Variation in volatile emission in *Mimulus guttatus* and implications for plant-insect interactions

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Abstract

The volatile compounds emitted from plants function as key host-location cues for their insect visitors. Floral volatiles guide pollinators to nectar and pollen, and in turn, pollinators initiate plant reproduction, potentially promoting a mutualistic interaction. Constitutive and herbivore-induced foliar volatiles function as an important plant defense by deterring herbivores and attracting natural enemies of herbivores. Given the importance of plant volatiles in insect behavior, variation in volatile phenotypes is expected to affect insect visitation patterns, and consequently, plant reproductive success and susceptibility to herbivores. Inbreeding is an important source of genetic variation that it is extremely common in natural populations of flowering plants. Since inbreeding increases homozygosity, inbreeding could increase expression of recessive alleles that disrupt biosynthesis of volatile compounds. In previous work, inbreeding in the mixed-mating herb *Mimulus guttatus* (yellow monkeyflower) decreased pollinator attraction and resistance to herbivory. However, the plant traits underlying these effects are unknown. This dissertation examined the effects of inbreeding on the floral and foliar volatile phenotypes of *M. guttatus* and the consequences of these effects for its pollinators and herbivores.

The first chapter of this dissertation investigates floral volatile profiles from experimentally inbred and outbred *M. guttatus*. Since olfactory cues are known to signal the quality of floral rewards, and since inbreeding in *M. guttatus* is known to reduce the quantity and quality of pollen (its only floral reward), it also investigates whether the floral volatile profile is associated with pollen rewards. Results demonstrated that inbreeding reduced emission of a sesquiterpene, β-trans-bergamotene. Six monoterpenes were positively associated with pollen rewards in *M. guttatus*, but there was no overlap between compounds exhibiting inbreeding effects and compounds associated with pollen rewards. The second chapter of this dissertation examines the foraging behavior of bumblebees (*Bombus impatiens*) in response to β-trans-bergamotene and compounds correlated with pollen rewards. Behavioral assays demonstrated that bumblebees are innately attracted to β-bergamotene. Moreover, after foraging on *M. guttatus*, bumblebees learned to prefer floral odors correlated with pollen rewards. However, bumblebees were only able to base foraging decisions on associative learning when presented floral scents that did not contain β-bergamotene, suggesting that its innate attractive properties could interfere with learned olfactory signals.

The third chapter of this dissertation investigates the effects of inbreeding and herbivory on foliar volatile emission in *M. guttatus*. Results demonstrated that herbivory significantly increased foliar volatile emission. In particular, herbivory increased emission of 1-octen-3-ol and neophytadiene, compounds that are known to function as indirect and direct defenses, respectively. This study also found an interaction between inbreeding and herbivory in the overall volatile profile, and that inbreeding reduced constitutive emission of individual components of the volatile blend. The fourth chapter of this dissertation examines performance and preferences of a generalist herbivore, *Helicoverpa zea*, and a specialist, *Junonia coenia*, in response to inbreeding and trichome density in *M. guttatus*. Results demonstrated that *H. zea* developed most efficiently on inbred plants with low trichome density, but did not exhibit a preference for inbred versus outbred plants. *Junonia coenia* performed best on outbred plants with low trichome density, and consistently preferred outbred over inbred plants, even when presented only olfactory cues. Both species consumed more leaf tissue in outbred plants, suggesting that inbred plants could have an advantage in defending against herbivores.

This dissertation is one of only a few studies to examine effects of inbreeding on plant volatile profiles, and it is the first study to demonstrate that innate olfactory preferences of bumblebees can override learned preferences for floral scents. It is also among the first to differentiate the responses of specialist and generalist herbivores to inbreeding effects in their host. The findings in this dissertation enhance our understanding of the ecology and evolution of plant-insect interactions, which can have important implications for the evolution of plant mating systems and the vulnerability of small populations.

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Chapter 1:

Inbreeding and variation in pollen rewards alter floral scent in *Mimulus guttatus*

Abstract

Inbreeding in plants is known to affect traits that mediate interactions between plants and insects. Previous studies of the mixed-mating herb, *Mimulus guttatus*, have demonstrated that pollinators prefer to visit outbred over inbred plants. Although inbreeding in *M. guttatus* reduces pollen viability and quantity, and therefore, reward quality for pollinators, this preference was independent of differences in pollen rewards. Floral scent is well-known in other species to mediate pollinator behavior. However, the floral volatile phenotype of *M. guttatus* has not been investigated. Here we examined effects of inbreeding in *M. guttatus* on floral scent and associations between floral scent components and pollen rewards. The strongest inbreeding effect was reduced emission of β-trans-bergamotene, the largest component of the overall volatile blend. The compounds α-pinene, β-pinene, myrcene, limonene, E-β-ocimene, and limonene oxide were positively correlated with pollen rewards, indicating the potential for these compounds to provide honest signals of rewards to pollinators. Our finding that inbreeding affects emission of a compound that is not directly associated with pollen rewards is consistent with previous work in which pollinators preferred outbred plants regardless of their pollen rewards. These results suggest that floral volatile signals from *M. guttatus* to its pollinators are highly complex, and they provide insight into the adaptive functions of different floral volatile components for plants as well as their pollinators.

Introduction

Inbreeding is extremely common in natural populations of flowering plants (Husband and Schemske 1996, Vogler and Kalisz 2001) and can greatly impact the distribution of genetic and phenotypic variation in a population. Since inbreeding increases homozygosity, exposing recessive alleles and decreasing the influence of overdominance, inbreeding reduces the fitness of most species (Falconer and Mackay 1996, Husband and Schemske 1996). Inbreeding may thus be expected to disrupt plant traits that mediate interactions with antagonists and mutualists. A number of studies have documented inbreeding effects on plant-herbivore interactions (Carr and Eubanks 2014). These studies have demonstrated that inbreeding affects plant defenses as well as host plant quality, thereby influencing susceptibility to herbivore damage. Only a few studies have examined inbreeding effects on plant-pollinator interactions (Ivey and Carr 2005, Carr et al. 2014, Carr et al. 2015), and the plant traits mediating these effects have been studied less extensively.

The current study is motived by seemingly paradoxical results from previous work that examined pollinator behavior in response to inbreeding and reward quality in the mixed mating, bumblebee-pollinated herb *Mimulus guttatus*. Inbreeding in *M. guttatus* consistently reduces pollen quantity and viability (Carr and Dudash 1995, 1997), and therefore pollen protein content (Yeamans et al. 2014). Since pollen is the only reward for pollinators in *M. guttatus* (Robertson et al. 1999), pollinators would be expected to preferentially visit outbred plants to optimize nutritional rewards. As expected, pollinators demonstrated preferences for outbred over inbred plants (Ivey and Carr 2005, Carr et al. 2014, Carr et al 2015). Furthermore, in pairwise choice tests in which artificial flowers were provisioned with either fertile or sterile anthers from *M. guttatus*, captive *Bombus impatiens* preferred fertile anthers. *Bombus impatiens* also preferred fertile over sterile anthers in an olfactometer test in which visual cues from anthers and pollen were absent (Carr et al. 2015). All of these results were obtained using bumblebees that had previous experience foraging on *M. guttatus*, and provided evidence that experienced bumblebees could make foraging decisions based on reward quality.

Counter to expectations, however, when foraging on live, intact flowers, experienced *B. impatiens* did not discriminate among outbred subpopulations of *M. guttatus* that differed significantly in reward quality (Wise et al. 2011, Carr et al. 2015). Moreover, experienced *B. impatiens* preferred outbred over inbred plants even when the pollen viability of outbred plants was as low on average as that of inbred plants (Carr et al. 2015), indicating that bumblebee preference for outbred plants was not directly driven by reward quality. Preferences for outbred plants were also independent of important visual cues, including corolla width and the number of open flowers (Ivey and Carr 2005, Carr et al. 2014, Carr et al. 2015).

Naïve *B. impatiens* (bees that had never foraged on *M. guttatus*) demonstrated a preference for outbred over inbred *M. guttatus* when given only olfactory cues from flowers (Carr et al. 2015), suggesting that a difference in floral scent could mediate the preference for outbred plants. Volatile compounds emitted from flowers are thought to be among the most important traits for attracting insect pollinators (Kessler et al. 2008, Schiestl 2010, Klahre et al. 2011). Floral volatiles function as long-distance attractants, as well as landing and feeding cues (Raguso 2008). Floral scent is thought to be important for foraging-naïve insects (Dotterl and Vereecken 2010), but insects also readily learn olfactory cues and modify preferences in favor of olfactory signals that are associated with floral rewards (Junker and Parachnowitsch 2015). For example, odor cues emanating directly from pollen or nectar are likely to provide reliable cues regarding reward quantity and quality and have been found to play an important role in pollinator attraction (Dobson et al. 1999, Ashman et al. 2005, Howell and Alcaron 2007). Floral volatiles that are not emitted directly from nectar or pollen may also serve as reliable cues if they exhibit patterns of emission that are consistently linked to the quality and quantity of nectar or pollen rewards. In particular, the volatile compound phenylacetaldehyde was found to be correlated with nectar sugar and pollen amount in *Brassica rapa* despite not being emitted from nectar or pollen, and bumblebees developed a preference for phenylacetaldehyde after foraging on *B. rapa* (Knauer and Schiestl 2015).

Given the importance of floral scent in the learning and discrimination of flowers by insect pollinators, variation in floral volatile phenotypes is expected to affect pollinator visitation patterns, and consequently, plant reproductive success. For example, pollinator visitation to *Hesperis matronalis* inflorescences was higher when inflorescences were augmented with floral scent extract, and higher scent emission led to higher seed production (Majetic et al. 2009). In a common garden study of bee-pollinated *Penstemon digitalis* (Parachnowitsch et al. 2012), phenotypic selection on floral scent was stronger than selection on flower size or color, and the volatile compounds under selection were common components of floral scent that have previously been found to attract bees.

Previous work in other plant species has demonstrated that inbreeding can alter volatile phenotypes. For example, inbreeding in *Cucurbita pepo* reduced total volatile emission and changed the relative composition of individual compounds (Ferrari et al. 2006). Inbreeding also altered foliar volatile emission in *Solanum carolinense,* with consequences for interactions between *S. carolinense* and its herbivores and even their natural enemies (Kariyat et al. 2012, 2013, 2014). Inbreeding appears to alter the floral volatile profile of *M. guttatus* in a way that affects pollinator foraging behavior (Carr et al. 2015), but to our knowledge, there are no published studies on volatile emission in *M. guttatus*.

In this study, we investigated variation in the floral volatile phenotype of *M. guttatus* using experimentally inbred and outbred plants. Specifically, we asked: 1) How does inbreeding alter floral volatile emission in *M. guttatus*? 2) Are compounds that show inbreeding effects associated with reward quality? This study builds on the previous work that demonstrated that pollinators discriminate against inbred *M. guttatus*, providing insight into patterns in floral scent variation that could potentially mediate pollinator behavior.

Materials and Methods

Study system: Mimulus guttatus

Mimulus guttatus (Phrymaceae, Beardsley and Olmstead 2002) is an herb native to open, wet habitats throughout western North America from Mexico to Alaska. It produces hermaphroditic, yellow, zygomorphic flowers paired at nodes along a raceme and measuring between 10 and 30 mm wide. Populations are usually annual, but can be perennial in areas that are wet year-round (Dole 1992). *M. guttatus* populations exhibit mixed mating systems with outcrossing rates that range from approximately 75% selfing ($t = 0.25$) to complete outcrossing ($t = 1.0$), averaging $t \approx 0.60$ (Ritland and Ritland 1989). Bumblebees (*Bombus* spp.) are its most important pollinator (Kiang 1972, Macnair et al. 1989, Vickery 1999), but *M. guttatus* also receives visits from other bees, syrphid flies, lepidopterans, and coleopterans (Macnair et al. 1989, Ivey and Carr 2005). Pollen is the primary, if not exclusive, floral reward for pollinators, as the flowers produce little or no nectar (Robertson et al. 1999).

Plants used in this study were descendants of seeds collected from over 500 random maternal families from an annual population in Napa County, CA (Snell Valley: 38° 42’ 02” N, 122° 24’ 29” W). One plant from each field-collected maternal family was randomly outcrossed in a greenhouse at Blandy Experimental Farm (Boyce, VA), thus providing a base generation of completely outcrossed plants. The plants used in the current study were derived from this base population and included two subpopulations, each comprising a different set of maternal families. One subpopulation was outbred with no biparental inbreeding, and the other was inbred. The inbred plants used in 2012 were self-pollinated for three generations (F= 0.875). Those used in 2013 were selfed for an additional generation (F=0.9375).

Approximately 20 seeds from each maternal family were sown into a 3-inch square pot filled with commercial growing medium (Fafard 3 Mix, Conrad Fafard, Agawam, MA). Pots were randomly arranged in trays (20 pots per tray) with bottom watering. After the first true leaves appeared (approximately 14 days), seedlings were transplanted into individual pots and were randomly arranged with 20 pots per tray and bottom watering. Plants were kept in a growth chamber (16:8 photoperiod, 22°C, 60-65% relative humidity) at the Pennsylvania State University.

Floral volatile collection and analysis

We collected volatiles in July 2012 and May 2013 from a total of 26 inbred plants from 19 maternal families (1-2 plants per maternal family) and 44 outbred plants each from a different maternal family. Immediately prior to each volatile collection period, we recorded the number of open flowers on each plant and the corolla width of each flower to account for effects of size on volatile emission.

Floral volatiles were collected from each plant from 10:00 to 18:00 in a growth chamber (26^{o}C, 60-65% relative humidity) under sodium and metal halide lights. The top flowering nodes of the raceme were enclosed in a 4-L glass chamber. We collected volatiles using a push-pull collection system (Analytical Research Systems, Gainesville, FL), which delivered filtered air through Teflon tubing into the chambers (2.5 L/min) and pulled air from the headspace using a vacuum (1.0 L/min) through a filter containing 40 mg of SuperQ (Alltech Associates). Within 24 hours of volatile collection, we eluted the trapped compounds with 150 µL dichloromethane and added 5 µL of a mix (dissolved in dichloromethane) containing the internal standards n-octane (40 ng/µL) and nonyl acetate (80 ng/µL). Samples were then injected in 1-µL aliquots using a splitless injector into an HP-1 column (250 µm internal diameter, 0.1 µm film thickness, 15 m length) in an Agilent 6890 gas chromatograph fitted with a flame ionization detector. The column was held at 0.5^{o}C for 0.5 minutes and then increased at 8^{o}C/min to 240^{o}C; helium was the carrier gas. Compounds were quantified (in ng) using MSD ChemStation software (Agilent Technologies) based on peak area relative to that of the internal standards. To identify compounds, samples were analyzed using an HP-1 column (0.25 mm internal diameter, 0.25 µm film thickness, 30 m length) in an Agilent 6890 gas chromatograph coupled with a mass spectrometer (electron ionization mode). Compound peaks were identified through comparison of mass spectra to the NIST 2008 and 2014 mass spectral libraries (National Institute of Standards and Technology) and identities were confirmed by comparison with mass spectra and retention times of commercial standards when possible. Beta-trans bergamotene was isolated from floral volatiles, elucidated by NMR, and confirmed by comparison with a biosynthetic standard.

Isolation and confirmation of β-trans-bergamotene

Raw material for isolation of β-trans-bergamotene was collected using the same push pull system as described for floral volatile collection; however, we collected volatiles from cut *M. guttatus* flowers in a water bath. We eluted the collected volatiles with dichloromethane and confirmed the presence of the peak of interest by GC-MS. We collected volatiles from ~18,000 flowers (~150 flowers per bath, 12 baths, 10 days). The entire sample was pooled and concentrated under a stream of N_2. The resulting solution was purified by preparatory gas chromatography using an Agilent 6890 GC coupled to a Gerstel Maestro preparative fraction collector (PFC). The separation was performed using an Agilent VF-1ms (30m × 53µm × 0.5µm film thickness) column in an oven gradient from 40°C to 250°C at 8°C/min and eluting peaks collected in the Gerstel PFC was cryo-cooled. This generated a pure peak of the unknown material that was analyzed by NMR in $CDCl_3$. Structure elucidation of the molecule form the NMR data suggested the identity as β-bergamotene. For further confirmation, β-trans-bergamotene was biosynthesized as a standard.

The standard of β-trans-bergamotene was biosynthesized as reported by Lin et al. (2013). The plasmid fma-TC was transformed into *Sacchromyces cerevisiae* BJ5464 (LGC Wesel, Germany) via electroporation. Transformed yeast was selected using Yeast Synthetic Drop-out media without uracil (Sigma) and four single colonies were further grown for compound production. A starter culture of 4 ml of Yeast Synthetic Drop-out media was grown for 72 hours at 28°C and shaking at 150rpm, then 1ml transferred to 1L yeast peptone dextrose media supplemented with 1% dextrose in a 2.8L fernbach flask. The 1L culture was grown at 28°C and shaking at 150rpm for 96h. After growth the cells were harvested via centrifugation (10 min at 3000rpm). The resulting cell pellet was extracted into acetone and the concentrated residue suspended in acetonitrile and partitioned with hexane. The hexane soluble fraction was concentrated en vacuao and further separated on a Discovery SPE DSC-Si silica column 100mg (Supelco) The sesquiterpene eluted in 100% hexane was measured by GC-MS and compared to the unknown sesquiterpene from *M. guttatus* volatile.

Association between floral volatiles and pollen rewards

We quantified reward quality (pollen viability and pollen quantity) for each plant from which we collected volatiles. Pollen viability and pollen quantity are known to be consistent within a plant in *M. guttatus* (Robertson et al. 1999). To determine pollen viability, the anthers from a single open flower from each plant were placed in lactophenol-aniline blue. Pollen viability was quantified as the proportion of pollen grains staining dark blue (Kearns and Inouye 1993). To determine pollen quantity, anthers from two buds from each plant were collected and stored separately in ethanol until they dehisced. We quantified the number of pollen grains in a single bud using an HIAC Royco particle counter and averaged replicates to obtain the pollen count per flower. We used the product of pollen quantity and pollen viability (number of viable pollen grains per flower) as the measure of reward quality in analyses.

Volatiles from pollen and anthers

To determine whether anthers or pollen could be the source of any floral volatile compounds, we collected volatile samples from isolated fertile anthers (i.e., anthers plus the pollen they contained), and sterile anthers (those that did not contain any pollen). Each sample included anthers from a median of 24 flowers from a single maternal family. We sampled from 6 outbred maternal families with sterile anthers, 6 outbred families with fertile anthers, and 2 inbred families with fertile anthers (we did not have inbred plants that were fully sterile). Anthers were removed from flowers and collected onto aluminum foil. The freshly collected anthers were enclosed in 750-ml glass chambers and volatiles were collected for 8 hours using a push-pull collection system with 1.0 L/min airflow into the chamber and 0.8 L/min vacuum. We eluted and analyzed anther volatile samples as described above (see *floral volatile collection and analysis*).

Data analysis

Floral volatile characterization. To determine which floral scent traits distinguished inbred from outbred *M. guttatus*, we tested for a difference in total volatile emission and for differences in individual compounds. To test for a difference in total volatile emission, we conducted a general linear mixed model using proc mixed in SAS 9.4 (SAS Institute Inc, 2013). Total volatile emission was square-root transformed to meet assumptions of homogeneous variances and normality. Pollination treatment (inbred or outbred) was used as the main fixed effect and flower number and corolla width were included as covariates. Year collected (2012 or 2013) and maternal family nested within pollination treatment were included as random effects.

We used two approaches to evaluate differences in emission of individual compounds. First, we evaluated the relative importance of each compound in determining differences in floral scent composition using the Random Forests classification algorithm (Ranganathan and Borges 2010). Random Forests is well suited for classifying multivariate data sets, and it has been successfully used to classify samples based on volatile composition (e.g., Junker et al. 2011, Parachnowitsch et al. 2012). Random Forests builds decision trees by bootstrapping from the set of samples and selects a minimum set of variables necessary to distinguish one group from another. It also provides a ranking of the relative importance of each variable in distinguishing between groups. In order to find the smallest set of compounds with which inbred plants could be distinguished from outbred, we ran 200 bootstrap iterations (Ranganathan and Borges 2010) using the package *VarSelRF* in R v. 3.2.0 (R Development Core Team, 2012). The minimum set of compounds needed to predict group membership (inbred or outbred) was based on the out-of-bag error (Ranganathan and Borges 2010) and included the smallest number of compounds whose error rate was within 1 standard error of the minimum error rate of all forests. The importance score for each compound was calculated as the decrease in classification accuracy when that compound was randomly permuted using the *'importance'* function in the *randomForest* package in R.

We also conducted general linear mixed models for all individual compounds using proc mixed in SAS. For each model, pollination treatment was the main fixed effect, flower number and corolla width were covariates, and year and maternal family nested within pollination treatment were random effects. We used the Bonferroni correction to adjust our alpha level to 0.003 to correct for conducting 17 tests. Compound emission was transformed as needed to meet ANOVA assumptions.

Association between volatile compounds and pollen rewards. To evaluate the relationship between each compound and reward quality, we conducted multiple regression analyses with the emission rate of each compound as the dependent variable and reward quality, flower number, and corolla width as independent variables. We selected the best model for each compound based on AIC with an all subset approach from 1000 bootstrap samples using proc glmselect in SAS. We considered a compound to be correlated with pollen rewards if reward quality was included in its best model.

Results

Floral volatile characterization

We detected 17 volatile compounds from *M. guttatus* flowers (Table 1). On average the volatile blend comprised mostly β-trans-bergamotene (approximately 25% of the total blend by mass) and unknown1 (approximately 24% by mass). Total volatile emission was about 19% greater from outbred than inbred plants. When we did not control for flower size, this difference was significant ($F_{1,61}$=8.06, P=0.0061), but when we controlled for corolla width by including it as a covariate, this difference was not significant ($F_{1,61}$=1.92, P=0.1712), indicating that higher volatile emission from outbred plants was largely explained by flower size.

Random Forests classified inbred and outbred plants with 76% accuracy and identified β-trans-bergamotene and α-bergamotene as the minimum set of compounds necessary to predict whether plants belonged to inbred or outbred treatments. β-trans-bergamotene, α-bergamotene, and 1-butanol, 3-methyl-benzoate had the highest ranked importance values, respectively. All of these compounds showed higher emission from outbred than inbred plants (Table 1).

General linear models supported the conclusions from the Random Forest analysis. The compounds with the three highest importance values in Random Forests also showed the strongest treatment effects in general linear models (Table 1). Emission of β-trans-bergamotene was 2.8 times higher from outbred than inbred plants. Five inbred families failed to produce β-trans-bergamotene at all. α-bergamotene, which accounted for approximately 1.2% of the total volatile profile by mass, had an emission rate that was 1.7 times higher in outbred than inbred plants. 1-butanol, 3-methyl-benzoate, which accounted for approximately 5% of the total volatile profile, exhibited an emission rate that was 2.3 times higher in outbred than inbred plants. After adjusting α for conducting 17 tests, these effects were significant for β-trans-bergamotene and 1-

butanol, 3-methyl-benzoate. No compounds exhibited significantly higher emission from inbred than outbred plants. In contrast with the Random Forests analysis, the general linear models controlled for flower size and number of flowers. Therefore, the general linear models demonstrate that the differences in emission are likely due to inbreeding effects on the synthesis or release of these compounds, and not simply due to reduced flower size or number of flowers in inbred plants.

Association between volatile compounds and pollen rewards

Plants in this study produced a median of 8,360 pollen grains per flower, ranging from 0 (male-sterile) to 25,760 pollen grains per flower. Of the flowers that produced pollen, the median viability was 88%, and these plants ranged from 10% to 99% viable pollen. None of the compounds that showed inbreeding effects included reward quality in its best supported model. However, reward quality was included in the best supported models with a positive slope for α-pinene, β-pinene, myrcene, limonene, E-β-ocimene, and limonene oxide (Table 2). In addition, linaloloxide and linalool included reward quality with a negative slope in their best supported models. These patterns persisted even when inbred plants were not included in the analyses, indicating that these correlations (and the lack thereof for other compounds) were not an artifact of inbreeding.

Volatiles from pollen and anthers

None of the volatile compounds detected in the floral volatile profile was present in anther volatile samples. Volatile samples from fertile anthers contained one compound, nonadecane (Figure 1), which was not found in the floral volatile headspace. No volatile compounds were detected in sterile anthers, indicating that nonadecane is emitted from pollen. This volatile profile was consistent for fertile and sterile anthers across all maternal families.

Discussion

We found that inbreeding altered the volatile blend of *M. guttatus* flowers. In particular, inbreeding significantly reduced emission of β-trans-bergamotene, the largest component of the volatile profile. This difference may explain the pollinator discrimination against inbred *M. guttatus* (Carr et al. 2015). Such discrimination could result in greater pollen limitation or higher rates of selfing in inbred plants, further reducing their fitness. To our knowledge, only one other study has examined inbreeding effects on floral volatile emission (Ferrari et al. 2006). In this study, inbreeding in *Cucurbita pepo* reduced total floral volatile emission and altered the relative composition of volatile compounds. This study did not examine the role of these differences in insect behavior, but the individual compounds affected by inbreeding have been found in other studies to be insect attractants. Inbreeding in *Solanum carolinense* was shown to alter foliar volatile emission in ways that compromised resistance to herbivory (Kariyat et al. 2012, 2013, 2014). For example, inbred plants exhibited higher constitutive volatile emissions than outbred plants, which made plants more apparent and attracted more herbivores. However, inbred plants exhibited weaker induction of volatiles in response to herbivory, and consequently, inbred plants attracted fewer herbivore natural enemies. Thus, previous work also suggests that inbreeding effects on plant volatile signals are generally deleterious for plants.

Our results demonstrated that inbreeding in *M. guttatus* reduced β-trans-bergamotene emission. If β-trans-bergamotene is found to be an attractant, this is a potential mechanism that could explain the previous discrimination by bumblebees against inbred plants (Carr et al. 2015). The functional role of β-trans-bergamotene in plant-pollinator interactions has not been examined. However, β-trans-bergamotene was identified as the major component of the male pheromone of the parasitic wasp *Melittobia digitata,* and this compound alone was as attractive as the whole male pheromone to female *M. digitata* (Consoli et al. 2002). Floral scents are often chemically similar to compounds produced by insects for their own communication (Schiestl 2010). This similarity may be due to selection imposed by innate biases in pollinator sensory systems and behaviors that lead to more reliable visitation to flowers. It is interesting to note that in previous work, the preference for olfactory cues from outbred *M. guttatus* was exhibited by bumblebees that had not previously foraged on *M. guttatus* (Carr et al. 2015), and so the preference for outbred plants cannot be explained by learning. The pheromone components of *B. impatiens* have not been documented, but it is possible that a pre-existing bias for β-trans-bergamotene or chemically similar compounds could mediate the preference for outbred *M. guttatus*.

Pollinators are known to develop preferences through associative learning for signals that are reliably correlated with rewards. Since plants benefit from multiple pollinator visits to conspecific individuals, pollinators are likely to select for scents that honestly signal the quantity and quality of food rewards (Wright and Schiestl 2009). We found that α-pinene, β-pinene, myrcene, limonene, E-β-ocimene, and limonene oxide are positively associated with the number of viable pollen grains per flower in *M. guttatus*, indicating the potential for these compounds to provide honest signals of rewards to pollinators. Three of these compounds—myrcene, limonene, and E-β-ocimene—were the most abundant compounds in the floral scent of the bumblebee-pollinated *Mimulus lewisii*, but almost nonexistent in its sister species, the hummingbird-pollinated *M. cardinalis*. Furthermore, these compounds mediated bumblebee preferences for *M. lewisii* over *M. cardinalis* (Byers et al. 2014a, b). Bees and hummingbirds likely impose different selection pressures on honest signals. In particular, hummingbirds are thought to have little sense of smell (Dobson et al. 2006) and forage exclusively for nectar in flowers (Thomson et al. 2000).

Thus, we should expect olfactory cues correlated with pollen rewards to be maintained in bumblebee-pollinated, but not hummingbird-pollinated, species. We do not know the roles of these compounds in interactions between bumblebees and *M. guttatus*. However, these patterns suggest that bumblebees might select for these compounds as honest signals of pollen rewards.

The most direct honest signals are those emitted by the reward itself (Wright and Schiestl 2009). Although studies on honest signals have largely focused on nectar as a floral reward (e.g., Raguso 2004, Wright and Schiestl 2009), anther and pollen scents often differ qualitatively from scents of other flower parts, and have been found to mediate pollinator behavior. For example, pollen volatiles in *Rosa rugosa* elicited landing and collecting in bumblebees (Dobson et al. 1999), and pollinator preference for hermaphrodite over female *Fragaria virginiana* flowers was explained primarily by volatiles in hermaphrodite anthers (Ashman et al. 2005). In previous work, bumblebees were able to discriminate fertile from sterile *M. guttatus* anthers when presented olfactory cues from anthers isolated from the rest of the flower (Carr et al. 2015), and our volatile collections from isolated *M. guttatus* anthers indicated that nonadecane was present in fertile, but not sterile, anthers. We did not detect nonadecane in the floral headspace, but nonadecane is relatively nonvolatile (melting point 32^{o}C), and it is possible that pollen needed to be fully exposed, rather than inside of the corolla, in order for nonadecane to be detectable in volatile collections. Insects have excellent olfactory acuity (Chapman 1998), however, and bumblebees are likely to be more sensitive than our sampling methods to chemical compounds. On the other hand, olfactory cues that are reliably correlated with rewards can provide honest signals even if they are not emitted from rewards themselves. For example, the volatile compound phenylacetaldehyde was correlated with nectar sugar and pollen amount in *Brassica rapa,* but not emitted from its pollen or nectar, and bumblebees developed a preference for phenylacetaldehyde over other volatile compounds after foraging on *B. rapa* (Knauer and Schiestl 2015). It is also possible that the compounds in *M. guttatus* that are correlated with, but not emitted from, its pollen rewards, function as honest signals to its pollinators.

The reliability of signals that are correlated with, but not emitted from, pollen could decline as pollen is depleted, if the signals do not change after visitation. Removal of pollen does not remove the source of these compounds, and this could weaken the associative learning of these compounds as pollen is removed. A previous study using *M. guttatus* found that when pollinator service was limited (low bee density), the number of viable pollen grains was a good predictor of bee visitation (Robertson et al 1999). Under high bee densities, however, this relationship disappeared. Signals that are correlated with rewards have the potential to be reliable at low foraging densities, when most of the available pollen is in flowers. At high foraging densities, most of the pollen is removed, potentially reducing signal reliability, but we do not know how floral signals might change at high foraging densities.

We found no overlap between compounds that were correlated with pollen rewards and compounds that were affected by inbreeding. Inbreeding is known to reduce pollen quantity and viability in *M. guttatus* (Carr and Dudash 1995, 1997), and so on average, inbred plants should provide lower-quality rewards than outbred plants. Since β-trans-bergamotene emission is higher in outbred plants, and since outbred plants have higher quality rewards on average, then pollinators who use β-trans-bergamotene as a foraging cue would, on average, obtain high quality pollen rewards. However, the compounds that are correlated with pollen rewards would be the most informative signals to pollinators in search of rewards, and it is possible that a pollinator would visit plants with high β-trans-bergamotene emission and low reward quality. In particular, male sterility is known to occur in natural populations of *M. guttatus* (Robertson et al.

1994, 1999), and in the current study we found that there were outbred male-sterile plants exhibiting high β-trans-bergamotene emission. In a previous study using fully outbred plants derived from the same population as those used in the current study, bumblebees were equally likely to visit male-sterile (rewardless) and fertile (rewarding) individuals (Wise et al. 2011). This suggests that even in a fully outbred population, bumblebees would not be cueing in on the most informative signals.

Honest signals of rewards are clearly beneficial to pollinators. However, the adaptive functions of honest signals from a plant's perspective are not as straightforward. For example, male-sterile or female plants gain an advantage if they attract pollinators despite their lack of pollen rewards. Furthermore, plants benefit reproductively if they receive multiple visits from pollinators (Karron et al. 2009). Thus, it might be advantageous for rewarding plants to produce signals that attract pollinators even after rewards have been depleted, although we do not know how floral volatile signals change after pollinator visitation. To date, little is known about the patterns of evolution and constraints on honest signaling.

Our findings indicate that the floral volatile signals from *M. guttatus* to pollinators are highly complex, and may convey mixed information to pollinators regarding reward status. Although it is known that insects are able to distinguish among complex volatile blends, we still know relatively little about how insects perceive individual components of floral volatile phenotypes (Dudareva et al. 2006). In this study, we established a link between sources of intraspecific variation and changes in floral volatile profiles, providing insight into components of floral scent that could be adaptive for plants as well as for pollinators. Future work should investigate insect behavior in response to these potentially key components of *M. guttatus* floral scent. These results could provide insight into the functional role of volatile signals, as well as the evolution of honest signals from plants to pollinators.

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Table 1. Volatile compounds emitted by *M. guttatus* (listed in ascending order by retention time), lsmean emission and 95% confidence limits in inbred and outbred plants, F-statistic for the main effect in general linear mixed models, and variable importance (normalized by standard deviation) as produced by Random Forests. Double asterisks indicate significant differences after adjusting α for multiple tests.

Compound	Lsmean Inbred (ng/8hr)	Lower 95% CL inbred	Upper 95% CL inbred	Lsmean outbred (ng/8hr)	Lower 95% CL outbred	Upper 95% CL outbred	$F_{1,61}$	Importance score
α-pinene	18.74	10.55	32.74	13.08	8.67	19.50	0.97	0.69
Benzaldehyde	8.23	1.71	30.48	6.24	1.26	22.26	0.49	-3.65
β-pinene	7.29	1.39	27.75	2.97	0.31	11.05	2.15	1.64
Myrcene	2.16	0.23	8.98	1.60	0.32	5.02	0.09	5.97
Limonene	11.38	1.59	42.34	1.68	0.14	7.70	3.01	3.18
E-β-ocimene	2.02	0.00	26.61	1.34	0.00	19.64	0.18	5.53
Linaloloxide	22.60	11.86	42.30	45.94	29.79	70.56	3.17	-1.97
Linalool	65.31	43.92	96.90	63.51	48.99	82.23	0.01	4.78
Unknown1	152.64	79.76	225.51	238.17	190.52	285.83	3.63	9.03
Trans-limonene oxide	11.53	4.57	21.65	6.78	2.33	13.54	1.82	5.69
α-copaene	13.26	6.75	21.94	16.24	11.19	22.23	0.36	10.38
1-butanol, 3-methyl-, benzoate	22.91	5.17	49.64	54.54	28.42	87.04	13.14**	19.35
α-bergamotene	6.25	1.82	12.18	11.08	5.74	17.48	5.31*	20.57†
Unidentified sesquiterpene 1	6.62	3.56	10.61	6.31	4.14	8.93	0.02	4.00
β-trans-bergamotene	80.20	32.93	148.17	224.25	150.69	312.38	12.73**	23.20†
Unidentified sesquiterpene 2	12.99	7.06	20.72	8.60	4.61	13.83	2.10	4.80
Unidentified diterpene	0.96	0.23	2.20	0.47	0.09	1.15	1.07	0.47

*, $p<0.05$; **, $p<0.003$

†Variables selected as most important for classifying inbred and outbred plants

Table 2. Compounds for which reward quality was included in the best-supported model in multiple regression analyses. Included are standardized regression coefficients and adjusted r^2 for the best supported models and the ratio of the Akaike weight for the best-supported model to that of the best model that did not include reward quality.

Compound	Number of Flowers	Corolla Width	Reward Quality	Adjusted r^2	Ratio of Akaike Weights
α-pinene	0.16	0.23	0.27	0.15	6.22
β-pinene		0.32	0.29	0.20	10.48
Myrcene	0.16	0.16	0.34	0.15	27.42
Limonene		0.21	0.35	0.16	26.89
E-β-ocimene		0.30	0.29	0.19	8.71
Linaloloxide	0.23	0.29	-0.31	0.16	17.75
Linalool	0.38		-0.17	0.15	1.22
Limonene Oxide		0.17	0.29	0.11	7.76

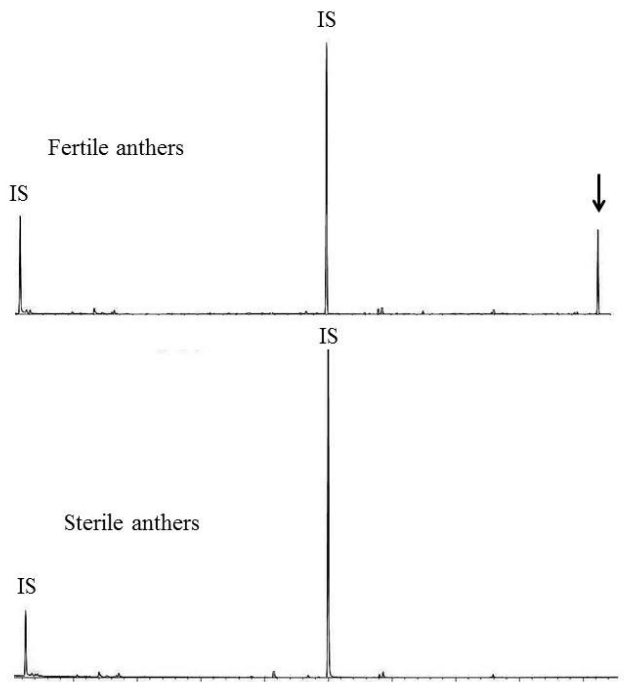

Figure 1. Gas chromatograms from fertile and sterile *M. guttatus* anther volatile samples. The arrow indicates nonadecane. IS indicates internal standards (n-octane and nonyl acetate).

Chapter 2:

Innately attractive and learned volatile cues mediate bumblebee preferences for *Mimulus guttatus* flowers

Abstract

Floral scent is a key trait that plants use to advertise rewards. Associative learning in pollinators is expected to select for production of honest signals of floral rewards in plants. However, evolution of and constraints on honest signaling are not well-studied. Previous work in the mixed-mating, bumblebee-pollinated herb *Mimulus guttatus* demonstrated that bumblebees prefer outbred over inbred plants, but this preference is independent of reward quality. Inbreeding in *M. guttatus* reduces emission of β-trans-bergamotene, but β-trans-bergamotene is not correlated with rewards. However, six compounds that are unaffected by inbreeding are positively correlated with rewards. We hypothesized that compounds that are correlated with rewards function as honest signals in *M. guttatus*, but that β-trans-bergamotene interferes with these signals. We conducted a series of pairwise choice tests using a Y-tube and using artificial flowers in a flight cage to determine whether 1) bumblebees are innately attracted to β-trans-bergamotene, 2) bumblebees develop a preference for compounds that are correlated with rewards, and 3) β-trans-bergamotene interferes with the ability of bumblebees to discriminate based on honest signals. To address objectives 2 and 3, we compared preferences for limonene, which was positively correlated with rewards, and linalool, which was slightly negatively correlated with rewards. Bees demonstrated an innate preference for β-trans-bergamotene in pairwise choice tests using live plants and using extracted floral volatile samples. Bumblebees did not innately prefer limonene or linalool. After foraging on live *M. guttatus* flowers with varying reward quality, bumblebees demonstrated a significant preference for limonene, but not linalool, indicating that bumblebees can form preferences for *M. guttatus* floral odors through associative learning. However, bumblebees only exhibited this learned preference when odor choices were presented over a background of *M. guttatus* floral scent that did not contain β-trans-

bergamotene. When β-trans-bergamotene was present in the background, bumblebees did not exhibit any preference. Thus, β-trans-bergamotene interferes with bumblebees' ability to make choices based on honest signals. We suspect that its innately attractive properties mediate bumblebee behavior in a way that is analogous to pheromone-mimicking compounds in deceptive orchids. Such deceptive signaling mechanisms have not previously been demonstrated in flowers that also offer rewards to pollinators. Perhaps reducing the covariance between signal and reward allows plants to minimize costs of reward production without causing avoidance learning in pollinators. These results demonstrate that volatile signals from plants to pollinators can be highly complex, and suggest that the evolution of honest signals is not straightforward.

Introduction

Floral volatiles play a prominent role in mediating the behavior of pollinating insects. Floral volatiles are known to attract pollinators by eliciting innate responses, and pollinators can also modify preferences through associative learning to favor odors associated with the highest-rewarding flowers (Schiestl and Johnson 2013). Variation in volatile blends among and within plant species can convey complex information about the reward status of these plants, and insects are able to distinguish among volatile profiles to locate their preferred host plants (Dudareva et al. 2006). To date, however, we know relatively little about the ways in which pollinators perceive and discriminate variation in complex volatile blends, or how innately attractive and learned volatile signals interact to influence pollinator behavior.

Floral scent is thought to be a key trait that plants use to advertise their floral rewards. Most insects can learn to associate olfactory cues with food, and are able to modify foraging preferences and behavior in response to learning. A pollinator's ability to learn floral scent depends on the reliability of the scent cue, and this is likely to select for the evolution of honest signals of floral rewards, potentially promoting a mutualistic interaction that maximizes both pollinator foraging efficiency and transfer of conspecific pollen among plants (Wright and Schiestl 2009).

The most obvious honest signals are those emitted from rewards themselves. Both pollen and nectar have been shown to emit blends of volatile compounds that are distinct from those of the whole flower, and these pollen and nectar odors play an important role in attracting pollinators (e.g., Dobson et al. 1999, Raguso 2004). Floral cues that are not emitted directly from rewards can also provide honest signals if they are positively correlated with floral rewards. For example, the compound phenylacetaldehyde was found to be positively correlated with nectar sugar and pollen amount in *Brassica rapa*, but not emitted by nectar or pollen (Knauer and Schiestl 2015). Bumblebees developed a preference for phenylacetaldehyde over other floral volatile compounds after foraging on *B. rapa*, demonstrating that phenylacetaldehyde functions as an honest signal in *B. rapa*. Furthermore, when foraging on scented artificial flowers, bumblebees learned to prefer volatile compounds that honestly signaled reward quality, suggesting that bees should be able to use any detectable signal correlated with rewards to improve foraging efficiency (Knauer and Schiestl 2015). However, this is the only study to demonstrate that bees are able to distinguish a signal that is correlated with rewards (honest signal) from a signal that is uncorrelated with rewards (dishonest signal).

Despite the benefits of honest signals to pollinators, plants can benefit from producing attractive signals without paying the costs of reward production (Gilbert et al. 1991). Since floral scent comprises a blend of compounds emitted from all floral tissues (Dudareva and Pichersky 2006), most olfactory signals are not emitted from rewards themselves, and this provides plants with the opportunity to cheat (Wright and Schiestl 2009). Innate sensory preferences of insects are known to select for floral volatiles that mimic chemical signals used by the insects themselves (Schiestl 2010), allowing plants to deceive pollinators by exploiting their innate biases (Schiestl 2005). For example, deceptive rewardless orchids attract pollinators by emitting volatile signals characteristic of sex pheromones or brood sites (Gaskett 2011, Urru et al. 2011). However, rewardless plants may represent only an extreme form of cheating. It is possible that rewarding plants could deceive pollinators as well, by reducing the covariance between olfactory signals and reward quality (Dafni 1984). A rewarding plant might produce signals that are variable with respect to the rewards obtained for a given signal, and it might even produce signals that elicit innate behaviors (e.g., pheromone mimics) in combination with signals that are more reliable. However, such subtle forms of cheating in plant-pollinator interactions are not well-studied.

The current study examines innate and learned preferences of bumblebees for different components of floral scent from the mixed-mating, bumblebee-pollinated herb, *Mimulus guttatus*. Previous studies have demonstrated that bumblebees discriminate against inbred *M. guttatus* in favor of outbred plants when foraging on live, intact flowers (Carr et al. 2014, Carr et al. 2015). Inbreeding in *M. guttatus* consistently reduces pollen quantity and viability (Carr and Dudash 1995, 1997), thereby decreasing protein content of pollen (Yeamans et al. 2014), the only floral reward in *M. guttatus* (Robertson et al. 1999). Thus, a preference for outbred plants might be expected due to their higher reward quality. However, experiments using outbred plants with variable levels of pollen quality, including male-sterile (rewardless) plants, have demonstrated that the preferences for outbred plants are not mediated by reward quality (Wise et al. 2011, Carr et al. 2015).

The preference for outbred plants persisted when naïve bumblebees (those that had never foraged on live flowers) were presented floral odors from outbred versus inbred *M. guttatus* (Carr et al. 2015). On the other hand, when anthers were presented in isolation from the rest of the flower, experienced bumblebees (those that had foraged on *M. guttatus*) demonstrated a preference for odors of fertile (rewarding) over sterile (rewardless) anthers (Carr et al. 2015), suggesting that bumblebees are able to use olfactory cues to discriminate on the basis of pollen rewards in the absence of other floral cues.

Our recent work on *M. guttatus* floral volatiles demonstrated patterns in *M. guttatus* floral scent that are analogous with previous patterns in bumblebee behavior (Chapter 1). Specifically, inbreeding reduces emission of β-trans-bergamotene, the highest-emitted compound overall in

M. guttatus flowers, and production of β-trans-bergamotene is not correlated with quantity of pollen rewards. β-trans-bergamotene has been identified as the major component of the male pheromone of the parasitic wasp *Melittobia digitata,* and plays a key role in attracting female *M. digitata* (Consoli et al. 2002). Signals such as sex pheromones that are involved in mating behavior are often processed with specific neuron receptors and elicit hard-wired behavior (Ayasse et al. 2001). This suggests the possibility that the preference of bumblebees for outbred plants (Carr et al. 2015) could be driven by innately attractive properties of β-trans-bergamotene. However, no studies have examined the role of β-trans-bergamotene in bumblebee behavior.

We also found that six compounds that are unaffected by inbreeding (α-pinene, β-pinene, myrcene, limonene, E-β-ocimene, and limonene oxide) are emitted in quantities that are positively correlated with the number of viable pollen grains in *M. guttatus* (Chapter 1). These compounds were not emitted from the pollen itself, but their positive correlation with pollen suggests the potential for these to function as honest signals of rewards (Knauer and Schiestl 2015). Moreover, three of these compounds (myrcene, limonene, and E-β-ocimene) were the highest-emitted compounds in the floral scent of the bumblebee-pollinated *Mimulus lewisii*, but almost nonexistent in its sister species, the hummingbird-pollinated *M. cardinalis*, and these compounds mediated bumblebee preferences for *M. lewisii* over *M. cardinalis* (Byers et al. 2014a, b). Since hummingbirds have little sense of smell (Dobson et al. 2006) and do not forage for pollen (Thomson et al. 2000), this further suggests that bees could select for these compounds as honest signals of pollen rewards.

M. guttatus pollen was found to emit only nonadecane, a compound that was not detected in the volatile blend from whole flowers (Chapter 1). This distinct odor profile could provide a direct honest signal to bumblebees. Although experienced bumblebees demonstrated the ability to distinguish between fertile and sterile anthers when anthers were isolated from the rest of the flower (Carr et al. 2015), this preference was not tested with naïve bees, and so these results did not indicate whether this preference was formed through associative learning.

Given that bumblebees are only able to discriminate on the basis of *M. guttatus* pollen rewards in the absence of other floral cues, bumblebees prefer outbred plants regardless of reward quality, and there is no overlap of volatile compounds affected by inbreeding and those associated with pollen rewards, we have hypothesized that β-trans-bergamotene could interfere with the ability of bumblebees to use honest signals when making foraging decisions. To test this hypothesis, we conducted a series of pairwise choice tests using a Y-tube and using artificial flowers in a flight cage to address the questions: (1) Do bumblebees exhibit a learned preference for olfactory cues from pollen? (2) Do olfactory cues from pollen play a role in the preference for floral odors from outbred plants? (3) Are bumblebees innately attracted to β-trans-bergamotene? (4) Do bumblebees learn volatile compounds that are correlated with pollen rewards? (5) Does β-trans-bergamotene interfere with bumblebees' ability to use reliable cues when foraging?

Materials and Methods

Study system: Mimulus guttatus and Bombus impatiens

Mimulus guttatus (Phrymaceae, Beardsley and Olmstead 2002) is an herb native to open, wet habitats throughout western North America from Mexico to Alaska. It produces hermaphroditic, yellow, zygomorphic flowers paired at nodes along a raceme and measuring between 10 and 30 mm wide. Populations are usually annual, but can be perennial in areas that are wet year-round (Dole 1992). *M. guttatus* populations exhibit mixed mating systems with outcrossing rates ranging from approximately 75% selfing (t = 0.25) to complete outcrossing (t = 1.0), averaging $t \approx 0.60$ (Ritland and Ritland 1989). Bumblebees (*Bombus* spp.) are its most important pollinator (Kiang 1972, Macnair et al. 1989, Vickery 1999), but *M. guttatus* also receives visits from other bees, syrphid flies, lepidopterans, and coleopterans (Macnair et al. 1989, Ivey and Carr 2005). Pollen is the primary, if not exclusive, floral reward for pollinators, as the flowers produce little or no nectar (Robertson et al. 1999).

Plants used in this study were descendants of seeds collected from over 500 random maternal families from an annual population in Napa County, CA (Snell Valley: 38° 42' 02" N, 122° 24' 29" W). One plant from each field-collected maternal family was randomly outcrossed in a greenhouse at Blandy Experimental Farm (Boyce, VA), thus providing a base generation of completely outcrossed plants. The plants used in the current study were derived from this base population and included two subpopulations, each comprising a different set of maternal families. One subpopulation was outbred with no biparental inbreeding, and the other was inbred. Inbred plants had passed through four generations of self-pollination (F=0.9375).

Plants were grown in a greenhouse at Blandy Experimental Farm (16:8 photoperiod). Approximately 20 seeds from each maternal family were sown into a 3-inch square pot filled with commercial growing medium (Fafard 3 Mix, Conrad Fafard, Agawam, MA). Pots were randomly arranged in trays (20 pots per tray) with bottom watering. After the first true leaves appeared (approximately 14 days), seedlings were transplanted into individual pots and were randomly arranged with 20 pots per tray and bottom watering.

We used a generalist pollinator, the bumblebee *Bombus impatiens*, for our behavioral experiments. We obtained commercial 'Class A' hives from Koppert Biological Systems (Ann Arbor, MI), and Standard Hives from Biobest USA, Inc. (Leamington, ON, Canada). All hives arrived with a queen, approximately 80 workers, and brood. Bees were provided with sugar water *ad libitum* and were supplemented inside of the hive with commercially available bee pollen (Y.S. Organic Bee Farms, Sheridan, IL).

Rewards and olfactory preferences

General Y-tube Procedures. For all Y-tube choice tests, odor sources were placed in glass chambers each randomized to the left or right arm of a 30 cm Y-tube. For tests that used whole flowers, both plants in a given pairwise test were matched for equal numbers of open flowers. Airflow was generated by a Syntech Stimulus Controller CS-55 and passed through the chambers and into the Y-tube. Individual *B. impatiens* were introduced at the base of the Y-tube and responded to odor cues by walking up one of the arms. A response for each bee was recorded when it crossed a designated line. Bees not reaching the decision line within 10 minutes were omitted from the data set. Each bee was used only once in a given test. Odor sources were replaced every 8-10 trials.

Reward quality and associative learning. We conducted two Y-tube olfactometer tests to assess the ability of bumblebees to learn to discriminate two different types of cues: 1) isolated anthers and 2) whole flowers, on the basis of reward quality.

For the first test, we presented *B. impatiens* a choice between fertile anthers isolated from one flower and sterile anthers isolated from another flower. To avoid any potential confounding inbreeding effects, we used anthers only from outbred plants. For the second test, we gave bees a choice between whole, intact flowers from a fertile outbred plant versus a sterile outbred plant.

We conducted each of these tests twice. The first time we conducted each test, we used bumblebees that were naïve (had never foraged on live flowers). After conducting each test with naïve bees, we allowed bees to forage on a greenhouse population of approximately 500 *M.*

guttatus plants that included inbred and outbred plants with varying pollen quantity, including male-sterile plants. We then repeated each of these tests using the experienced bees to examine learned preferences.

Role of rewards in the preference for outbred plants. Naïve bumblebees previously demonstrated a preference for olfactory cues of flowers from outbred over inbred plants, and experienced bees demonstrated a preference for fertile over sterile isolated anthers (Carr et al. 2015). To evaluate whether outbred plants were inherently more attractive than inbred plants, we tested whether bees would prefer floral odors from outbred plants even when they were less rewarding than inbred plants. We conducted a Y-tube choice test using the experienced bees that we used in the learning test (described above) in which we presented each bee with a choice between whole, intact flowers from a sterile (rewardless) outbred plant versus a rewarding (>85% viable pollen) inbred plant.

Data analysis for rewards and olfactory preferences. Since each pair of odor sources was used for 8-10 trials, thereby introducing potential non-independence among these trials, we conducted log-linear models that included odor source and pair as variables to ensure that bumblebee preference was not influenced by the particular pair of odor sources used in its trial. For all of these tests, the odor × pair interaction was nonsignificant. Thus, we concluded that bumblebee choice was independent of the particular pair of odor sources used in a given trial, and so we eliminated this interaction term in further analyses.

We conducted chi-squared goodness-of-fit tests of the null hypotheses that bees chose anthers and plants at random. To test the null hypothesis that bumblebee choice for fertile versus sterile anthers was independent of experience, we conducted a log-linear model with odor source (sterile or fertile anthers), experience level (naïve or experienced), and odor × experience as variables.

Attractiveness of β-trans-bergamotene

We conducted two sets of Y-tube olfactometer tests to evaluate the ability of *B. impatiens* to discriminate between floral volatile phenotypes with different amounts of β-trans-bergamotene. These bees had not previously foraged on *M. guttatus*.

We first presented bees with a choice between intact flowers from an inbred full sib of a high emitter and an inbred full sib of a low emitter of β-trans-bergamotene. Although we had volatile data only for full sibs of these plants, they had inbreeding coefficients of 0.9375, and so we expect that the volatile data were representative of the volatile production of plants in choice tests.

Next, we gave bees a choice between extracted floral volatile samples re-evaporated from silicone septa that contained high amounts versus low amounts of β-trans-bergamotene. Volatile samples had been collected using a push-pull volatile collection system and extracted with dichloromethane (described in Chapter 1), and were stored at -80^{o}C until used in behavioral assays. For a given pairwise test, we used samples that had approximately equal total volatile mass and for which β-trans-bergamotene was the compound that showed the largest difference in mass between samples (ranging from 18% to 42% greater by mass in the high than the low β-trans-bergamotene blend). Immediately before each pairwise test, septa were treated with dichloromethane. Once the dichloromethane was absorbed, we treated septa with 10 µl of the volatile extract. Each septum was randomized to the left or right of the Y-tube for each trial, and trials were conducted as described in *General Y-tube procedures*.

Data analysis for attractiveness of β-trans-bergamotene. To ensure that bumblebee preferences were not influenced by the pair of plants or volatile samples used in their respective trials, we conducted log-linear models that included bumblebee preference and the pair of odor sources as variables. For all of these tests, the odor × pair interaction was nonsignificant. Thus, we concluded that bumblebee preferences were independent of the particular pair of odor choices used in a given trial, and so we eliminated this interaction term in further analyses. To test the null hypothesis that bees chose plants and volatile samples at random (i.e., that bumblebee choice was independent of the amount of β-trans-bergamotene in an odor source), we conducted chi-squared goodness-of-fit tests.

Effects of β-trans-bergamotene on learned responses

General Procedures. We conducted pairwise choice tests with artificial flowers to investigate whether β-trans-bergamotene could interfere with components of *M. guttatus* floral scent that reliably indicate high quality pollen rewards. We made artificial flowers from yellow construction paper (Y-HUE from Color Aid Corporation, Hudson Falls, NY) cut into a 6-petal shape using a 'Cuttlebug Scribble Flower™ and a Sizzix© press (Lake Forest, CA). To make a "corolla", we removed the cap of a clear 1.5-ml microcentrifuge tube and inserted it into a hole cut into the center of the petals. We also clipped off the bottom of the microcentrifuge tube so that scented air could flow from the base through the opening of the artificial flower (Figure 1).

To assess the role of β-trans-bergamotene in bumblebees' ability to discriminate between odors, we provided a background floral scent from live *M. guttatus* flowers (both artificial flowers were given the same background scent). We conducted each pairwise test using live flowers that produced β-trans-bergamotene and using flowers that did not produce β-trans-bergamotene. Since the only plants that did not emit β-trans-bergamotene were inbred, we used inbred plants for all assays so that potentially more vigorous outbred plants did not confound our results.

We conducted behavioral tests in a flight cage (L x W x H approximately 80cm x 60cm x 60cm) with gray walls and floor to provide a neutral background (Russell et al. 2015). In one wall there were 2 ports spaced 13 cm apart into which we placed artificial flowers. Air from a pressurized tank was filtered through a 2-Channel Air Delivery System (Analytical Research Systems, Gainsville, FL) and flowed at 2 L/min through Teflon tubing into a 900-ml glass chamber containing live, intact *M. guttatus* flowers (isolated from the rest of the plant with aluminum foil). Air flowed out of the chamber (to provide the background scent) through Teflon tubing that was split 2 ways, each into a different artificial flower (Figure 2). Each artificial flower was augmented with a different odor source that was introduced after the air split.

For each trial, one bee was introduced into the flight cage through an opening at the opposite end from the artificial flowers. We recorded each bee's preference as the first artificial flower that the bee landed on. Any bee that failed to land on an artificial flower within 15 minutes was excluded from the data set. Each bee was used only once in a given experiment, but the same bee could have been tested in more than one experiment. The plants used to provide the background floral scent were replaced every 5-6 trials.

β-trans-bergamotene and presence of pollen. We conducted pairwise choice tests to investigate whether presence of β-trans-bergamotene in the background affected preferences of experienced bumblebees for fertile versus sterile anthers. These bees had foraged on a greenhouse population of *M. guttatus* that included inbred and outbred plants with varying pollen quantity, including male-sterile plants (approximately 500 plants total).

We conducted one set of trials in which the background floral scent was from inbred lines that were known to produce β-trans-bergamotene, and a second set in which the background scent was from inbred lines that we previously identified as unable to produce β-trans-bergamotene. Following the air split, fertile anthers were placed inline to allow their odors to flow into one of the artificial flowers, and sterile anthers were placed in the airflow to the second artificial flower. Anthers were held inline by inserting them into a connector in between two pieces of tubing.

β-trans-bergamotene and reliable cues. We conducted a series of pairwise choice tests to determine whether bumblebees could learn particular compounds based on their reliability as indicators of reward quality, and to determine whether β-trans-bergamotene could interfere with any learned responses. We tested preferences for select compounds by presenting bees with artificial flowers augmented with a compound versus a control. We focused on two compounds whose signals were expected to provide contrasting information to bumblebees with respect to reward quality. To represent the group of compounds that should be reliable indicators of high reward quality, we chose to test limonene because it showed the highest correlation with reward quality (Chapter 1). To contrast with limonene, we tested linalool. Linalool was slightly negatively correlated with reward quality in *M. guttatus*, but had the third-highest emission rate of all compounds, was emitted by every plant, and was not affected by inbreeding.

To augment artificial flowers with different odor sources, we treated silicone septa with volatile compounds that had been diluted with dichloromethane, and placed them inside of the "corollas" of the artificial flowers. For assays testing preferences for limonene, we treated one septum with 100 ng limonene (the quantity necessary to emit 40 ng over 2 hours, approximately the mean + 2SD limonene emission in *M. guttatus*). We treated a second septum with an equivalent volume of dichloromethane so that we could test preferences for limonene versus the control. For assays testing preferences for linalool, we treated one septum with 200 ng linalool (because linalool was emitted from septa at approximately half the rate of limonene), and treated the other septum with an equivalent volume of dichloromethane to serve as a control. We replaced septa every 2 hours to ensure a consistent emission rate across all trials.

We tested naïve and learned preferences for each compound on backgrounds of floral scent that did and that did not contain β-trans-bergamotene (8 tests total; Table 1). We expected that if bumblebees form a positive association with only compounds that are reliable indicators of high reward quality, then after experience, they should exhibit a preference for limonene, but not linalool. If β-trans-bergamotene interferes with learned responses, then bumblebees should exhibit a less intense preference when β-trans-bergamotene is in the background than when β-trans-bergamotene is absent from the background.

We conducted all tests first using naïve bees (those that had never foraged on live flowers). We repeated each test using experienced bees. To provide experience, we placed 4-8 live, outbred *M. guttatus* plants (ranging 24-60 flowers total) into a second flight cage (L x W x H approximately 80cm x 60cm x 80cm) with gray floor and walls. Approximately 50% of the flowers in the cage at any given time were male-sterile. One bee at a time was introduced into this cage and allowed to forage. Once the bee had made a number of visits at least equal to 50% the number of flowers available in the cage, the bee was caught with a vial and immediately introduced into the testing cage to record its response to the scented artificial flowers. Bees that did not forage or did not complete the minimum number of visits were not tested. All bees that were tested had visited a combination of sterile and fertile flowers during training, and completed the choice test within 15 minutes. Plants in the training cage were replaced after each bee.

Data analysis for assays with artificial flowers. Since we used each plant to provide the background volatile blend for multiple trials, we conducted log-linear models using SAS Proc Catmod to determine whether bumblebee preference was dependent on the particular plant used for its trial. Since all of these tests indicated a nonsignificant odor × plant interaction, we concluded that bumblebee preference did not depend on which plant was in the background, and thus we eliminated this interaction term for further tests.

To test the null hypotheses that bees chose artificial flowers at random, we conducted chi-squared goodness-of-fit tests. To assess whether learning played a role in bumblebee preferences, we tested for an interaction between odor and experience level (naïve or experienced) using log-linear models in SAS proc catmod.

Results

Rewards and olfactory preferences

Reward quality and associative learning. In pairwise tests for the ability of bumblebees to learn anther or pollen scents (Figure 3a), only 42% of inexperienced bees chose fertile over sterile isolated anthers (χ^2_1=1.67, P=0.1967). However, after experience, 75% chose fertile over sterile isolated anthers (χ^2_1=10.0, P=0.0016). The log-linear model showed a significant odor × experience interaction (χ^2_1=10.20, P=0.0014), indicating that the preference for fertile versus sterile anthers depended on whether a bee was experienced.

When given whole, intact flowers from outbred plants with fertile versus sterile anthers (Figure 3b), neither naïve nor experienced bees showed any preference (naïve: χ^2_1= 0.07, *P*=0.7893; experienced: χ^2_1= 0.1; *P*=0.7518). These results suggest that bumblebees are only able to distinguish fertile from sterile anthers in the absence of other floral odors from outbred plants.

Role of rewards in preference for outbred plants. When presented sterile outbred plants versus fertile inbred plants, 85% of experienced bees chose sterile outbred plants (χ^2_1=19.6, *P*<0.0001; Figure 4), indicating that the preference for outbred plants did not depend on these plants having greater pollen rewards.

Attractiveness of β-trans-bergamotene

In pairwise tests using the floral odors of inbred lines of high versus low emitters of β-trans-bergamotene, 69% of naïve bees preferred full sibs of high emitters (χ^2_1=6.75, *P*=0.0094; Figure 5a). In pairwise tests for isolated volatile samples with high versus low amounts of β-trans-bergamotene, 80% preferred volatile blends with high amounts of β-trans-bergamotene (χ^2_1=18.0, *P*<0.0001; Figure 5b). These results demonstrate that *B. impatiens* is innately attracted to β-trans-bergamotene.

Effects of β-trans-bergamotene on learned responses

β-trans-bergamotene and presence of pollen. When β-trans-bergamotene was in the background, only 52% of experienced bumblebees preferred fertile anthers (χ^2_1=0.04, *P*=0.8415; Figure 6a). However, when β-trans-bergamotene was not in the background, 84% of experienced bumblebees preferred fertile anthers (χ^2_1=12.46, *P*=0.0004; Figure 6b). This suggested that β-trans-bergamotene interferes with the ability of experienced bumblebees to discriminate among flowers based on pollen rewards.

β-trans-bergamotene and reliable cues. When presented a background of floral scent containing β-trans-bergamotene, 59% of naïve bees and 53% of experienced bees showed a preference for limonene (known to be positively correlated with pollen rewards) over the control (naïve: χ^2_1=0.93, *P*=0.3359; experienced: χ^2_1=0.13; *P*=0.7150; Figure 7a). When presented a background of floral scent that did not include β-trans-bergamotene, 50% of naïve bees preferred limonene over the control (χ^2_1=0.00, *P*=1.0). However, 86% of experienced bees exhibited a preference for limonene (χ^2_1=15.21, p<0.0001; Figure 7b). The log-linear model indicated a significant odor × experience interaction (χ^2_1=7.57; *P*=0.0059). Thus, bumblebees developed a preference for limonene through associative learning, but they were only able to exhibit this preference when β-trans-bergamotene was not present in the background.

When the background floral scent contained β-trans-bergamotene, 43% of naïve bees and 60% of experienced bees showed a preference for linalool versus the control (naïve bees: χ^2_1=0.53, *P*=0.4652; experienced bees: χ^2_1=1.20; *P*=0.2733; Figure 8a). When the background floral scent did not include β-trans-bergamotene (Figure 8b), 40% of naïve bees preferred linalool (χ^2_1=1.20; *P*=0.2733). However, only 33% of experienced bees chose linalool, suggesting a marginal preference for the control over linalool (χ^2_1=3.33; *P*=0.0679). However, the effect of avoidance learning on this preference was nonsignificant (odor × experience: χ^2_1=0.29; *P*=0.5925).

Discussion

Our findings demonstrate that bumblebees show a learned preference for compounds that are reliable indicators of pollen rewards, and they show a weak avoidance of linalool, a compound with a weak negative association with pollen rewards (Chapter 1). Bumblebees were innately attracted to β-trans-bergamotene, a compound uncorrelated with rewards (Chapter 1) and only exhibited their learned preferences in the presence of floral scent that did not contain β-trans-bergamotene. Together, our results demonstrate that reliable floral volatile cues in *M. guttatus* can provide honest signals to bumblebees, but β-trans-bergamotene interferes with the ability of bumblebees to use these signals when foraging. These findings may help explain the paradoxical responses of bumblebees to inbreeding and rewards (Wise et al. 2011; Carr et al. 2015).

Learned and innately attractive volatile cues

Insects often perceive a whole volatile blend differently from how they perceive the sum of its parts (Smith et al. 2006), and there is evidence of competition among scent components (Smith 1998). We found that experienced bumblebees preferred odors from fertile over sterile anthers when cues from other floral parts were absent, but when presented odors from intact flowers from outbred plants with fertile versus sterile anthers, their choice was random with respect to reward quality. We observed similar patterns when we assessed preferences for honest signals on odor backgrounds with and without β-trans-bergamotene. When β-trans-bergamotene was present in the background, learned preferences for honest signals disappeared. Experienced bumblebees even preferred floral odors from rewardless outbred plants over those from rewarding inbred plants, which produced little to no β-trans-bergamotene. This could explain why bumblebees discriminated so strongly against inbred plants even when they offered more pollen rewards than outbred plants (Carr et al. 2015).

Our results demonstrated that naïve bees favored floral odors containing high amounts of β-trans-bergamotene, demonstrating that β-trans-bergamotene is innately attractive to *B. impatiens*. The only study to date on the role of β-trans-bergamotene in insect behavior demonstrated that β-trans-bergamotene was the largest component of the male pheromone of the parasitic wasp, *Melittobia digitata*, and female *M. digitata* were as attracted to β-trans-bergamotene alone as they were to the whole male pheromone (Consoli et al. 2002). Pheromones involved in mating behavior are often detected with specific olfactory receptors and are known to elicit hard-wired behavior (Ayasse et al. 2001). The pheromone components of *B. impatiens* are not known. However, such a hard-wired response to β-trans-bergamotene could distract *B. impatiens* from honest signals and may explain the patterns we observed in bumblebee preferences.

Honest signals

Plant-pollinator interactions are often thought of as mutualistic, and honest signals are predicted to play a key role in mediating these interactions (Wright and Schiestl 2009). Most studies on honest signals focus on nectar as a floral reward, but honest signals associated with pollen rewards can also play an important role in pollinator behavior. For example, pollen and anthers often have scent profiles that are distinct from those of other floral tissue. Pollen odors were found to be important in bumblebee discrimination among *Rosa rugosa* flowers (Dobson et al. 1999), and odors of hermaphrodite anthers were found to explain preferences of generalist pollinators for hermaphrodite over female *Fragaria virginiana* flowers (Ashman et al. 2005). In the current study, bumblebees learned to prefer isolated fertile anthers over sterile anthers. The only detectable compound in *M. guttatus* pollen is nonadecane, a compound that was not detected in the volatile profile of whole flowers (Chapter 1). The learned preference for fertile anthers therefore suggests that nonadecane could have provided an honest signal to bumblebees that pollen is present in a flower.

Bees can also evaluate the correlation between the intensity of a volatile cue and the amount of floral reward, indicating that an olfactory cue that is consistently correlated with rewards can provide an honest signal even if it is not emitted from rewards themselves (Knauer and Schiestl 2015). Neither limonene nor linalool is emitted from pollen in *M. guttatus*. Limonene is positively correlated with pollen (Chapter 1), and we found that after foraging on live *M. guttatus*, bumblebees developed a preference for limonene. In contrast, linalool exhibits a slight negative correlation with pollen in *M. guttatus* (Chapter 1), and after bumblebees foraged on *M. guttatus*, they exhibited a marginal preference for the control over linalool, suggesting either indifference or avoidance learning of linalool. Similarly, phenylacetaldehyde was found to be positively correlated with nectar sugar and pollen amount in *Brassica rapa* even though it was not present in pollen or nectar, and bumblebees developed a preference for phenylacetaldehyde after foraging on *B. rapa* (Knauer and Schiestl 2015). Thus, consistent with the results of Knauer and Schiestl (2015), our findings demonstrate that bumblebees develop preferences through associative learning specifically for compounds that are positively associated with rewards.

One caveat of honest signals not emitted directly from floral rewards is that as floral rewards are depleted, the reliability of these signals may decrease. The reduced reliability of these signals could impede learning of these signals because foraging bees will not necessarily encounter flowers at the peak of their ability to reward pollinators. Bumblebees previously demonstrated the ability to discriminate among *M. guttatus* plants based on quantity of viable pollen when foraging densities were low (Robertson et al. 1999). However, at high foraging densities, this discrimination disappeared. When we initiated learning in our choice tests with limonene and linalool, we used unvisited plants for each bee, and so our study did not account for environments that experience high foraging densities and reward depletion. We might expect the reliability of limonene to decline at high foraging densities because removal of pollen does not remove the source of limonene, but we do not know how *M. guttatus* volatile signals change with pollinator visitation.

Dishonest signals

Since β-trans-bergamotene mediates bumblebee behavior even though it provides no information to pollinators about reward status, we might consider β-trans-bergamotene to be deceptive. The response of bumblebees in previous work to male-sterile outbred *M. guttatus*, which exhibited high β-trans-bergamotene emission, demonstrates a clear example of cheating. These plants were as attractive as rewarding outbred plants (Wise et al. 2011). They were even more attractive than rewarding inbred plants (Carr et al. 2015), which generally produced little to no β-trans-bergamotene. This suggests that β-trans-bergamotene deceived bumblebees into visiting outbred male-sterile plants despite their lack of rewards. Such deceptive pollination of rewardless flowers is well-documented in the Orchidaceae, several species of which are known to attract pollinators by producing chemical signals that exploit reproductive stimuli of their pollinators (Schiestl 2005). The innate attractiveness of β-trans-bergamotene to bumblebees in our study and its importance in female attraction in another hymenopteran suggest that β-trans-bergamotene could attract bumblebees to male-sterile *M. guttatus* through a similar mechanism as deceptive orchids.

Unlike deceptive orchids, however, most *M. guttatus* flowers do offer rewards in the form of pollen. Bumblebees were even able to learn and prefer signals that are positively associated with rewards, indicating that *M. guttatus* produces honest signals of rewards. Despite this associative learning, bumblebees were unable to discriminate between odor profiles in which β-trans-bergamotene was present. This suggests that bumblebee behavior is primarily driven by an unreliable signal, even in rewarding plants. This could represent a subtle form of deception in which the signal that a pollinator uses to decide which flower to visit (β-trans-bergamotene) is not indicative of the available rewards in that flower.

Although the benefits of honest signals to pollinators are clear, dishonest signals could be adaptive for plants in a number of ways. Dishonest signals could attract pollinators while enabling plants to minimize the resources allocated to reward production. Dishonest signals could also benefit plants reproductively if they enable multiple pollinator visits to a given flower (Karron et al. 2009). On the other hand, deceptive signaling could be selected for because it encourages pollinators to leave a plant after fewer visits, thereby reducing geitonogamous self-pollination (Schiestl and Johnson 2013).

It is also possible that deception is driven by interspecific competition. In a natural plant community, species compete with each other for pollinators. Since different species likely have different levels of floral rewards, it may not be advantageous for the more poorly rewarding species to be completely honest about the rewards they have to offer pollinators. Given that *M. guttatus* produces almost no nectar, it is likely less rewarding than many of its co-flowering plant species. Nectar production can be costly for plants (Pyke 1991), and it could be advantageous for a plant to attract pollinators without having to produce nectar. We found that β-trans-bergamotene overrode bumblebee preferences for all honest signals that we tested, whether they were in the pollen itself or simply correlated with pollen. It is possible that the innate attractiveness of β-trans-bergamotene could also distract pollinators from visiting more rewarding species in the plant community, thereby giving *M. guttatus* a competitive advantage for pollinators.

Despite the expectation that pollinators should select for honest signals in plants, our findings suggest that the innate sensory preferences of insects could be an important factor in imposing constraints on the evolution of honest signals. We found that *M. guttatus* emits honest signals, and these signals can play a role in bumblebee foraging preferences. However, an innately attractive signal, β-trans-bergamotene, seems to override preferences for honest signals. Bees given a choice of colors that were different from those that were learned previously demonstrated innate color preferences even after learning (Gumbert 2000), but to our knowledge, the current study is the first to demonstrate that innate olfactory preferences can persist after learning. The disruption of honest signals by an innately attractive signal could represent a subtle form of deceit-pollination that enhances plant fitness without causing avoidance learning in pollinators. This could be adaptive for a plant species that offers lower quantities of floral rewards than other co-flowing species in its community, suggesting the possibility that dishonest signals in rewardless orchids may simply be extreme examples of a more widespread phenomenon.

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Table 1. Floral scent profiles presented in choice tests for limonene and linalool and expected outcome of each test if 1) bees learn compounds that are reliable signals and 2) β-trans-bergamotene interferes with reliable signals. Each row represents one test. Artificial flower treatments were randomized to the left or right for each trial.

β-trans-bergamotene in background floral scent?	Experience level	Artificial flower treatment 1	Artificial flower treatment 2	Hypothesized preference
Yes	Naïve	Limonene	Control	No Preference
Yes	Experienced	Limonene	Control	No Preference
No	Naïve	Limonene	Control	No Preference
No	Experienced	Limonene	Control	Limonene
Yes	Naïve	Linalool	Control	No Preference
Yes	Experienced	Linalool	Control	No Preference
No	Naïve	Linalool	Control	No Preference
No	Experienced	Linalool	Control	No Preference or Control

Figure 1. Artificial flower used in choice tests

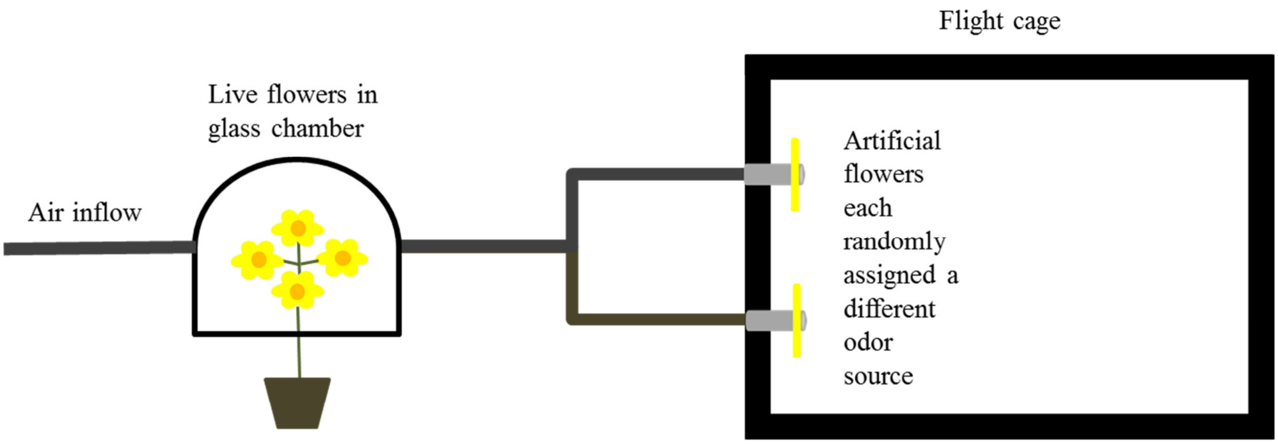

Figure 2. Set-up for choice tests with artificial flowers. Air flowed into a glass chamber containing live *M. guttatus* flowers. Air flowed out of the chamber and split two ways, each into a different artificial flower. For choice tests using fertile versus sterile anthers, anthers were placed inline after the split in air flow. For choice tests using limonene and linalool, septa treated with these compounds were placed directly inside of the artificial flowers.

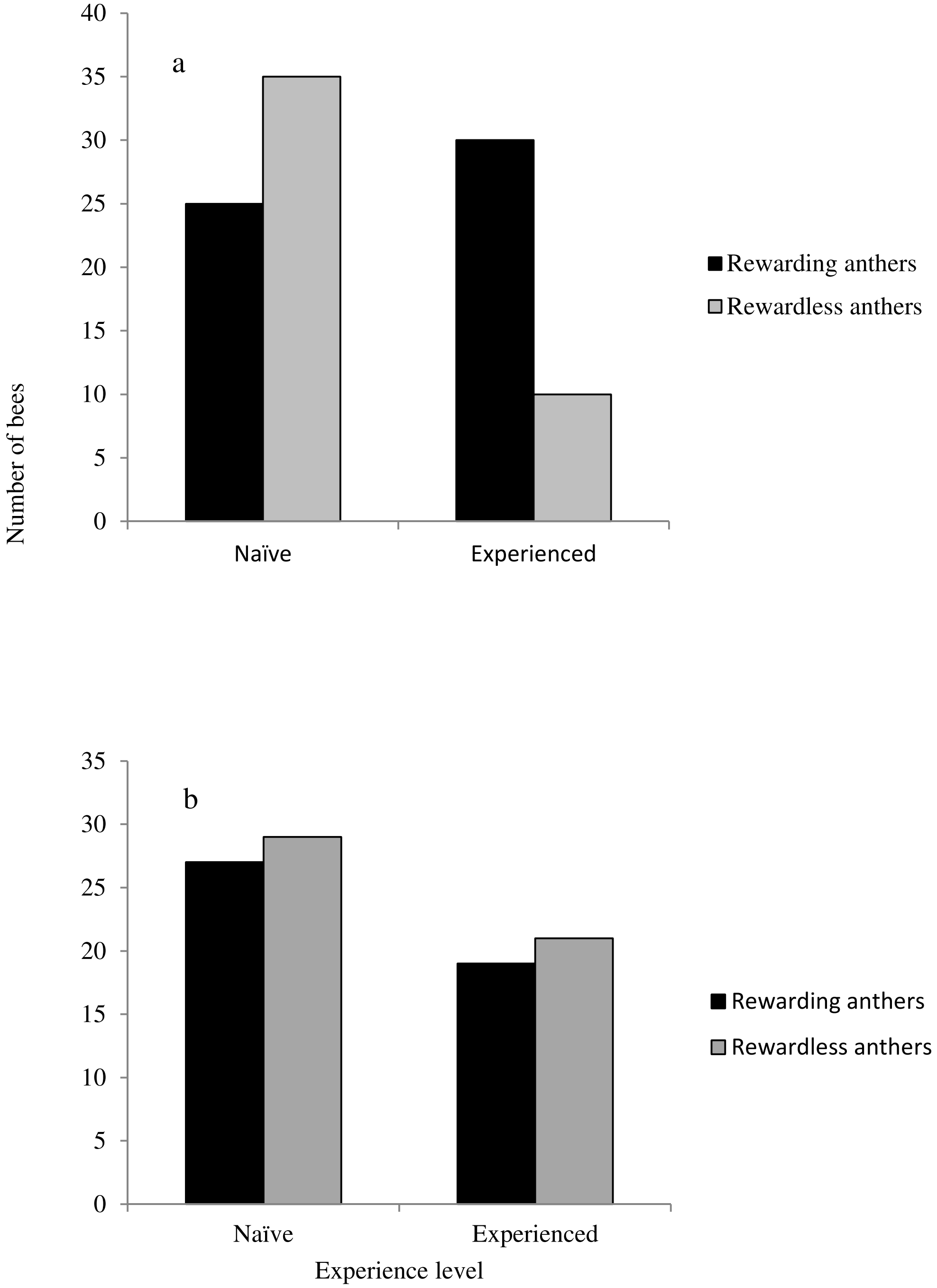

Figure 3. Number of bees that chose rewarding (fertile) and rewardless (sterile) anthers in pairwise choice tests when (a) anthers were presented in isolation from the rest of the flower, and (b) live, intact flowers from outbred plants were presented.

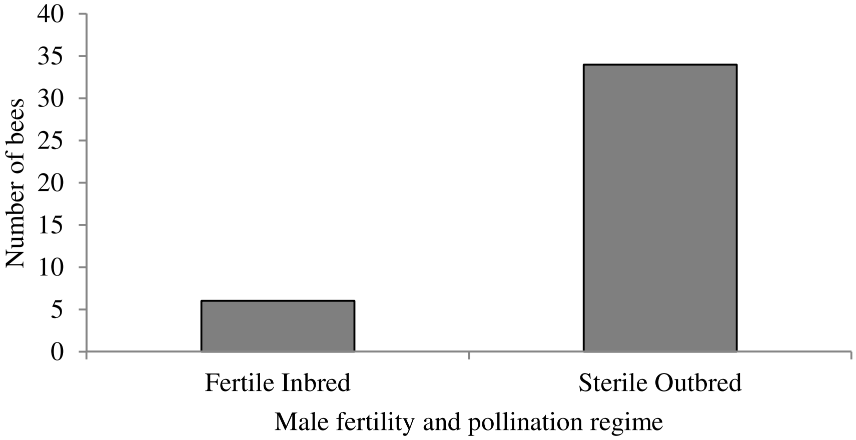

Figure 4. Number of experienced bees that chose rewarding flowers from inbred *M. guttatus* versus sterile flowers from outbred *M. guttatus* in pairwise choice tests.

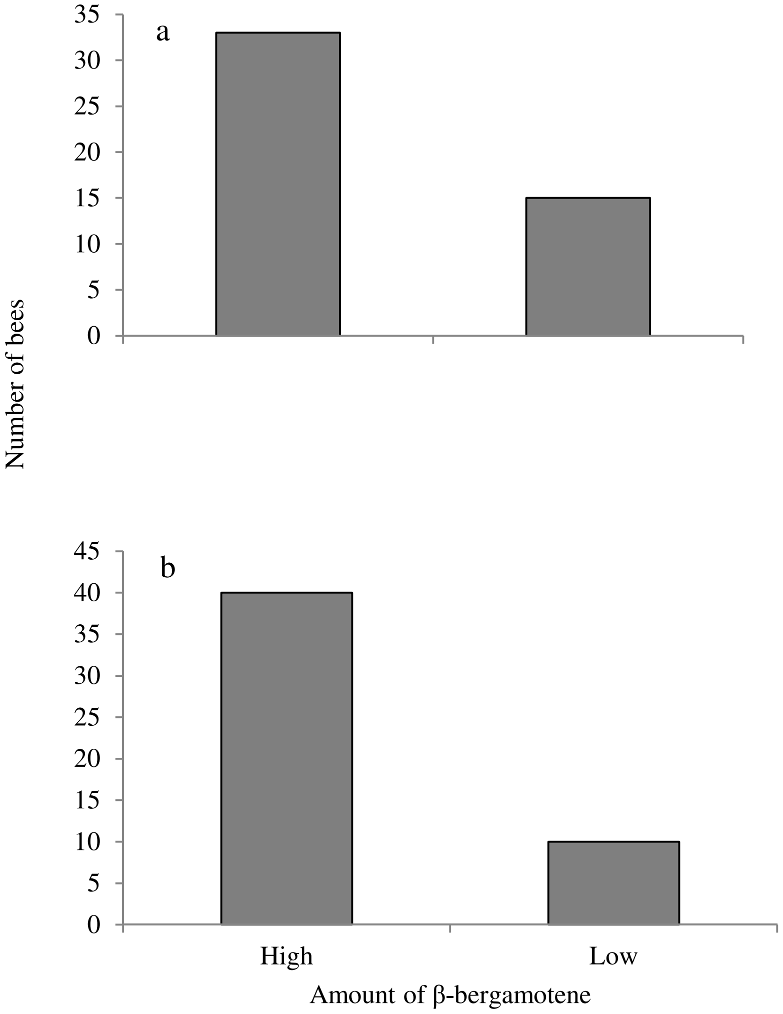

Figure 5. Number of bees that chose (a) Full sibs of plants, and (b) extracted volatile samples from plants that produced high versus low amounts of β-trans-bergamotene in Y-tube olfactometer tests.

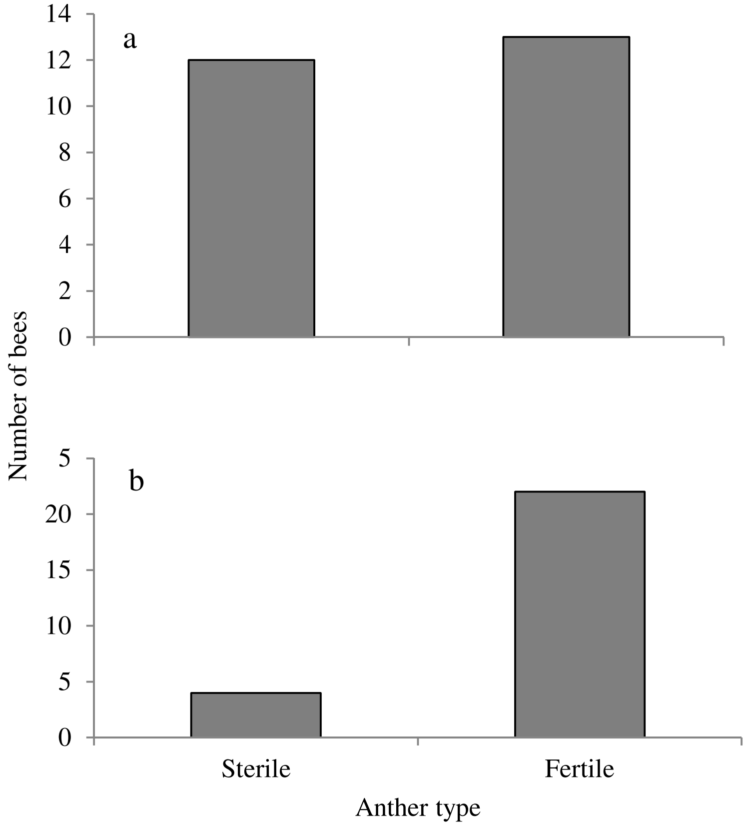

Figure 6. Number of experienced bees that chose artificial flowers augmented with fertile versus sterile anthers in pairwise choice tests in which (a) β-trans-bergamotene was in the background, and (b) β-trans-bergamotene was not in the background.

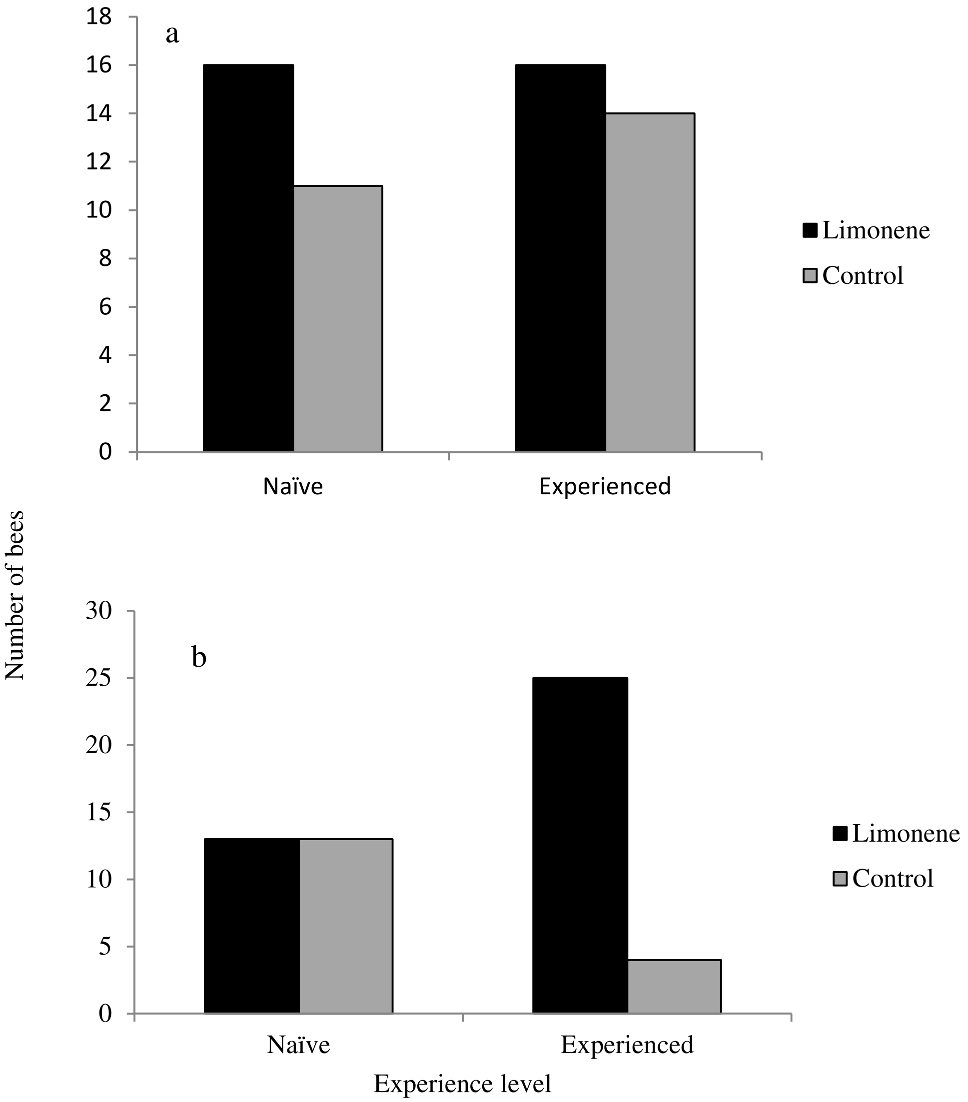

Figure 7. Number of bees that chose artificial flowers augmented with limonene versus control flowers in pairwise choice tests in which (a) β-trans-bergamotene was in the background, and (b) β-trans-bergamotene was not in the background.

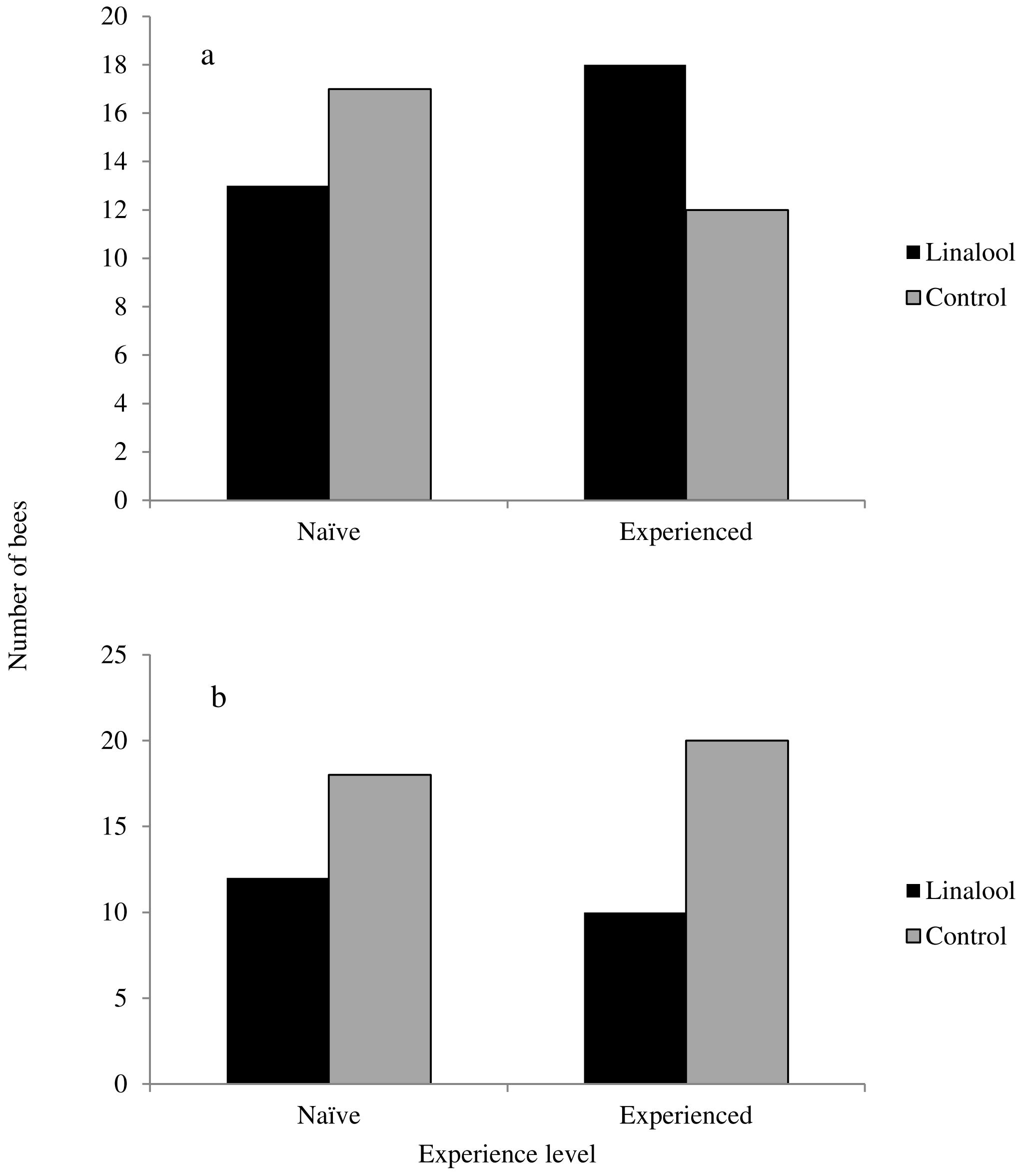

Figure 8. Number of bees that chose artificial flowers augmented with linalool versus control flowers in pairwise choice tests when (a) β-trans-bergamotene was in the background, and (b) β-trans-bergamotene was not in the background.

Chapter 3:

Herbivory and inbreeding alter foliar volatile emission in *Mimulus guttatus*

Abstract

Foliar volatiles play a prominent role in plant-herbivore interactions, and can function to attract or deter herbivores. Herbivory can also induce quantitative and qualitative changes in foliar volatile profiles that are well-known to attract natural enemies of herbivores, mediating a tritrophic interaction that provides an indirect defense. Inbreeding occurs frequently in natural plant populations and can alter the distribution of genetic variation in a plant population. Since inbreeding increases homozygosity, inbreeding could alter biosynthetic pathways involved in production and release of volatile compounds. However, few studies have examined effects of inbreeding on volatile emission. The current study used a factorial design to investigate the effects of herbivory and inbreeding on the foliar volatile profile of the mixed-mating herb, *Mimulus guttatus*. Herbivory significantly increased total volatile emissions and increased production of 1-octen-3-ol and neophytadiene, compounds that have previously been found to function in plant defense. We also found a significant herbivory × inbreeding interaction, and three compounds exhibited higher constitutive emission from outbred relative to inbred plants. If these compounds are deterrent or toxic to herbivores, this suggests that inbreeding reduces constitutive defenses. However, if these compounds are attractive to herbivores, this suggests that inbred plants could have an advantage that promotes mixed-mating. These results provide insight into the evolution of plant mating systems and the ecology and evolution of plant-herbivore and tritrophic interactions.

Introduction

Volatile compounds emitted from plant foliage function as key host-location cues for insect herbivores and their natural enemies. Insects are able to distinguish among complex blends of compounds based on compound ratios (Bruce et al. 2005), a subset of compounds in the blend (Tasin et al. 2007), or even a single compound (Hern and Dorn 2004). Variation in volatile profiles among and within plant species can convey information about a plant's physiology and stresses (Dudareva et al. 2006), and these signals can elicit attraction or avoidance in insects. Moreover, plants respond to herbivory by altering emission rates and the composition of volatile blends (Pare and Tumlinson 1999), thereby signaling that they have been attacked. Volatile profiles are known to exhibit a substantial amount of genetic variation (Hare 2011). In particular, inbreeding changes the distribution of genetic variation in a population, and this could have important ecological implications. However, only a few studies have examined how volatile profiles are influenced by inbreeding.

Volatile compounds emitted constitutively are often byproducts of a plant's physiological activity, but evidence suggests that constitutive volatiles can also be an important plant defense trait by directly deterring herbivores (Das et al. 2013). However, most studies of foliar volatiles have focused on emission from herbivore-damaged plants. Since these induced defenses are only activated if an herbivore attacks, they can reduce the physiological and ecological costs associated with volatile emission (Dicke and Hilker 2003). Herbivore-induced volatiles have been found to act as a direct defense against herbivores themselves. For example, *Nicotiana tabacum* released herbivore-induced volatiles at night that deterred females of the nocturnal moth *Heliothis virescens* from ovipositing (De Moraes et al. 2001), and herbivore-induced volatiles released from *Nicotiana attenuata* decreased oviposition rates of *Manduca quinquemaculata*

(Kessler and Baldwin 2001). Induced volatiles are also known to attract predators and parasitoids of herbivores, mediating a tritrophic interaction that provides an indirect plant defense (Arimura et al. 2009). For example, induced volatiles in *N. attenuata* increased egg predation by *Geocoris pallens* (Kessler and Baldwin 2001). The parasitic wasp *Cotesia marginiventris* was attracted to induced volatiles in *Zea mays*, indicating that *C. marginiventris* can use these cues to locate plants infested with their hosts (Turlings et al. 1990). In addition to mediating insect behavior, volatile signals can be perceived by neighboring undamaged plants as warning signals that indicate the presence of potential attackers. These signals can induce volatile emission in neighboring undamaged plants (Arimura et al. 2002, Ruther and Kleier 2005), and can even prime defense responses in neighboring undamaged plants so that these plants have a faster and stronger response when they are attacked (Frost et al. 2007, Heil and Bueno 2007). Induced volatiles are not always beneficial to plants, however, as some herbivore species can be attracted to these signals (Bolter et al. 1997, Horiuchi et al. 2003). Thus, natural selection on foliar volatile profiles can be dependent on the species of herbivores, parasitoids, and predators in a plant community.

Previous work has shown that there is heritable variation in foliar volatile profiles (Delphia et al. 2009), demonstrating that volatile profiles can respond to selection. Given the prominent role of foliar volatiles in plant defense, variation in volatile phenotypes could have important implications for the fitness of plants and the insects that interact with them. Inbreeding occurs frequently in natural populations of flowering plants, as over 70% of animal-pollinated plants self-pollinate more than 20% of the time (Goodwillie et al 2005). Inbreeding can play a key role in the distribution of genetic and phenotypic variation in a population and is thought to be important in the evolution of plant mating systems and the vulnerability of small populations

(Charlesworth and Charlesworth 1987). Since inbreeding increases homozygosity, inbreeding might be expected to disrupt biosynthetic pathways involved in the synthesis and release of volatile compounds.

Studies in *Solanum carolinense* have demonstrated that inbreeding alters foliar volatile emissions, with adverse effects on its resistance to herbivory. Inbreeding led to higher levels of constitutive emissions, and this attracted more generalist herbivores in the field, likely because inbred plants were more apparent. However, inbred plants exhibited weaker induction in response to herbivory, and consequently, inbred plants attracted fewer herbivore enemies than outbred plants (Kariyat et al. 2012). Furthermore, outbred *S. carolinense* emitted more total constitutive nighttime volatiles, including individual compounds previously known to deter ovipositing moths, and *Manduca sexta* moths preferred to lay eggs on inbred plants (Kariyat et al. 2013). In pairwise choice tests, *M. sexta* larvae consistently preferred inbred plants, whether both plants in a pair were damaged or undamaged (Kariyat et al. 2014).

The herb *Mimulus guttatus* has become a model system for ecological and evolutionary studies due to its broad ecological diversity and mating system variation (Wu et al. 2008). Previous studies have demonstrated that inbreeding in *M. guttatus* affects its resistance to herbivores, but these effects vary depending on which population plants come from. In one *M. guttatus* population, *Philaenus spumarius* nymphs reared on inbred plants matured as significantly larger adults than those reared on outbred plants, indicating that inbreeding reduced resistance. In a second *M. guttatus* population, *P. spumarius* took significantly longer to develop into adults when reared on inbred plants, indicating that inbreeding increased resistance (Carr and Eubanks 2002). Evidence suggests that resistance to herbivory could be heritable in *M. guttatus* (Ivey et al. 2009), but the specific traits mediating resistance are unknown. We previously found that inbreeding alters the floral volatile profile of *M. guttatus*, with negative effects on pollinator visitation (Chapter 1, Chapter 2). No studies have investigated foliar volatile emissions in *M. guttatus*.

The current study examines the foliar volatile phenotype of *M. guttatus*, focusing on variation created by inbreeding and herbivory. We collected foliar volatile samples from *M. guttatus* using a factorial design to address the questions, 1) How does herbivory affect foliar volatile emissions? 2) What are the effects of inbreeding on the foliar volatile phenotype? 3) Is there an interaction between herbivory and inbreeding effects?

Materials and Methods

Study system: Mimulus guttatus

Mimulus guttatus (Phrymaceae, Beardsley and Olmstead 2002) is an herb native to open, wet habitats throughout western North America from Mexico to Alaska. It produces hermaphroditic, yellow flowers that are paired at nodes along a raceme and can produce over 100 flowers in a season. Populations are usually annual, but can be perennial in areas that are wet year-round (Dole 1992). *M. guttatus* populations exhibit mixed mating systems with outcrossing rates that range from approximately 75% selfing ($t = 0.25$) to complete outcrossing ($t = 1.0$), averaging $t \approx 0.60$ (Ritland and Ritland 1989). *M. guttatus* leaves contain glandular trichomes. Trichome density is variable within and among *M. guttatus* populations, and evidence suggests that trichomes in *M. guttatus* function to defend against herbivores (Holeski 2007).

The *M. guttatus* used in this study were descendants of seeds collected from over 500 random maternal families from an annual population in Napa County, CA (Snell Valley: 38° 42’ 02” N, 122° 24’ 29” W). One plant from each field-collected maternal family was randomly outcrossed in a greenhouse at Blandy Experimental Farm (Boyce, VA), thus providing a base generation of completely outcrossed plants. The current study used two subpopulations of plants derived from this base population, each comprising a different set of maternal families. Each subpopulation represented a different pollination treatment. One subpopulation comprised inbred plants that had passed through four generations of self-pollination, so that these plants had an inbreeding coefficient of F=0.9375. The other subpopulation comprised outbred plants had been produced without biparental inbreeding (F=0).

Approximately 20 seeds from each maternal family were sown into a 3-inch square pot filled with commercial growing medium (Fafard 3 Mix, Conrad Fafard, Agawam, MA). Pots were randomly arranged in trays (20 pots per tray) with bottom watering. After the first true leaves appeared (approximately 14 days), seedlings were transplanted into individual pots and were randomly arranged with 20 pots per tray and bottom watering. Plants were kept in a growth chamber (16:8 photoperiod, 22°C, 60-65% relative humidity) at the Pennsylvania State University.

Volatile collection and analysis

We collected volatiles from rosettes from 22 maternal families from each pollination treatment. We collected from two individuals per maternal family: one was randomly selected to receive a damage treatment, and the other served as an undamaged control. Plants were damaged by allowing a third-instar larva of the generalist lepidopteran, *Helicoverpa zea* (starved for 12 hours prior), to feed on their leaves beginning 24 hours before and lasting through the 8-hour collection. The experimental design included 11 blocks of 8 plants (2 maternal families per pollination × damage treatment in each block).

Glandular trichomes can contain volatile compounds (Paré and Tumlinson 1997, Laue et al. 2000), and therefore have the potential to be an additional source of variation in volatile profiles. Following volatile collection, we quantified trichome density of each plant so that we could control for any possible variation due to differences in trichome density. We cut four 3.6-mm leaf disks from each plant using a cork borer, and counted the number of trichomes on the dorsal surface of each disk. We averaged the four counts to obtain a per-plant trichome density for analyses.

Since plant size was variable within and between pollination treatments, we quantified leaf area of each plant to control for differences in plant size. We harvested plants, scanned the leaves, and calculated leaf area from digital images.

We collected volatiles from plants in their rosette stage (prior to flowering), as this is when they are most vulnerable to herbivory. We collected volatiles from each plant from 10:00 to 18:00 in a growth chamber (26^{o}C, 60-65% relative humidity) under sodium and metal halide lights. Each rosette was enclosed in a 4-L glass chamber. We collected volatiles using a push-pull collection system (Analytical Research Systems, Gainesville, FL), which delivered filtered air through Teflon tubing into the chambers (2.0 L/min) and pulled air from the headspace using a vacuum (1.0 L/min) through a filter containing 40 mg of SuperQ (Alltech Associates).

Within 24 hours of volatile collection, we eluted the trapped compounds with 150 µl dichloromethane and added 5 µL of a mix containing the internal standards n-octane (40 ng/µL dichloromethane) and nonyl acetate (80 ng/µL dichloromethane). Samples were then injected in 1-µl aliquots using a splitless injector into an HP-1 column (250 µm internal diameter, 0.1 µm film thickness, 15 m length) in an Agilent 6890 gas chromatograph fitted with a flame ionization detector. The column was held at 0.5^{o}C for 0.5 minutes and then increased at 8^{o}C/min to 240^{o}C, and helium was the carrier gas. Compounds were quantified (in ng) using MSD ChemStation software (Agilent Technologies) based on peak area relative to that of the internal standards. To identify compounds, samples were analyzed using an HP-1 column (0.25 mm internal diameter, 0.25 µm film thickness, 30 m length) in an Agilent 6890 gas chromatograph coupled with a mass spectrometer (electron ionization mode). Compound peaks were identified through comparison of mass spectra to the NIST 2008 and 2014 mass spectral libraries (National Institute of Standards and Technology) and identities were confirmed by comparison with mass spectra and retention times of standards when possible.

Statistical analysis

We analyzed total volatile emission using a general linear mixed model using Proc Mixed in SAS 9.4 (SAS Institute Inc. 2012). We included pollination treatment (inbred or outbred), herbivory treatment (damaged or undamaged), and their interaction as fixed effects and block as a random effect. To control for variation due to trichomes and differences in plant size, we also included number of trichomes, leaf area, and all pairwise interactions between each of these variables and pollination level and herbivory treatment as covariates. Total volatile emission was log-transformed to meet ANOVA assumptions.

To analyze the overall volatile profile, we conducted a permutational multivariate analysis of variance (permanova) using the Permanova+ package in Primer 6 (Primer-e 2008). We conducted the analysis on Bray-Curtis similarities using a reduced model with pollination, damage, and their interaction as fixed effects, block as a random effect, and trichome density, leaf area, and all pairwise interactions as covariates. We conducted 999 permutations of residuals and calculated Monte Carlo P-values based on pseudo-F tests. Since the permanova identified the damage and pollination × damage effects as significant, we conducted a separate general linear mixed model for each individual compound in SAS 9.4. The emission of each compound was transformed as necessary to meet ANOVA assumptions. Each model included the same terms as those used in the permanova, but we considered only damage and pollination × damage effects (because the permanova identified these as significant) for hypothesis tests of individual compounds. For compounds with a significant pollination × damage interaction, we conducted the contrasts, 1) inbred versus outbred damaged, and 2) inbred versus outbred undamaged.

Results

Mimulus guttatus foliage emitted 21 volatile compounds (Table S1). The most dominant compounds in the volatile profile were neophytadiene (43% of the total emission), followed by 1-octen-3-ol (13% of the total emission) and nonanal (8% of the total emission). Neither pollination treatment nor pollination × damage had an effect on total volatile emission (pollination: $F_{1,65}$=0.02, P=0.8895; pollination × damage: $F_{1,65}$=0.61, P=0.4395). However, the effect of herbivory was significant. Total volatile emission was 3.2 times higher from damaged than undamaged plants ($F_{1,65}$=5.65, P=0.0204; Figure 1).

The permanova indicated that damage level is a significant predictor of the volatile profile and that the interaction between damage level and pollination treatment is significant (Table 1). Thus, herbivory significantly alters the foliar volatile blend of *M. guttatus*, and the effect of herbivory is dependent on whether plants are inbred or outbred. Univariate analyses indicated that the effect of herbivory was significant for the compounds 1-octen-3-ol and neophytadiene (Figure 2). Damaged plants emitted 9.1 times more 1-octen-3-ol than undamaged plants ($F_{1,65}$=5.65, P=0.0205). Emission of neophytadiene was 5.4 times higher in damaged than undamaged plants ($F_{1,65}$=7.54, P=0.0078).

The pollination × damage interaction was significant for two unknown compounds and 2,4-bis(1,1-dimethylethyl)-phenol (unknown4: $F_{1,65}$=7.13, P=0.0096; unknown7: $F_{1,65}$=6.84, P=0.0111; 2,4-bis(1,1-dimethylethyl)-phenol: $F_{1,65}$=8.19, P=0.0056). Contrasts indicated that for each of these compounds, undamaged outbred plants had significantly higher emissions than undamaged inbred plants. Specifically, emission of unknown4 was 5.9 times higher from undamaged outbred plants ($F_{1,65}$=6.75, P=0.0116; Figure 3a). Unknown7 exhibited 3.7 times higher emission from undamaged outbred plants ($F_{1,65}$=5.94, P=0.0175; Figure 3b). Emission of 2,4-bis(1,1-dimethylethyl)-phenol was 1.5 times higher from outbred plants ($F_{1,65}$=4.65, P=0.0347; Figure 3c).

Discussion

Our findings demonstrated that herbivory significantly altered the foliar volatile profile of *Mimulus guttatus*. Herbivory in other species is known to induce emission of additional compounds, increase the quantities of constitutively-emitted compounds, and alter the ratios of compounds in the blend (Dicke and van Loon 2000). Our results add to the well-established literature demonstrating that herbivory causes changes in foliar volatile emission. Permanova results indicated a significant damage effect, suggesting that herbivory altered aspects of the overall volatile phenotype, such as ratios or composition. We also found that *M. guttatus* plants responded to herbivory by increasing total volatile emissions, and this was driven primarily by increased emission of the compounds 1-octen-3-ol and neophytadiene. Inbreeding did not affect total volatile emission, but permanova results demonstrated an interaction between inbreeding and herbivore damage.

Herbivory in *M. guttatus* resulted in substantial increases of the compounds 1-octen-3-ol and neophytadiene. Evidence from studies in other species suggests that both of these compounds could play an important role in defense, either directly or through tritrophic interactions. The larval parasitoid *Microplitis croceipes* preferred cotton-fed *Heliothis virescens* larvae over artificial diet-fed larvae, and the presence of 1-octen-3-ol in cotton-fed larvae was found to play a key role in this preference (Morawo and Fadamiro 2016). Thus, the increased emission of 1-octen-3-ol by *M. guttatus* in response to herbivory could increase parasitism of herbivores, which has been found to increase seed production, thereby enhancing plant fitness (van Loon et al. 2000). Neophytadiene is known to be a component of the bio-oil of *Solanum lycopersicum*, and it decreased feeding and increased mortality of the Colorado potato beetle (Cáceres 2015).

Despite the significant increases in emission of potentially key components of the *M. guttatus* volatile blend, it is important to note that induced volatile responses can be specific to the type of herbivory as well as the species of herbivore and its developmental stage. For example, feeding-induced volatiles are known to differ from oviposition-induced volatiles (Hilker and Meiners 2002). Moreover, the specialist parasitic wasp *Cardiochiles nigriceps* was able to distinguish between volatiles induced by its host, *Heliothis virescens*, and a non-host, *Helicoverpa zea*, in tobacco, cotton, and maize (De Moraes et al. 1998). In addition, corn leaves infested with early (first-fourth) instar *Pseudaletia separata* larvae produced higher quantities of herbivore-induced volatiles than leaves infested by late (fifth-sixth) instar larvae, and females of the parasitic wasp *Cotesia kariyai* were attracted only to leaves damaged by early-instar larvae (Takabayashi et al. 1995). The species we used to inflict damage, *Helicoverpa zea*, is a highly generalized herbivore, but a different generalist or even a different instar of *H. zea* could have elicited a different response in *M. guttatus*. In addition, feeding by multiple herbivore species is known to induce a different response than feeding by a single herbivore (de Boer et al. 2008), and so using multiple herbivore species for our damage treatment may have provided a better representation of a natural setting.

Our permanova results also indicated a significant pollination × damage interaction, suggesting that the effect of herbivory depended on whether plants were inbred and outbred. Since permanova takes into account differences in the overall volatile profile, it could be sensitive to changes in the composition and ratios of compounds, whereas analyses of variance can only detect differences in emission rates. Since the total volatile emissions did not exhibit a pollination × damage interaction, this suggests that the significant interaction in the permanova could be explained by minor differences in emission rates that altered the volatile blend as a whole.

When we evaluated the pollination × damage interaction in individual compounds, we found that this interaction was significant in three compounds. However, this interaction was not caused by a difference in inducibility. For all three compounds, contrasts demonstrated that this significance was explained by higher constitutive emission in outbred than inbred plants. Our findings contrast with those from a study on inbreeding effects in *Solanum carolinense* (Kariyat et al. 2012). In this study, constitutive volatile emissions were higher in inbred plants. Undamaged inbred plants attracted more herbivores than undamaged outbred plants, likely because inbred plants were more apparent. Induction was higher in outbred plants, and this included induction of key compounds known to attract natural enemies of herbivores. On the other hand, outbred *S. carolinense* exhibited higher nighttime constitutive volatile emissions than inbred plants, and several of the compounds exhibiting higher emission in outbred plants deterred ovipositing nocturnal moths (Kariyat et al. 2013).

One of the compounds exhibiting higher constitutive emission in outbred *M. guttatus* plants, 2,4-bis(1,1-dimethylethyl)-phenol, is in the venom of the ant *Oecophylla smaragdina*, an important species for controlling pests (Das et al. 2014). The effects of 2,4-bis(1,1-dimethylethyl)-phenol by itself on insect herbivores are not known, but since *O. smaragdina* uses its venom to paralyze its prey, this suggests the potential for 2,4-bis(1,1-dimethylethyl)-phenol in plants to function as a direct defense against herbivores. In this case, outbred *M. guttatus* would be constitutively better defended than inbred plants, which could be advantageous in environments with high herbivore densities.

Chemical compounds can also function in plant-pathogen interactions, and 2,4-bis(1,1-dimethylethyl)-phenol has been demonstrated in other species to act as a defense against pathogens. For example, it has been found to exhibit antibacterial properties in mango oil (Abdullah et al. 2011) and to inhibit growth of the oomycete *Phytophthora cinnamomi* and germination of *Aspergillus* spores in avocado roots (Rangel-Sánchez et al. 2014). A previous study investigating effects of inbreeding in *Silene alba* on its interaction with the anther-smut fungus *Microbotryum violaceum* found that effects of inbreeding varied by genotype (Ouborg et al. 2000). However, if 2,4-bis(1,1-dimethylethyl)-phenol increases *M. guttatus* resistance to pathogens, then lower constitutive production of this compound in inbred plants suggests that they are constitutively less resistant than outbred plants with respect to this trait.

Herbivory alters plant traits that influence selfing rate, and could therefore play an important role in mating system evolution in plants (Ashman 2002). Strong inbreeding depression in *M. guttatus* for ovule number and pollen production, (Carr and Dudash 1995, 1997), floral displays and flower size (Ivey and Carr 2005), and floral scent (Chapter 1) is likely to impede the spread of selfing alleles (Charlesworth and Charlesworth 1987). Inbred *M. guttatus* also tend to be less tolerant of herbivory (Carr and Eubanks 2002, Ivey et al. 2004), which should reinforce selection against selfing, but the effects of inbreeding on resistance to herbivory are more variable. Xylem-feeding spittlebugs (*Philaenus spumarius*) performed better on inbred plants in one population and outbred plants in another (Carr and Eubanks 2002), whereas two species of caterpillars (a specialist, *Junonia coenia*, and a generalist, *Helicoverpa zea*) both consumed less leaf tissue on inbred plants relative to outbred plants (Chapter 4). *Junonia coenia* performed better on outbred plants and preferred undamaged outbred plants over undamaged inbred plants in paired choice tests, even when presented only olfactory cues from plants (Chapter 4). The ability of *J. coenia* to locate outbred plants by olfactory cues alone suggests a difference in constitutive volatile cues. We found three compounds that showed significantly higher constitutive emission from outbred relative to inbred *M. guttatus*. If this difference results in outbred plants attracting more herbivores, this could create an advantage for offspring of self-pollinated plants (Ashman 2002).

Our results demonstrate that herbivory elicits changes in the foliar volatile profile of *M. guttatus*, increasing emission of compounds that are potentially important as defenses. Moreover, we found evidence that inbreeding and herbivory interact to alter the volatile profile, but the implications of this effect are less clear. The susceptibility of *M. guttatus* to herbivores is likely driven by the composition and densities of herbivore species and their natural enemies in its community, and the inbreeding effects we observed could be positive or negative for *M. guttatus*, depending on its environment. Future work should explore the role of these volatile emission patterns in foraging behavior and performance of herbivores and their natural enemies. This will provide insight into the adaptive components of foliar volatile profiles, which could have important implications for the evolution of plant mating systems, plant-herbivore dynamics, and tritrophic interactions.

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Table 1. Permanova results for analysis on the overall foliar volatile profile.

Variable for analysis	df	MS	Pseudo-*F*	Monte Carlo *P*
Block	10	5855.5	3.35	0.001
Leaf area	1	1167.2	0.67	0.718
Trichomes	1	2276	1.30	0.241
Pollination	1	3510.4	2.01	0.056
Damage	1	7309.8	4.18	0.001
Pollination × trichomes	1	1945.1	1.11	0.352
Damage × trichomes	1	2571.5	1.47	0.144
Pollination × leaf area	1	1990.6	1.14	0.340
Damage × leaf area	1	4647.6	2.66	0.012
Pollination × damage	1	4086.8	2.34	0.021

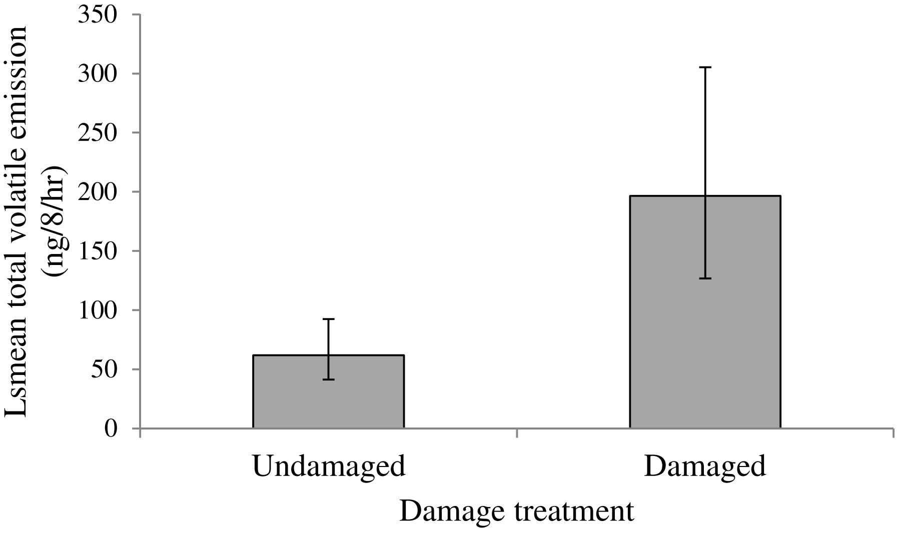

Figure 1. Lsmean total volatile emission from undamaged and herbivore-damaged *Mimulus guttatus* plants. Error bars represent 95% confidence intervals.

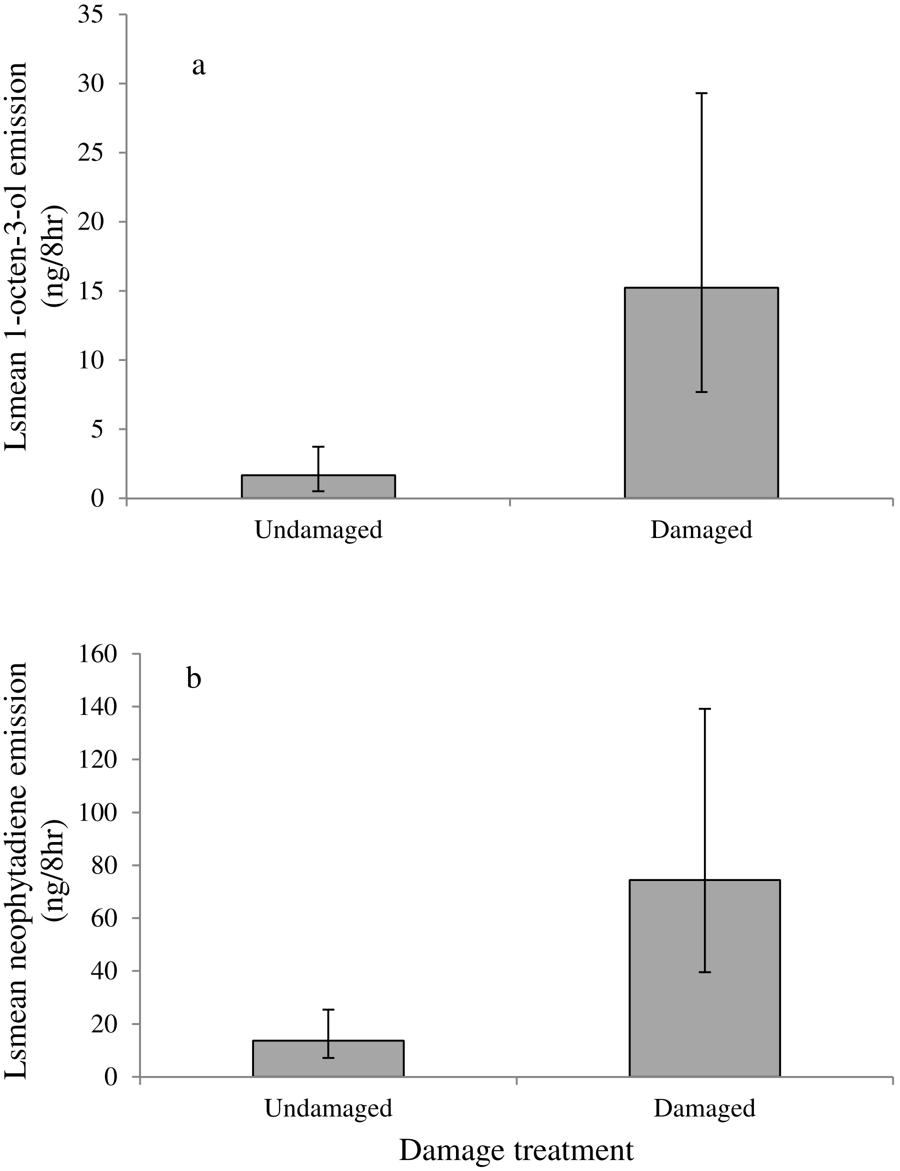

Figure 2. Lsmean emission from undamaged and damaged plants of compounds that showed significant herbivory effects: a) 1-octen-3-ol, and b) neophytadiene. Error bars indicate 95% confidence intervals.

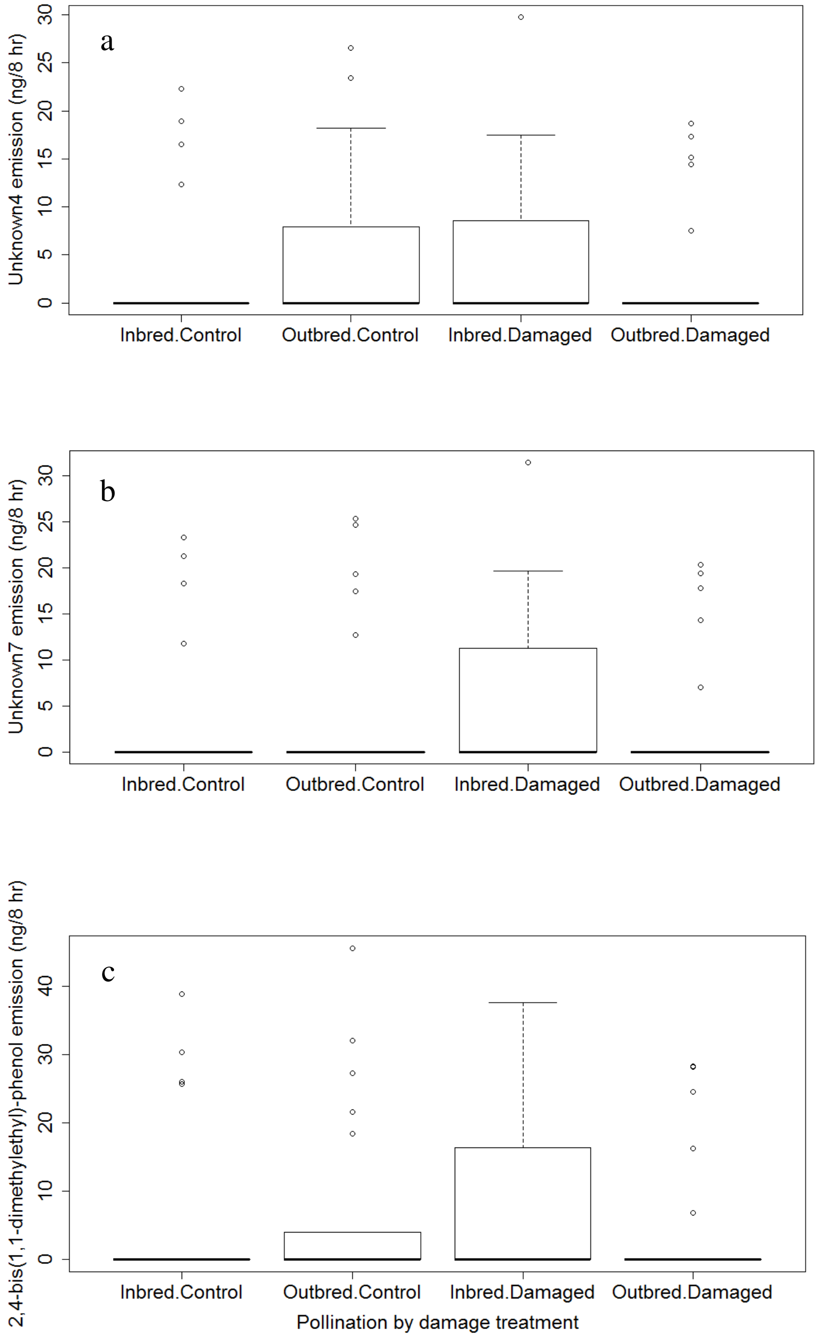

Figure 3. Distributions of emission rates by pollination and damage level of compounds that exhibited significant pollination × damage interactions: a) unknown4, b) unknown7, c) 2,4-bis(1,1-dimethylethyl)-phenol.

Supplementary Material

Table S1. Foliar volatile compounds emitted by *M. guttatus* (listed in ascending order by retention time), lsmean emission, and 95% confidence limits in undamaged and damaged plants.

Compound	Lsmean undamaged (ng/8hr)	Lower 95% CL undamaged	Upper 95% CL undamaged	Lsmean damaged (ng/8hr)	Lower 95% CL damaged	Upper 95% CL damaged
1-octen-3-ol	1.67	0.51	3.73	15.23	7.69	29.30
Octanal	0.31	-0.19	1.12	1.05	0.21	2.47
Decane	0.23	-0.19	0.88	0.45	-0.09	1.30
Unknown1	0.40	-0.17	1.37	5.71	2.79	10.87
Unknown2	0.99	0.22	2.24	1.63	0.56	3.44
Unknown3	0.43	-0.07	1.20	0.76	0.10	1.81
Unknown4	2.06	0.24	5.68	1.92	0.16	5.64
Unknown5	1.30	0.33	3.00	1.79	0.55	4.02
Nonanal	10.40	5.53	16.77	20.85	13.05	30.47
4,8-dimethyl-1,3,7nonatriene[1]	2.09	0.59	5.00	6.66	2.78	14.54
Unknown6	-0.02	-0.09	0.06	-0.05	-0.12	0.04
Decanal	0.33	-0.15	1.10	0.42	-0.13	1.34
E-2-decenal[1]	1.36	0.29	3.31	1.61	0.36	4.01
Unknown7	1.54	0.29	4.00	1.69	0.33	4.46
3-hydroxy-2,2,4-trimethylpentyl isobutyrate[1]	0.49	-0.01	1.24	0.88	0.21	1.94
Unknown8	1.41	-4.40	7.23	3.75	-2.57	10.07
Unknown9	0.07	-2.62	2.76	1.69	-1.23	4.61
unknown10	0.02	0.08	0.28	0.06	0.04	0.47
2,4-bis(1,1-dimethylethyl)-phenol	9.58	3.14	16.01	10.00	3.28	16.71
Neophytadiene[1]	13.71	7.20	25.38	74.44	39.60	139.18
unknown11	1.81	0.15	5.88	3.58	0.84	10.37

[1] Identified by library search rather than comparison with standards

Chapter 4:

A generalist and a specialist herbivore are differentially affected by inbreeding and trichomes in *Mimulus guttatus*

Abstract

Inbreeding in plants is well-known to affect plant-herbivore interactions, but these effects can vary by herbivore species. The phenotypic effects of inbreeding in plants are similar to the phenotypic effects of environmental stress. Thus, plant defense theories that are used to predict the effects of stress on plant-herbivore interactions might also be able to predict effects of inbreeding on plant-herbivore interactions. If inbred plants exhibit similar phenotypes as plants under environmental stress, the plant stress hypothesis predicts that generalist herbivores will perform better on inbred relative to outbred plants because inbred plants have lower levels of chemical defense. If outbred plants exhibit high levels of defense and high nutritional quality similar to unstressed plants, the plant vigor hypothesis predicts that specialist herbivores, which are more tolerant of plant defenses, will perform better on outbred plants due to their higher nutritional quality. However, these plant defense theories do not account for structural defenses. We used a factorial design to examine effects of inbreeding and trichome density in *Mimulus guttatus* on performance of the generalist, *Helicoverpa zea,* and the specialist, *Junonia coenia*. We also examined preferences of each herbivore for inbred versus outbred plants when given leaf disks and when given only olfactory cues. The generalist *H. zea* developed most efficiently on inbred plants, and the specialist *J. coenia* performed best on outbred plants. Trichomes negatively affected performance of both species, even within the pollination treatment on which each herbivore performed best. *H. zea* did not exhibit strong preferences for inbred versus outbred plants, but *J. coenia* consistently preferred outbred over inbred plants. Our results support the predictions of plant defense theories on the effect of inbreeding on herbivore performance, but trichomes were able to reduce the performance of both herbivores. We

conclude that the ability of plant defense theories to predict inbreeding effects on plant-herbivore interactions may depend on which plant traits are affected by inbreeding.

Introduction

Inbreeding in plants is expected to alter traits that mediate resistance to herbivores, and several recent studies have documented that inbred plants suffer more herbivore damage than outbred plants and herbivore development is enhanced on inbred relative to outbred plants (Carr and Eubanks 2014). This pattern is likely explained by increased homozygosity that results from inbreeding (Charlesworth and Charlesworth 1987, Husband and Schemske 1996), which could express recessive alleles that reduce chemical and structural defenses and nutritional quality. However, inbreeding effects on resistance can vary depending on the species of herbivore. In particular, herbivores exhibiting different host breadths are thought to exhibit different levels of tolerance to plant chemical defenses, and could therefore impose contrasting selection pressures on plant defenses (Strauss and Irwin 2004, Ali and Agrawal 2012). Despite the number of studies that document negative effects of inbreeding on plant resistance, we currently know little about how inbreeding affects resistance to herbivores that differ in their host breadth.

Generalist herbivores are often negatively impacted by plant secondary compounds, whereas many specialists have evolved counterdefenses to these chemicals (Gatehouse 2002). If inbreeding reduces production of secondary compounds, then generalists might respond more positively than specialists to these reduced defenses. Hull-Sanders and Eubanks (2005) noted that the phenotypic effects of inbreeding, such as reduced growth, biomass, and reproductive success (Charlesworth and Charlesworth 1987, Husband and Schemske 1996) are similar to the phenotypic effects of environmental stress, which are also known to alter resistance and tolerance of plants to herbivory (Koricheva and Larsson 1998). Plant defense theories have been developed to help predict interactions between stressed plants and herbivores (Price 1991, Stamp 2003), and perhaps these theories could also be used to predict interactions between inbred plants and herbivores. Stressed plants are expected to have low levels of defense and therefore greater vulnerability to herbivores (plant stress hypothesis; White 1969, Stamp 2003). A similar pattern might be expected with inbred plants. On the other hand, the plant vigor hypothesis predicts that unstressed, vigorous plants should have high nutritional quality and high levels of defense (Price 1991). Specialist herbivores are more tolerant of plant defenses and are therefore more affected by nutritional quality than defenses. Thus, if phenotypes of outbred plants are analogous to phenotypes of unstressed plants, then specialist herbivores might be expected to perform better when reared on outbred relative to inbred plants.

Hull-Sanders and Eubanks (2005) tested the plant stress and plant vigor hypotheses using two specialist tortoise beetles, a generalist aphid species, and a generalist moth species on inbred and outbred *Ipomoea hederacea*. As expected, both specialists performed better when reared on outbred plants, and the generalist aphid populations performed better on inbred plants. However, the larvae of the generalist moth performed better on outbred plants, contradicting the plant stress hypothesis. To our knowledge, this is the only study to assess effects of plant inbreeding on herbivores with different degrees of specialization.

Herbivore larvae maximize their fitness if they are able to locate and identify the plants on which they will perform best. Thus, if inbreeding affects host plant quality for a particular herbivore, inbreeding might also be expected to affect attractiveness of a plant to that herbivore. Several lines of evidence suggest that the specialist herbivore *Manduca sexta* is able to discriminate between inbred and outbred *Solanum carolinense* plants on the basis of host plant quality. For example, larvae of *M. sexta* were found to prefer, consume more, and perform better on inbred *Solanum carolinense* (Delphia et al. 2009). Foliar volatile signals provide key host-location cues for insect herbivores, and in pairwise choice tests, *M. sexta* larvae exhibited a preference for olfactory cues from inbred *S. carolinense* (Kariyat et al. 2014). In addition, inbreeding reduced phenolic investment and diversity in *S. carolinense*, and this attenuated defense in inbred plants led to greater larval mass and consumption by *M. sexta* on inbred plants (Campbell et al. 2012).

Since *M. sexta* is a specialist on members of the Solanaceae, the findings that demonstrate that it prefers and performs best on inbred plants directly contradict the plant stress and plant vigor hypotheses. However, consistent with these plant defense theories, generalist herbivores were more attracted to volatile phenotypes of inbred relative to outbred *S. carolinense* (Kariyat et al. 2012). Other work in *S. carolinense* has demonstrated that inbreeding affects plant defenses that are not accounted for in the plant stress and plant vigor hypotheses. Stellate trichomes and internodal spines may be key to mitigating interactions between *M. sexta* and *S. carolinense*. These structural defenses were found to deter feeding, negatively impact growth, and damage the peritrophic membrane of *M. sexta* (Kariyat et al. 2017), but inbreeding reduced their production in *S. carolinense* (Kariyat et al. 2013). If the benefits to *M. sexta* of the reduced structural defenses outweighed the potential costs of reduced host vigor and altered chemistry, this could explain why this example runs counter to plant stress and vigor hypotheses' predictions.

The herb *Mimulus guttatus* exhibits broad ecological diversity and mating system variation, and is therefore an excellent model system for evolutionary and ecological studies (Wu et al. 2008). In natural populations, *M. guttatus* experiences herbivory from a variety of generalist herbivores (Holeski 2007a), and *M. guttatus* exhibits a number of defense traits that could affect interactions with its herbivores. One of these traits is the presence of glandular trichomes on its leaves. In previous work, higher trichome density did not deter feeding by the generalist xylem-feeding spittlebug, *Philaenus spumarius,* in a no-choice experiment, and in choice tests, *P. spumarius* did not discriminate against plants with high trichome density (Holeski 2007a). However, *P. spumarius* feeds on xylem fluid and does not cause direct foliar damage. Trichomes in other species have been shown to deter leaf-feeding (Loe et al. 2007), inhibit insect movement (Medeiros and Moreira 2002), and in glandular form they can also contain volatile and nonvolatile secondary metabolites that are repellant or toxic to herbivores (Paré and Tumlinson 1997, Laue et al. 2000). To our knowledge, no studies have examined effects of *M. guttatus* trichomes on foliar herbivores.

M. guttatus also synthesizes a variety of phenylpropanoid glycosides (PPGs) in its foliage (Holeski et al. 2013, Keefover-Ring et al. 2014), which have been found to act as feeding deterrents to generalist herbivores (Mølgaard 1986). However, one of these PPGs, verbascoside, functions as a feeding stimulant and increases performance of *Junonia coenia* (Holeski et al. 2013), a specialist lepidopteran herbivore that commonly feeds on *M. guttatus* (Bowers 1984). In previous work, larval mass of *J. coenia* fed artificial diets that contained verbascoside was four times greater than larval mass of *J. coenia* reared on diets without verbascoside (Holeski et al. 2013). This suggests that generalist and specialist herbivores could exert different selection pressures on foliar chemistry in *M. guttatus* (Lankau 2007).

Previous work has examined effects of inbreeding in *M. guttatus* on performance of the generalist, *P. spumarius,* and found that inbreeding effects can vary by population (Carr and Eubanks 2002). In particular, *P. spumarius* reared on inbred plants from one population emerged as significantly larger adults than those reared on outbred plants, indicating that in this population, inbreeding increased performance. However, in a second *M. guttatus* population, *P. spumarius* nymphs took significantly longer to develop into adults when reared on inbred plants, indicating that in this population, inbreeding decreased performance. No studies have examined response to inbreeding in *M. guttatus* of herbivores with different levels of host breadth.

The current study examines larval preferences and performance of a generalist lepidopteran herbivore, *Helicoverpa zea*, and the specialist, *J. coenia*, in response to inbreeding and trichome density in *M. guttatus*. Trichome density is not affected by inbreeding in *M. guttatus* (Carr *unpublished data*), and this allowed us to examine effects of trichomes independently from other inbreeding effects. We used a factorial design with experimentally inbred and outbred *M. guttatus* plants selected for low or high trichome densities to address the questions: 1) How do inbreeding and trichomes affect herbivore performance? 2) Do herbivores exhibit preferences for inbred vs. outbred plants? 3) Do olfactory cues play a role in herbivore preferences? 4) Do *H. zea* and *J. coenia* respond in different ways to inbreeding and trichomes?

Materials and Methods

Study species

Mimulus guttatus (Phrymaceae, Beardsley and Olmstead 2002) is an herb native to open, wet habitats throughout western North America from Mexico to Alaska. Populations are usually annual, but can be perennial in areas that are wet year-round (Dole 1992). It produces hermaphroditic, yellow flowers, and can produce over 100 flowers in a season. *M. guttatus* populations exhibit mixed mating systems with outcrossing rates that range from approximately 75% selfing ($t = 0.25$) to complete outcrossing ($t = 1.0$), averaging $t \approx 0.60$ (Ritland and Ritland 1989). Its leaves contain glandular trichomes. Trichome density is variable within and among *M. guttatus* populations (Holeski 2007b).

The *M. guttatus* plants used in this study were descendants of seeds collected from over 500 random maternal families from an annual population in Napa County, CA (Snell Valley: 38° 42’ 02” N, 122° 24’ 29” W). We randomly crossed one plant from each field-collected maternal family in a greenhouse at Blandy Experimental Farm (Boyce, VA), thus providing a base generation of completely outcrossed plants. The current study used two subpopulations of plants derived from this base population, each comprising a different set of maternal families. One subpopulation was subjected to three generations of selection for high leaf trichome density, and the other was subjected to three generations of selection for low leaf trichome density. For each of 19 maternal families, we used plants that had been fully outcrossed (F=0) and plants that had passed through one generation of self-pollination (F=0.5). We performed cross-pollinations by rubbing pollen from a randomly chosen, unrelated plant onto the stigma of an emasculated recipient. We performed self-pollinations by using pollen from a flower to pollinate its own stigma. We collected seeds when fruits began to dehisce.

We sowed approximately 20 seeds from each pollination treatment within each maternal family into a 3-inch square pot filled with commercial growing medium (Fafard 3 Mix, Conrad Fafard, Agawam, MA). We arranged pots randomly in trays (20 pots per tray) with bottom watering. After the first true leaves appeared (approximately 14 days), we transplanted seedlings into individual pots that we arranged randomly with 20 pots per tray and bottom watering. We kept plants in a greenhouse at Blandy Experimental Farm.

We used one generalist herbivore, *Helicoverpa zea* (Lepidoptera: Noctuidae), and one specialist, *Junonia coenia* (Lepidoptera: Nymphalidae), in our preference and performance assays. *Helicoverpa zea* is an herbivore that can be found throughout North America except northern Canada and Alaska. Its larvae feed on a wide range of host plants, including many cultivated crops. We purchased *H. zea* larvae from Frontier Agricultural Sciences (Newark, DE) that were reared on a wheat germ and soy flour-based diet prior to behavioral assays. *Junonia coenia* is a specialist herbivore that inhabits much of the United States and Mexico. Its larvae commonly feed on *M. guttatus* (Holeski et al. 2013), as well as on plants that contain iridoid glycosides, such as *Plantago lanceolata* (Bowers 1984). We purchased *J. coenia* larvae from Shady Oak Butterfly Farm (Brooker, FL) that were reared on *P. lanceolata* prior to behavioral assays.

Performance Assays

We conducted no-choice tests to assess the performance of *H. zea* and *J. coenia*. At the start of this experiment, *H. zea* larvae were at approximately fourth instar and *J. coenia* were approximately third instar. This experiment used a factorial design with pollination (inbred or outbred) and trichome density (high or low) as treatments. We made cages from 710-ml up&up^{TM} containers by replacing a portion of the lid with breathable mesh. We put each individual caterpillar into its own cage and randomly assigned it to one of the four pollination × trichome treatments (15 caterpillars per pollination × trichome treatment for each species). We gave leaves to each caterpillar in its cage from plants of its assigned treatment from a combination of maternal families allowed it to feed *ad libitum* until pupation.

We evaluated the performance of each herbivore species by quantifying survival, larval mass gain, development time, and pupal mass. We recorded the mass of each larva at the beginning of the no-choice assay, and once each day thereafter until it pupated. We also recorded whether or not each larva survived to pupation, and if so, the number of days until pupation and the mass of each pupa. To assess whether larval performance could be driven by consumption versus digestive efficiency, we also quantified the amount of leaf tissue consumed by each individual from digital images using ImageJ, and we collected and quantified the mass of frass from each larva daily.

Preference Tests

We conducted pairwise choice tests using *H. zea* and *J. coenia* larvae with leaf disks and with olfactory cues from whole plants. For leaf disk assays, we used 16.8-mm diameter leaf disks from the youngest fully-expanded leaf of each plant. For each trial, we randomized paired inbred and outbred disks from the same maternal family to opposite sides of a 150-mm diameter petri dish on moistened filter paper. We placed one caterpillar at the edge of the petri dish equidistant from each disk, and we recorded which disk (inbred or outbred) the caterpillar contacted. We conducted two separate choice tests (one test for each trichome density treatment) for each species. We used a different caterpillar for each trial in a given test (n=40 larvae for each test with *H. zea;* n= 50 larvae in each test with *J. coenia*).

To assess preferences of *H. zea* and *J. coenia* larvae on the basis of olfactory cues, we conducted pairwise choice tests using a Y-tube. We matched pairs of inbred and outbred whole plants from the same maternal family by size and placed them into separate 5-L glass chambers, each randomized to a different arm of the Y-tube. A pressurized tank delivered air through a 2-Channel Air Delivery System (Analytical Research Systems, Gainsville, FL) and through each chamber into the Y-tube. We introduced individual caterpillars at the base of the Y-tube, and caterpillars responded to olfactory cues by walking up one of its arms. We recorded a response once a caterpillar had crossed a designated line. For *H. zea*, we conducted separate tests for high and low trichome plants (n= 40 larvae for each test). Due to limited numbers of *J. coenia* larvae, we were only able to test *J. coenia* olfactory preferences for low trichome plants (n=23 larvae). We used a different caterpillar for each trial within a given test.

Trichome Density

To ensure that the high and low trichome treatments each were comparable between inbred and outbred treatments, we quantified trichome density for inbred and outbred plants in each maternal family. We counted trichomes on the adaxial side of two 3.6-mm diameter disks from each of the two oldest fully-expanded leaves in each plant. Mean ± standard error trichome counts for inbred high trichome, outbred high trichome, inbred low trichome, and outbred low trichome plants, respectively, were 123 ± 14, 121 ± 16, 51 ± 10, and 44 ± 8. A factorial ANOVA with pollination and trichome treatments and their interactions as fixed effects demonstrated that trichome densities were significantly different between the high and low trichome treatments ($F_{1,28}$=38.47, P<0.0001). Pollination treatment did not affect trichome density ($F_{1,28}$=0.14, P=0.7081), nor was there a pollination × trichome interaction ($F_{1,28}$=0.05, P=0.8321). Thus, we concluded that trichome densities in both the high and low trichome density treatments were comparable in inbred and outbred plants.

Statistical Analysis

Performance Assays. We performed all analyses in SAS 9.4 (SAS Institute Inc, 2012). To test the null hypothesis that survival was independent of pollination treatment and trichome density, we conducted separate log linear models for each species in which survival (either survived to pupation or died), pollination treatment, and trichome density were variables.

We analyzed larval total mass gain, days to pupation, pupal mass, leaf area consumption, and frass mass for *H. zea* using general linear mixed models as a factorial design with pollination treatment (inbred or outbred), trichome density (high or low), and their interaction as fixed effects. We log-transformed leaf area consumption and frass mass and we Box-Cox transformed number of days to pupation to meet ANOVA assumptions. When analyses indicated a significant interaction, we conducted the following a-priori orthogonal contrasts: 1) inbred high trichome versus outbred high trichome, and 2) inbred low trichome versus outbred low trichome. Since none of the *J. coenia* in the inbred high trichome treatment survived to pupation, we analyzed larval mass gain, days to pupation, pupal mass, leaf area consumption, and frass mass of *J. coenia* using general linear mixed models with pollination × trichome treatment as the independent variable (3 treatments total: inbred low trichome, outbred low trichome, and outbred high trichome). When these analyses indicated a significant treatment effect, we conducted the following a-priori contrasts: 1) inbred low trichome versus outbred low trichome, and 2) outbred low trichome versus outbred high trichome. Since this pair of contrasts was not orthogonal, we adjusted our alpha level to 0.025 for these contrasts. For all analyses of larval mass gain and days to pupation, we included initial larval mass as a covariate to control for differences in starting larval stages of each individual. Since consumption and defecation are expected to be greater for larvae that take longer to pupate, we included days to pupation as a covariate in analyses of leaf area consumption and frass mass.

Preference Tests. To test the null hypothesis that caterpillars chose plants randomly in choice tests with leaf disks and in Y-tube choice tests, we conducted chi-squared goodness-of-fit tests with the null expectation that an equal number of caterpillars would choose inbred and outbred plants.

Results

Helicoverpa zea performance

On average, 71% of *H. zea* larvae survived to pupation, and survival did not differ significantly among pollination or trichome treatments (survival × pollination: χ^2_1=1.37, *P*=0.24;

survival × trichomes: χ^2_1=0.57, *P*=0.45; Table S1). Larval mass gain, number of days to pupation, and pupal mass of *H. zea* also did not exhibit significant differences between pollination treatments (mass gain: $F_{1,55}$= 1.31, *P*=0.26; days to pupation: $F_{1,37}$=0.53, *P*=0.47; pupal mass: $F_{1,38}$=0.00, *P*=0.98; Table S1), trichome treatments (mass gain: $F_{1,55}$= 0.03, *P*=0.87; days to pupation: $F_{1,37}$=1.18, *P*=0.29; pupal mass: $F_{1,38}$=1.69, *P*=0.20; Table S1), or their interaction (mass gain: $F_{1,55}$= 2.15, *P*=0.15; days to pupation: $F_{1,37}$=2.93, *P*=0.10; pupal mass: $F_{1,38}$=1.26, *P*=0.27; Table S1). However, pollination treatment, trichome density, and their interaction had a significant effect on leaf area consumption (pollination: $F_{1,37}$=22.03, *P*<0.0001; trichome density: $F_{1,37}$=18.43, *P*=0.0001; pollination × trichome density: $F_{1,37}$=21.81, *P*<0.0001; Figure 1a). Pairwise contrasts demonstrated that within the low trichome density treatment, *H. zea* consumed 1.90 times more leaf area when they were fed outbred than inbred plants ($F_{1,37}$=49.23, *P*<0.0001), but there was no difference in leaf consumption from inbred and outbred plants with high trichome density ($F_{1,37}$=0.01, *P*= 0.92). Effects of pollination and trichome treatments on frass production of *H. zea* exhibited a similar pattern to leaf area consumption (pollination: $F_{1,37}$=4.26, *P*=0.046; trichome density: $F_{1,37}$=6.30, *P*=0.017; pollination × trichome density: $F_{1,37}$=5.05, *P*=0.031; Figure 1b). Within the low trichome density treatment, larvae fed outbred plants defecated 1.58 times more than those fed inbred plants ($F_{1,37}$=10.44, *P*=0.0026), but there was no difference in frass production when feeding on inbred and outbred plants with high trichome density ($F_{1,37}$=0.03, *P*= 0.86). Since *H. zea* reared on inbred low trichome plants consumed significantly less leaf tissue but mass gain, development time, and pupal mass were equivalent across all treatments, this indicated that *H. zea* fed inbred low trichome plants developed more efficiently than those fed other treatments.

Junonia coenia performance

Survival to pupation of *J. coenia* larvae was dependent on pollination (χ^2_1=4.40, *P*=0.036) and trichome density (χ^2_1=5.41, *P*=0.02). In particular, none of the caterpillars fed inbred high trichome plants survived to pupation (Figure 2). Survival was 53% in the inbred low trichome treatment, 47% in outbred high trichome plants, and 93% in outbred low trichome plants. Larval mass gain, number of days to pupation, and pupal mass of *J. coenia* all exhibited significant differences between pollination × trichome treatments (mass gain: $F_{2,23}$=19.57, *P*<0.0001; development time: $F_{2,24}$=29.57, *P*<0.0001; pupal mass: $F_{2,24}$=30.36, *P*<0.0001; Figure 3). Pairwise contrasts indicated significant differences between inbred and outbred plants within the low trichome treatment. Specifically, larvae gained 2.31 times more mass ($F_{1,23}$=33.80, *P*<0.0001), took 46% less time to pupate ($F_{1,24}$=58.85, *P*<0.0001), and exhibited a pupal mass that was 1.54 times greater ($F_{1,24}$=57.73, *P*<0.0001) on outbred than inbred plants. Contrasts also indicated that trichome density had a significant effect within outbred plants. Mass gain was 1.99 times greater ($F_{1,23}$=20.95, *P*=0.0001), development time was 26% faster ($F_{1,24}$=8.17, *P*=0.0087), and pupal mass was 1.28 times higher ($F_{1,24}$=18.10, *P*=0.0003) for larvae fed low trichome than high trichome plants. Leaf area consumption was also greatest on outbred low trichome plants ($F_{2,24}$ =15.47, *P*<0.0001; Figure 4a). Contrasts indicated that within low trichome plants, leaf consumption was 1.59 times greater on outbred plants ($F_{1,24}$=27.52, *P*<0.0001), and within outbred plants, leaf consumption was 1.24 times greater on low trichome plants ($F_{1,24}$=9.94, *P*=0.0043). Larvae also defecated the most when fed outbred low trichome plants ($F_{2,24}$ =14.59, *P*<0.0001; Figure 4b). Contrasts indicated 2.4 times more frass from larvae fed outbred than inbred plants within the low trichome treatment ($F_{1,24}$=28.95, *P*<0.0001) and 1.2 times more frass from larvae fed low than high trichome outbred plants ($F_{1,24}$=3.47, *P*=0.075). Thus, *J.*

coenia performed best on outbred low trichome plants, and this is likely explained in part by greater consumption of these plants.

Helicoverpa zea preference

H. zea showed little evidence of a preference for either inbred or outbred plants. In pairwise choice tests using leaf disks from high trichome plants, 26 of 40 *H. zea* caterpillars chose disks from inbred plants (χ^2_1=3.6, *P*=0.058). When presented leaf disks from low trichome plants, 19 of 40 caterpillars chose inbred plants (χ^2_1=0.10, *P*=0.75). In pairwise tests for olfactory cues from whole plants with high trichome density, 22 of 40 caterpillars chose inbred plants (χ^2_1=0.4, *P*=0.53). When given a choice between olfactory cues from an inbred or outbred plant with low trichome density, 18 of 40 caterpillars chose inbred plants (χ^2_1=0.4, *P*=0.53).

Junonia coenia preference

J. coenia showed a fairly consistent pattern of preference for outbred plants. In pairwise choice tests for leaf disks from high trichome plants, 32 of 50 *J. coenia* caterpillars chose leaf disks from outbred plants (χ^2_1=3.92, *P*=0.048). In choice tests for leaf disks from low trichome plants, 33 of 50 caterpillars chose outbred plants (χ^2_1=5.12, *P*=0.024). When presented olfactory cues from low trichome plants, 16 of 23 caterpillars chose outbred plants (χ^2_1=3.52, *P*=0.061).

Discussion

We found that the generalist, *Helicoverpa zea*, consumed and defecated more when it fed on outbred *Mimulus guttatus*. Plants that experience greater leaf consumption are often considered to be less resistant to herbivores (e.g., Delphia et al. 2009). However, our study demonstrated that the larvae that consumed the greatest leaf area (those reared on outbred plants) exhibited the same survival rate, growth rate, pupal mass, and development time as those reared on inbred plants. Herbivores have been found to increase consumption of low-quality host plants to compensate for low post-ingestive efficiency (Kause et al. 1999). Larvae were allowed to feed *ad libitum* in our study, but in a natural setting, the reduced development efficiency on outbred plants could require spending more time feeding and slow development time, thereby increasing exposure and vulnerability to natural enemies. We do not know the mechanisms underlying the reduced efficiency on outbred *M. guttatus*, but inbreeding in *Solanum carolinense* is known to reduce production of defense-related secondary metabolites (Campbell et al. 2012). If inbreeding reduces production of defensive chemical compounds such as enzyme inhibitors, then an herbivore that is negatively affected by these compounds would be expected to develop most efficiently on inbred plants.

In contrast with our results from *H. zea*, inbreeding in *M. guttatus* reduced performance of the specialist, *Junonia coenia.* Larvae of *J. coenia* exhibited greater mass gain and pupal mass and a faster development time when reared on outbred plants. Specialist herbivores are known to have evolved counterdefenses to secondary compounds in plants, and are expected to be more affected by plant nutritional quality than by plant defenses (Price 1991). As mentioned earlier, inbreeding is known to reduce defensive compounds in *S. carolinense* (Campbell et al. 2012), but inbreeding effects on *M. guttatus* secondary chemistry are unknown. *M. guttatus* is known to contain phenylpropanoid glycosides (PPGs; Holeski et al. 2013, Keefover-Ring et al. 2014), and one of these PPGs, verbascoside, has been shown to act as a feeding stimulant and increase larval mass of *J. coenia* (Holeski et al. 2013). Leaf consumption by *J. coenia* was also greatest on outbred plants, suggesting that there may be greater levels of feeding stimulant in these plants. If inbreeding reduces production of feeding stimulants, such as verbascoside, this could explain the lower consumption and performance of *J. coenia* on inbred plants.

The performance of both *H. zea* and *J. coenia* was negatively affected by trichomes, but our results suggest that trichomes could have interfered with each herbivore through a different mechanism. *Helicoverpa zea* reared on high trichome plants required higher consumption of leaf tissue to achieve the same mass gain and pupal mass and to develop as quickly as those reared on low trichome plants. Glandular trichomes in other species often secrete organic compounds that are toxic to herbivores (Ranger et al. 2004). Since trichomes seemed to interfere with development and did not inhibit feeding, this suggests that glandular trichomes in *M. guttatus* may contain compounds that negatively affect *H. zea* development. *Junonia coenia* reared on high trichome plants consumed less leaf tissue and exhibited lower performance, which suggests that trichomes could have interfered with feeding. It is possible that, unlike compounds in foliar tissue that may be attractive and stimulate feeding, there are compounds in trichomes that deter *J. coenia*. Furthermore, high densities of trichomes can impede movement and feeding (Wilkens et al. 1996), and thus, trichomes could function as a physical defense to *J. coenia*. Although *H. zea* performed better on inbred plants and *J. coenia* performed better on outbred plants, trichomes seemed to lessen the benefits of feeding on inbred and outbred plants for *H. zea* and *J. coenia*, respectively. Specifically, the development efficiency of *H. zea* reared on inbred high trichome plants was reduced to the efficiency of *H. zea* reared on outbred plants. Performance of *J. coenia* was consistently better in low than high trichome outbred plants, and survival of *J. coenia* was almost equivalent in outbred high trichome and inbred low trichome plants, suggesting that trichomes prevented *J. coenia* from taking advantage of the otherwise high-quality outbred plants. Moreover, in the lower-quality inbred plants, *J. coenia* did not survive at all when plants had high trichome densities. Thus, trichomes could function as an additional line of defense to foliar secondary metabolites that is effective even against specialist herbivores.

The generalist *H. zea* demonstrated a marginal preference for inbred plants in pairwise choice tests using leaf disks from plants that were selected for high trichome density. However, *H. zea* did not exhibit any preference for inbred versus outbred plants when given leaf disks from low trichome plants or when given olfactory cues from *M. guttatus* plants. The specialist *J. coenia* preferred outbred plants in leaf disk tests using both high and low trichome plants. Although our sample size in Y-tube choice tests with *J. coenia* was low, *J. coenia* also exhibited a marginal preference for olfactory cues from outbred plants. Previous studies have found that specialist herbivores are better than generalists at choosing their best hosts. For example, specialist species of butterflies chose to oviposit on individual *Urtica dioica* plants that yielded the highest survival and fastest growth rate, but generalist butterfly species failed to demonstrate a preference for higher-quality plants (Janz and Nylin 1997). In addition, specialist populations of *Uroleucon ambrosiae* chose their hosts faster than generalist populations of this species (Bernays and Funk 1999). On the other hand, our preference tests only considered larvae that were naïve (they had never been exposed to *M. guttatus*). Previous work indicates that lepidopteran larvae are able to modify host plant preferences through associative learning (Pszczolkowski and Brown 2005, Blackiston et al. 2008). Since generalist herbivores have a larger range of host plants from which to choose, associative learning is expected to have a greater fitness benefit to generalists than specialists (Bernays 2001). It is possible that *H. zea* would have preferred their best host plants if they had been given an opportunity to learn cues from *M. guttatus*. Moreover, our preference tests included only undamaged *M. guttatus* plants, but evidence suggests that herbivore damage alters the foliar volatile profile of a number of plant species (e.g., De Moraes et al. 1998, Kessler and Baldwin 2001, Kariyat et al. 2012), including *M. guttatus* (Haber et al. *unpublished data*). Thus, we would have a more complete picture of how inbreeding affects herbivore preferences if we had investigated preferences for damaged in addition to undamaged plants.

To our knowledge, only one other study has examined the effects of plant inbreeding on herbivores with different levels of specialization. Specialist herbivores consistently performed best on outbred *Ipomoea hederacea* (Hull Sanders and Eubanks 2005), just as we observed in the specialist *J. coenia* on outbred *M. guttatus*, supporting predictions from the plant stress hypothesis (Stamp 2003) and the plant vigor hypothesis (Price 1991). Hull-Sanders and Eubanks (2005) also tested for preferences for one of the two specialists and as we found with *J. coenia*, these herbivores preferred outbred over inbred plants. One of two generalists in the Hull-Sanders and Eubanks (2005) study performed best on inbred plants, and *H. zea* in our study developed most efficiently on inbred plants. Although these studies suggest that plant defense theories can be useful to predict plant-herbivore dynamics, a number of other studies demonstrate patterns that are variable and sometimes even conflicting with these defense theories. The generalist *Philaenus spumarius* performed best on inbred plants from one *M. guttatus* population, but performed best on outbred plants in a second *M. guttatus* population (Carr & Eubanks 2002). The specialist *Abrostola asclepiadis* did not differ in biomass when reared on inbred versus outbred *Vincetoxicum hirundinaria* (Muola et al. 2011). Studies on *Solanum carolinense* and its specialist *Manduca sexta* demonstrate that *M. sexta* performs best on inbred plants (Delphia et al. 2009, Campbell et al. 2012). Our results demonstrate that herbivore responses to physical defenses, which are not addressed in these plant defense theories (Price 1991, Stamp 2003), could limit their predictive power. Furthermore, inbreeding effects on plant resistance to herbivores have been found to be influenced by a population's history of inbreeding and herbivory (Leimu et al. 2008). This also suggests the potential for these factors to influence the response of herbivore performance to inbreeding in ways that conflict with existing defense theories.

Inbreeding depression is thought to play a key role in preventing the evolution of self-pollination in plants (Charlesworth and Charlesworth 1987). Models of evolution from outcrossing to selfing predict that in an initially outcrossing population, evolution of selfing is prevented if progeny from self-pollination experience at least a 50% reduction in fitness relative to fully outbred progeny (Lande and Schemske 1985). In these cases, self-fertilization is thought to be maintained for reproductive assurance in environments where pollinator visitation is unpredictable (Kalisz and Vogler 2003, Kalisz et al. 2004). However, self-pollination is known to also affect traits that mediate resistance to herbivores (Carr and Eubanks 2014). If inbreeding decreases plant resistance, then herbivores might select against self-pollination in a plant population. However, if inbreeding increases plant resistance to herbivores that are present at high densities, herbivory could select for high frequencies of self-pollination (Ashman 2002). The effect of inbreeding on the amount of damage inflicted on a plant may differ between specialist and generalist herbivores, and this could therefore produce different selection pressures on mating system evolution. In the current study, however, inbred plants received less damage from both the specialist and generalist, although for apparently different reasons. This suggests both could select for self-pollination in *M. guttatus*.

Our findings demonstrate that inbreeding in *M. guttatus* has opposing effects on a generalist versus a specialist herbivore, and these patterns support the Plant Vigor and Plant Stress Hypotheses. As expected, the generalist developed most efficiently on inbred plants, and the specialist performed best on and preferred outbred plants. Moreover, we investigated effects of trichomes independently from other inbreeding effects and demonstrated that inbreeding effects on herbivore performance were muted at high trichome densities. These results suggest that plant defense theories can be useful to predict effects of inbreeding on plant-herbivore interactions, but that the relationship between these theories and inbreeding may depend on which specific plant traits are affected by inbreeding.

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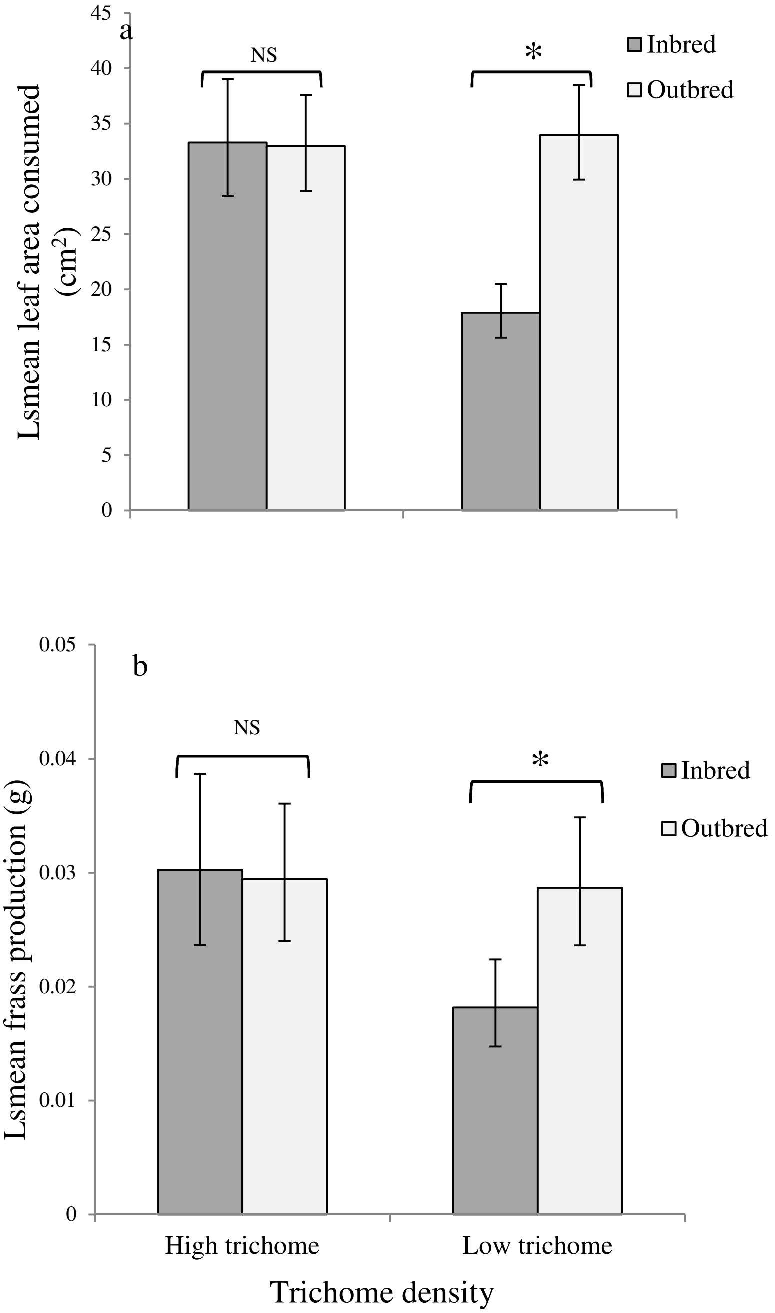

Figure 1. (a) Leaf area consumed and (b) frass production of *H. zea* fed each *M. guttatus* treatment. Error bars indicate 95% confidence limits. Asterisks indicate pairwise contrast is significant; NS indicates pairwise contrast is nonsignificant

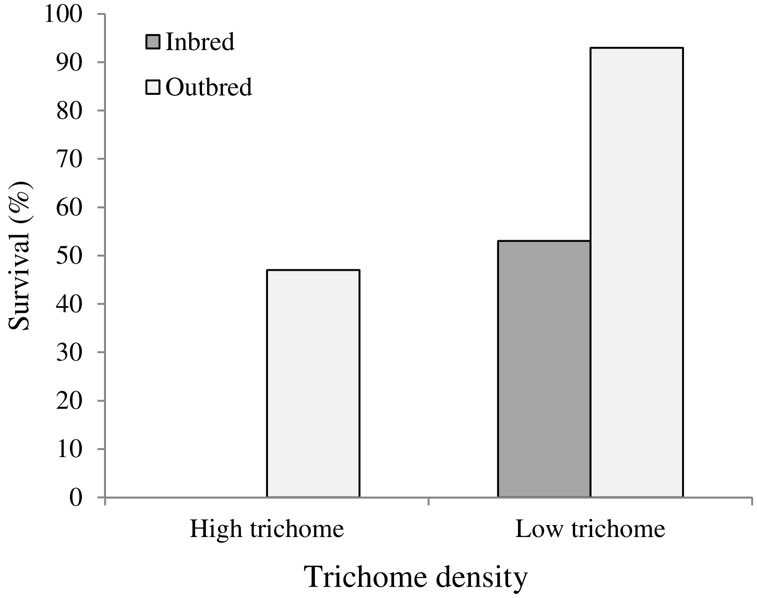

Figure 2. Percent of *J. coenia* that survived to pupation when fed each treatment of *M. guttatus*. Fifteen caterpillars were used in each pollination × trichome treatment. The inbred, high trichome treatment yielded 0% survival

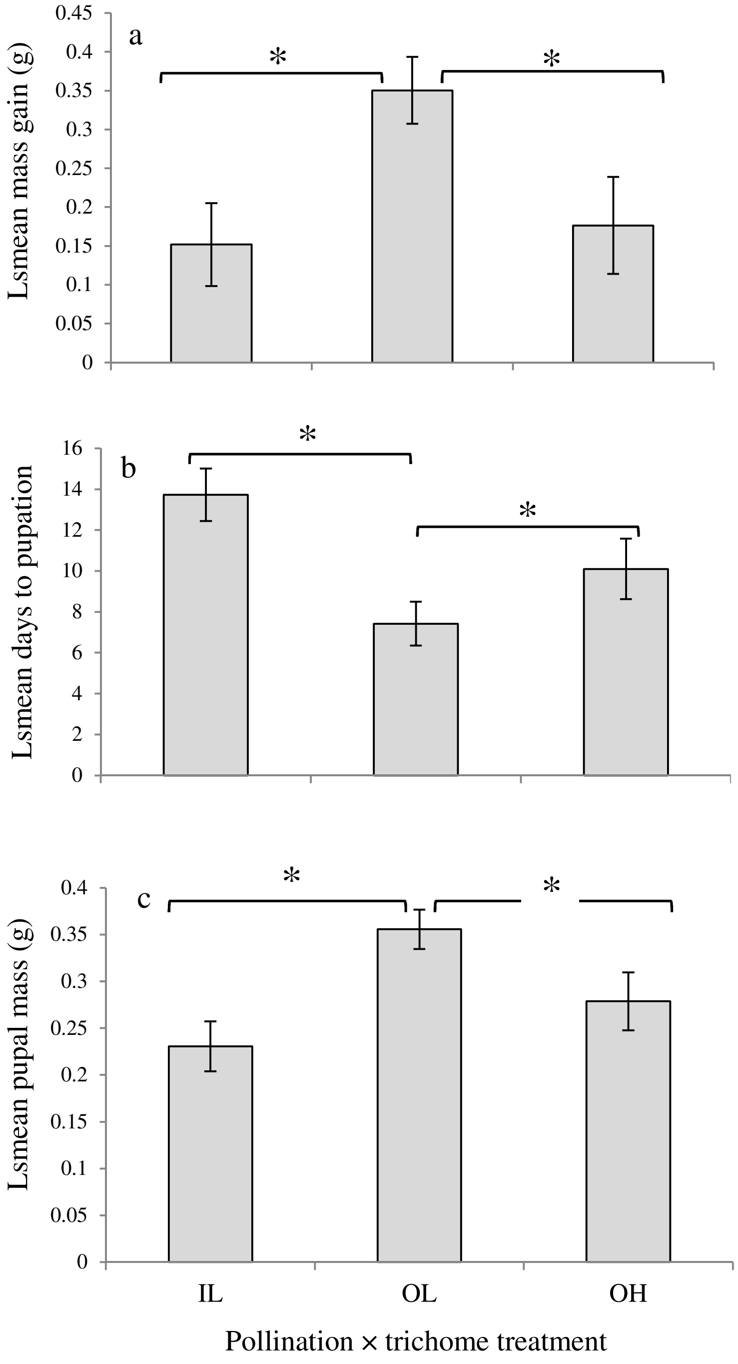

Figure 3. (a) Mass gain, (b) days to pupation, and (c) pupal mass of *J. coenia* larvae fed each *M. guttatus* treatment. Treatments include inbred low trichome (IL), outbred low trichome (OL), and outbred high trichome (OH). Error bars indicate 95% confidence limits. Asterisks indicate significant pairwise contrasts

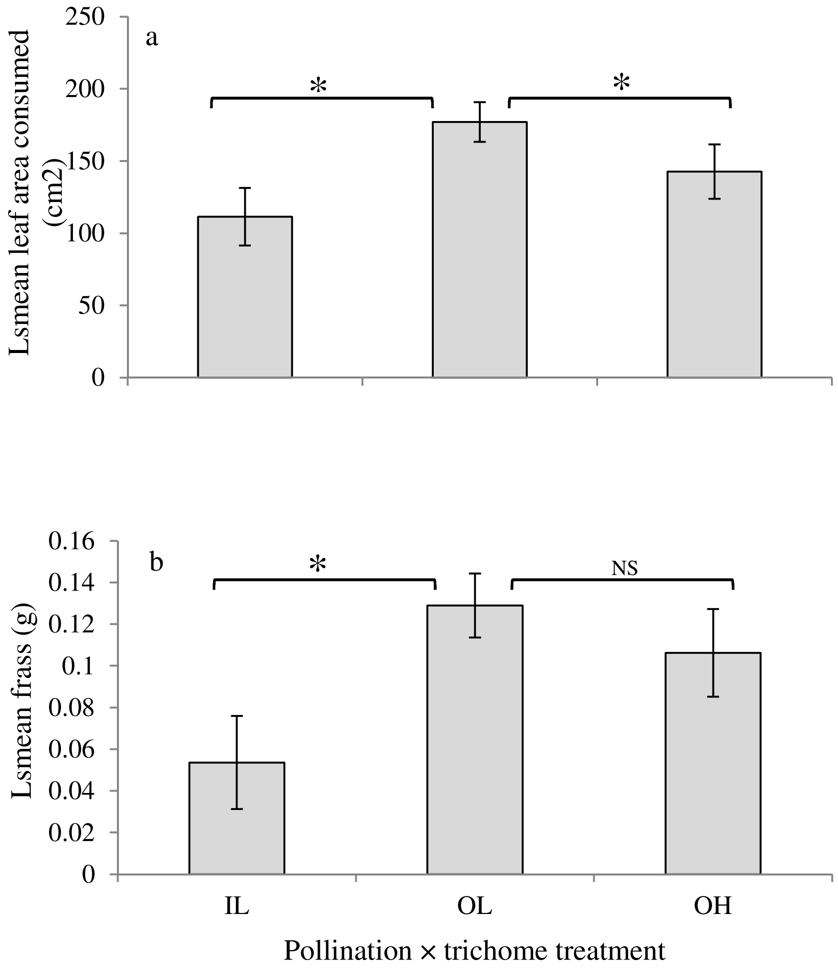

Figure 4. (a) Leaf area consumed, and (b) frass production of *J. coenia* fed each *M. guttatus* treatment. Treatments include inbred low trichome (IL), outbred low trichome (OL), and outbred high trichome (OH). Error bars indicate 95% confidence limits. Asterisks indicate significant pairwise contrasts; NS indicates a nonsignificant pairwise contrast

Supplementary Material

Table S1. Percent survival to pupation and lsmeans (and 95% confidence intervals) for mass gain, development time, and pupal mass of *Helicoverpa zea* reared on each pollination × trichome treatment of *Mimulus guttatus* plants.

	Inbred high trichome	Inbred low trichome	Outbred high trichome	Outbred low trichome
Survival (%)	53.3	73.3	78.6	80.0
Mass gain (g)	-0.1605 (-0.1738, -0.1471)	-0.1519 (-0.1651, -0.1386)	-0.1433 (-0.1564, -0.1301)	-0.1540 (-0.1672, -0.1408)
Days to pupation	5.59 (5.07, 6.09)	4.95 (4.47, 5.41)	5.04 (4.56, 5.49)	5.18 (4.74, 5.60)
Pupal mass (g)	0.2008 (0.1679, 0.2337)	0.2356 (0.2075, 0.2636)	0.2166 (0.1885, 0.2446)	0.2191 (0.1922, 0.2459)