

The effects of floral fragrance manipulations of *Ipomopsis aggregata* on the seed predator *Hylemya*

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I. INTRODUCTION

Since the inception of research on plant-animal interactions, numerous studies have analyzed the effects of floral characteristics on the behavior of different classes of animal associates in order to gauge selection pressures on flowers (Campbell 2009; Harder and Johnson 2009). However, the majority of these projects have focused on visual cues, especially in the context of pollination systems and often in artificial settings. The recent advent of better, more cost-effective technology has allowed an expansion within this field of research to include olfactory cues, and since the first analysis in 1966 by Dodson and Hills, over 1700 unique volatile organic compounds have been isolated from nearly 1000 angiosperm species (Raguso 2008).

Subsequent descriptions of how fragrance functions in plant-animal interactions have shown its importance in the manipulation of behavior (see Schiestl 2010). The ability of volatiles to disperse long distances allows them to signal far in advance of visual cues. Additionally, volatile assemblages that have a unique compound or proportion of compounds, or that are presented in a novel context, are able to elicit a range of behaviors (Raguso 2008). Therefore, the scents may be generalized across many organisms or specific to one or a few associates.

The direction of plant communication may be positive or negative; for example, a compound may attract pollinators or deter herbivores (see Piechulla and Pott 2003). Identifying individual components that drive these actions is difficult, requiring thorough knowledge of floral fragrance chemistry and behavioral assays (Pichersky and Gershenzon 2002). One such example of a successful experiment is the recent elucidation of the interaction between certain orchid species and male solitary bees in which a combination of deceptive appearance and scent mimicry convinces the bees to “copulate” with the blossoms, effectively pollinating them (Pichersky and Gershenzon 2002). Williams and Dodson (1971) further showed that Euglossine bees are attracted to pure compounds from a floral fragrance suite, indicating that a single scent among many may have a substantial influence on behavior.

The floral traits of members of the Polemoniaceae (Phlox) family have been a popular subject for trait manipulation in plant-animal interaction studies (Melendez et al. 1997; Melendez and Campbell 1998; Caruso 2000). However, relatively little is known about the variation in volatile assemblage and emission rates from species of this

family, especially in regards to their effect on insects (but see Galen et al. 2011). Irwin and Dorsett (2002) analyzed the chemical components and proportions of fragrance in the two confamilial species—*Ipomopsis aggregata* (Scarlet Gilia) and *Polemonium foliosissimum* (Towering Jacob's Ladder)—using solid-phase microextraction, gas chromatography, and mass spectrometry. The buds and corollas produce a diverse set of compounds, including monoterpenoids, sesquiterpenoids, and alcohols. The largest proportion of bud volatiles in both species was comprised of the structural isomers α -pinene and β -pinene, both of which also have enantiomeric (hereafter designated as +/-) forms (Table 1). A further analysis of *I. aggregata* employing dynamic headspace sampling and thermal desorption analysis confirmed the dominance of α -pinene for *I. aggregata* (Bischoff, unpublished).

Pinenes belong to the chemical class of terpenes, and the immense diversity and plasticity of this group of compounds is well known for its potential to mediate plant-environment interactions (Tholl 2006). Because they are volatile, they are also important signaling compounds, and their function has undergone major changes as insects have come to recognize them as cues (Theis and Lerdau 2003). In this case, I examined whether or not pinene emission had an effect on seed predation.

Both *I. aggregata* and *P. foliosissimum* serve as host plants of the anthomyiid fly, *Hylemya* sp., which oviposits on the interior of the sepals. Oviposition occurs when the buds of each species have corollas whose length exceeds those of its sepals (Brody et al 2007). The egg then hatches and consumes the full seed load of that flower, so years of high oviposition may result in a dramatic decrease in seed set. While the effects of characteristics such as plant size, population density, and floral morphology on this relationship have been explored in depth (Brody 1992), it is unknown whether *Hylemya* responds to any fragrance compound. However, one study examining enantioselectivity found that *Manduca* oviposition behavior is highly dependent upon enantiomeric composition (Reisenman et al 2010).

The purpose of this experiment was to determine the individualistic effects of all four pinene forms-- alpha-(+)-pinene, alpha-(-)-pinene, beta-(+)-pinene, and beta-(-)-pinene – on the oviposition preferences of *Hylemya*. The questions I asked were:

1. Do any of these forms of pinene affect the oviposition rate of *Hylemya*?

2. If there is an effect, what are its direction and strength?

I hypothesize that at least one of the four compounds will negatively affect oviposition (i.e. serve as a deterrent).

If one of the compounds has a strong negative effect, then plants that produce it in greater abundance than average may have an advantage in seed set. If there is a weak effect or none at all, then this suggests that the chemicals may have a different target or that they are byproducts of other chemical processes.

II. METHODS

i. *Location*

Two sites by Gothic, CO, were chosen for this experiment, each with a large population of *I. aggregata*. The Vera Falls site (lat 38.961836, long -106.992311) was used for the first two experimental sessions (Jul 13-17, Jul 18-22, 2011), whereas the Rustler's Gulch site (lat 38.994755, long -107.015034) was used for the remaining two weeks (Jul 27-31, Aug 2-6, 2011). The experiment was moved from the first to the second site in order to continue working with plants in full bloom. By moving the plants up-valley, I was able to find less phenologically-advanced plants. The seed predator's life cycle is synchronized to that of its host, so this way I was able to maintain a more consistent rate of oviposition for each treatment. Additionally, background rates of oviposition were calculated using twelve plants from the Vera Falls site and nine plants from the Rustler's Gulch site. This was to confirm that the initial buds—those with corollas extending beyond the sepals—chosen for treatment would not have any prior oviposition. The background oviposition rate for the mature flowers was calculated by censusing all of the fully-opened flowers on twelve stalks of different plants and dividing the total number of eggs by the total number of blossoms.

ii. *Experimental Sessions*

I initially tracked bud phenology and determined that the average time between when a bud was suitable for *Hylemya* oviposition and when it began to bloom was 4-5 days. Each experimental session thus consisted of a five-day cycle in which the first four were exclusively for treatment applications and the fifth day was for the scoring

of *Hylemya* eggs. In the laboratory, treatments were prepared by mixing either alpha-(+)-pinene, alpha-(-)-pinene, beta-(-)-pinene (GC-grade standards for pinene identification, Sigma-Aldrich), or beta-(+)-pinene (Santa Cruz Biotechnology), in a 1:10 ratio with purified paraffin oil (Sigma-Aldrich). Each treatment was applied at the plant level, and 1 μ L of each solution (including a control treatment with only paraffin oil) was pipetted onto rubber septa twice a day with an average time interval of 4.5 hours between treatments. The application times ranged from 8:15 AM to 3:00 PM to cover the activity period of the flies.

The first two sessions were conducted at Vera Falls with only the control, alpha-(+), and alpha-(-) pinene. Each treatment consisted of twenty plants, yielding sixty total plants, each with a unique, randomly-assigned number designation. Nine number designations were moved to different plants at the start of the second session due to foraging damage. The second two sessions utilized the same treatments as the first two, but with the addition of beta-(+) and beta-(-)-pinene. Each treatment at this site involved 15 plants. Between the two latter sessions, two number designations were moved due to a lack of buds.

Unscented PTFE rubber septa (Supelco, Sigma-Aldrich) were used as the base for the pinene treatments. These were prepared by cutting a slit in the middle, allowing attachment of the disk-shaped septa to the peduncle of each inflorescence cluster. I used scent-free nylon-6 oven bags (Reynold's) for handling the septa during attachment to each plant. Three septa were placed on each plant, with each septum assigned to a top, middle, and bottom position along the main stalk. These were further arranged so that the non-laminated side of each septum faced the buds, and applications were exclusively performed on this side. For the three septa per plant, I recorded the number of buds for which the corolla length exceeded the sepal length. For weeks 2-4, I additionally recorded the number of advanced buds or flowers and the number of emerging buds so that I could distinguish between buds and flowers present throughout the treatment and those that appeared after the start of the experiment.

In between sessions, the septa were thoroughly cleaned to eliminate the odor compounds. The septa were subjected to two rounds of soaking in GC-grade methanol and then baked in a drying oven for three or more hours. After this procedure, they were handled with forceps that were likewise treated and stored in a methanol-cleaned petri dish in order to avoid contamination by other volatiles until their attachment in the field.

At the end of the treatment period, each plant was scored for the number of *Hylemya* eggs. Scoring was done in the early morning in order to avoid further oviposition by the flies. With forceps, the sepals of each of designated bud were pulled back completely, and the number of eggs found was recorded according to the position of the associated septum. I did not distinguish between eggs on separate buds and multiple eggs on the same bud. In other words, two buds, each with one egg, was considered equal to one bud with two eggs.

iii. *Statistics*

The data were organized at the plant level for calculations, so totals were summed over multiple experimental sessions on the same plant. The overall oviposition rate, defined as the total number of eggs divided by the total number of buds per cluster, was then calculated for each plant, and each plant served as a data point for a given treatment. The data were analyzed in two different groupings. First, I lumped plants from both sites and compared the control, alpha-(+), and alpha-(-)-pinene treatments. The overall ANOVA was supplemented by testing of two a priori contrasts: alpha-(+) versus alpha-(-), and the average of these two treatments compared to the control. In the second grouping, I looked only at the data from the second site, comparing all five treatments. This analysis was supplemented by a comparison of the two beta pinene treatments. All data were arcsine-square root-transformed and analyzed using a one-way block ANOVA in the statistical program, JMP9. Specific contrasts were done in SAS 9.1.

III. RESULTS

i. *Background rates*

At the Vera Falls site, the twelve plants censused had a total of 144 buds and 147 mature flowers that were checked for eggs. The immature buds had no observed eggs. The background rate calculated from the mature flowers was 51.22% with a standard error of 8.595% across the twelve plants. This rate was not significantly different from the control treatment for the first two weeks ($F_{1,80} = 0.14$, $p = 0.709283$). At the second site, 70 buds were censused and also had an oviposition rate of 0%. A total of 135 mature flowers yielded a background

oviposition rate of 39.58% with a standard error of 8.570% across nine plants. I found no significant difference between the average oviposition rate of these plants and the control plants at Site 2 ($F_{1,85}=0.74$, $p = 0.392109$).

ii. *Treatment rates*

a. Grouping 1: Control, alpha-(+)-pinene, alpha-(-)-pinene treatments

Comparing across all sessions, I found that although there was no significant difference among these three treatments, the trend was suggestive ($F_{2,112} = 2.57$, $p = 0.0810$; Fig. 1). However, a specific contrast between the two alpha-pinene treatments did show a significant difference ($F_{1,112} = 4.42$, $p = 0.0377$), though neither treatment was significantly different from the control ($p > 0.05$). Alpha-(-)-pinene consistently had a higher oviposition rate than alpha-(+)-pinene across sites and weeks.

b. Grouping 2: All treatments at Site 2

The oviposition rate did not differ significantly among the five treatments ($F_{4,72} = 1.18$, $p = 0.3269$; Fig. 2). However, when I compared just the beta-pinene treatments, the difference was nearly significant ($F_{1,72} = 3.00$, $p = 0.0873$). The trend for this analysis was that the beta-(+)-pinene had an average oviposition rate about 50% greater than that of beta-(-)-pinene.

IV. DISCUSSION

i. *Treatment effects*

Hylemya showed a strong preference for the alpha-(-)-pinene over the alpha-(+)-pinene, laying about 33% more eggs when alpha-(-) volatiles were present. This result indicates that the flies are able to distinguish between enantiomeric forms. Assuming that the energetic cost is similar between the two forms and that alpha-pinene does not confer benefits through attracting pollinators, the production of alpha-(-) rather than alpha-(+) by *I. aggregata* may be detrimental to seed set. Within beta-pinene, application of the beta-(-) led to a mean oviposition rate of approximately one-third less than beta-(+)-pinene, although this difference was not statistically significant.

The production of floral fragrances is a balance between benefit, detriment, and energy costs. While the relationship between *I. aggregata* and *Hylemya* is negative, *I. aggregata* also interacts with other organisms:

negatively with nectar robbers and herbivores, and positively with pollinators. The floral fragrance that it has evolved thus represents a balance between all of these interactions and the energetic cost of producing each chemical form for individual plants. It may be very expensive for the plant to synthesize some compounds while in other instances, compounds may be produced as a byproduct of necessary metabolic processes. Although this experiment examined each enantiomer individually, the actual picture is much more complicated, likely involving ratios of the enantiomers instead of exclusively one form or another.

The effect of alpha and beta pinene manipulations on the relationship between *Hylemya* and *I. aggregata* may also be relevant to the interactions between *Hylemya* and *Polemonium foliosissimum* since both members of the Polemoniaceae family produce these compounds and are used as hosts by *Hylemya*. Because beta pinene is produced in much higher concentrations in *P. foliosissimum*, one explanation for the apparently lower oviposition in the beta-(-)-pinene treatment is that the flies associate beta pinene with *P. foliosissimum*, and the experimental association here with *I. aggregata* presented a novel context for the scent. The disparity between the fragrance signal and the visual signals by the treated *I. aggregata* may have then deterred *Hylemya* from ovipositing on a treated bud.

There are also potential sources of error to consider from this experiment. The first is that there was accidental and biased contamination of the septa by other volatiles. Since the septum container was open and the septa exposed during septum application, contact with other plants or insects may have compromised the lack of scent of the cleaned septa. Secondly, herbivory damage to the plant may have caused the production of defense compounds that could have affected oviposition. During this experiment, if the top of the inflorescence was eaten during a treatment session but the original septa positions remained, the herbivory was noted, but the septa were not moved to another plant until the beginning of the next session.

ii. Future research

Much information still needs to be collected about this system to understand the relationship between *I. aggregata* and *Hylemya*. Using dynamic headspace sampling, I collected samples for treated septa after 15, 75, 35,

185, and 245 minutes. However, the results from a gas chromatography and mass spectrometry analysis are not yet available, so the degradation of the scent augmentation treatments is unknown. Additionally, laboratory work on the exact enantiomeric composition and fieldwork to determine natural variation in production of pinenes will tell us which of the pinene treatments applied here was most similar to that normally produced by *I. aggregata*.

Multiple-trait manipulations with scent and *Hylemya* for characteristics such as floral tube width and color could reveal more about the differences in *Hylemya* attractiveness between *P. foliosissimum* and *I. aggregata* and may reveal correlational selection for traits specific to one of these two hosts of *Hylemya* (Campbell 2009), meaning that two or more traits have non-additive effects on fitness and may influence speciation. Much work has also been done on the responses of animals to the physical characteristics, such as corolla size, corolla length, inflorescence height, of *I. aggregata* (Mitchell 1994, Brody 1992). It will thus be interesting to perform single-trait manipulations with scent to examine the behavior of *I. aggregata*'s other animal associates. Determining how other species respond to single compounds will help to explain the tradeoffs between attraction and deterrence faced by *I. aggregata* and the composition of its floral fragrance.

In general, the results of this experiment suggested several trends. However, the small sample size likely limited their significance. Further work to expand the oviposition dataset is recommended. In addition, it may be worthwhile to perform behavioral observations to see if the flies may be initially attracted to the scent but then deterred by other characteristics.

Table 1: Fragrance profiles

Species	% α -pinene	% β -pinene
<i>I. aggregata</i>	93.17 $\pm$ 1.30	5.01 $\pm$ 0.94
<i>P. foliosissimum</i>	25.91 $\pm$ 0.79	60.50 $\pm$ 1.08

Figure 1: Comparison of the control, alpha-(+), and alpha-(-)-pinene treatments across both sites. Means and 95% confidence intervals are plotted after backtransforming the data.

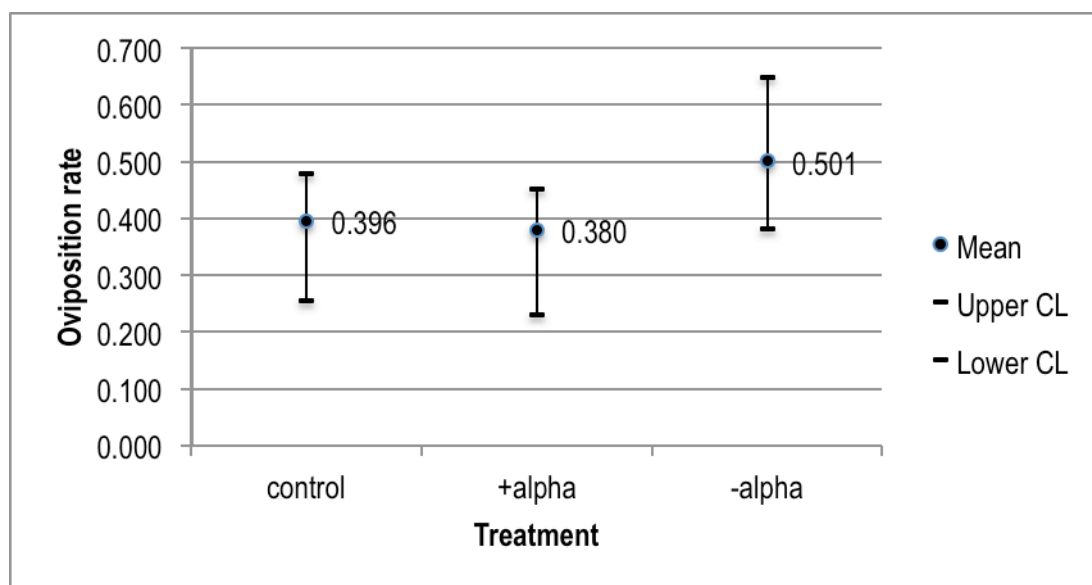
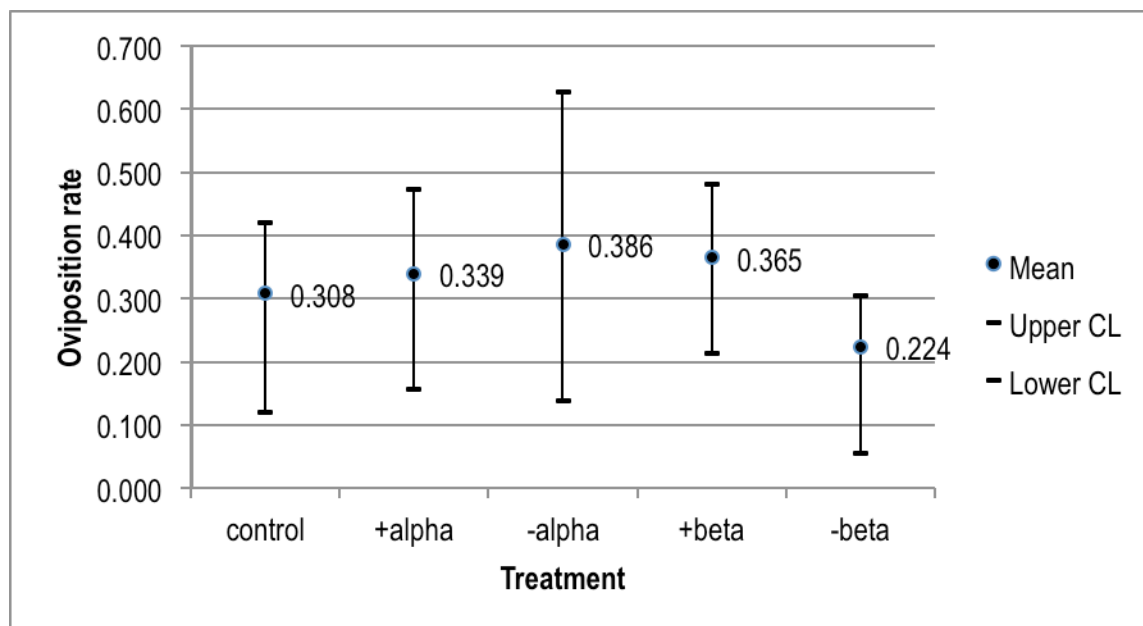


Figure 2: Comparison of all treatments within Site 2. Conventions as in Fig. 1.



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