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The European wool-carder bee (*Anthidium manicatum*) eavesdrops on plant volatile organic compounds (VOCs) during trichome collection

Kelsey K. Graham[a],[*], Steve Brown[b], Stephanie Clarke[a], Ursula S.R. Röse[c], Philip T. Starks[a]

[a] Department of Biology, Tufts University, Medford, MA 02155, USA
[b] Northern Essex Community College, Haverhill, MA 01830, USA
[c] Department of Biology, University of New England, Biddeford, ME 04005, USA

ABSTRACT

The plant-pollinator relationship is generally considered mutualistic. This relationship is less clear, however, when pollinators also cause tissue damage. Some Megachilidae bees collect plant material for nests from the plants they pollinate. In this study, we examined the relationship between *Anthidium manicatum*, the European wool-carder bee, and the source of its preferred nesting material − *Stachys byzantina,* lamb's ear. Female *A. manicatum* use their mandibles to trim trichomes from plants for nesting material (a behaviour dubbed "carding"). Using volatile organic compound (VOC) headspace analysis and behavioural observations, we explored (a) how carding effects *S. byzantina* and (b) how *A. manicatum* may choose specific *S. byzantina* plants. We found that removal of trichomes leads to a dissimilar VOC bouquet compared to intact leaves, with a significant increase in VOC detection following damage. *A. manicatum* also visit *S. byzantina* plants with trichomes removed at a greater frequency compared to plants with trichomes intact. Our data suggest that *A. manicatum* eavesdrop on VOCs produced by damaged plants, leading to more carding damage for individual plants due to increased detectability by *A. manicatum*. Accordingly, visitation by *A. manicatum* to *S. byzantina* may incur both a benefit (pollination) and cost (tissue damage) to the plant.

1. Introduction

Insect damage can cause cascading chemical changes within plants that help with healing and protect against further herbivore damage (Karban and Myers, 1989). However, these chemical changes are not just within the plant. Plants can change the volatile organic compounds (VOCs) they emit in response to damage, which can affect the surrounding community of plants, herbivores, and beneficial plant visitors such as predatory and parasitic wasps (Holopainen and Gershenzon, 2010; Pare and Tumlinson, 1999). Here, we investigate a relatively understudied plant-insect relationship − flowering plants and Hymenopteran visitors that can act as both mutualists and antagonists.

Many Hymenoptera have evolved to use changes in plant VOCs for their benefit. For example, predatory and parasitic wasps can use changes in VOCs as a way to locate lepidopteran hosts (reviewed in Pare and Tumlinson, 1999). Herbivore damage induces changes in plant VOCs, either causing an increase in production of VOCs, or a change in compounds emitted. Wasps have evolved to use this plant response as a signal for presence of potential prey (predatory wasps) or hosts (parasitic wasps) (Turlings et al., 1990). Following herbivore

damage, plants such as corn and cotton have been shown to produce VOCs unique to herbivore damage that are distinguishable from background odours. The release of these VOCs coincides with periods when parasitoids are most likely to be foraging, contributing to recruitment of beneficial parasitoids by the plant (Turlings et al., 1995).

Bees foraging for nectar and pollen have also evolved the ability to use plant VOCs as a signal of resource quality. However, unlike predatory and parasitic wasps, foraging bees generally associate these cues with a lower quality resource (pollen and nectar), and avoid these herbivore damaged plants (Kessler and Halitschke, 2007, 2009; Mothershead and Marquis, 2000). For instance, wild tomato flowers (*Solanum peruvianum*) were visited less frequently by pollinators following both real herbivore damage and a jasmonic acid treatment to induce plant response to herbivore damage (Kessler et al., 2011).

Accordingly, changes in plant VOCs may be repellent or attractive to Hymenoptera, largely based on what they are searching for. Generally, we expect changes in plant VOCs following damage to be repellent to Hymenoptera seeking floral resources (typical plant-pollinator relationship), but attractive to Hymenoptera in search of prey or hosts (typical plant-parasitoid relationship). Furthermore, we would expect

VOCs produced by damaged plants to be an evolved signal to attract beneficial parasitoids that can lower herbivore loads. Conversely, we would expect a change in plant VOCs to be a cue for pollinators (non-directed passive transfer of information) since changes in VOCs are often repellent to beneficial pollinators (Bradbury and Vehrencamp, 2001; Smith and David, 2003).

Plant-pollinator interactions are often thought of as mutually beneficial, but the relationship can be complicated when the pollinator also causes damage to the plant. These opposing roles can occur during different life stages of the pollinator; caterpillars might consume plant tissues but later pollinate the same plant as adults (Kessler et al., 2010; Pettersson, 1991; Thompson and Pellmyr, 1992). Conflicts can also occur during the same life stage. Mason bees, leaf-cutter bees, and other members of the family Megachilidae serve as pollinators, but their collection of nesting material can damage the plants they visit.

In this study, we focus on one member in the family Megachilidae, *Anthidium manicatum,* that collects plant trichomes for nest construction. Trichomes are small hairs protruding from the plant epidermis used in water regulation (Fahn, 1986), herbivore deterrence, and storage of VOCs (Levin, 1973). *A. manicatum*, the European wool-carder bee, is a solitary bee species whose common name derives from the females' "carding" behaviour − female bees cut plant trichomes with their mandibles, ball them up (a behaviour reminiscent of carding wool), and fly them back to a cavity where they will use the trichomes to line their nest (Eltz et al., 2015; Müller et al., 1996; Payne et al., 2011). Male *A. manicatum* do not collect nesting material, but instead guard territories of floral resources, including plants used for nesting material by females (Severinghaus et al., 1981). Male *A. manicatum* will aggressively defend these territories from both conspecific males and heterospecific pollinators (Wirtz et al., 1988), while female *A. manicatum* are allowed to enter the floral territories unimpeded, where they can collect nectar, pollen and nesting material. Because female *A. manicatum* are visiting plants for pollen and nectar, as well as for nesting material, the relationship between *A. manicatum* females and their trichome sources is complicated. A local population of *A. manicatum* can both fill the role of pollinator and leaf tissue herbivore through collection of trichomes from the same plants they visit for floral resources. Here, we look at the relationship between *A. manicatum* and its most commonly cited source of nesting material, *Stachys byzantina* (Garbuzov and Ratnieks, 2014; Gibbs and Sheffield, 2009; Hicks, 2011; Miller et al., 2002; Payette, 2001; Payne et al., 2011; Severinghaus et al., 1981; Strange et al., 2011; Wirtz et al., 1988). *S. byzantina* has both dense glandular trichomes (Salmaki et al., 2009) and long simple trichomes (Salmaki et al., 2011). In addition to visiting *S. byzantina* for nesting material, *A. manicatum* also visit *S. byzantina* for food resources, possibly providing a significant contribution to the plant's reproductive fitness (Payette, 2001; Severinghaus et al., 1981). Predicting how *S. byzantina* may have evolved to respond to *A. manicatum* damage is therefore complicated, as attraction of *A. manicatum* could yield both a benefit and a cost to the plant. Likewise, understanding how *A. manicatum* may have evolved to use VOCs produced by *S. byzantina* is also of interest, as the upregulation of VOCs may be either attractive or repellent.

Here, we explore this complicated plant-pollinator relationship, by specifically looking at the relationship between *A. manicatum* and *S. byzantina* as a source of nesting material. We first investigated if carding damage changes the VOC output of *S. byzantina*. We performed headspace VOC collection and used GC–MS and GC-FID for identification and relative quantification of VOCs. Second, we determined how trichome damage impacted *A. manicatum* visitation to *S. byzantina*. We quantified the distribution of bee carding damage in semi-natural *S. byzantina* populations, and compared visitation rates of *A. manicatum* to damaged (trichomes removed) versus undamaged (trichomes intact) *S. byzantina* plants. Through these studies, we aimed to gain a clearer understanding of the plant-pollinator relationship; specifically, we wished to better understand the relationship when a pollinator also
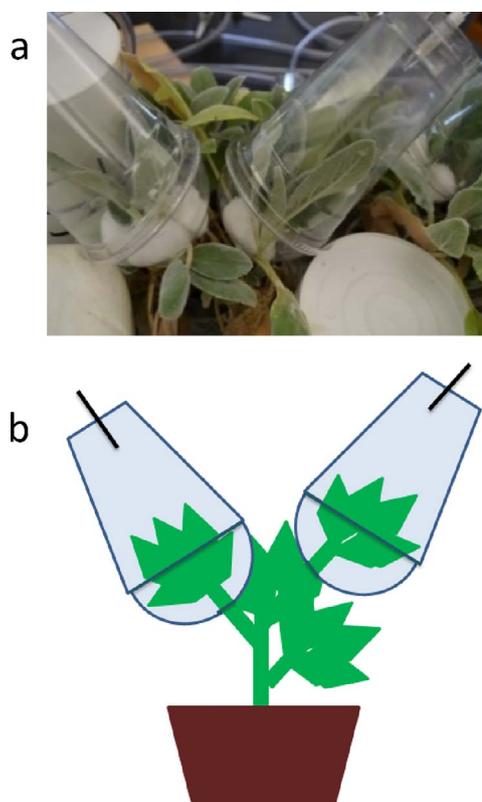

Fig. 1. Headspace VOC collection on one *S. byzantina* plant. (a) Picture; (b) Schematic. VOCs were collected from eleven plants total. Each plant had both treatments − VOCs collected from a mechanically carded and an uncarded region.

serves a role more commonly filled by plant pests.

2. Methods

2.1. Collection and analysis of VOCs released by mechanically carded Stachys byzantina plants

We compared differences in VOCs emitted between mechanically carded and uncarded *S. byzantina* leaves. Eleven *S. byzantina* plants of approximately equal size were purchased from a local plant nursery. All plants were checked for general health and lack of previous bee carding damage. We then collected and analysed headspace VOCs from both mechanically carded and uncarded leaves, as well as from ambient air (negative control), at the University of New England (Biddeford, ME). Volatile headspace collections were taken from a mechanically carded region (one stalk consisting of about five leaves, two of which were mechanically carded) and compared to volatile headspace from an uncarded region (one stalk consisting of about five leaves) on the same plant (see Fig. 1). Mechanical carding was performed using a razor blade (Fig. 2). Two healthy leaves, one younger and one older, were chosen haphazardly and the trichomes on the top surface of their leaves were removed using the razor blade. Razor blades were cleaned, or a new razor blade was used between trials. We confirmed similarity of mechanical damage to natural carding damage by visual comparison under a dissection microscope; however, mechanical carding can only be assumed as a representation of actual *A. manicatum* carding damage. Additionally, *A. manicatum* carding is found most commonly on the underside of leaves, though also found on the tops of leaves; however, for mechanical carding, only the trichomes on the top of the leaves were removed due to the delicate nature of the underside of the leaf.

To collect headspace volatiles, each stalk was enclosed in a clear PET plastic cup with an open dome lid and a volume of 850 ml. Purified air was allowed to flow into the chamber through the bottom at a rate

Fig. 2. Mechanical removal of trichomes on *Stachys byzantina* using a razor blade. Only the trichomes from the tops of the leaves were removed to avoid damage to the leaf tissue on the more irregular undersides of the leaves.

of 850 ml/min. At the top of the chamber, we inserted a volatile collection trap containing 50 mg of Super-Q adsorbent (Alltech Associates, Inc, Deerfield, IL). We then attached this trap to a vacuum pump pulling air through the trap at a rate of 850 ml/min for three hours. We extracted the filters with 100 μl dichloromethane, and 600 ng of nonylacetate was added as an internal standard. Samples were subsequently analysed by Gas Chromatography-Mass Spectrometry (GC–MS) and quantified using Gas Chromatography-Flame Ionization Detector (GC-FID).

We analysed all samples quantitatively on an Agilent Technologies model 7890A gas chromatograph equipped with model 7693 auto sampler (Agilent Technologies, Wilmington, DE) and flame ionization detection. One μl of each extracted sample was injected on-column and analysed on an Agilent J & W HP-5 30 m × 320 μm × 0.25 μm 19091J-413 capillary column (J & W Scientific, Folsom, CA) with helium as a carrier gas at a constant linear velocity of 32 cm s^{-1}. The oven temperature was maintained at 40 °C for 5 min and then increased at a rate of 5 °C min^{-1} to 280 °C and held for 5 min. The injector temperature was set to track the oven and the detector temperature was 300 °C. Data were analysed on MSD ChemStation DS software (Agilent). Estimated abundance was calculated by comparison to the internal standard.

For qualitative analyses, the same GC was connected to an Agilent Technologies MS5975C mass spectrometer operating in electron impact mode. We analysed one μl of each sample using split less injection at 250 °C. An Agilent J & W HP-5MS 30 m × 250 μm × 0.25 μm column was used with helium as a carrier gas at a constant velocity of 40 cm s^{-1}. The oven temperature was maintained at 40 °C for 5 min and then increased at a rate of 5 °C min^{-1} to 280 °C and held for 5 min. The transfer line temperature was set to at 280 °C and the ion source

temperature to at 230 °C. Data were analysed on MSD ChemStation DS software. We identified VOCs by comparison of mass spectra with spectra in the Wiley 9th and NIST 11 MS Library, and spectra obtained of authentic compounds. We also compared GC retention times of VOCs with GC retention times of the authentic compounds on the HP-5MS column.

Comparison of the patterns of VOC composition between treatments was performed using multivariate analysis. A data matrix of pairwise Bray-Curtis dissimilarity indices between samples was built, and non-metric multidimensional scaling (NMDS) (R package vegan) was used to visualize patterns of dissimilarity (see similar methods in Soler et al., 2012). Estimated VOC abundance (described above) was used for calculation of Bray-Curtis similarities. NMDS finds the best two-dimensional representation of the distance matrix, allowing for visualization of grouping between treatments. A Permutational Multivariate Analysis of Variance (PERMANOVA) was then used to test the null hypothesis − no difference between treatments (mechanical carding and no carding). The PERMANOVA was based on 1000 permutations, and is nonparametric (with only one factor). Similarity percentage (SIMPER) was then used to identify which compounds were responsible for differences between treatments. All analyses were performed using R version 3.3.1 (R Core Team, 2016).

2.2. Within plant distribution of bee carding damage on Stachys byzantina

We assessed 54 *S. byzantina* plants for within plant distribution of bee carding damage. We visited five plant nurseries in eastern Massachusetts between June and July 2012. All *S. byzantina* plants at each nursery were checked for carding damage; however, only plants with more than 18 mature leaves and with visible carding damage were included in the study (39 plants total) due to the required minimum number of leaves needed for our methods. We identified three "reference" leaves on each plant: an uncarded leaf, a minimally carded leaf (only one carding track, "singly carded"), and a heavily carded leaf (two or more carding tracks, "multiply carded"; see Fig. 3). The five closest leaves surrounding the reference leaf were then checked for carding damage, without any overlap of leaf groupings, and the damage on these five leaves was recorded. Selection of reference leaves was almost entirely random. Most of the bee carding damage is on the undersides of leaves. Therefore, on approach to the plant, we could not visually assess damage before turning over leaves (at random). Reference leaves were identified as the first leaf we found on the plant to have the level of damage characterized above.

We used generalized linear mixed models to compare the number of bee carded leaves occurring around each type of reference leaf. The response variable, carding damage on the five leaves surrounding a reference leaf, was considered as a binomial response (0–uncarded leaf, 1–carded leaf) for each of the five leaves. We included type of reference leaf as the factor of interest, and individual plant and nursery location
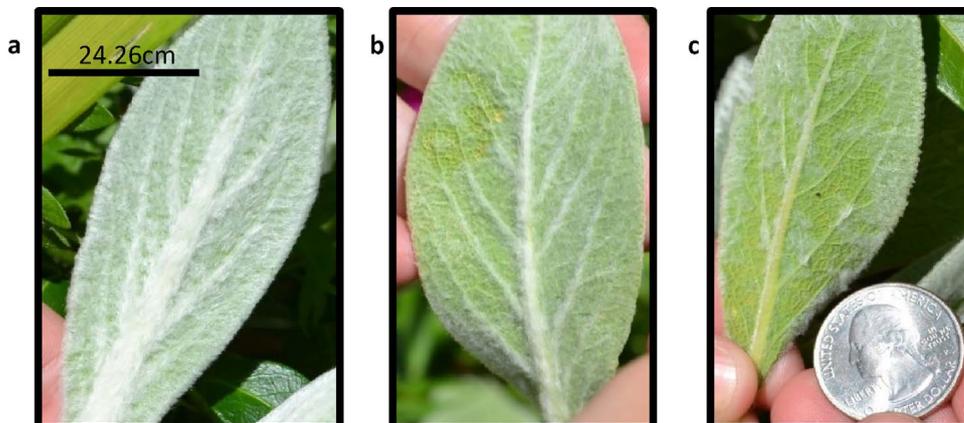

Fig. 3. Carding distribution methods. Three reference leaves were chosen on each *Stachys byzantina* plant − (a) uncarded, (b) singly bee carded and (c) multiply bee carded. Five surrounding leaves were then checked for damage around each reference leaf, with no overlap between leaf groupings.

were included as random effects. The most parsimonious model was chosen through comparison of AICc scores (Table S1). If competing models were within 2.0 ΔAICc, the simplest model was chosen. Both the marginal R^2 (R^2GLMM(m)) and the conditional R^2 (R^2GLMM(c)) are reported (as calculated in Nakagawa and Schielzeth, 2013). The marginal R^2 describes the proportion of variance explained by the fixed factor alone. The conditional R^2 describes the proportion of variance explained by both the fixed and random factors (Nakagawa and Schielzeth, 2013). Data had a binomial distribution, and we used a logit link function. All analyses were performed using R version 3.3.1 (R Core Team, 2016).

"Small" *S. byzantina* plants, those with fewer than 18 mature leaves, could not be used in the analysis above, but were still checked for bee carding damage. Percent of total leaves with any carding damage was recorded. This gave us an estimate of average carding damage per plant in semi-natural populations.

2.3. Anthidium manicatum attraction to mechanically carded versus uncarded Stachys byzantina plants

To further test if carding damage is attractive to *A. manicatum*, we observed differences in visitation rates to mechanically carded *S. byzantina* plants versus uncarded *S. byzantina* plants. We performed 32 trials at seven locations at or around Tufts University in Medford, MA. Locations were picked based on presence of foraging *A. manicatum* and presence of *Nepeta cataria* (catmint), a preferred plant for pollen and nectar collection (Payette 2001; pers. obs.). These locations were considered optimal observation areas as they were likely to have high traffic of *A. manicatum* to the area.

Trials were carried out between August and September, 2014 between 11:00-16:00 h on days with no precipitation when the temperature was between 21 and 32 °C (Couvillon et al., 2010). *A. manicatum* will activity forage during this time and temperature window (pers. obs.). In addition, we also checked to see that *A. manicatum* were actively foraging in the area before we started each trial. For each trial, we placed two non-flowering *S. byzantina* plants of similar size at the testing location. For this study, we were focused on visits related to collection of nesting material, therefore, only non-flowering plants were used. Before trials, *S. byzantina* plants were housed in the Tufts University greenhouse and had no prior carding damage. Plants ranged in size but typically had approximately 20–25 leaves. One *S. byzantina* plant was haphazardly chosen to be mechanically carded; approximately 25% of its leaves were carded with a razor. Only the tops of the leaves were carded to minimize damage to the delicate backs of the leaves. Twenty-five percent was chosen as it is the average amount of bee carding damage we found naturally on "small" *S. byzantina* plants in local plant nurseries (see previous methods). The plants were placed about 1.5 m from each other, and equidistant from the focal flowering plant (catmint), within 0.5 m. We observed visitation and behaviour of *A. manicatum* near the *S. byzantina* plants for thirty minutes from about 1.5 m away.

Testing was repeated at each site (four or five times), but at least 24 h apart. The possible effect of pseudo-replication from the same individual visiting several times could not be completely removed from the study, particularly for territorial males who are likely to visit several times if guarding that area. However, we do not anticipate this significantly effecting the data as trials were done at seven locations far enough apart that an established territorial male would not be guarding multiple locations.

During the testing period, we recorded the number of visits by *A. manicatum* to each *S. byzantina* plant. A visit was counted whenever an *A. manicatum* came in close proximity to the test plant (generally within 15 cm) and hovered over or landed on the plant. Hovering near the plant was included as a visit, because this is a common behaviour for *A. manicatum* near plants, and this behaviour is likely associated with investigation of resources (Severinghaus et al., 1981). Unfortunately, differentiation between female and male *A. manicatum* was often not possible due to the speed of visitations and relative similarity in appearance between small males and females. Therefore, because sex of the visitor could not be determined every time, sex was not included in the analysis. Any incidences of carding at plants was also recorded.

We used generalized linear mixed models (R package lme4) to compare number of visits to mechanically carded *S. byzantina* plants to number of visits to uncarded plants. Model response variable was number of *A. manicatum* visits, the fixed effect was treatment, and location was included as a random effect. Due to the paired design of the experiment, trial ID was also included as a random effect. Models used a Poisson distribution with a log link function. The same parameters were then used to compare number of bee carding events during the trial period between carded and uncarded plants. The same methods were used to select the most parsimonious model as described previously (Table S1). All statistical analyses were performed using R version 3.3.1 (R Core Team, 2016).

Table 1
Identified volatile organic compounds (VOCs) detected through headspace collection of *Stachys byzantina* leaves that underwent two treatments − mechanical removal of trichomes (carded) or no manipulation (uncarded). Collections ran for three hours and VOCs were quantified and qualified through GC–MS and GC-FID. Non-metric multi-dimensional scaling (NMDS) was used to visualize differences between the relative proportions of VOCs detected from the two treatments (carded and uncarded leaves) (Fig. 4). Similarity percentage (SIMPER) was then used to determine contribution of each VOC to dissimilarity between treatments.

| VOC	Classification	Carded (ng over 3 h)	Uncarded (ng over 3 h)	NMDS Average contribution to dissimilarity	SD of contribution
(Z)-3-Hexenyl acetate	Green leaf volatile	372.2	16	0.16542	0.11035
β-cubebene[a]	Sesquiterpene	603.5	33.27	0.14645	0.13771
Homosalate[a]	Ester of Salicylic acid	183.4	24.64	0.1028	0.11178
(Z)-3-Hexenol	Green leaf volatile	202.5	3.91	0.09818	0.08414
β-pinene	Monoterpene	321.7	43.45	0.0951	0.08771
(+) valeranone[a]	Sesquiterpenoid	167.6	24.55	0.05321	0.02861
α-pinene	Monoterpene	141.7	30	0.04418	0.04298
(E)-2-Hexenal	Green leaf volatile	100.5	1	0.03811	0.0392
Limonene	Monoterpene	146	2.45	0.03503	0.0343
Hexyl acetate	Green leaf volatile	45.7	1.64	0.021	0.01654
(Z)-2-Hexenal	Green leaf volatile	36.4	1	0.01902	0.01758
(E)-2-Hexenol	Green leaf volatile	26.2	1.45	0.01187	0.0117
(E)-2-Hexenyl acetate	Green leaf volatile	19	1.09	0.00834	0.00589

[a] Tentative identification.
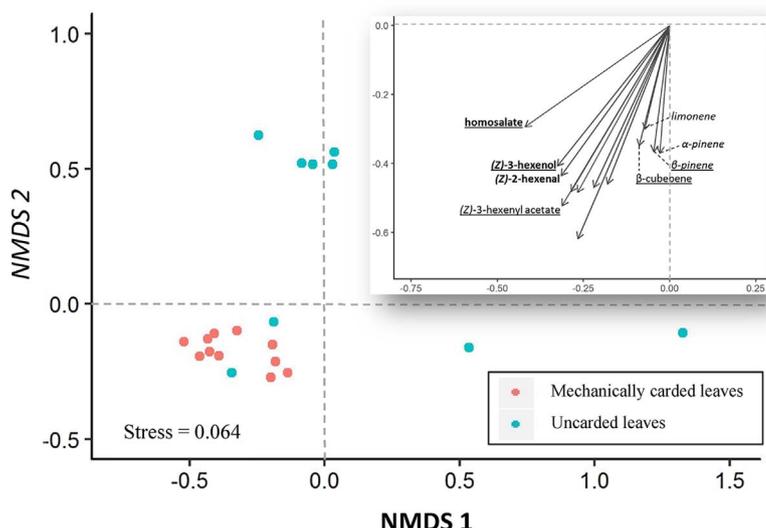
Fig. 4. Non-metric multi-dimensional scaling (NMDS) of the relative proportions of VOCs detected under two treatments − uncarded and mechanically carded *Stachys byzantina* leaves. NMDS based on Bray-Curtis dissimilarity index. Inset graph shows average VOC contribution to dissimilarity (no points under the inset picture). Compounds in bold were the most important loadings for NMDS 1, and compounds in italics were the most important loadings for NMDS 2. Underlined compounds were the top five compounds driving dissimilarity between the treatments.

3. Results

3.1. Measure of VOCs released by mechanically carded Stachys byzantina plants

We were able to identify ten compounds detected from *S. byzantina* headspace collection, as well as tentatively identify three more (Table 1). Most of the VOCs were green leaf volatiles or terpenes. The VOCs detected from mechanically carded leaves were significantly dissimilar compared to those detected from uncarded leaves (PERMANOVA; R^2 = 0.31; f = 8.96, df = 1, p < 0.001) (Fig. 4). Abundance of five compounds explained most of the dissimilarity between carded and uncarded leaves: β-pinene (72.5%), *(Z)*-3-hexenol (61.1%), homosalate (49.4%), β-cubebene (37.2%), and *(Z)*-3-hexenyl acetate (19.7%). It should also be noted that the NMDS analysis grouped the VOCs from carded leaves, while VOCs from uncarded leaves were much more scattered (Fig. 4). Overall, there was an 83.9% dissimilarity between treatments, with greater abundance of VOCs detected in the mechanically carded treatment (Table 1). Generally, there were also more unidentified VOCs detected from carded leaves compared to uncarded leaves (Fig. S1).

3.2. Within plant distribution of bee carding damage on Stachys byzantina plants

Analyses showed that the bee carded reference leaves (both singly and multiply carded) had significantly more carded leaves around them than the uncarded reference leaves (R^2GLMM(m) = 0.03, R^2GLMM(c) = 0.42; X^2 = 18.63, df = 2, p < 0.001; Fig. 5). Given the amount of carding damage on each plant, it is unlikely that it resulted from one individual making several return trips. However, extensive damage from one individual cannot be ruled out.

3.3. Anthidium manicatum visits to mechanically carded versus uncarded Stachys byzantina plants

More *A. manicatum* visits were made to plants with mechanical carding compared to uncarded plants (R^2GLMM(m) = 0.15, R^2GLMM (c) = 0.77; X^2 = 44.64, df = 1, p < 0.001, Fig. 6). The simplest model within 2.0 ΔAIC removed location ID as a random effect. However, due to the territorial behaviour of male *A. manicatum*, location is an important effect to control for. We would expect locations with territorial males to have higher visitation rates compared to locations without a territorial male. Therefore, we kept location as a random effect included in the model (as this model also was within the 2.0 ΔAIC
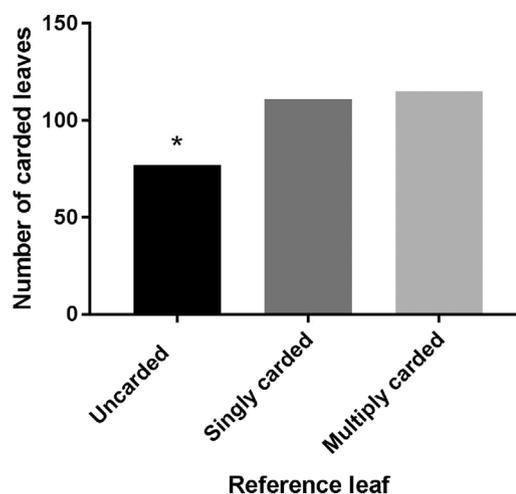

Fig. 5. Number of carded leaves around each type of reference leaf. There were significantly fewer total carded leaves around an uncarded reference leaf than either type of carded reference leaf (singly or multiply carded) (GLMM; X^2 = 18.63, df = 2, *p < 0.001, 54 plants).
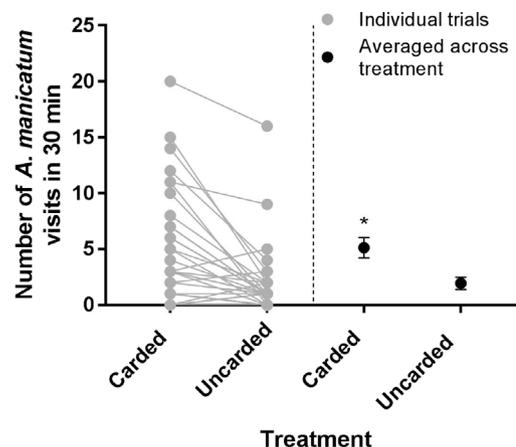

Fig. 6. Average number of *A. manicatum* visits to mechanically carded *S. byzantina* versus uncarded *S. byzantina*. Each observational trial lasted 30 min. Each trial is represented by the grey dots and lines, with almost all trials have more visits at the carded plant than the uncarded plant. Means by treatment are shown by the black dots, with standard error. Overall, there were significantly more *A. manicatum* visits to mechanically carded *S. byzantina* than uncarded *S. byzantina* (GLMM; X^2 = 44.65, df = 1, *p < 0.001).

threshold) (Table S1).

There was no difference in occurrences of carding by *A. manicatum* between plants that were mechanically carded (7) and those that were uncarded (4) (R^2GLMM(m) = 0.00, R^2GLMM(c) = 0.76; X^2 = 0.80, df = 1, p = 0.372).

4. Discussion

Here, we have shown that (1) *S. byzantina* leaves with their trichomes removed (mechanically carded) released a significantly different bouquet of VOCs compared to leaves with their trichomes intact (uncarded); (2) bee carding damage is grouped in distribution on *S. byzantina* plants; and (3) mechanical carding of plants increases *A. manicatum* visitation. Taken together, our results support the hypothesis that *A. manicatum* carding damage induces changes in *S. byzantina* VOCs, and that these chemical changes are attractive to other *A. manicatum*.

When comparing the headspace VOCs of mechanically carded *S. byzantina* leaves compared to uncarded leaves, we were able to detect significant differences in the emitted compounds. Additionally, four of the five compounds that explained the most dissimilarity between treatments have previously been associated with changes in Hymenoptera behaviour: *(Z)*-3-Hexenyl acetate (Bruinsma et al., 2009; Whitman and Eller, 1990), β-cubebene (Belz et al., 2013; Patricio et al., 2004), *(Z)*-3-hexenol (Turlings et al., 1995; Whitman and Eller, 1990), and β-pinene (Hoebeke et al., 2011). Furthermore, the VOC profile of carded treatments was similar across individual plants. This suggests that individual *S. byzantina* respond similarly to carding damage, and therefore produce a stable cue which could be used by *A. manicatum*. However, to better understand whether *A. manicatum* use this cue, and whether they would deem this change in VOCs as attractive or repellent, we needed to observe patterns in carding damage and *A. manicatum* visits.

By observing patterns in carding damage on individual *S. byzantina* plants, we provide evidence that *A. manicatum* carding damage is grouped in distribution, with more carding damage occurring around reference leaves with bee carding damage. This suggests that changes in VOCs following carding damage are attractive to female *A. manicatum* foraging for nesting material, resulting in additional carding damage to that area of the plant. However, we should make note that the marginal R^2 value for this model was relatively low, indicating that only 3% of the variability is explained by the factor (reference leaf) alone. The conditional R^2 value is higher (0.42) suggesting between plant variation and between location variation was high. However, we believe the observed grouping in carding distribution represents a real trend as results from our behavioural trials provide additional support for this hypothesis. More *A. manicatum* visited mechanically carded *S. byzantina* plants than uncarded *S. byzantina* plants. These data further suggest that *A. manicatum* are attracted to changes in the *S. byzantina* VOC profile following damage to trichomes.

An alternative hypothesis is that *A. manicatum* are using visual cues to identify plants with or without carding damage. However, we do not think this is likely given the amount of carding damage on the undersides of leaves. Additionally, it is not clear what advantage carded plants would have for *A. manicatum*. Using VOCs to identify plants more easily therefore seems like a more likely explanation for increased visitation at carded plants.

We provide evidence that *A. manicatum* use plant VOCs as a behavioural cue, but why would this relationship have evolved? Exploitation of plant VOCs is not a unique trait among Hymenoptera (De Moraes et al., 1998; Dicke and Baldwin, 2010; Kessler and Baldwin, 2001; Kessler and Halitschke, 2007; Kessler et al., 2011). However, the relationship between *A. manicatum* and *S. byzantina* does not fit the typical plant-pollinator or plant-parasitoid models. Individual *A. manicatum* can be both a pollinator and a pest to the same plant.

Since *A. manicatum* are attracted to previously carded *S. byzantina*,

this system seems to follow trends more similar to plant-parasitoid systems, rather than plant-pollinator systems where changes in VOCs are usually repellent (Kessler and Halitschke, 2007, 2009; Mothershead and Marquis, 2000). But attraction of predatory or parasitic wasps often benefit the plant through direct reduction in herbivores. In contrast, attraction of *A. manicatum* following plant tissue damage does not appear to bring any similar benefits; in fact, emitting VOCs that attract *A. manicatum* will likely increase the amount of damage a plant sustains due to additional *A. manicatum* carding. *A. manicatum* visitation is also unlikely to significantly increase the plant's reproductive fitness through pollination services. *A. manicatum* are generally considered poor pollinators (Soper and Beggs, 2013) and *S. byzantina* often reproduce clonally (Legkobit and Khadeeva, 2004). Therefore, since the benefits (pollination services) to the plant in attracting *A. manicatum* are minimal at best, a change in VOCs by the plant likely did not evolve as a signal in response to *A. manicatum*.

Instead, *A. manicatum* have likely evolved to eavesdrop on plant signals intended for other uses, e.g. tissue damage repair, beneficial parasitoid attraction, or defence priming (Heil and Karban, 2009; Turlings et al., 1995). *(Z)*-3-hexenyl acetate is commonly associated with herbivore damage in many systems, and its production is thought to increase plant defence (De Moraes et al., 2001; Loughrin et al., 1994; Rodriguez-Saona et al., 2002; Röse et al., 1998; Röse et al., 1996). This supports the idea that *S. byzantina* is emitting VOCs, such as *(Z)*-3-hexenyl acetate, in response to the damage caused by *A. manicatum*, not due to an evolved benefit of attracting *A. manicatum*. This interaction could therefore be considered eavesdropping, as the receiver (*A. manicatum*) likely gains a benefit while the sender (*S. byzantina*) does not.

The benefit to *A. manicatum* in eavesdropping is relatively straightforward. Due to increased production of VOCs following damage, *A. manicatum* can likely detect VOCs from a damaged plant more easily than those given off by an undamaged plant. Female *A. manicatum* would therefore benefit by using this cue as a way to reduce their search time for nesting material. Male *A. manicatum* are also likely to benefit from eavesdropping on plant VOCs. Male *A. manicatum* would greatly benefit by using this cue in association with female *A. manicatum* presence. Similar to parasitoid wasps, if male *A. manicatum* associate a change in plant VOCs with presence of female *A. manicatum*, they should be able to decrease their search time for potential mates. It is likely that many of the visits to *S. byzantina* in our behavioural trial that included only hovering were male *A. manicatum*. Males spend the majority of their time actively patrolling their territory, which is punctuated with frequent hovering behaviour (Severinghaus et al., 1981). Males patrol in search of females, heterspecific intruders, and competing males. If males are able to associate a change in plant VOCs following carding damage with the presence of a female *A. manicatum*, this could greatly increase their mating frequency.

Both male and female *A. manicatum*'s use of VOCs would appear to increase the density of *A. manicatum* around *S. byzantina*. However, what effect increased presence of *A. manicatum* (male or female) has on *S. byzantina* can only be speculated. Trichomes are important for plant water regulation (Fahn, 1986) and herbivore deterrence (Levin, 1973). Anecdotally, we have noticed that herbivore damage is common on naturally carded sections of *S. byzantina* plants. We therefore hypothesize that removal of trichomes would incur a fitness cost to the plant; however, direct effects of trichome removal on the plant should be examined. A change in VOCs is also likely to decrease flower attractiveness to other pollinators, which would be an additional cost (Kessler and Halitschke, 2007, 2009; Mothershead and Marquis, 2000). Increased presence of territorial male *A. manicatum* might also decrease pollinator visitations. Male *A. manicatum* actively keep away heterospecific pollinators attempting to gain access to guarded plants. Therefore, guarded plants could face pollinator limitations, though this remains to be explored.

An additional area of future research is understanding how bee damage and mechanical damage differ. A caveat to any study using

mechanical damage to replicate natural animal behaviour is that we do not know the differences that may exist between mechanical damage and animal damage. While the mechanical removal of trichomes is visually similar to removal of trichomes by bees, additional effects that bee damage may have on the plant cannot be accounted for. For instance, bees may leave a chemical cue on the plants they visit that can be recognized by other bees (e.g. scent marking; Gawleta et al., 2005), or perhaps leave behind a signal that is changing the chemistry of the plant (e.g. insect saliva's manipulation of plant defensive chemicals; Musser et al., 2006). Optimally, in this study, we would have been able to collect headspace VOCs from bee carded plants. However, there were significant limitations in our ability to do this. *A. manicatum* females would not card plants in a laboratory setting, and we were not able to locate enough *A. manicatum* actively carding in the field to attempt field VOC collections. Nonetheless, our data support the attractive role of VOCs in both damage types (mechanical and bee carding), though the intricacies of how these two types of damage influence both *S. byzantina* and *A. manicatum* remains to be explored.

This study explores the relationship between *A. manicatum*, a worldwide invasive species (Russo, 2016; Strange et al., 2011), and *S. byzantina*, its most commonly cited source of nesting material. Much is still left unknown in this system − fitness effect of carding damage to plants, importance of pollination services provided by *A. manicatum*, and importance of specific VOCs in both plant fitness and *A. manicatum* attraction, to name a few. Given the prevalence of *A. manicatum* (Strange et al., 2011) and *S. byzantina* throughout temperate regions, we propose this system as one particularly well suited for further exploration of this type of complicated plant-pollinator relationship.

Acknowledgements

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.beproc.2017.08.005.

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