator and Herbivore Attraction to *Cucurbita* Floral Volatiles

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**Abstract** Mutualists and antagonists may place conflicting selection pressures on plant traits. For example, the evolution of floral traits is typically studied in the context of attracting pollinators, but traits may incur fitness costs if they are also attractive to antagonists. Striped cucumber beetles (*Acalymma vittatum*) feed on cucurbits and are attracted to several volatiles emitted by *Cucurbita* blossoms. However, the effect of these volatiles on pollinator attraction is unknown. Our goal was to determine whether pollinators were attracted to the same or different floral volatiles as herbivorous cucumber beetles. We tested three volatiles previously found to attract cucumber beetles in a factorial design to determine attraction of squash bees (*Peponapis pruinosa*), the specialist pollinators of *cucurbita* species, as well as the specialist herbivore *A. vittatum*. We found that 1,2,4-trimethoxybenzene was attractive to both the pollinator and the herbivore, indole was attractive only to the herbivore, and (*E*)-cinnamaldehyde was attractive only to the pollinator. There were no interactions among volatiles on attraction of squash bees or cucumber beetles. Our results suggest that reduced indole emission could benefit plants by reducing herbivore attraction without loss of pollination, and that 1,2,4-trimethoxybenzene might be under conflicting selection pressure from mutualists and antagonists. By examining the attraction of both mutualists and antagonists to *Cucurbita* floral volatiles, we have demonstrated the potential for some compounds to influence only one type of interaction, while others may affect both interactions and possibly result in tradeoffs. These results shed light on the potential evolution of fragrance in native *Cucurbita*, and may have consequences for yield in agricultural settings.

**Keywords** *Acalymma vittatum* · *Cucurbita* · (*E*)-cinnamaldehyde · Floral volatiles · Herbivore · Indole · *Peponapis pruinosa* · Pollinator · 1,2,4-Trimethoxybenzene

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## Introduction

Interactions among plants, their herbivores, and their pollinators are thought to have led to the diversification of both plants and insects (e.g., Becerra 1997; Farrell and Mitter 1998; Dodd et al. 1999). Historically, studies of plant–herbivore and plant–pollinator interactions have focused either on pollinator attraction to floral traits or herbivore deterrence by plant defenses. However, most plants interact simultaneously with a variety of pollinators and herbivores that could exert reinforcing or conflicting selection pressure on plant traits (Strauss and Whittall 2006; Adler 2007). The study of pair-wise interactions in isolation may not give us an adequate understanding of the costs and benefits of phenotypic traits that mediate interactions with both herbivores and pollinators (Gomez and Zamora 2000; Herrera 2000; Adler et al. 2006).

An increasing number of studies have demonstrated that floral display and rewards may attract antagonists as well as mutualists. For example, wider corollas have made *Polemonium viscosum* flowers more attractive to pollinators but also more vulnerable to ant nectar thieves (Galen and Cuba 2001), and *Ipomopsis aggregata* plants with larger inflorescences attract higher rates of predispersal seed predation (Brody and Mitchell 1997). Hermaphrodites of *Fragaria virginiana* experience more herbivory by the bud-clipping weevil *Anthonomus signatus* compared to female morphs, and this difference is thought to be due to pollen rewards offered by hermaphrodites (Ashman et al. 2004a). Greater nectar rewards in *Datura stramonium* increased oviposition of herbivorous larvae by pollinating hawkmoths (Adler and Bronstein 2004). Therefore, attracting pollinators without also attracting herbivores may be a challenge for flowering plants that require animal pollinators to reproduce.

Scent plays a near-ubiquitous role in plant–insect interactions. Plant volatiles can attract pollinators, provide learning cues for foraging insects, serve as herbivore repellants or attractants, or attract natural enemies of herbivores (De Moraes et al. 1998; Pichersky and Gershenzon 2002; Cunningham et al. 2004; Ashman et al. 2005; Huber et al. 2005). Despite the many ways that scent influences interactions between plants and insects, we know relatively little about how herbivores and pollinators respond to the same scent volatiles. Such information may provide insight into the evolution of the composition, strength, and timing of scent emission. For example, both *Cirsium arvense* and *Nicotiana attenuata* have high floral volatile emission rates during peak reproductive periods that decline after pollination, suggesting a possible cost to emission after pollination is effected (Euler and Baldwin 1996; Theis and Raguso 2005; Theis et al. 2007). However, there are few data to assess the costs and benefits of scent emission in most systems.

Our study addressed the role of floral scent mediating interactions among squash plants, their herbivores, and their pollinators. Most *Cucurbita* species are monoecious, rely on pollinator visitation for reproduction, and are often pollinated by specialist squash bees (Hurd et al. 1974). Several floral volatiles from *Cucurbita* blossoms are attractive to many species of herbivorous cucumber beetles (Andersen and Metcalf 1986; Lampman and Metcalf 1987; Lewis et al. 1990; Metcalf et al. 1995). However, the effect of these volatiles on pollinator attraction is unknown. The goal of this study was to determine whether pollinators and cucumber beetles were attracted to the same floral volatiles to provide a greater understanding of how mutualistic and antagonistic interactions may affect reproductive fitness and selection on *Cucurbita* floral scent.

## Methods and Materials

**Study System and Site** The study was conducted in a butternut squash field located in the town of Dighton, Bristol County, in southeastern Massachusetts, USA (41°49'06.65" N, 71°07'57.19" W).

*Cucurbita moschata* Duchesne (butternut squash, Cucurbitaceae) is an annual broad-leaved vine crop. Plants are monoecious with separate male and female flowers that last for 1 day, opening before sunrise and wilting by early afternoon (McGregor 1976). The scent of male and female *Cucurbita* blossoms attracts *Acalymma vittatum* Fabricius (striped cucumber beetle, Chrysomelidae) (Andersen and Metcalf 1986; Lewis et al. 1990; Metcalf and Lampman 1991; Metcalf et al. 1995; Granero et al. 2004; Ferrari et al. 2006), a specialist herbivore that is responsible for extensive damage to northeastern cucurbit crops (Hoffmann et al. 1996). Leaves and roots of some *Cucurbita* species also produce volatiles that may attract cucumber beetles and other insects (Peterson et al. 1994; Cosse and Baker 1999). However, we focused on floral volatiles because these are most likely to be attractive to pollinators as well as herbivores.

*Acalymma vittatum* overwinters as adults in diapause that emerge in late spring, feed on cotyledons and new leaves, and then oviposit in early summer. Larvae are specialists on cucurbit roots. The next generation of adults emerges in midsummer and feeds on cucurbit leaves, blossoms, and fruit (Metcalf and Metcalf 1992). Cucumber beetles are often found in cucurbit flowers, where they feed on pollen and floral tissue (Bach 1977; Andersen and Metcalf 1986, 1987; Metcalf and Metcalf 1992).

The squash bee *Peponapis pruinosa* Say (squash bee, Apidae) is one of the most abundant pollinators of squash and pumpkin (Hurd et al. 1974; Shuler et al. 2005). This specialist species collects pollen exclusively from *Cucurbita* blossoms (Hurd et al. 1974). *Apis mellifera* L. (honeybee, Apidae) pollinates cucurbits but does not have a particular affinity (Shuler et al. 2005). Other pollinators that visit *Cucurbita* crops include *Bombus* spp. Latreille (bumblebee, Apidae), various halictid species (*Lasioglossum* spp., *Agapostemon* spp., and Augochlorinae, Halictidae), and *Melissodes bimaculata* Lepeletier (two-spotted miner bee, Apidae) (Shuler et al. 2005).

**Treatments** Three volatiles, 1,2,4-trimethoxybenzene (T hereafter, Sigma-Aldrich, St. Louis, MO, USA, T68802, 97% pure), indole (I hereafter, Sigma-Aldrich, 13408, 99+% pure), and (*E*)-cinnamaldehyde (C hereafter, Sigma-Aldrich, C80687, 99% pure), are components of *Cucurbita* blossoms that, in combination, were attractive to *A. vittatum* in previous studies and have been used to monitor beetle abundance (Andersen and Metcalf 1986; Lewis et al. 1990; Metcalf et al. 1995). Our purpose was to determine whether these volatiles are also attractive to pollinators to assess whether plants might experience a tradeoff in terms of their ability to attract pollinators while deterring herbivores. The presence and absence of the three volatiles was manipulated in a  $2 \times 2 \times 2$  factorial design for a total of eight combinations. For each treatment, volatiles were placed separately onto cotton wicks in 20- $\mu$ l amounts and were applied additively to follow the method of Lewis et al. (1990). Due to its solid state at room temperature, 20 mg of I was dissolved in 20  $\mu$ l of acetone. Acetone was not used for the other volatiles, which were liquids. Therefore, we used two control treatments, 20  $\mu$ l of acetone and a dry cotton wick, for a total of nine treatments.

Emission rates vary widely within and among species, and our treatment emission rate is approximately 100-fold higher than what is emitted from various *Cucurbita* on a daily basis (Andersen and Metcalf 1987; Theis, Adler, and Hazzard, personal observation). These rates may be high, but they were selected to mirror those used previously (Lewis et al. 1990). Further, *A. vittatum* attraction had a tightly positive log-linear response to concentrations of both indole and TIC between 0.3 and 30 mg per trap (Lewis et al. 1990). While the slope of the response was higher for TIC than indole, this suggests that beetle response may change in magnitude, but not direction, due to higher concentrations of volatiles. However, similar dose–response curves have not been conducted for *P. pruinosa*. There was 20  $\mu$ l of volatile

in treatments with a single component (T, I, and C alone), 40  $\mu\text{l}$  of total volatile in treatments with two components (T + I, T + C, I + C), and 60  $\mu\text{l}$  of total volatiles in treatments with all three components (T + I + C). The processing of mixtures of distinct volatiles in the insect brain appears to be close to additive, or even slightly suppressive (Deisig et al. 2006), and so the increase in total volatiles in the combined treatments should not by itself increase attraction.

**Trap Design** Based on preliminary tests of white, silver, and yellow bowls (Solo Cup, Highland Park, IL, USA) in two sizes (540 ml vs. 4.8 l), we selected yellow (stock no. SPB18), to serve as the trap base because it attracted the most insects (data not shown), which is consistent with previous research using other trap types (Hoffmann et al. 1996). Preliminary trapping indicated that bowl size did not affect insect trapping (data not shown), so the 540-ml bowls were used because they were similar in size to a butternut squash flower. Traps were composed of a bowl with a 20-ml scintillation vial attached to the bottom with silicone-based glue (Permatex, clear RTV silicone adhesive sealant, model 66B, Permatex, Hartford, CT, USA). A 2-ml graduated microcentrifuge tube was placed inside the 20-ml vial. A piece of cotton roll (TIDI Brand, nonsterile, #2-medium, TIDI Products, Neenah, WI, USA) was cut to 3 mm  $\times$  1 cm and placed into each centrifuge tube. Traps were filled with 520 ml of a soap–water solution (unscented Dawn<sup>®</sup> soap, Proctor and Gamble, Cincinnati, OH, USA).

**Experimental Design** Twenty-seven traps were placed in the field on July 26, 2005. Traps were arranged in three blocks, each of which was a row that included one trap in each treatment combination. Traps within blocks were approximately 3 m apart, alternating every other plant, and positioned underneath a broad leaf to protect from rain and mimic flower position. Volatiles were replaced everyday between 1800 and 1900 hours, just before sunset, which allowed us to monitor squash bee pollinators that began to forage before sunrise (Hurd et al. 1974). To avoid bias due to edge or positional effects, traps were moved over one position within the block each day.

**Insect Collection and Identification** Insects were collected on two separate dates, July 31 and August 4, so that each collection represented multiple days of cumulative insect trapping. Butternut squash plants began to bloom on July 31, reducing the number of pollinators found in traps in the second collection. Insects were cleaned and dried following a protocol from the USDA Bee Biology and Systematics Laboratory (2002). Pollinators were identified to genus using Michener et al. (1994) and to species using Mitchell (1960, 1962).

**Data Analysis** The number of *P. pruinosa* and *A. vittatum* per trap were used as response variables in separate analyses. Other insects were not caught in sufficient numbers to be analyzed (see “Results” section, “Insect Composition and Abundance” subsection). The two controls (acetone vs. no solvent) were combined into one treatment because there was no difference between them for either insect group ( $F_{1, 11} < 0.1$ ,  $P > 0.75$  for *A. vittatum*; no *P. pruinosa* caught in either control). We then determined the effect of T, I, and C as fixed main effects (presence/absence of each), their interactions, block, and collection date on *P. pruinosa* and *A. vittatum* abundance by using separate ANOVAs. Trap was the unit of replication. The number of *A. vittatum* was  $\log(x+1)$  transformed to meet assumptions of normality; *P. pruinosa* abundance was normal without transformation. Mean position was used as a covariate for the pollinator analysis but dropped from the beetle analysis because it was not significant. All analyses were performed with SAS v. 9.1.

We expect that synergistic effects of individual compounds will be reflected by significant interaction terms in our ANOVA analysis. However, synergism was defined by Metcalf et al. (1995) as a trap catch that is more than the additive components of the mixture, and they reported the synergistic ratios (SR). SR is the ratio of the mean trap catch of the mixture (minus the control) to the total mean trap catches of each component in isolation (minus the control). We have calculated these values as well.

## Results

**Insect Composition and Abundance** The primary pollinator trapped was *P. pruinosa* (75% of all bees). *Apis mellifera* (3.3%), *Bombus* spp. (1.7%), *M. bimaculata* (6.6%), *Halictus rubicundus* Christ (Halictidae, 1.7%), *Halictus ligatus* Say (Halictidae, 5%), *Augochlorella striata* Provancher (1.7%), and *Lasioglossum* spp. (5%) were also collected from the traps. *Acalymma vittatum* (62.7% of all beetles) was the primary beetle trapped, followed by *Cyclocephala borealis* (northern masked chafer, Scarabaeidae, 34%) and click beetles (Elateridae, 3.3%).

**Beetle Attractants** *Acalymma vittatum* was significantly attracted to T and I (Table 1). There were 2.7 times as many *A. vittatum* caught in traps baited with T compared to traps without T, and traps baited with I were 3.3 times more attractive than those without (Fig. 1a). C was not attractive to *A. vittatum*. The effect of the volatiles on *A. vittatum* attraction was additive; there were no significant interactions. However, the combination of TIC trapped twice as many beetles as T + I + C (SR ratio 2.0; Fig. 2a). Other combinations did not appear to be more attractive than the summation of single components (TI vs. T + I: SR ratio 1.2; TC vs. T + C: SR ratio 0.4; IC vs. I + C: SR ratio 0.1; see Fig. 2a for visual comparisons).

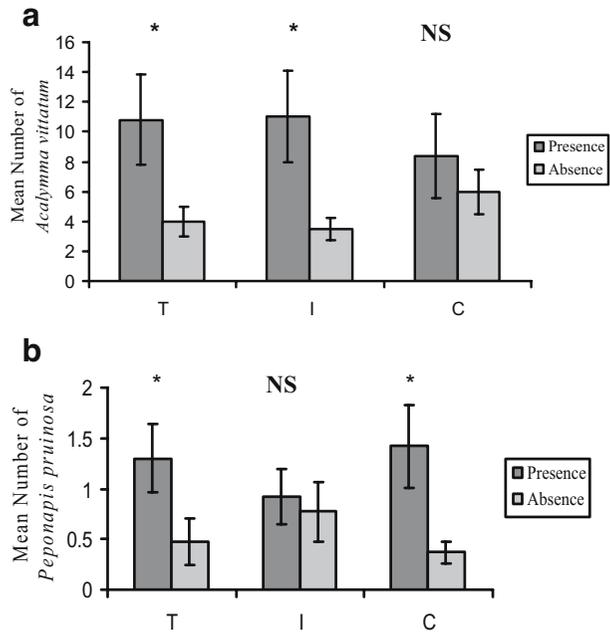
**Pollinator Attractants** *Peponapis pruinosa* was significantly attracted to T and C, but not to I (Table 2, Fig. 1b). Traps baited with T attracted 2.7 times more *P. pruinosa* than those without T, and traps baited with C were 3.8 times more attractive than those without. There were no interactive effects of any volatiles on pollinator attraction, and the SR ratio was between 0.4 and 1.1 for all volatile combinations (Fig. 2b).

**Table 1** Effects of floral volatiles on the number of *A. vittatum* collected in traps

Source	df	SS	F	P <sup>a</sup>
T	1	6.53	15.05	<0.001
I	1	8.79	20.27	<0.001
C	1	0.01	0.02	0.888
T × I	1	1.11	2.56	0.117
T × C	1	0.09	0.21	0.649
I × C	1	0.34	0.78	0.382
T × I × C	1	0.44	1.02	0.319
Block	2	1.32	1.52	0.230
Date	1	10.89	25.12	<0.001
Error	43	18.65	–	–

<sup>a</sup> Significant *P* values are shown in bold.

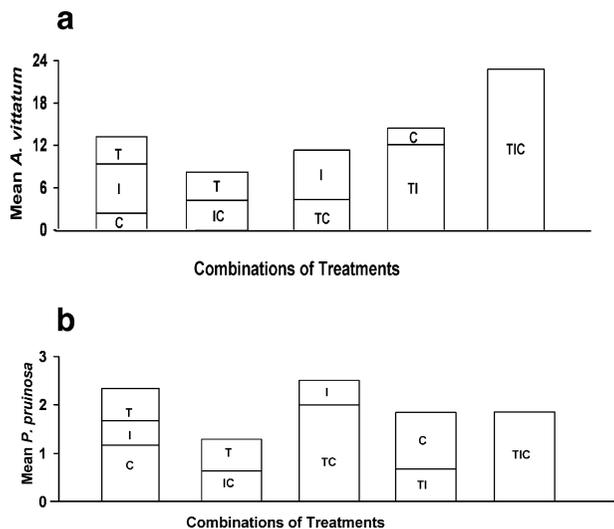
**Fig. 1** Mean number of *A. vittatum* (a) and *P. pruinosa* (b) collected per trap with each volatile treatment. Significant data at  $P < 0.05$  are indicated by asterisks; non-significant data are indicated by NS. Error bars represent standard error



**Discussion**

We found that each of the three volatiles tested had different effects on the attraction of pollinators and herbivores. (*E*)-cinnamaldehyde was attractive to the pollinator *P. pruinosa* but not the herbivore *A. vittatum*, indole was attractive to *A. vittatum* but not *P. pruinosa*, and 1, 2, 4-trimethoxybenzene was attractive to both (Fig. 1). Because *Cucurbita* rely on pollinators for reproduction (McGregor 1976), the attraction of pollinators has fitness consequences. Furthermore, *A. vittatum* and related diabroticite beetles can have

**Fig. 2** Number of *A. vittatum* (a) and *P. pruinosa* (b) caught in traps baited with single volatiles (T, I, or C) as compared to combinations (T + IC, C + TI, I + TC, or TIC together) to illustrate potential synergistic effects



**Table 2** Effects of floral volatiles on the number of *P. pruinosa* collected in traps

Source	df	SS	<i>F</i>	<i>P</i> <sup>a</sup>
T	1	6.87	4.64	<b>0.037</b>
I	1	0.003	0.00	0.960
C	1	12.36	8.35	<b>0.006</b>
T × I	1	0.9	0.61	0.440
T × C	1	0.6	0.41	0.527
I × C	1	0.36	0.24	0.626
T × I × C	1	0.23	0.16	0.696
Block	2	2.94	0.99	0.379
Date	1	17.46	11.79	<b>0.001</b>
Mean position	1	12.3	8.31	<b>0.006</b>
Error	42	16.18	–	–

<sup>a</sup> Significant *P* values are shown in bold.

devastating effects on cucurbit growth, reproduction, and survival (Metcalf and Metcalf 1992; Hoffmann et al. 1996). Thus, the attraction of both pollinators and cucumber beetles is likely to have fitness consequences for native cucurbits and implications for crop yield in managed cucurbit systems.

While a growing number of studies have demonstrated that floral display or reward can be costly due to the attraction of antagonists (Brody and Mitchell 1997; Adler and Bronstein 2004; Ashman et al. 2004b; Strauss and Irwin 2004), the current study is one of few to address the role of scent in attracting pollinators compared to herbivores. Similar to our study, specific floral volatiles present in *C. arvense* scent attracted just pollinators, just herbivores, or both pollinators and herbivores (Theis 2006). In addition to having two different corolla shapes, *P. viscosum* has two distinct scent morphs that attract different types of pollinators depending on the presence of floral antagonists (Galen et al. 1987; Galen and Cuba 2001). In areas with low populations of nectar-robbing ants, *P. viscosum* flowers have a wide corolla and a sweet scent that attracts bumblebee pollinators. In areas with high ant populations, flowers have a narrow corolla that deters ants, at the cost of attracting bumblebee pollinators. These flowers emit a skunky scent that attracts fly pollinators that can fit in the narrow corolla.

We found that *A. vittatum* was attracted to 1,2,4-trimethoxybenzene and indole, but not (*E*)-cinnamaldehyde, and that there were no interactive effects of volatiles on attraction. This is contrary to what was observed by Lewis et al. (1990), who found in a field study in Illinois that *A. vittatum* was attracted to indole and (*E*)-cinnamaldehyde, but not 1,2,4-trimethoxybenzene. Similar to their result, we found the combination of TIC to be more attractive than the sum of the parts, with an SR ratio of 2.0. However, our statistical analysis (factorial rather than one-way ANOVA) suggests that this difference is not significant. There are a number of potential explanations for the disparity between our results and those of Lewis et al. (1990). For example, there are many differences in habitat, including microclimate, soil type, and vegetation, between Massachusetts and Illinois. Experimental outcomes can vary over space and time (e.g., Thompson and Cunningham 2002), and the differences between our results and those of Lewis et al. (1990) suggests that this may be the case for the response of *A. vittatum* to *Cucurbita* floral volatiles.

While this research was conducted in an agricultural setting, our results could be relevant to wild cucurbit systems. Native *Cucurbita* species, such as *C. pepo* subsp. *texana* in the southwestern USA, are pollinated by squash and gourd bees of the genus *Peponapis* (Hurd et al. 1971) and are fed upon by *A. vittatum* and other species of diabroticite beetles (Quesada et al. 1995). Pollinators and herbivores could exert different selective pressures on

floral scent, which may influence the evolution of scent composition. Further research could involve manipulative field experiments with *C. pepo* subsp. *texana* in its native habitat to determine if changes in scent volatiles cause appreciable differences in herbivory, pollination, and plant reproduction. Such research should also address the role of scent concentration, which was relatively high in our study, to determine if lower levels that reflect natural blossom emission have similar effects on attraction of both pollinators and herbivores.

Our findings could have important implications for the cucurbit industry. *Cucurbita* fragrance components were not attractive to all visiting specialist insects. For example, we found that only pollinators are attracted to (*E*)-cinnamaldehyde and only herbivores were attracted to indole. The next step would be to determine whether incremental changes in these volatiles lead to differential attraction of pollinators or herbivores. However, there could be unanticipated consequences of a manipulation in volatile levels if these compounds have important functions in other plant parts (e.g., De Moraes et al. 1998). On the other hand, if variation in scent led to increases in pollination or decreases in damage, plant breeders might benefit from selecting plants that produce more (*E*)-cinnamaldehyde or less indole. Decreasing herbivory without the use of pesticides might have the added benefit of limiting damage to local bee populations (Allen-Wardell et al. 1998).

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