Herbivore specificity and the chemical basis of plant–plant communication in *Baccharis salicifolia* (Asteraceae)

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Summary

- It is well known that plant damage by leaf-chewing herbivores can induce resistance in neighbouring plants. It is unknown whether such communication occurs in response to sap-feeding herbivores, whether communication is specific to herbivore identity, and the chemical basis of communication, including specificity.
- We carried out glasshouse experiments using the California-native shrub *Baccharis salicifolia* and two ecologically distinct aphid species (one a dietary generalist and the other a specialist) to test for specificity of plant–plant communication and to document the underlying volatile organic compounds (VOCs).
- We show specificity of plant–plant communication to herbivore identity, as each aphid-damaged plant only induced resistance in neighbours against the same aphid species. The amount and composition of induced VOCs were markedly different between plants attacked by the two aphid species, providing a putative chemical mechanism for this specificity. Furthermore, a synthetic blend of the five major aphid-induced VOCs (ethanone, limonene, methyl salicylate, myrcene, ocimene) triggered resistance in receiving plants of comparable magnitude to aphid damage of neighbours, and the effects of the blend exceeded those of individual compounds.
- This study significantly advances our understanding of plant–plant communication by demonstrating the importance of sap-feeding herbivores and herbivore identity, as well as the chemical basis for such effects.

Introduction

Communication among plants in response to herbivory is a phenomenon that has intrigued ecologists for > 30 yr (Baldwin & Schulz, 1983; Rhoades, 1983; Heil & Karban, 2010; Karban *et al.*, 2014a,b; Moreira *et al.*, 2016). Plant–plant communication occurs when undamaged plants ('receivers' hereafter) respond to airborne cues (volatile organic compounds, VOCs) from herbivore-damaged neighbours ('emitters' hereafter) by increasing anti-herbivore defences (Karban, 2008; Heil, 2014; Karban *et al.*, 2014a), potentially enhancing fitness (Karban & Maron, 2002). Although methodological problems in early studies generated controversy (Fowler & Lawton, 1985), this phenomenon has now been demonstrated with field and laboratory experiments for > 30 plant species (reviewed by Heil & Karban, 2010; Karban *et al.*, 2014a).

Upon herbivore attack, plants deploy a wide variety of induced responses (Agrawal, 1999; Bingham & Agrawal, 2010; Xiao *et al.*, 2012; Moreira *et al.*, 2015; Rowen & Kaplan, 2016). The level of induced defences in plant tissues are dependent on the plant genetic, ontogenetic, as well as environmental conditions, but also vary as a function of the abundance, diet breadth and feeding guild of herbivores (Mithöfer & Boland, 2008). Biotic stimuli from herbivores (e.g. oviposition secretions and saliva) can trigger biochemical and physiological responses specific to the attacker (Mithöfer & Boland, 2008). Such specificity of induced responses is believed to be adaptive because it enhances traits that effectively increase plant resistance and reduces investment in less effective traits (Bingham & Agrawal, 2010). Despite substantial evidence for specificity of induced plant responses, it is unknown whether this specificity extends to the responses of (and thus communication with) neighbouring plants. Herbivore-mediated specificity in plant–plant communication should similarly reduce damage and increase the fitness of receiver plants, as the identity of a neighbour's herbivores is presumably predictive of the identity of the receiver's future herbivores.

Although a wide range of herbivore-induced VOCs have been documented (De Moraes *et al.*, 1998; Rasmann & Turlings, 2008; Clavijo McCormick *et al.*, 2012; Xiao *et al.*, 2012; Rowen & Kaplan, 2016), few studies have experimentally documented which volatiles underlie plant–plant communication, including specificity of communication. A number of studies from a breadth of plant–herbivore systems have identified specific VOCs associated with both intra- and inter-specific plant–

communication, including methylated forms of plant hormones, green leaf volatiles and terpenoids (Karban *et al.*, 2000; Erb *et al.*, 2015; Quintana-Rodriguez *et al.*, 2015; Rowen & Kaplan, 2016). Nevertheless, few studies have experimentally exposed undamaged receivers to herbivore-induced VOCs to demonstrate definitively the chemical basis to plant–plant communication. One exception is work by Erb *et al.* (2015), which showed that the emission, and subsequent perception, of indole by maize plants can increase resistance against future herbivory episodes in neighbouring conspecifics. Although it is well known that herbivore-induced VOCs can be specific to herbivore identity (De Moraes *et al.*, 1998; Rasmann & Turlings, 2008; Clavijo McCormick *et al.*, 2012; Xiao *et al.*, 2012; Rowen & Kaplan, 2016), no past study has tested for specificity of plant–plant communication, or the VOCs mediating such specificity.

Past work on plant–plant communication has been based upon damage by leaf-chewing herbivores (about one quarter of studies) or artificial damage (three quarters of studies), rather than that by sap-feeding herbivores (Karban *et al.*, 2014a). Nevertheless, sap-feeding herbivores (e.g. aphids) are ecologically important, being among the most destructive insect pests on cultivated and wild plants in temperate regions (Blackman & Eastop, 2006). Leaf-chewing and sap-feeding herbivores elicit VOCs via separate plant signalling pathways (jasmonic acid and salicylic acid, respectively; Staudt *et al.*, 2010; Saad *et al.*, 2015). Accordingly, the strength and underlying chemical basis of plant–plant communication may differ between these two herbivore guilds.

We tested for specificity in plant–plant communication with respect to two sap-feeding herbivores and the VOCs underlying such effects. We carried out a glasshouse experiment using the dioecious woody shrub *Baccharis salicifolia* (Asteraceae) and two common and ecologically distinct aphid species. In doing so, we build towards a more realistic and complete understanding of the ecological and evolutionary consequences of the herbivore specificity of plant–plant communication and the chemical mechanisms underlying such specificity.

Materials and Methods

Natural history

Baccharis salicifolia (Ruiz & Pav.) Pers. is a long-lived, dioecious, woody shrub that grows in riparian or otherwise mesic habitats throughout the southwestern United States and northern Mexico. This species reproduces exclusively via seed, with flowering occurring between January and May. The present study was based upon a natural population of *B. salicifolia* occurring in 80 ha of habitat within the University of California San Joaquin Marsh Reserve (33.66°N, 117.85°E; Orange County, CA, USA; Mooney *et al.*, 2012; Abdala-Roberts *et al.*, 2016).

Several characteristics of this plant species make it an ideal model system to address questions regarding the nature, magnitude, mechanisms and specificity of plant communication. First, vascular connections among stems in *B. salicifolia* are limited due to its growth form of having many co-dominant stems emerging

from a single root mass. Therefore, signalling among neighbouring plants could have evolved as a byproduct of adaptation for within-plant communication to coordinate systemic induction, as was shown in other species with similar inefficient vascular connections (Frost *et al.*, 2007; Karban *et al.*, 2014a). Second, because of putative medicinal properties, the secondary chemistry of *B. salicifolia* has been well characterized, and is known to be dominated by several mono- and sesquiterpenes volatile compounds (e.g. Loayza *et al.*, 1995; García *et al.*, 2005), which have been proposed to mediate plant–plant communication in other systems. Third, *B. salicifolia* frequently grows in large, highly dense monospecific stands, allowing for plant–plant communication even if VOC dispersal is limited to short distances. Finally, *B. salicifolia* can be cloned easily from cuttings, thus allowing for experimental controls of genetic variation in plant–plant communication (see later).

At our study site *B. salicifolia* is colonized extensively by two aphid species (Mooney *et al.*, 2012). The specialist aphid *Uroleucon macolai* Blanchard (Hemiptera: Aphididae) feeds on nonwoody terminal stems (Mooney *et al.*, 2012; Abdala-Roberts *et al.*, 2016) and has an exceptionally narrow diet breadth, feeding only upon *B. salicifolia* and *B. polifolia* Griseb (Blackman & Eastop, 2006). The generalist aphid *Aphis gossypii* Glover (Hemiptera: Aphididae) feeds on the underside of leaves or on the growing tips of *B. salicifolia* shoots (Mooney *et al.*, 2012; Moreira & Mooney, 2013; Abdala-Roberts *et al.*, 2016) and is reported to feed on numerous host plant species across the angiosperm phylogeny, including a number of important crops (Blackman & Eastop, 2006). These two aphid species differ in size, with *U. macolai* being *c.* three-fold larger than *A. gossypii,* the aphids being 1.27 ± 0.08 mg and 0.45 ± 0.07 mg per adult, respectively (mean $\pm$ SE; $n = 10$). Both species of aphids are extremely tractable as experimental subjects because they are viviparous, parthenogenetic and have very short generation times (1–2 wk; Dixon, 1998).

Expt 1: Herbivore specificity of plant–plant communication

This experiment was carried out in two replicate iterations, with minor differences in methodology based upon the plant material available in each instance. On November 2012, the first iteration, we cloned two male and two female *B. salicifolia* genetic lines ('genotypes' hereafter) from our study site to be able to statistically control for genetic variation in plant–plant communication. Clonal copies of parental genotypes originated from 10-cm-long stem cuttings of mature plants. To obtain distinct genotypes, we collected cuttings from four plants distributed over the 80 ha of our study area. We placed fresh cuttings in perlite medium under a misting bench for 4 wk, and then transplanted them to 2-l pots with a soil mixture composed of equal parts peat moss, redwood compost, silica sand and pumice mixed with slow-release fertilizer at a concentration of 0.5 g l^{-1} of soil. At the same time, we collected aphids of both species from a single stem of two different plants and reared them on potted *B. salicifolia* in a separate glasshouse. These plants supporting aphids were watered regularly and maintained at 22–25°C. In May 2013 (6 months later),

when plants reached *c.* 140 cm in height (main stem), we assigned two *B. salicifolia* plants of the same genotype to a mesh fabric cage (69 × 69 × 122 cm) in order to prevent aphid dispersal and intrusion of aphid natural enemies. One plant acted as an emitter and the other one served as receiver. We assigned emitter plants to one of three treatments: subjected to *U. macolai* feeding; subjected to *A. gossypii* feeding; and control (untreated plants). In total, there were 38 plants corresponding to 19 cages (eight control, five with *U. macolai*, and six with *A. gossypii*) and two plants per cage. We separated emitter and receiver plants inside the cages so that plants were separated by a minimum of 20 cm. We separated adjacent cages by 2 m to prevent cross-communication among the different treatments: 60 cm was considered the distance for plant–plant signalling in other systems (Karban *et al.*, 2006; Heil & Adame-Álvarez, 2010).

We added unwinged, mature (reproductive) aphids to a single growing tip of each emitter plant using a fine paintbrush. Because *U. macolai* is *c.* three-fold larger than *A. gossypii* (see earlier), we added aphids at a density of 30 *U. macolai* and 90 *A. gossypii* individuals in emitter treatments in order to provide a similar herbivore biomass on all plants. Aphids fed and reproduced on the emitter plants for 15 d, after which emitter plants were removed (keeping the receivers inside the cages). During this period, aphid densities remained sufficiently low to avoid the induction of winged morphs.

Upon removal of the emitter plants, each receiver plant was inspected to guarantee that it was aphid-free (all were) and then inoculated with two unwinged adult aphids of each species using a fine paintbrush. The aphid species were placed on the growing tips of separate branches, and the length of each branch was recorded. Although we assume that there were no competitive interactions between aphid species, any such dynamics were consistent among treatments. After the aphids reproduced (between 24 and 48 h), the inoculate adults and all but two nymphs of each species were removed. We then monitored the two nymphs of each species for survival, age at first reproduction and reproductive rate daily (number of nymphs per day and aphid) until Day 5 of reproductive maturity (Mooney *et al.*, 2012).

The second iteration of this experiment was nearly identical to the first one, with the following exceptions: different *B. salicifolia* genotypes from the same population were used; plants were grown for 3 months (from September to December 2013) to reach a height of *c.* 30 cm (vs 6 months and 140 cm above); there were 30 cages (10 per treatment) and 60 plants (vs 19 cages and 38 plants above); and because plants were smaller in size, aphid adults were added at a density of 15 *U. macolai* and 45 *A. gossypii* (vs 30 *U. macolai* and 90 *A. gossypii* above). All other experimental procedures were identical to those performed for the first iteration.

We separately analysed age at first reproduction and reproductive rate (nymphs produced) at Day 5 of reproductive maturity as metrics of performance for each aphid species on receiver plants with linear models using PROC GLM in SAS (SAS 9.4 System; SAS, Cary, NC, USA; Littell *et al.*, 2006). We treated the main effects of emitter induction treatment (three levels: emitters as control, *A. gossypii* feeding and *U. macolai* feeding) and experimental iteration as fixed factors. To account for size differences among plants, we included the total length of the respective aphid-bearing branches as a covariate. There was no effect of plant sex in preliminary analyses, and we removed this from our statistical model. We did not include the effect of plant genotype due to insufficient replication.

Expt 2: Effects of herbivory on VOC emission

On January 2014, we cloned three male and three female genotypes of *B. salicifolia* from our same study site. Three months later (April 2014) we assigned plants among three induction treatments: control (untreated), subjected to *U. macolai* feeding (15 individuals per plant), and subjected to *A. gossypii* feeding (45 individuals per plant). We assigned 18 plants each to control and *A. gossypii* treatments, and 20 plants for the *U. macolai* treatment, with plant genotypes represented approximately equally among and within the treatments. Each plant was individually enclosed in a mesh fabric bag. Two weeks after initiating induction treatments, we collected aboveground VOCs emitted from each plant following Rasmann *et al.* (2011). Briefly, plants were bagged with a 2-l Nalophan bag, and VOCs were adsorbed on a 100 mg super-Q trap for 6 hr at a rate of $0.25 \, l \, min^{-1}$. Traps were eluted with 150 µl dichloromethane (Merck, Dietikon, Switzerland) to which we had previously added the internal standard (IS) (tetraline; Sigma-Aldrich, CAS number: 119-64-2, 198 ng in 10 µl dichloromethane). Five microlitres of each sample was subsequently injected onto a GC-MS (Agilent 6890 Gas Chromatograph coupled with a 5973N Mass Selective Detector; Agilent, Santa Clara, CA, USA) fitted with a 30 m × 0.25 mm × 0.25 µm film thickness HP-5MS fused silica column (Agilent). We operated the GC in splitless mode with helium as the carrier gas (flow rate $1 \, ml \, min^{-1}$). The GC oven temperature program was: 1 min hold at 50°C, $10°C \, min^{-1}$ ramp to 130°C, $5°C \, min^{-1}$ ramp to 180°C, $20°C \, min^{-1}$ ramp to 230°C and 1 min hold at 300°C. We identified volatile terpenes using Kovats retention index from published work (Loayza *et al.*, 1995; Zunino *et al.*, 1997) and by comparison with commercial standards when available. We measured the richness (total number of compounds) and total emission of individual VOCs as a proportion to the internal standard.

We compared induction treatments for the richness of VOCs (number of compounds), the emission of each individual VOC, and total VOC emission by using linear models using PROC GLM in SAS (SAS 9.4 System; Littell *et al.*, 2006). For these analyses, we treated the main effect of induction treatment (control, *A. gossypii* feeding, and *U. macolai* feeding) as a fixed factor. We also included plant sex and plant genotype (nested within sex) in order to account for sex and genotype identity differences between individual plants. Using the same statistical model, we then compared induction treatments for chemical composition (abundances of each VOCs) using a permutational multivariate analysis of variance (PERMANOVA; Anderson, 2001; Pratt *et al.*, 2014). A PERMANOVA is analogous to an ANOVA, but partitions similarity matrices between treatments and uses permutation tests with pseudo-*F* ratios. We then calculated

similarity percentage (SIMPER) to determine which compounds contribute to the observed pairwise differences in VOC profile among herbivore-induction treatments. SIMPER analysis calculates the average contribution of individual components to the average dissimilarity between samples or groups that are known to differ among treatments based upon PERMANOVA results. PERMANOVA and SIMPER tests were based on 10 000 permutations of a Bray–Curtis dissimilarity matrix for VOC emission. This matrix was calculated using the GC-MS volatile peak emissions with a square-root transformation to reduce the influence of highly abundant compounds. Differences in VOC composition among herbivore-induction treatments were visualized using nonmetric multidimensional scaling (NMDS). Ordination was achieved in two dimensions after three runs (stress = 0.138; $R^2 = 0.92$). Both PERMANOVA and NMDS were executed using the 'VEGAN' package (Oksanen *et al.*, 2015) in R v.3.2.3 (R Core Team, 2015).

Expt 3: Effects of VOCs exposure on aphid performance

Based on the findings from Expt 2, we sought to explore the role of specific VOCs in mediating plant–plant communication. On April 2015 in the glasshouse we exposed 70 individuals of *B. salicifolia* belonging to 11 genotypes (six males and five females) to seven different synthetic blends of VOCs; five individual herbivore-induced VOCs that were highly inducible after herbivore damage (based upon findings from Expt 2; see the Results section), a blend of all five VOCs and a mock control. Each plant was individually enclosed in a mesh fabric bag. Experimental plants were grown from cuttings as described above, using six of the same genotypes used in Expt 2 as well as five new genotypes from the same field site. Within each treatment, 10 individual plants from either nine or 10 different genotypes were used, half male and half female.

The five chemical compounds used were: ethanone (Sigma-Aldrich, CAS number: 122-00-9), limonene (Sigma-Aldrich, CAS number: 5989-27-5), methyl salicylate (Sigma-Aldrich, CAS number: 119-36-8), myrcene (Sigma-Aldrich, CAS number: 123-35-3) and ocimene (Sigma-Aldrich, CAS number: 13877-91-3). We also observed that (E)-4,8-dimethyl-1,3,7-nonatriene was induced markedly after herbivore damage, but we omitted it due to a lack of exact identification with pure standard.

Volatile exposure treatments consisted of exposing plants to artificial emitters containing one individual compound, five artificial emitters together (all compounds), or empty artificial emitters for control plants. Artificial emitters were placed within 10 cm of *B. salicifolia* plants. Our artificial emitters were similar to those of Hiltpold *et al.* (2010) and Erb *et al.* (2015). Briefly, 2 ml glass chromatographic vials were topped by screw thread caps with a Teflon septa, which had a small hole cut in the centre to pass a 12.5-cm capillary tube (100 µl ringcaps). Each vial contained a small piece of cotton inoculated with 100 µl of the pure compound. The multiple compounds treatment was performed by attaching multiple artificial emitters to each plant, with 20 µl of a single compound in each vial.

Capillary tubes were sealed around the vial entrance and lid using parafilm to ensure volatiles were released only through the upright end of the capillary tube. These emitters were then secured to the sides of pots of experimental plants within mesh cages for an exposure period of 5 d. During the exposure period, all plants within a volatile treatment were grouped together to avoid airborne mixing of compounds among volatile treatments, with a spacing of 1 m between plants within treatment groups, and at least 3 m between plants among treatment groups. After exposure, the emitter vials were removed, and all plants were intermixed and randomized at a minimum spacing of 1 m. We then conducted an aphid performance bioassay with *U. macolai* (only) using the same methodology as described above (Expts 1, 2).

We separately analysed age at first reproduction and reproductive rate (nymphs produced) at Day 5 of reproductive maturity as metrics of performance for *U. macolai* with linear models using PROC GLM in SAS (SAS 9.4 System; Littell *et al.*, 2006). For these analyses, we conducted orthogonal *a priori* contrasts comparing the means of each volatile treatment against the control. We treated the main effect of exposure treatments (control, each individual compound and all five compounds combined) as a fixed factor. We also included plant sex and the length of the aphid-bearing branch as covariates. We did not include the effect of plant genotype due to insufficient replication.

Results

Expt 1: Herbivore specificity of plant–plant communication

Overall, we detected herbivore-specific induction of resistance in receiver plants with respect to reproductive rate (at Day 5), but not to age at first reproduction; feeding by each aphid on emitter plants induced resistance to the same, but not the alternate aphid species on neighbouring plants.

Emitter plants damaged by *U. macolai* did not significantly affect the age at first reproduction of *U. macolai* on receivers (Table 1a; Fig. 1a). Emitter plants damaged by *U. macolai* (but not by *A. gossypii*) significantly affected the *U. macolai* reproductive rate at Day 5 of reproductive maturity on receiver plants (Table 1a; Fig. 1b). The reproductive rates of *U. macolai* on receiver plants adjacent to emitters damaged by *U. macolai* were *c.* 30% lower compared with receiver plants that were adjacent to control (undamaged) and *A. gossypii* damaged emitters, and these latter two did not differ significantly from each other (Fig. 1b).

Emitter plants damaged by *A. gossypii* did not significantly affect the age at first reproduction of *A. gossypii* on receivers (Table 1b; Fig. 1c). Emitter plants damaged by *A. gossypii* (but not by *U. macolai*) marginally affected *A. gossypii* reproductive rate at Day 5 of reproductive maturity on receiver plants (Table 1b; Fig. 1d). *Aphis gossypii* reproductive rate on receivers that were adjacent to *A. gossypii* damaged emitters were *c.* 23% lower compared with receivers that were adjacent to control and *U. macolai*-damaged emitters, and these latter two did not differ significantly from each other (Fig. 1d).

Table 1 Herbivore specificity of plant–plant communication

	df$_{num,den}$	Age at first reproduction		Reproductive rate at reproductive Day 5	
		F	P	F	P
(a) *Uroleucon macolai*					
Treatment	2,44	1.96	0.153	12.87	**< 0.001***
Experiment iteration	1,44	19.55	**< 0.001**	0.89	0.350
Branch length	1,44	1.21	0.278	0.12	0.733
(b) *Aphis gossypii*					
Treatment	2,41	2.18	0.126	3.17	**0.053***
Experiment iteration	1,41	28.74	**< 0.001**	5.34	**0.026**
Branch length	1,41	12.04	**0.001**	0.47	0.498

Summary of the linear model for the effects of emitter induction treatment on emitter *Baccharis salicifolia* plants (three levels: emitters as control, *Uroleucon macolai* feeding and *Aphis gossypii* feeding) on the performance of (a) *U. macolai* and (b) *A. gossypii* on receiver plants. Because this experiment was carried out in two replicate iterations, the effect of the experimental iteration was included in the statistical model. Aphid performance was measured as age at first reproduction (days) and the reproductive rate at reproductive Day 5. Branch length was used as a covariate. *F*-values, degrees of freedom and associated significance levels (*P*) are shown. Significant (*P* < 0.05) or marginal (0.05 < *P* < 0.10) *P* values are shown in bold.
*Results of *post-hoc* Tukey–Kramer Honest Significant Difference tests are shown in Fig. 1.

Expt 2: Effects of herbivory on VOCs emission

Overall, we found that plants damaged by both aphid species increased the richness and the total amount of VOCs relative to control plants. Moreover, plants damaged by *U. macolai* induced not only all compounds induced by *A. gossypii*, but also some additional ones.

Emitter induction treatments significantly affected the emission of volatile compounds by *B. salicifolia* emitter plants (Table 2). VOC richness from plants damaged by *U. macolai* and *A. gossypii* was 25% higher than in control plants (Fig. 2a). Similarly, the total emission of VOCs in plants damaged by *U. macolai* and *A. gossypii* was 223% and 125% higher, respectively, than in control plants (Fig. 2b). Although the total emissions induced by *U. macolai* was higher than that of *A. gossypii*, this difference was not significant (Fig. 2b). Concerning the emission of individual VOCs, plants damaged by *A. gossypii* and *U. macolai* significantly increased the emission of (E)-β-ocimene, (E)-4,8-dimethyl-1,3,7-nonatriene and ethanone compared with control plants (Table 3). Plants damaged by *U. macolai* also significantly increased the emission of three additional VOCs (β-myrcene, limonene and an unidentified compound) compared with control and *A. gossypii*-damaged plants (Table 3). Both emitter induction treatments also induced the production of methyl salicylate (not present in control plants), and this compound was significantly more induced in plants damaged by *U. macolai* than in plants damaged by *A. gossypii* (Table 3).

We found that VOC profiles varied with herbivore-induced treatments (PERMANOVA; Table 4; Fig. 3). The similarity percentage analysis revealed that in all pairwise comparisons between emitter induction treatments (E)-β-ocimene, limonene, (E)-4,8-dimethyl-1,3,7-nonatriene and an unknown compound (Kovats retention index = 11.59) were the main compounds
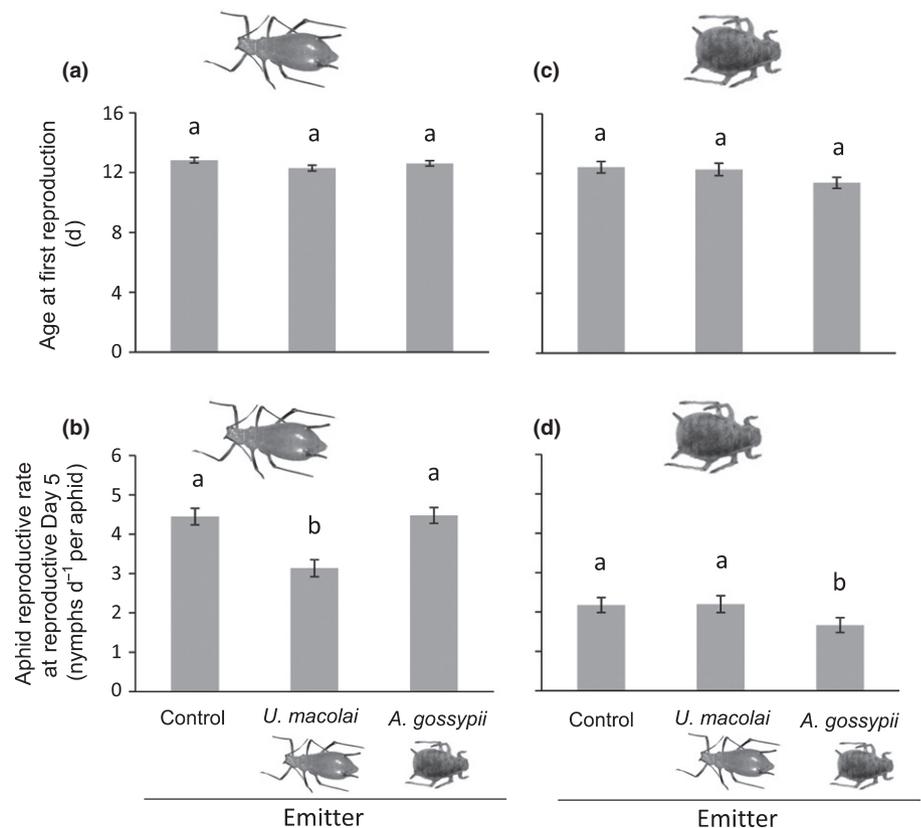

Fig. 1 Herbivore specificity of plant–plant communication. Effect of emitter induction treatments in the emitter *Baccharis salicifolia* plants (three levels: emitters as control, *Uroleucon macolai* feeding and *Aphis gossypii* feeding) on the performance (age at first reproduction and reproductive rate at reproductive Day 5) of both aphid species on receiver plants. Left panel (a, b) shows the performance of the specialist aphid *U. macolai*, whereas the right panel (c, d) shows the performance of the generalist aphid *A. gossypii*. Least-square means ± SE (*n* = 18 for control, 15 for *U. macolai* and 16 for *A. gossypii*). Different letters indicate significant differences within treatments at *P* < 0.05, based on *post-hoc* Tukey–Kramer Honest Significant Difference tests.

Table 2 Effects of herbivory treatments on volatile richness and emission

		Richness		Emission	
	$df_{num,den}$	F	P	F	P
Treatment	2,48	4.44	**0.017***	10.26	**< 0.001***
Sex	1,48	1.54	0.220	0.00	0.947
Genotype (sex)	4,48	1.93	0.120	1.49	0.221

Summary of the linear model for the effects of induction treatment (three levels: control, *Aphis gossypii* feeding, and *Uroleucon macolai* feeding), plant sex (male vs female) and plant genotype (nested within sex) on the richness and total emission of volatile organic compounds (VOCs) of *Baccharis salicifolia* plants. F-values, degrees of freedom and associated significance levels (P) are shown. Significant P values (P < 0.05) are shown in bold.
*Results of *post-hoc* Tukey–Kramer Honest Significant Difference tests are shown in Fig. 2.

contributing to the observed differences in VOC composition (Fig. 3b). These four compounds collectively explained > 55% of dissimilarity between treatment groups in all pairwise comparisons and were significantly correlated with the NMDS configuration (Supporting Information Table S1).

Expt 3: Effects of VOC exposure on aphid performance

Overall, aphid performance was drastically reduced in plants exposed to artificial emitters in comparison with control plants, but such effects were largely limited to the mixture of the five VOCs.

Specific contrasts between each volatile treatment and the control revealed that the age at first reproduction was 23% higher in plants exposed to all the VOCs combined (ethanone, limonene, methyl salicylate, β-myrcene and β-ocymene) than in control plants (Fig. 4a; Table S2). Age at the first *U. macolai* reproduction in plants exposed to individual VOCs did not significantly differ in comparison with control plants (Fig. 4a; Table S2). Aphid reproductive rate at reproductive Day 5 was 42% lower in plants exposed to all the VOCs combined than in control plants (Fig. 4b). Finally, aphid reproductive rate at Day 5 was lower by 33% (marginal effect) and 30% (marginal effect) in plants exposed to limonene and methyl salicylate, respectively, than in control plants (Fig. 4b).

Discussion

Our results demonstrate plant–plant communication in response to aphid herbivory and that this communication is specific to herbivore identity. Further, the induced resistance observed was underlain by a mixture of volatile organic compounds (VOCs), whose effects exceeded those of individual compounds. Aphid damage to emitter plants only reduced subsequent herbivory by the same aphid species on neighbouring plants. Moreover, we also identified multiple compounds underlying specificity in plant–plant communication in this system. In this sense, the composition of emitted VOCs was markedly different in plants attacked by the dietary generalist (*Aphis gossypii*) and specialist
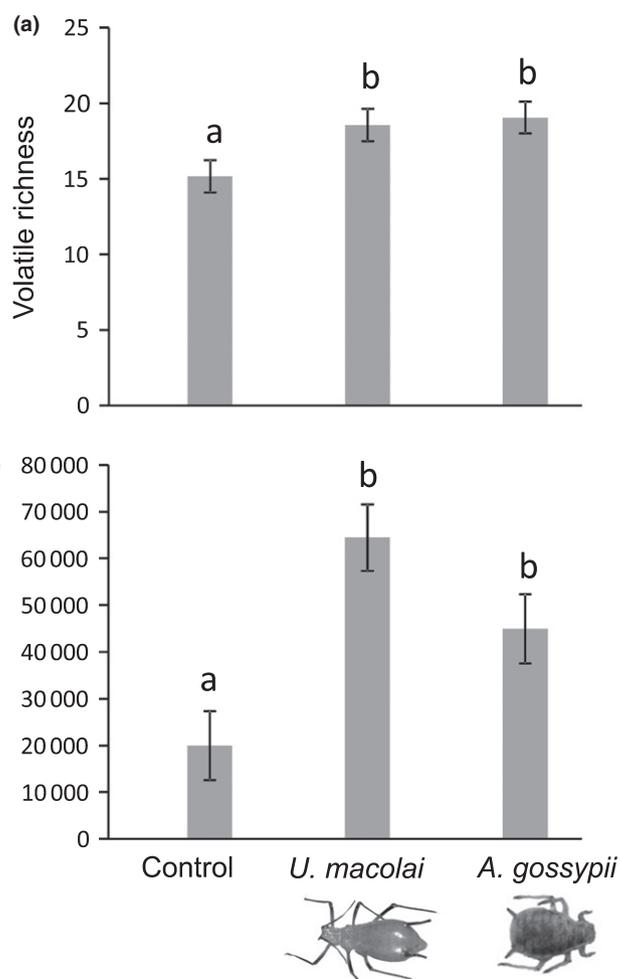

Fig. 2 Effects of herbivory treatments on volatile richness and emission. Effect of induction treatments (three levels: control, *Uroleucon macolai* feeding and *Aphis gossypii* feeding) on the (a) richness (number of compounds) and (b) total emission (ng h⁻¹) of volatile organic compounds (VOCs) of *Baccharis salicifolia* plants. Least-square means ± SE (n = 18 for control and *A. gossypii* treatments and 20 for *U. macolai* treatment). Different letters indicate significant differences within treatments at P < 0.05, based on *post-hoc* Tukey–Kramer Honest Significant Difference tests.

(*Uroleucon macolai*) aphid species, both of which, in turn, differed from undamaged (control) plants. Furthermore, we show that artificial exposure of plants to the mixture of VOCs associated with *U. macolai* feeding (ethanone, limonene, methyl salicylate, myrcene, ocimene) induces resistance in receiver plants of comparable magnitude to real aphid herbivory. Together, these findings provide a better understanding of the ecological and evolutionary consequences of the herbivore specificity of plant–plant communication and the chemical compounds underlying such specificity.

Our results showed that there is herbivore specificity of plant–plant communication, because herbivore resistance on receiver *Baccharis salicifolia* plants increased only when emitter and receiver plants were attacked by the same aphid species. Specificity of induced defences against herbivores has been found across several plant–herbivore systems (Agrawal, 1999; Bingham & Agrawal, 2010; Xiao *et al.*, 2012; Moreira *et al.*, 2013, 2015;

Table 3 Effects of herbivory on individual volatile emission

Compound	Control	A. gossypii	U. macolai
α-pinene (9.37)	78.50 ± 15.90 a	106.03 ± 15.28 a	113.33 ± 16.02 a
β-pinene (9.79)	380.05 ± 118.01 a	590.25 ± 97.61 a	415.20 ± 98.36 a
β-myrcene (9.93)	258.22 ± 84.05 a	384.31 ± 78.67 a	864.37 ± 130.05 b
δ-carene (10.11)	541.32 ± 167.81 a	888.40 ± 167.81 a	821.72 ± 177.80 a
Limonene (10.31)	9434.38 ± 3776.80 a	16 065.06 ± 3421.42 a	25 466.00 ± 3629.13 b
(E)-β-ocimene (10.51)	3774.42 ± 2851.64 a	15 862.00 ± 2851.64 b	11 567.00 ± 2791.60 b
Acetophenone (10.71)	64.56 ± 12.10 a	48.79 ± 11.69 a	59.09 ± 11.35 a
γ-terpinolene (10.89)	90.74 ± 51.01 a	166.02 ± 34.39 a	157.32 ± 34.39 a
Linalool (11.00)	239.05 ± 58.81 a	288.56 ± 86.94 a	191.64 ± 88.46 a
(E)-4,8-dimethyl-1,3,7-nonatriene (11.19)	2083.93 ± 949.62 a	3394.73 ± 919.10 b	5575.21 ± 881.43 b
Unknown (11.59)	8625.20 ± 4629.06 a	6082.50 ± 3492.37 a	24 444.00 ± 3498.25 b
Methyl salicylate (11.98)	–	133.99 ± 70.38 a	462.85 ± 69.27 b
Verbenone (12.08)	71.69 ± 23.54 a	114.88 ± 28.47 a	94.05 ± 22.06 a
Valeric acid (12.38)	327.13 ± 61.62 a	205.65 ± 61.51 a	172.90 ± 69.60 a
Geranial (12.67)	333.22 ± 115.44 a	409.38 ± 118.91 a	392.73 ± 116.96 a
Ethanone (12.86)	209.16 ± 50.42 a	434.84 ± 58.74 b	493.80 ± 62.04 b
Butanoic acid (13.99)	116.14 ± 23.56 a	76.10 ± 22.12 a	74.38 ± 16.38 a
Germacrene D (14.87)	211.14 ± 168.39 a	352.30 ± 142.20 a	635.55 ± 123.34 a
α-selinene (14.96)	143.12 ± 49.46 a	104.60 ± 44.00 a	82.36 ± 28.52 a
γ-cadinene (15.13)	113.72 ± 98.76 a	129.92 ± 82.51 a	338.87 ± 70.89 a
α-cadinene (15.38)	79.46 ± 26.42 a	43.19 ± 19.15 a	60.31 ± 15.13 a

Effect of emitter induction treatments (three levels: control, *Aphis gossypii* feeding and *Uroleucon macolai* feeding) on individual volatile compounds emitted by *Baccharis salicifolia* plants (ng h^{-1}). Least-square means ± SE ($n = 18$ for control and *A. gossypii* treatments and 20 for *U. macolai* treatment). Different letters indicate significant differences within treatments at $P < 0.05$, based on *post-hoc* Tukey–Kramer Honest Significant Difference tests. Compounds follow an ascending order of Kovats retention index, as shown in brackets.

Table 4 Effects of herbivory on volatile profiles

Source	df$_{num,den}$	Pseudo-F	P
Treatment	2, 48	6.02	**< 0.001**
Sex	1, 48	1.15	0.299
Genotype (sex)	4, 48	1.93	**0.013**

Permutational analysis of variance analysis (PERMANOVA) of volatile organic compound (VOC) composition emitted by *Baccharis salicifolia* plants, with the main effects of emitter induction treatment (control, *Aphis gossypii* feeding and *Uroleucon macolai* feeding), plant sex and plant genotype (nested within sex). Analysis is based on Bray–Curtis distances of square-root transformed data, using 10 000 permutations. Significant P values ($P < 0.05$) are shown in bold.

Rowen & Kaplan, 2016). Such specificity could have evolved by means of natural selection, and may thus be adaptive (Bingham & Agrawal, 2010). However, few previous studies have tested whether herbivore specificity extends to the responses of (and thus communication with) neighbouring plants. In one of the few available studies, and contrary to our findings, Helms *et al.* (2013) found that tall goldenrod (*Soligado altissima*) plants that were infested by a galling insect elicited resistance to a chewing herbivore in the neighbouring plants, thus suggesting a broad spectrum effect of VOCs emitted by *S. altissima*. In this sense, specificity can be predicted to be adaptive in that receiver plants are preparing specifically for attack by the herbivore associated with neighbouring (emitter) plants. Such specificity suggests a likely larger role for plant–plant communication (than nonspecific response) as it increases the magnitude of ecologically relevant resistance.

Herbivore-mediated specificity in plant–plant communication must have been underlain by specificity in the induction of VOCs from aphid-damaged *B. salicifolia* plants. In particular, we found that the amount and relative contribution of (E)-β-ocimene, limonene, (E)-4,8-dimethyl-1,3,7-nonatriene, and one unknown compound largely differed between emitter induction treatments (i.e. control and feeding by both aphids). Upon herbivore attack, plants release complex blends of herbivore-induced VOCs from many of their tissues (De Moraes *et al.*, 1998; Clavijo McCormick *et al.*, 2012; Dudareva *et al.*, 2013). Herbivore species vary in their oral secretions, timing, intensity and pattern of damage (Agrawal, 2000; Mithöfer & Boland, 2008), and this, in turn, is likely to drive the considerable herbivore-specific VOC profiles (Clavijo McCormick *et al.*, 2012). Arimura *et al.* (2000) provide evidence for the mechanistic basis of specificity in plant–plant communication. Leaves of uninfested *Phaseolus lunatus* plants exposed to spider mite-induced VOCs (including β-ocimene and (E)-4,8-dimethyl-1,3,7-nonatriene) activated five defence genes, but no gene expression was observed following exposure to VOCs from artificially damaged plants (Arimura *et al.*, 2000). This was likely to be due to a more complex blend of VOCs emitted after spider mite infestation, and reflects our result that the highest resistance was induced from the synthetic mixture of VOCs.

The VOCs underlying plant–plant communication are largely unknown (Heil & Karban, 2010), but past studies of herbivore-induced VOCs are suggestive of the likely agents (e.g. Karban *et al.*, 2014b; Erb *et al.*, 2015). We found that a synthetic blend of five VOCs (ethanone, limonene, methyl salicylate, β-myrcene
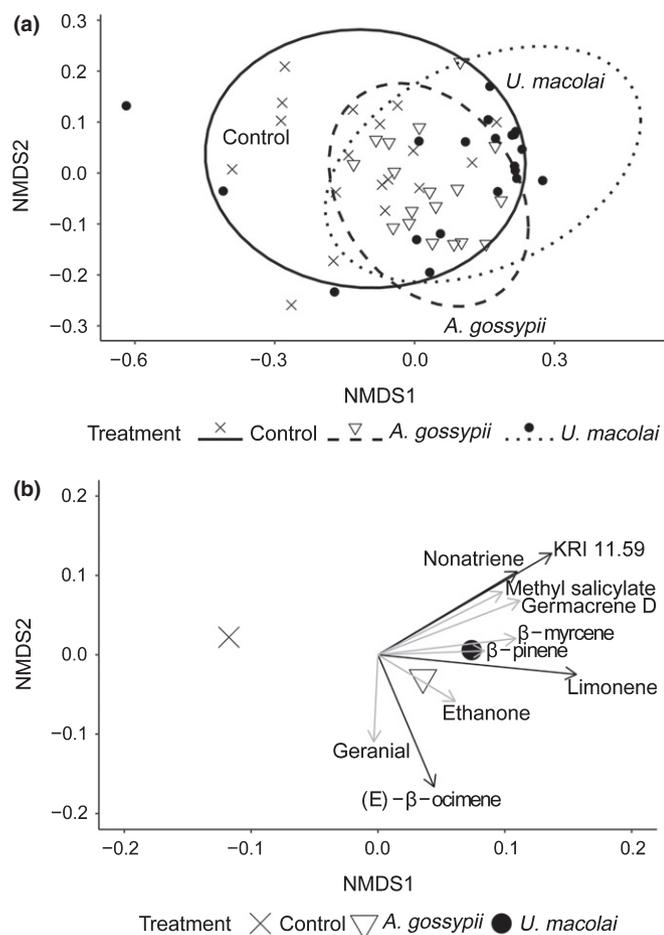
Fig. 3 Effects of herbivory on volatile profiles. (a) Nonmetric multidimensional scaling (NMDS) of *Baccharis salicifolia* volatile profiles for plants of three emitter induction treatments (×, control; triangles, *Aphis gossypii* feeding; circles, *Uroleucon macolai* feeding). Scaling is based on pairwise Bray–Curtis dissimilarity, calculated from the square-root transformation of individual compound emissions from each experimental plant (stress = 0.138). Ellipses indicate 95% confidence intervals around the centroid for each emitter induction treatments (solid line, control; dashed, *A. gossypii* feeding; dotted, *U. macolai* feeding). (b) Volatile organic compound (VOC) vectors indicate influence in NMDS figure, scaled relative to one another by R^2 values. Vectors in black indicate four main compounds contributing to > 55% (in all pairwise comparisons) of dissimilarity in VOC composition among emitter induction treatments, and grey vectors indicate the additional top six compounds that collectively explained > 70% of dissimilarity in all pairwise comparisons.

and (E)-β-ocymene) induced resistance to *U. macolai* at similar magnitude to herbivore-induced volatiles, reducing fecundity by 30% and 43%, respectively. Weaker herbivore resistance was induced by individual compounds, demonstrating that these compounds act synergistically, as has been demonstrated previously for plant recruitment of predators (e.g. D'Alessandro *et al.*, 2006). β-ocimene and (E)-4,8-dimethyl-1,3,7-nonatriene were each observed previously to underlie induced resistance against herbivores (Arimura *et al.*, 2000). Similarly, previous studies have documented that monoterpenes (e.g. (E)-β-ocimene and β-myrcene) play a central role in plant–plant signalling processes (Arimura *et al.*, 2000; Godard *et al.*, 2008) and methyl salicylate

has been shown to prime resistance to bacterial pathogens and sap-feeding herbivores (e.g. Shulaev *et al.*, 1997; Yi *et al.*, 2009; Thaler *et al.*, 2010). Our study thus adds to the growing understanding of the nature and identity of airborne signals involved in plant–plant communication.

To our knowledge, our study is the first demonstration of plant–plant communication mediated by sap-feeding herbivores (i.e. aphids). Previous research on plant communication has focused exclusively on patterns of induction by chewing herbivores or artificial damage mimicking those herbivores (Karban *et al.*, 2014a). The fundamental differences in mouthparts between aphids and chewing herbivores result in different feeding patterns, forms of plant injury and subsequent hormonal responses (Howe & Jander, 2008; Rowen & Kaplan, 2016). Broadly, chewing herbivores tend to elicit the jasmonic acid signalling pathway, whereas aphids and other sap-feeders generally induce the salicylic acid pathway (Thaler *et al.*, 2012). The sap-feeding mouthparts of aphids cause relatively little mechanical damage to plant tissues (Züst & Agrawal, 2016) and past studies have suggested that they induce relatively low concentrations of VOCs (see review by Rowen & Kaplan, 2016). Nevertheless, in the present study feeding by both the specialist and generalist aphid increased the total concentration of VOCs by several fold (3.2- and 2.2-fold, respectively) in comparison with undamaged plants, levels of induction similar to chewing herbivores in other systems (Dudareva *et al.*, 2013). Only direct comparisons of VOC induction between sap-feeding and chewing herbivores on the same plant species will shed more light on this question.

Whether or not plant–plant communication is the by-product of within-plant signalling has been a controversial topic. It is difficult to imagine an advantage of communication to emitter plants, given that the benefit of communication is entirely to the receiver (Heil & Karban, 2010). In this sense, Karban & Shiojiri (2009) have proposed that the emitters might increase their inclusive fitness if receivers are genetically closely related to the emitter (i.e. a sort of kin selection as described for animals). There is indeed rising evidence that plants, like many animals, are able to distinguish VOCs emitted from plants with different degrees of relatedness, in which they increase herbivore resistance in response to VOCs from their close relatives compared to those from unrelated neighbours (Karban & Shiojiri, 2009; Karban *et al.*, 2013, 2014b; Pearse *et al.*, 2013; Moreira *et al.*, 2016). Accordingly, our results showing strong plant–plant communication could be a consequence of using clones of *B. salicifolia* plants as emitters and receivers (i.e. within-plant signaling). Further studies should thus investigate if plant–plant communication in *B. salicifolia* also exists by using different genetic entries as emitters and receivers (i.e. between-plant signalling).

Finally, we point to several key areas of future research that will provide a more comprehensive understanding of ecological and evolutionary consequences of plant–plant communication. First, further studies should test for genetic variation in the strength of plant–plant communication, that is whether genotypes differ significantly as emitters or receivers of closely and distantly related individuals. Evaluating this genetic variation would be the basis for testing for genotypic selection and evolution of

Fig. 4 Effects of volatile exposure on aphid performance. Effect of volatile organic compound (VOC) exposure treatment (E, ethanone; L, limonene; MS, methyl salicylate; M, β-myrcene; O, β-ocymene; ALL, all five compounds) on the performance (age at first reproduction and reproductive rate at the reproductive Day 5) of the specialist aphid *Uroleucon macolai* on *Baccharis salicifolia* plants. The dashed line represents the mean value for control plants and the shaded area represents the standard error around that mean. Least-square means ± SE ($n = 11$). Asterisks indicate significant (*, $P < 0.05$) or marginal ((*), $P < 0.10$) differences between control and the rest of VOC exposure treatments, based on *post-hoc* Tukey–Kramer Honest Significant Difference tests. ns, nonsignificant.
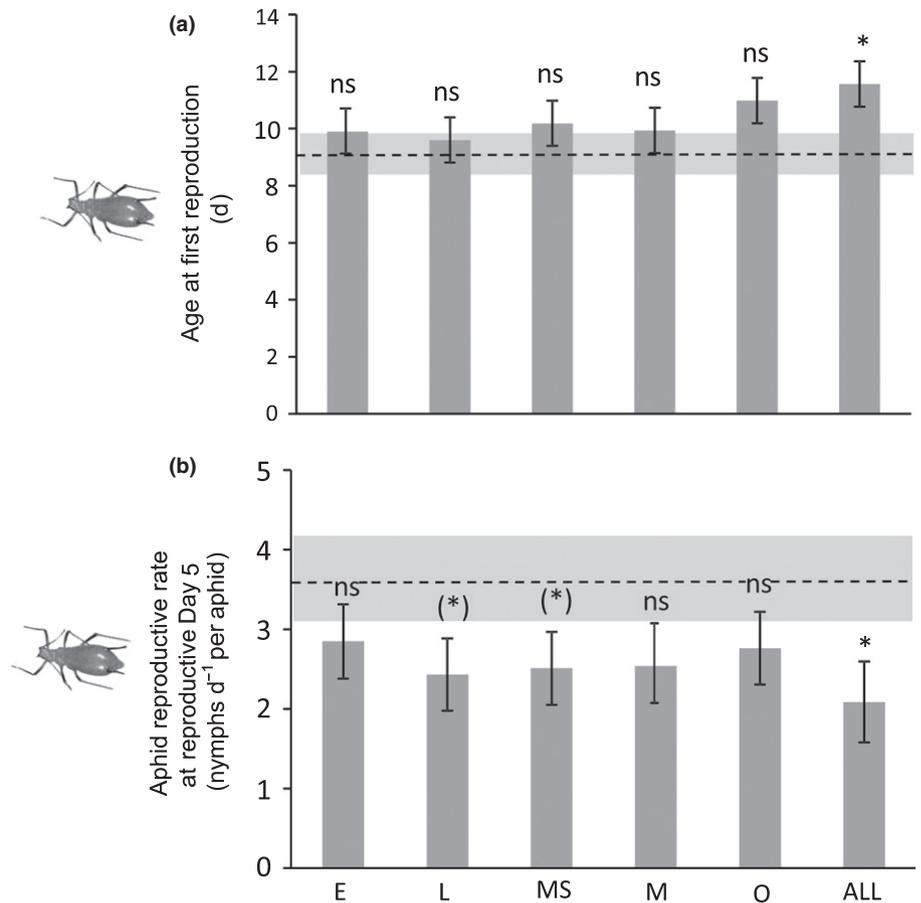
plant–plant communication mechanisms. Second, further studies should address not only the chemical mechanisms underlying the herbivore specificity in plant communication, but also the molecular mechanisms behind this specificity, for example specific changes in the expression of different resistance genes on receiver plants. Third, studies are needed to evaluate how receiver plants may benefit with respect to indirect defence from herbivore natural enemies. Indirect defence could be mediated by associational effects of emitter VOCs recruiting enemies that attack herbivores on receivers, or through emitter VOCs inducing the production of VOCs on receivers that recruit natural enemies. Finally, studies documenting the strength of plant–plant communication in comparison to other ecological dynamics will work to establish the ecological relevance of these dynamics.

Author contributions

X.M., K.A.M. and S.R. planned and designed the research; X.M., C.S.N., A.K. and S.R. performed the experiments; X.M. and C.S.N. analysed the data; X.M. wrote the first draft of the manuscript and C.S.N., S.R. and K.A.M. provided comments on earlier versions.

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Supporting Information

Additional Supporting Information may be found online in the Supporting Information tab for this article:

Table S1 Correlation results of VOCs in relation to NMDS configuration.

Table S2 Summary of the linear model for the effects of VOC exposure treatments on aphid performance.

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