RESEARCH ARTICLE

Attack and aggregation of a major squash pest: Parsing the role of plant chemistry and beetle pheromones across spatial scales

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Abstract

1. Successful management of insect crop pests requires an understanding of the cues and spatial scales at which they function to affect rates of attack of preferred and non-preferred host plants. A long-standing conceptual framework in insect–plant ecology posits that there is hierarchical structure spanning host location, acceptance and attack that could be exploited for integrated pest management.

2. We investigated how plant- and insect-derived chemical cues affect successive decisions of host choice in aggregating insects, and tested predictions in the *Cucurbita pepo*–*Acalymma vittatum* system. *Acalymma vittatum* is an aggregating specialist beetle pest that strongly prefers zucchini (*C. p. pepo*) to summer squash (*C. p. ovifera*), two independent domesticates of *C. pepo*. We hypothesized that subspecies-specific plant traits, especially volatile cues, interact with the male-produced aggregation pheromone to amplify beetle preference for *C. p. pepo*.

3. Differential beetle attack of *C. pepo* subspecies in the field is not determined by plant traits that affect host finding or differential aggregation due to pheromones: across two years, beetles had strong density-dependent attraction to both subspecies when male beetles were feeding, and no interactions between plant volatiles and the male-produced pheromone were detected. In the absence of male pheromone emission, beetles were equally unattracted to plants with or without beetle feeding.

4. In contrast, plant traits that mediate insect acceptance appear to underlie differences in preference. At a local scale, beetles did not accept and emigrated from *C. p. ovifera* compared to *C. p. pepo*. Distinct volatile emissions were observed between subspecies, but further work is needed to identify if these volatiles promote emigration.

5. *Synthesis and applications*. By dissecting pest preference during successive host choice decisions, we isolated a trait with implications for pest management. Beetles on cucurbits can be managed by employing cultivars with differential susceptibility (e.g. trap cropping), and the mechanistic knowledge presented here informs best practices and limitations for on-farm applications. More broadly, pest management in diversified cropping systems can be enhanced through understanding

how plant preference gradients affect herbivore movement and behaviour, and plant breeders can target traits to reduce herbivory in such systems.

1 | INTRODUCTION

Herbivory by insect pests follows from successive decisions (host finding, local examination of plant cues and acceptance, attack) that can be differentially impacted by plant and insect factors (Miller & Strickler, 1984; Thorsteinson, 1960). The contributions of plant and insect cues intersect when aggregating insects rely upon plant chemistry to initiate or enhance aggregations (Reddy & Guerrero, 2004; Tumlinson, 1988), making it challenging to isolate a single factor affecting aggregation and preference. Nonetheless, the successive steps of host choice provide a framework to test if and how plant chemistry contributes to scenarios leading to proliferation of and herbivory by aggregating insects on plants.

For beetle pests, once an individual arrives on a host, plant chemistry can be incorporated into (Renwick, Hughes, & Krull, 1976), or promote release of (Hughes & Renwick, 1977; Jaffé et al., 1993), aggregation pheromones. Responding conspecific beetles may then be attracted to induced plant volatiles from beetle feeding (Bolter, Dicke, Van Loon, Visser, & Posthumus, 1997; Sun et al., 2010), pheromone alone or amplification of pheromone attractiveness by plant volatiles (Beran et al., 2011; Dickens, 2006). Finally, upon arriving, beetles may use local cues to reject non-preferred or accept preferred host plants (Finch & Collier, 2000; Silva & Clarke, 2019). Dissecting the role of plant chemistry throughout the process of host choice would enhance our understanding of mechanisms of preference in aggregating beetles and inform ecological management strategies.

Given the extensive natural variation in plant chemistry, aggregation outcomes may be distinct on preferred and non-preferred plants. Indeed, aggregating beetles have shown differences in pheromone emission (Edde, Phillips, Robertson, & Dillwith, 2007) and attraction (Dickens, 2000) on different host plant species. This may be caused by non-preferred plants lacking chemistry to facilitate pheromone release, having less attractive plant volatiles or other traits that promote emigration. Plant variation in precursors and synergists are known in pine-feeding bark beetle systems (Seybold, Huber, Lee, Graves, & Bohlmann, 2006), and interspecific chemical differences quantitatively alter pheromone output (Erbilgin et al., 2014). However, there is limited knowledge of how intraspecific plant trait variation affects aggregation (Birgersson, Schlyter, Löfqvist, & Bergström, 1984; Loughrin, Potter, Hamilton-Kemp, & Byers, 1996, 1997), especially in agricultural systems. This information could allow growers to choose cultivars that modify aggregation behaviour as a means of pest management

on diversified farms. Thus, resolving the role of intraspecific plant chemical variation in preference and aggregation may lead to improved strategies for management.

Cucurbitaceae and the major specialist beetle pest *Acalymma vittatum* (Coleoptera: Chrysomelidae), provide an excellent system to dissect the relationship between plant cues and herbivore aggregation in preference. Cucurbitaceae specialist beetles have been extensively studied for their association with cucurbitacins, non-volatile and bitter triterpenoids of the Cucurbitaceae that cause compulsive feeding behaviour (Metcalf, Metcalf, & Rhodes, 1980). However, release and perception of the *A. vittatum* male-produced aggregation pheromone, vittatalactone, is not affected by cucurbitacin content (Smyth & Hoffmann, 2002, 2003). This suggests that other aspects of plant chemistry, including plant volatiles, may affect *A. vittatum* host plant preferences resulting in beetle aggregation.

Acalymma vittatum preferentially consumes leaves of cultivars of *Cucurbita pepo* ssp. *pepo* over *C. pepo* ssp. *ovifera* (syn. *C. p. texana*; Brzozowski, Leckie, Gardner, Hoffmann, & Mazourek, 2016; Hoffmann, Robinson, Kyle, & Kirkwyland, 1996), although the chemical mechanistic bases remain elusive (Brzozowski, Mazourek, & Agrawal, 2019; Theis, Barber, Gillespie, Hazzard, & Adler, 2014). We thus tested if subspecies-specific factors affect beetle behaviour through which observed differences in preference may be explained. Specifically, does plant subspecies alter attraction of conspecifics, aggregation pheromone release, or promote beetle behaviours like emigration? And which has a greater contribution to ultimate differences in attack?

In this study, using representative cultivars from each subspecies, we dissected the successive steps in *A. vittatum* host choice that result in *C. p. pepo* sustaining more damage than *C. p. ovifera* at the seedling stage (pre-flowering). We tested the relative contributions of *C. pepo* volatiles, beetle male-produced aggregation pheromone release, and potential for synergistic action between those in preferred and non-preferred *C. pepo*. We predicted that *C. p. pepo* would be more attractive to *A. vittatum* during host location and more conducive to density-dependent attraction than *C. p. ovifera*, whereas *C. p. ovifera* may be deterrent (or promote emigration). Over the 3 years of field experiments, we tested the effect of subspecies on (a) density-dependent beetle aggregation, (b) relative contributions of plant and beetle chemical cues to aggregation during field colonization, (c) beetle host acceptance, and (d) attack and (e) foliar headspace volatiles. We use these findings to recommend management practices for *C. pepo* crops in particular, and generally for diversified farming systems.

2 | MATERIALS AND METHODS

2.1 | Plants

Two cultivars, *C. p. ovifera* cv. Success PM (yellow summer squash) and *C. p. pepo* cv. Golden Zucchini were previously established to be, respectively, non-preferred and preferred by *A. vittatum* (Brzozowski et al., 2016). Untreated seeds were acquired from commercial sources in 2016 (High Mowing Organic Seeds; Seed Savers Exchange), and Cornell University seed stocks were used in all other experiments. All seeds were started in 72-cell flats with McEnroe Organic Lite Growing mix, and then transplanted into 1.74 L pots 10 days after sowing at the Cornell University Agricultural Experiment Station (CUAES) greenhouses. The greenhouses were maintained with a 14 hr light, 10 hr dark photoperiod with supplemental metal halide lighting, with 27°C day and 21°C night temperatures. Plants were watered daily, and *Amblyseius cucumeris* (BioBest, Westerlo) was applied to prevent establishment of thrips. All experiments were conducted on pre-flowering plants both to focus on initial beetle colonization of seedlings (1–3 fully expanded true leaves), and also because it is known that floral volatiles are highly attractive to beetles (Andersen & Metcalf, 1986). Plant damage was visually estimated as percent leaf defoliation (0%–100% scale with 5% increments) at the end of all experiments by a single observer (LB).

2.2 | Insects

Adult *A. vittatum* were collected from mixed *Cucurbita* spp. crops at the CUAES Homer C. Thompson Organic Vegetable Farm (42°31′05.7″N 76°20′07.1″W). When appropriate, *A. vittatum* were separated by sex by visually examining abdominal apex morphology (White, 1977). Assays were conducted when *A. vittatum* are abundant in the region (mid June–July).

2.3 | Beetle host location

Traps were used to test the effects of subspecies, beetle infestation type (male or female beetles, as only males produce vittatalactone) and density (number of beetles) on *A. vittatum* host colonization. Traps were similar to those used by Smyth and Hoffmann (2002, 2003), are pictured in Figure S1, and detailed below.

Plants were enclosed in opaque mesh bags (25 cm by 30 cm), and beetles were added to the appropriate treatments and allowed to establish for 24 hr (Figure S1a). Plants were then brought to Thompson Farm and spaced 3 m apart in an empty field (Figure S1b). Wire cages (30.5 cm × 51 cm) were placed around the pots and secured with bamboo poles (Figure S1c,d). Then, five 5-cm-wide strips of white spunbonded polyethylene (Tyvek HomeWrap, Dupont) were attached to the outside of the cages with binder clips at equal intervals, and coated with Tangle-trap sticky coating (Tanglefoot; Figure S1c,d). Overall, this design obscured visual cues for colonizing beetles. In both years (2016, 2017), each week from mid-June through mid-July, a new set of plants and insects were assembled and assayed as a temporal experimental block with 30 total traps per block. The design is shown in Figure S2 and detailed as follows.

In the first assay year (2016), beetles were caught on traps with one of four density treatments per subspecies ('discrete treatments' hereafter, Figure S2a): four males, four females, eight males and controls (plants without beetles). Four female beetles were chosen because we expected females to consume more tissue than males, and thus produce leaf damage intermediate to the male beetle treatments. There were five temporal experimental blocks with four replicate traps of each of the beetle treatments, and three replicate control traps per subspecies. One block was dropped from analysis after an organic pesticide application on an adjacent squash field lead to insect dispersal overwhelming nearby traps. Over the course of 3 days, beetles trapped were counted daily but not removed.

The following year (2017), the treatments were adjusted so that the quantitative relationship between infestation density by male and female beetles and number of beetles trapped could be assessed ('quantitative treatments' hereafter, Figure S2b). Over three temporal blocks, there were two replicates of controls (plants without beetles), one trap each of nine male treatments (1, 2, 3, 4, 6, 7, 8, 10, 12) and one trap each of four female treatments (2, 4, 8, 10) per subspecies. After 3 days, trapped beetles were recorded. Then, a subset was retained and modified ('separated treatments' hereafter, Figure S2c) to test the attractiveness of the components of plants with prior beetle feeding (beetles removed), and of beetles that had previously fed (and placed on a pot of fresh soil) compared to beetles actively feeding. The treatments with 6, 8, 10 and 12 male, and 8 female beetles feeding on plants were thus separated to form ten new traps per subspecies. In addition, the 3, 4 and 7 male, and 4 female beetle treatments, and one control (plant only) per subspecies were retained as positive controls. The number of trapped beetles was counted after 2 days. Data from one of the three blocks of the separated treatments were not analysed due to the low number of beetles caught (10 total).

2.4 | Beetle host acceptance

Beetle emigration from (rejection of) each subspecies was measured at a CUEAS site isolated from known squash production to reduce confounding beetle immigration (Varna, NY, USA, 42°27′52.9″N 76°26′41.7″W). The experiment was conducted in three temporally separated blocks in 2018 with 10 replicates of each subspecies per block. Plants were enclosed in a mesh bag and infested with eight beetles. After allowing the beetles to establish for at least 8 hr, the plants were brought to the field, and the bags were opened after sunset, when the beetles were less active. The following day, beetles were counted four times (08:00, 10:00, 14:00, 16:00).

2.5 | Beetle attack

Beetle preference in attack between subspecies, measured as defoliation, was evaluated at a local scale in the greenhouse. Since cucurbitacins are present in some *C. pepo* cotyledons (Ferguson, Metcalf, Metcalf, & Rhodes, 1983), cotyledons were removed from some plants 24 hr prior to infestation to test leaf chemistry alone. One plant of each subspecies was placed 25 cm apart in mesh bags (25 by 30 cm, two plants per bag, both with or both without cotyledons). Five beetles were released and fed for 48 hr. Beetle preference for leaf tissue alone was tested in 2017 in two temporally separated blocks with 22 total replicates, and for intact plants in three blocks with 43 total replicates in 2018.

2.6 | Chemical analyses

In 2017, for each subspecies, headspace volatiles were collected from plants with no beetles, plants with a range of males (two replicates of 2, 3, 4, 8 and one instance of 1, 6, 10), plants with a range of females (one instance of 2, 4, 10) and two replicates of 'soil controls' that consisted solely of the pot with soil and bag. The samples were prepared in the same fashion as the traps used in the quantitative treatment field assays, and the appropriate treatments were infested with beetles 24 hr prior to volatile collection. Oven bags (Reynolds) were placed over each sample, and a small hole was punctured in the bags for an ORBO^{TM} 32S activated charcoal 100/50 mg ampoule (Supelco). Volatiles were collected from 11:00 to 16:00 with two collection manifolds connected to separate 12 V vacuum pumps (Gast™ Manufacturing Inc. at a flow rate of 350 ml/min). Ampoules were stored at –20°C.

Ampoules were spiked with 4 μl of 90 ng/μl tetraline (internal standard) and eluted twice with 200 μl dichloromethane into glass vials. The samples were then run on a GC-MS (Varian Saturn 2200 GC/MS/MS, Agilent Technologies), equipped with a DB-WAX FAME

column (Agilent J&W GC Column, 30 m × 0.25 mm ID, df = 0.25). Peak area was quantified in Varian MS Workstation Version 6.9.1 (Agilent Technologies) and normalized by tetraline peak area. Unfortunately, multiple samples were lost during preparation (remaining samples are shown in Table 1; Section 3).

2.7 | Statistics

Data from field experiments with beetle traps were analysed as linear mixed models in the LME4 package (Bates, Mächler, Bolker, & Walker, 2015) in R (R Core Team, 2016).

For tests of beetle colonization by subspecies, the number of beetles caught or plant defoliation was the response variables, block was a random effect, and ANOVA was conducted on the fixed effects. For the discrete treatment experiment, the categorical variables of subspecies, density treatment (n = 4) and their interaction were fixed effects. In the quantitative treatment experiment with all intact traps, the quantitative variable of beetle density and the categorical variables of beetle sex and subspecies with all pairwise interactions were fixed effects. In the separated treatments, the fixed effects of treatment (male beetles only, plants previously fed upon by male beetles, or positive controls; traps with female beetles were excluded from the analysis), plant subspecies and their interaction were tested. The effect of plant damage on number of beetles caught was also tested in the discrete treatment experiment, where linear mixed models with fixed effects of subspecies, defoliation and their interaction were used for each beetle treatment separately.

The beetle host acceptance assay was analysed by categorizing the plants as either with or without complete emigration (zero beetles), summing each category across all three blocks and comparing between subspecies with a William's corrected G-test for a 2 × 2 table. The beetle host attack choice assays were analysed by categorizing all of the plants as either with or without damage, summing the

Treatment	*Cucurbita pepo* ssp. *pepo*			*Cucurbita pepo* ssp. *ovifera*		
	Linalool	RT11.8	Vittatalactone	Linalool	RT11.8	Vittatalactone
Field cages						
1M	0.7	N.D.	<LOQ	6.2	1.8	N.D.
2M	1.5	N.D.	<LOQ	4.5	1.0	<LOQ
3M	1.2	N.D.	<LOQ	6.0	0.8	<LOQ
4M	—	—	—	4.9	0.9	<LOQ
6M	0.8	N.D.	<LOQ	—	—	—
8M	5.0	0.4	1.07	13.0	0.8	<LOQ
10M	—	—	—	9.0	0.7	0.660
2F	1.3	N.D.	N.D.	—	—	—
10F	1.8	0.5	N.D.	8.5	0.8	N.D.

TABLE 1 Normalized signal intensity (*k*-counts) of selected plant volatiles (linalool, RT11.8) and male aggregation pheromone (vittatalactone) detection and quantification by GC-MS

Notes: The treatments are male beetles 'M' and female beetles 'F', and the abbreviations are defined as follows: 'N.D.', not detected; '<LOQ', less than limit of quantification; '—', no sample. All values are a single instance, except the values reported for two males of *C. p. pepo*, and three males of *C. p. ovifera* are the mean of two samples.

number in each category across all blocks, and comparing between subspecies with Fisher's exact test for a 2 × 2 contingency table.

Differences in subspecies headspace volatiles were analysed ($n = 8$ samples per subspecies; $n = 6$ volatiles above limit of quantification in at least two samples). VOCs present in soil controls ($n = 2$) were removed from analysis. Difference in total plant VOC emission was tested with an ANOVA and fixed effects of subspecies, beetle number, their interaction and manifold. Subspecies differences in specific compounds were tested with a one-tailed t-test. Compositional differences in beetle induced volatiles between subspecies were assessed with permutational multivariate analysis of variance using Bray–Curtis dissimilarities with 100 permutations in R/vegan (Oksanen et al., 2019) where all induced treatments were pooled. Random forest analysis was conducted in R/randomForest (Liaw & Wiener, 2002), and variable selection on out-of-box error rate was conducted in R/varSelRF (Diaz-Uriarte, 2007), with 5,000 trees.

3 | RESULTS

3.1 | Host location

Field insect traps were first used to test the effects of volatile cues from visually masked plants and discrete levels of beetle density on *A. vittatum* host location. In total, 3,824 beetles were trapped. Traps of both *C. pepo* subspecies infested with female beetles and controls (no beetles) were equivalent in beetle catch, and caught 56% fewer beetles than traps infested with four or eight pheromone-producing male beetles (Figure 1a; Table S1). There was a 71% increase in beetle capture from traps containing four males to those containing eight males for less preferred *C. p. ovifera*. In contrast, similar numbers of beetles were caught in four and eight male traps for preferred *C. p. pepo* (8% more in traps with eight males; no subspecies-by-treatment interaction, Table S1). Analyses of counts from earlier time points (1, 2 days) were consistent with results from 3 days (Table S2).

Percent leaf defoliation within traps was similar for both of the four beetle density treatments (male and female) and increased for the eight beetle treatment (Figure 1b). Caged beetle density was the

best predictor of beetle damage ($F_{1,87} = 81.33$, $p < 0.001$), but there were also significant effects of subspecies ($F_{1,87} = 4.75$, $p = 0.032$), and their interaction ($F_{1,87} = 9.14$, $p = 0.003$); this interaction was driven by unexpectedly higher damage on *C. p. ovifera* plants infested with eight male beetles (Figure 1b). However, there was no effect of damage, or interaction between damage and subspecies, on number of beetles caught (Table S3).

The following year, traps were set up with an expanded range of caged beetles of each sex to quantitatively test for interactions between beetle infestation density, beetle sex, foliar damage and subspecies. Fewer total beetles were trapped (592), but traps infested with female beetles at any density and plant controls were again equivalently unattractive, and increasing the number of male beetles in cages linearly increased the number of beetles trapped (Figure 2a). There were no significant interactions between subspecies, damage or beetle density in the trap (Table S4). The interaction between beetle density and sex was significant, where exclusively more males increased trap catch (Table S4). Plant defoliation was similar between subspecies, increased with beetle density and female beetles caused more damage than males (Figure 2b; Table S4).

At the end of the quantitative treatment assay, a subset of the cages were separated into traps with male beetles alone, plants with previous male feeding alone and male beetles and plants together (positive control) and beetles were trapped for 2 days. While fewer beetles were caught (150), there was an effect of treatment ($F_{2,41} = 12.18$, $p < 0.001$) where traps with male beetles alone or plants previously fed by male beetles caught 72% and 79% fewer beetles than positive controls (male beetles feeding on plants), respectively (Figure 3). Higher numbers of male beetles only increased trap catch in positive controls ($F_{1,7} = 8.48$, $p = 0.023$), not the other treatments (male beetles alone, $F_{1,11} = 0.20$, $p = 0.665$; plants alone, $F_{2,41} = 4.00$, $p = 0.071$; Figure 3).
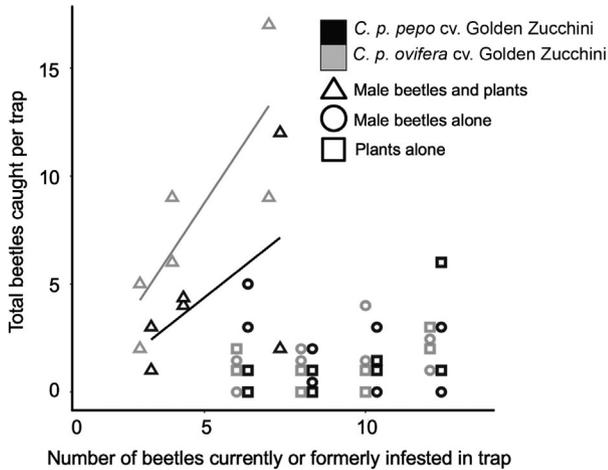
3.2 | Host acceptance

When beetles were given the opportunity to emigrate, fewer *C. p. pepo* plants were deserted (rejected) by beetles compared
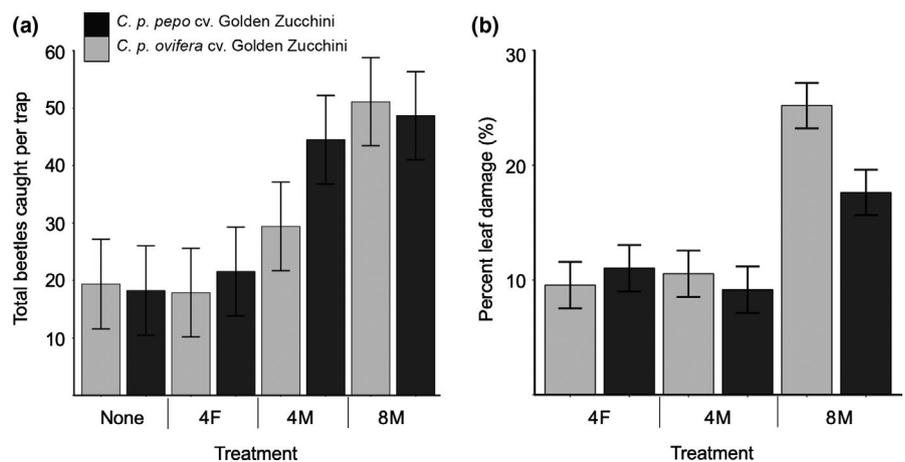
FIGURE 1 Beetle attraction to volatiles emitted from discrete plant-beetle treatment combinations in visually masked field cages: (a) number of beetles caught and (b) leaf damage inside the traps, grouped by plant subspecies and treatment. Treatment refers to level of infestation: four female ('4F'), four male ('4M') or eight male beetles ('8M'), or control (no beetles; 'none') on both plant subspecies (differentiated by colour). The height of bars is the least-squared mean, and error bars represent one standard error

(a)

(b)
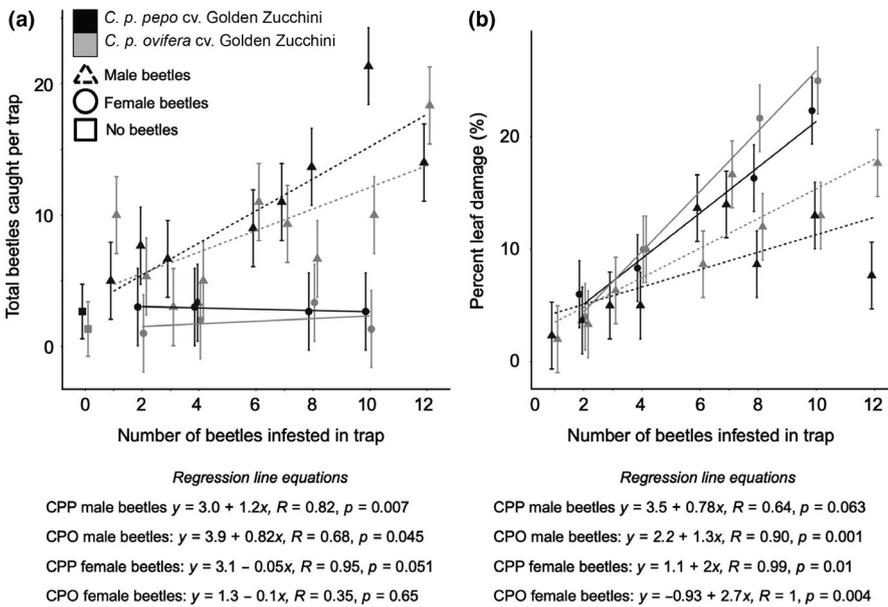
FIGURE 2 Beetle attraction to volatiles emitted from quantitative plant-beetle treatment combinations in visually masked field cages: (a) number of beetles caught and (b) the leaf damage inside the traps, grouped by plant subspecies and beetle sex. Plant subspecies are differentiated by colour, and sex of beetles in trap is indicated by shape. Points of all shapes represent the least-squared mean, and bars represent one standard error. The lines are linear regressions for each plant subspecies (*C. p. pepo*, 'CPP'; *C. p. ovifera*, 'CPO') and beetle sex combination

Regression line equations

CPP male beetles $y = 3.0 + 1.2x$, $R = 0.82$, $p = 0.007$

CPO male beetles: $y = 3.9 + 0.82x$, $R = 0.68$, $p = 0.045$

CPP female beetles $y = 3.1 - 0.05x$, $R = 0.95$, $p = 0.051$

CPO female beetles: $y = 1.3 - 0.1x$, $R = 0.35$, $p = 0.65$

Regression line equations

CPP male beetles $y = 3.5 + 0.78x$, $R = 0.64$, $p = 0.063$

CPO male beetles: $y = 2.2 + 1.3x$, $R = 0.90$, $p = 0.001$

CPP female beetles: $y = 1.1 + 2x$, $R = 0.99$, $p = 0.01$

CPO female beetles: $y = -0.93 + 2.7x$, $R = 1$, $p = 0.004$

FIGURE 3 Beetle attraction to volatiles emitted from separated plant-beetle combinations (traps containing male beetles alone, plants alone or positive controls of male plants and beetles together) in visually masked field cages. The points (circles, triangles and squares) are raw values, and lines are linear regressions for each plant subspecies and beetle sex combination where the there was a significant effect of number beetles (current, or previously feeding; see Section 3.1)

to *C. p. ovifera* over 1 day (William's corrected *G*-test 08:00 hr $G = 5.56$, $p = 0.018$; 10:00 hr $G = 3.25$ $p = 0.072$; 14:00 hr $G = 9.74$ $p = 0.002$; 16:00 hr $G = 13.03$ $p < 0.001$). Less than 50% of leaf tissue was removed from any plant, and percent defoliation was consistent between subspecies (*C. p. pepo* 10.7%; *C. p. ovifera* 11.7%).

3.3 | Beetle attack

Beetle damage to leaves was assessed in neighbouring *C. p. ovifera* and *C. p. pepo* choice assays. Significantly more *C. p. ovifera* had no

damage, both when cotyledons were intact (plants with no damage: CPO 13/22, CPP 0/22, Fisher's exact $p < 0.001$) or removed (plants with no damage: CPO 38/43, CPP 2/44, Fisher's exact $p < 0.001$).

3.4 | Beetle aggregation pheromone and plant volatiles

Headspace volatiles were collected to determine if aggregation pheromone (vittatalactone) release from beetles or plant volatile emissions differed between subspecies. We detected vittatalactone only in samples with at least one male beetle (Table 1). Six additional volatiles were detected in the headspace of plants under field conditions (Table S5). *C. p. ovifera* samples had 2.5-fold greater volatile emission than *C. p. pepo* ($F_{1,11} = 20.43$, $p < 0.001$), and more beetles enhanced volatile emissions in both subspecies ($F_{1,11} = 7.86$, $p = 0.017$). Neither the interaction between subspecies and beetle number ($F_{1,11} = 0.07$, $p = 0.802$) nor effect of manifold ($F_{1,11} = 0.05$, $p = 0.827$) were significant. Headspace composition differed between subspecies (PERMANOVA subspecies pseudo-$F_{1,13} = 7.69$, $p = 0.001$; manifold pseudo-$F_{1,13} = 0.89$, $p = 0.535$). Random forest classification had an out-of-bag error rate of 12.5%, and found linalool and an unidentified compound ('RT11.8') were sufficient to parse subspecies through variable selection (Table S5). Most *C. p. pepo* samples lacked RT11.8 and linalool concentration was fourfold higher in *C. p. ovifera* than *C. p. pepo* (Table 1; Figure S3).

4 | DISCUSSION

Disentangling the roles of plant chemistry and pheromone production or perception on the hierarchy of host choice can provide insight as to why aggregating insects demonstrate preference for particular plants and inform management strategies. Here, we sought to

determine how plant chemistry affects successive steps in *A. vittatum* aggregation, and if this differed between preferred *C. pepo* ssp. *pepo* and non-preferred *C. pepo* ssp. *ovifera*. Unexpectedly, we found no differences in conspecific attraction to plant volatiles from *C. p. pepo* or *C. p. ovifera* during field colonization under any treatment tested—controls (constitutive plant volatiles), female-infested plants (induced plant volatiles, lacking pheromone), plants previously infested with males (induced plant volatiles, lacking pheromone) and male-infested plants (induced plant volatiles, with pheromone). Instead, the density of feeding male beetles was the strongest predictor of beetle attraction and plants lacking active male beetle feeding were unattractive. Thus, we tentatively conclude that active male feeding is a sufficient stimulus to release aggregation pheromone and resulted in density-dependent field-scale attraction and colonization on seedlings of both plant subspecies (pre-flowering). However, *A. vittatum* rejected *C. p. ovifera* through emigration, likely due to deterrent plant volatiles.

4.1 | Beetle attraction to plant chemistry, pheromones in host location

Our work compared *A. vittatum* aggregation behaviour between two cultivated *C. pepo* subspecies. Given the established dichotomy in *A. vittatum* preference between subspecies across multiple scales (Hoffmann et al., 1996; Brzozowski et al., 2016), we hypothesized that vittatalactone release, plant volatiles, or the interactions thereof, influenced *A. vittatum* preference during field colonization. These functions are non-exclusive as, for instance *Leptinotarsa decemlineata* is attracted to plant volatiles alone, and volatiles synergize with the aggregation pheromone (Dickens, 2006).

While we lacked sufficient sensitivity to quantitatively characterize vittatalactone concentrations, we confirmed that vittatalactone was released by male beetles feeding upon both *C. pepo* subspecies, as is true for both cucurbitacin-rich and -poor cucumber plants (Smyth & Hoffmann, 2002, 2003). Food sources commonly increase aggregation responses across diverse beetles (reviewed in Wertheim, van Baalen, Dicke, & Vet, 2005), and further work is needed to understand how ingesting food facilitates release in this species. Nonetheless, our results indicate that differences in pheromone production while *A. vittatum* feeds upon *C. pepo* is not the cause of observed preference differences between subspecies.

There was also no evidence that plant volatiles themselves or differential synergism with aggregation pheromone affect host plant attraction. While attraction to volatiles (especially from damaged tissue) is widespread in aggregating beetles (Bolter et al., 1997; Loughrin et al., 1996, 1997; Sun et al., 2010), our result is consistent with previous work that constitutive and induced cucumber volatiles were unattractive to *A. vittatum* (Smyth & Hoffmann, 2003). Furthermore, the consistent density-dependent attraction of conspecifics to both *C. pepo* subspecies when infested with male beetles indicates that volatiles from both subspecies had either equal positive effects or were neutral. In the discrete treatment assay,

there was greater attraction to the preferred *C. p. pepo* infested with fewer male beetles than non-preferred *C. p. ovifera*. However, when we assayed a quantitative range of male beetles the following year, conspecific attraction was proportional to male beetle density.

We thus conclude that beetles do not demonstrate preference for *C. p. pepo* during host location due to long distance plant volatile cues or aggregation pheromone (vittatalactone), indicating that observed differences in attack are due to other behaviours.

4.2 | Local cues, and host acceptance

Local cues that lead to individual movement decisions are an important component of insect host acceptance (Finch & Collier, 2000; Silva & Clarke, 2019), and appear to affect *A. vittatum* preference. There was greater *A. vittatum* emigration from the non-preferred *C. p. ovifera* even when undamaged leaf tissue remained. Similarly, *A. vittatum* was equally attracted to cucumber monocultures (preferred) and cucumbers in a multi-species polyculture (corn, broccoli; less preferred; Bach, 1980a, 1980b). However, *A. vittatum* remained longer in monocultures, and more frequently moved from polycultures to monocultures (Bach, 1980a, 1980b). Thus, emigration after host plant quality assessment appears to be a key component of *A. vittatum* preference. However, mechanistically, previous work found no associations between common metrics of leaf quality like nitrogen, trichomes, or water content and *A. vittatum* damage across diverse Cucurbitaceae (Theis et al., 2014).

In local choice assays, *A. vittatum* almost exclusively damaged the preferred *C. p. pepo*. Most *C. p. ovifera* had no indications that the beetles sampled the plant before choosing to consume *C. p. pepo*, potentially due to locally acting deterrents. While specific compounds were not measured, induced deterrent volatiles to generalist caterpillars were previously reported in other *C. pepo* cultivars (Brzozowski et al., 2019).

4.3 | Scale-dependent cues?

We found that two volatiles, linalool and an unidentified compound, had differential emission between subspecies. Such differences are often sufficient cues for insects about host plant identity (Parachnowitsch, Raguso, & Kessler, 2012) and may indicate plant resistance status (but see Pearse, Gee, & Beck, 2013). Although we cannot establish causality, the greater emission of linalool in *C. p. ovifera* may be responsible for short distance deterrence and host rejection. Plant-produced linalool has been reported to deter insect herbivory (Aharoni et al., 2003) and oviposition (Kessler & Baldwin, 2001), and have contrasting scale-dependent effects (Yang, Stoopen, Thoen, Wiegers, & Jongsma, 2013) in other systems. Increased replication and assays with isolated linalool would provide stronger evidence for deterrence by *C. p. ovifera*. However, short-range repellency is still consistent with our host location findings, as insects typically rely on scale-dependent cues (Finch & Collier, 2000; Silva & Clarke, 2019).

4.4 | Implications for pest management on diversified farms

Our work advances efforts to reduce beetle damage on *C. pepo* crops via influencing *A. vittatum* movement by plant germplasm deployment. First, if exclusively non-preferred *C. p. ovifera* crops are grown, there may be aggregation hotspots: beetles confined on *C. p. ovifera* and *C. p. pepo* generate similar amounts of overall damage, but it is concentrated on fewer *C. p. ovifera* plants (Brzozowski et al., 2016), indicating that beetles will eventually aggregate on some *C. p. ovifera* plants after emigrating. Plant breeding for enhanced *C. p. ovifera* deterrence could further promote beetle movement, and should be paired with trap cropping to provide a destination for emigrating beetles. Trap cropping often relies on flowering cucurbits (Gardner, Hoffmann, & Mazourek, 2015), as floral volatiles are highly attractive to beetles (Andersen & Metcalf, 1986). For *C. pepo*, we show that trap cropping to minimize damage on *C. p. pepo* likely relies on the attractiveness of *C. maxima* alone, whereas the trap effectiveness may be amplified by beetles emigrating from *C. p. ovifera*. Similarly, beetle movement should also be investigated on farms with successional plantings, where there are *C. pepo* at both seedling and flowering stages, as we expect different drivers of beetle colonization behaviour (deterrence and attraction, respectively). While our work further confirms that vittatalactone is sufficient for beetle host location on diverse *C. pepo*, data on the effectiveness of synthetic lures are mixed and require additional development (JG and MH, unpubl. data, 2012; Weber, 2018).

This study provides mechanistic information on how *A. vittatum* behaviour is affected by *Cucurbita* spp. germplasm, and broadly highlights the value of crop diversification and suggests strategies by which it improves pest management. Approaches like push-pull, where non-preferred and preferred cultivars are used in spatial arrangements to 'push' pests away from the main crop and 'pull' towards the trap crop (Miller & Cowles, 1990) already target insect movement behaviour as a means of pest management. For farms without an explicit push-pull design and management, our work emphasizes that pest movement along a preference gradient could have substantial impacts on pest densities and ultimately damage. Thus, by considering interactions between diverse crop cultivars in the system, management efforts may be more successful. Importantly, and complementary to management efforts, plant breeders should seek to augment this range of traits from attraction to deterrence to increase performance of the system as a whole, not only for individual crops.

5 | CONCLUSIONS

We found that *A. vittatum* demonstrated no preference in field colonization for *C. p. pepo* over *C. p. ovifera* seedlings and released pheromone if confined on either plant. Instead, beetles more readily rejected and emigrated from the non-preferred *C. p. ovifera* and attacked preferred *C. p. pepo* at local scales, potentially due local

perception of plant volatiles. These results add to our understanding of why *C. p. pepo* cultivars are more consistently damaged by *A. vittatum*: it is not because of aggregation behaviours, but likely due to deterrent cues from *C. p. ovifera* that beetles respond to when close to plants. Broadly, variation in factors that affect host preference may be commonly limited to cues that mediate insect behaviour at local scales and host acceptance, not field colonization. These preference differentials should be applied in diversified farming systems to modulate pest movement and reduce crop damage.

CONFLICT OF INTEREST

A.A.A., A.K., J.G., L.J.B. and M.P.H. declare that they have no conflict of interest. M.M. is the co-founder of Row 7, an organic seed company.

AUTHORS' CONTRIBUTIONS

A.A.A., L.J.B., M.P.H. and M.M. designed the project; J.G. and L.J.B. collected the data; A.A.A., A.K., L.J.B. and M.M. analysed the data; L.J.B. led the writing of the manuscript with contributions and approval from all authors.

DATA AVAILABILITY STATEMENT

Data available via the Dryad Digital Repository: https://doi.org/10.5061/dryad.69p8cz8zg (Brzozowski et al., 2020).

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