

# Manipulating two olfactory cues causes a biological control beetle to shift to non-target plant species

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## Summary

**1.** Olfactory cues can determine the host preferences of herbivorous insects, but their role in host shifting is unclear. Host specificity and the potential for host shifts are important criteria for screening and post-release evaluation of biological control agents for invasive plants. However, the role of olfactory cues in mediating host shifts in biological control agents is not well understood.

**2.** To investigate the role of olfactory cues in host selection of a reportedly monophagous flea beetle (*Agasicles hygrophila*), an important biocontrol agent for invasive alligator weed (*Alternanthera philoxeroides*), we extracted and analysed the volatiles produced by the host-plant *A. philoxeroides* and the non-host plants *Alternanthera sessilis*, *Beta vulgaris* and *Amaranthus mangostanus*. Moreover, we used electrophysiological techniques, behavioural bioassays and field trials to test the antennal responses and behavioural preferences of *A. hygrophila* to combinations of different plant volatiles and treatments, and pure compounds in different dosages and combinations.

**3.** We show that *A. hygrophila* female beetles indeed use olfactory cues to select plants for feeding and oviposition and that the survivorship of larvae on the second preferred non-host plant *A. sessilis*, a close relative of the first preferred host plant *A. philoxeroides*, was over 75% in a field trial. Although female beetles responded to many volatile compounds from host and non-host plants, (*E*)-4,8-dimethyl-1,3,7-nonatriene (DMNT) positively encouraged the beetle's feeding and oviposition preferences, whereas (*Z*)-3-hexenol displayed repellent effect. Remarkably, complementation assays with (*Z*)-3-hexenol on host plant or DMNT on non-host plants significantly shifted *A. hygrophila* host preferences to non-host plants and resulted in oviposition and egg hatching on the non-host plant *A. sessilis* in field trials.

**4. Synthesis.** We demonstrate an olfactory mechanism by which a specialized herbivorous beetle uses the ratio of two common plant volatiles, (*E*)-4,8-dimethyl-1,3,7-nonatriene and (*Z*)-3-hexenol, to discriminate between its host and non-host plants in nature. This study highlights an important mechanism by which olfactory cues could lead to undesired host range expansion in biocontrol agent, thus representing an important warning of the potential for a host shift and development of invasiveness in a common biocontrol agent, the flea beetle.

**Key-words:** (*E*)-4,8-dimethyl-1,3,7-nonatriene, (*Z*)-3-hexenol, *Agasicles hygrophila*, *Alternanthera philoxeroides*, attraction and repulsion, host plant preference, host shift, non-target effect, plant volatiles

## Introduction

Host plant shifting is among the most common mechanisms of speciation in the phytophagous insects, and is thus an important evolutionary force for the origin and maintenance

of biodiversity in this group (Farrell 1998; Berlocher & Feder 2002; Dres & Mallet 2002). Shifting hosts can lead to reproductive isolation and behavioural variation by promoting divergent adaptation that impede gene flow for sympatrically differentiated host-plant races of herbivores, resulting in speciation (Hatanaka 1993; Feder *et al.* 1994; Hawthorne & Via 2001; Smadja & Butlin 2009). For the biological control of weeds, the possibility of host shifting raises an important

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concern in cases where highly specialized herbivores are deliberately introduced into new environments. These environments of course contain not only target weedy species but also potential new hosts. In addition, newly introduced herbivores also experience different selective pressures in the new environments compared to their original habitats (van Klinken & Edwards 2002; Louda *et al.* 2003). Host specificity and the potential for host shifts are important criteria for screening and post-release evaluation of biological control agents for invasive plants (Ma 2001; van Klinken & Edwards 2002; Lu & Ding 2012).

Most phytophagous beetles feed on a limited range of taxonomically related plant species, and some are highly specialized on one or a few host plant species (Winkler & Mitter 2008; Chapman 2009; Xue *et al.* 2011). Studies on host specialization and adaptation have revealed that plant chemistry plays an important role in the evolution of plant–beetle associations (Feeny 1975; Städler 1992; Landolt & Phillips 1997; Rasmann & Agrawal 2011). Moreover, host specificity and host shifts correlate with the capability of beetles to respond to specialized plant metabolites; for instance, *Phratora* and *Ophraella* leaf beetles have shifted amongst chemically analogous, but taxonomically distinct, plant hosts (Futuyma & McCafferty 1990; Köpf *et al.* 1998).

Attraction or repulsion of herbivores may occur at a distance from the plant source when the critical cues are volatile, or upon contact with plants when the cues are non-volatile. Numerous studies have shown that plant volatiles are important olfactory cues in host selection processes in the herbivorous insects (Bruce, Wadhams & Woodcock 2005; Webster *et al.* 2008; Dicke & Baldwin 2010; Bruce & Pickett 2011; Loreto *et al.* 2014). The contribution of beetles' olfactory (preingestive) preferences to host shifting remains unclear, although likely important, given the role of olfaction in host selection. Meanwhile, the specific cues underlying host preference for particular plant–herbivore pairs often remain unknown. Identifying to what extent common plant volatiles might contribute to host shifting would improve our understanding of chemosensory speciation and permit more accurate evaluations of the ecological risks of host range expansion of biological control agents to non-target species.

*Agasicles hygrophila* Selman & Vogt (Coleoptera: Chrysomelidae, *Altiticinae*) is a reportedly monophagous flea beetle and an effective biocontrol agent for the destructive and invasive alligator weed *Alternanthera philoxeroides* (Mart.) Griseb (Amaranthaceae). Host specificity and potential host shifting were considered the most important factors for evaluation of *A. hygrophila* in many countries where it was introduced for biological control of *A. philoxeroides*. An early study found that *A. hygrophila* fed only on *A. philoxeroides* in Argentina and Uruguay (Zielske, Simons & Silverstein 1972). Extensive laboratory feeding assays supported the hypothesis that *A. hygrophila* feeds only on *A. philoxeroides* in China (Wang *et al.* 1988; Wu *et al.* 1994). However, recent laboratory work and field surveys by us and others have shown that adult *A. hygrophila* flea beetles can survive on the co-occurring non-host plant *Alternanthera sessilis* and

can feed on the important crop plants *Portulaca oleracea*, *Beta vulgaris* variety *saccharifera* and *B. vulgaris* variety *cicla* plants (Wang *et al.* 1988; Wu *et al.* 1994; Lu J *et al.* 2012; Lu X *et al.* 2013). Furthermore, the overlap in geographical range of the target *A. philoxeroides* and non-host *A. sessilis* is projected to increase in response to environmental alterations caused by climate change (Lu J *et al.* 2010, 2012, 2015; Lu X *et al.* 2013, 2015). Our previous studies also indicated a role of olfaction in host selection by this beetle in multiple-plant choice experiments (Lu J *et al.* 2012, 2015).

To date, little is known about the chemical ecology of the *A. philoxeroides*–*A. hygrophila* interaction, and it is not known whether this specialized beetle distinguishes between olfactory cues typical of its host plant and olfactory cues from non-host plants. A previous study demonstrated that a particular flavone in *A. philoxeroides* functions as a feeding stimulant for *A. hygrophila* and contributes to its host specificity (Vogt, Quimby & Kay 1992). However, we hypothesized that volatiles characteristic of *A. philoxeroides* could also play an important role in host selection and host use by this specialized beetle.

To address this hypothesis, we tested the following predictions:

1. Olfactory cues from host and non-host plants play a decisive role in host location and assessment by *A. hygrophila* flea beetles.
2. *Agasicles hygrophila* uses specific volatile cues to distinguish between its host plant and non-host plants.
3. Altering volatile cues associated with host and non-host plants can shift the host preference and oviposition of *A. hygrophila* adults in the field.
4. *Agasicles hygrophila* adults can oviposit and produce surviving offspring on certain non-host plants when the host plant is supplemented with repellent volatiles and non-host plants are supplemented with attractive volatiles.

We tested these predictions by conducting a series of multiple-choice preference tests, olfactometer assays, volatile collections and analyses, and electrophysiological measurements in both field and laboratory conditions.

## Materials and methods

### PLANTS AND GROWTH CONDITIONS

Alligatorweed, *A. philoxeroides* (Mart.) Griseb (Amaranthaceae) were planted individually in plastic pots (12 cm in diameter) containing a mixture of peat and vermiculite (3 : 1). We also selected 12 non-target (non-host) plant species that occur in areas in which *A. philoxeroides* and *A. hygrophila* either also occur, or are expected to occur under projected climate change scenarios. The non-target species were chosen as follows: (i) closely related species or the same family as *A. philoxeroides*: *A. sessilis*, *Amaranthus mangostanus* and *Amaranthus retroflexus* plants (Amaranthaceae); (ii) similar morphology to *A. philoxeroides* that provides similar visual cues for host selection by *A. hygrophila* adults (i.e., both plants possess prostrate stems and succulent alternate leaves clustered at stem joints and ends) and similar phenological period (i.e., periodic and seasonal growth and

development) to *A. philoxeroides*: *P. oleracea* (Portulacaceae); and (iii) crops with significant economic value such as *B. vulgaris* variety *saccharifera*, *B. vulgaris* variety *cicla* and *Chenopodium album* (Chenopodiaceae); *Brassica pekinensis* (Cruciferae); *Solanum melongena* and *Lycopersicon esculentum* (Solanaceae); *Vicia faba* (Leguminosae); and *Cucumis sativus* (Cucurbitaceae). The 12 non-host species were bought as either seeds or seedlings from commercial suppliers (Seedlings Trading Market, Taigu, China). All plants were cultured to the standardized developmental stage (approximately 10–15 leaves and 30–40 cm in height after 4–6 weeks) in a greenhouse (under  $25 \pm 3$  °C, 16/8-h light/dark (L/D) photoperiod and  $85 \pm 5\%$  RH).

## INSECTS

*Agasicles hygrophila* flea beetles were reared on *A. philoxeroides* in a greenhouse under the same conditions as described for the 13 plant species. Adults and 2nd-instar larvae were used for the experiments. All experiments were conducted at  $26 \pm 1$  °C,  $85 \pm 5\%$  RH and with a 16/8-h L/D photoperiod in a growth chamber (Safe, PRX-450 C, Ningbo Safe Experimental Instrument Co., Ltd, Ningbo, China).

## MULTIPLE-CHOICE PREFERENCE TESTS IN THE OPEN FIELD

To assess the host plant selection of uninfested plants by colonizing adult *A. hygrophila* beetles under natural conditions, we conducted multiple-choice behaviour tests in the open field of Taigu county, Shanxi Province, China (37.4°N, 112.5°E; a temperate monsoon climate) from 1 June to 30 September 2015. This is a region in which *A. philoxeroides* and *A. hygrophila* do not yet occur, but are expected to occur under projected climate change scenarios. The 13 host and non-host plants, grown as described above in the greenhouse, were transferred in pots into the field. The pots were buried into the soil and the plants were allowed to acclimate for 1 week, during which time the plants were watered regularly. To avoid damage from unwanted insects in the field, plants were manually checked daily and buried with pots into the soil 12 h before the experiments. Any plants visibly damaged by other insects (<5% of those originally transferred) were replaced with undamaged plants that had also acclimated for 1 week. The 13 plant species were arranged randomly in the field to form replicated circular plots (each c. 16 m<sup>2</sup>) where three plants of the same species were arranged in a group and the distance between adjacent groups was 0.2 m. This randomly arranged plot was replicated six times alongside each other at a 2 m distance in a 2 plot by 3 plot design (Fig. 1a). The total area of this experiment was about 432 m<sup>2</sup> (including six small plots and the isolated spaces between plots).

Although no insect-derived pheromones of *A. hygrophila* beetles have been reported so far, to eliminate the potential role of beetle pheromones in host preferences of conspecifics, we only used females in the field trials, as our previous studies have shown that the host preferences of females and males are not different (Lu J *et al.* 2012, 2015). One-day-old unmated virgin females were transferred into the rearing cages (25 × 25 × 25 cm), and positioned outdoors for 2 days to allow them to adapt to the field environment, during which time the insects were deprived of food but supplied with water by a sprayer at 3-h intervals during the daytime from 06.00 until 18.00 h. We then released these insects in the centre of the circles, with 50 females released in the each plot every time. On average, 95% of the released beetles chose a plant. This experiment was repeated six times on different days (three times with insects released in the morning and three times with insects released in the afternoon) with different

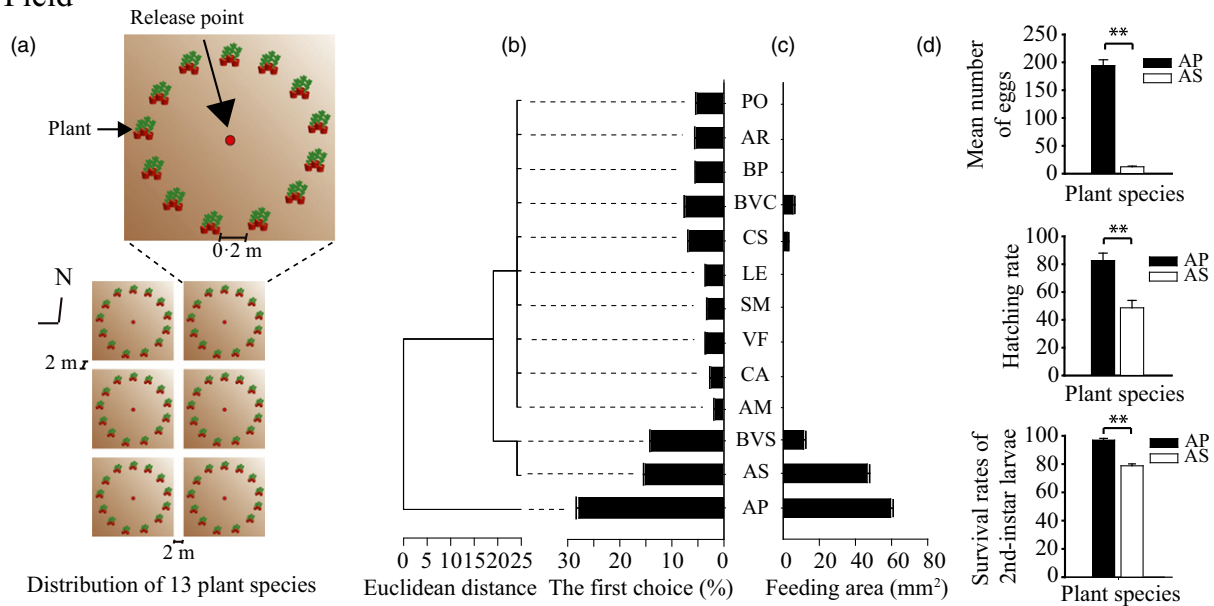
plantations of the 13 plant species over 1 month. In total, 1800 adults were released into the field. Their choices were recorded between 07.00 and 21.00 h or between 17.00 and 19.00 h because at other times of day the beetles are less mobile and more likely to settle on plants, based on 24 h of monitoring in a preliminary experiment. This experiment also indicated that the beetles did not leave the circles in which they were released to move to other circles. The numbers of insects on the plants and the amount of feeding damage was recorded every 12 h for 2 days. Circles were monitored over the period of the assay to prevent beetles from escaping, and beetles were re-collected at the end of each assay. To measure the extent of feeding damage, the individual leaf area and the total leaf area of the plant were measured by taking photographs of the plant leaves with a camera (Canon, EOS 1200D) and calculating the damaged areas using Image J v1.48 (National Institutes of Health). We also monitored local temperature and humidity throughout the experiment (Fig. S1, Supporting Information).

For oviposition preference of mated females, 180 mated females were transferred into the rearing cage about 3–6 days post-emergence and the cage (25 × 25 × 25 cm) was placed outdoors for 2 days, during which the insects were provided with their host plant, *A. philoxeroides*, allowing adaptation to the field environment. These satiated mated females were then used in experiments. We monitored the ovipositional preference and the number of egg masses every 30 min over 2 h post-release. Plants with egg masses were then monitored for hatching rate and larval survival in the following days up to 2nd-instar larvae stage. This experiment was also repeated six times in the same plots on 15 different days and circles were monitored, beetles collected and plants replaced after each repetition as described for feeding preference above.

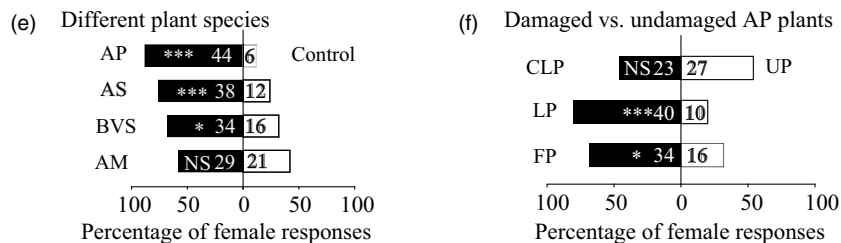
## PLANT TREATMENTS FOR VOLATILE COLLECTION AND BEHAVIOURAL ASSAYS

To test our second prediction, which is that there are volatile cues emitted from host and non-host plants and which are used by adult *A. hygrophila*, we collected and analysed the volatile compounds produced by undamaged and damaged plants, including the most-preferred (AP, *A. philoxeroides*), second-most-preferred (AS, *A. sessilis*), intermediately preferred (BVS, *B. vulgaris* variety *saccharifera*) and least-preferred (AM, *A. mangostanus*) species, and determined which compounds are perceived and used in host selection in a series of behavioural assays. These host and non-host plants were subjected to one of six treatments to simulate relevant states in nature including undamaged, mechanical damaged and insect-damaged conditions: (i) control (CTRL), clean glass jars (40 cm high × 17 cm internal diameter); (ii) healthy undamaged host *A. philoxeroides* and non-host plants (*A. sessilis*, *B. vulgaris* variety *saccharifera* and *A. mangostanus*) cultured and protected from any insect feeding and mechanical damage in an environmental chamber; (iii) the same host and non-host plants on which leaves were cut with a blade as a mechanical damage control (CLP, 200 cuts per plant); (iv) an adult female beetle feeding damage treatment where the same plants were enclosed in groups of five intact conspecifics in an insect-rearing cage (40 × 40 × 40 cm) and exposed to 100–150 *A. hygrophila* females in an environmental chamber for 24 h (FP); (v) the same beetle feeding treatment but with male instead of female *A. hygrophila* adults (MP); or (vi) the same beetle feeding treatment but with *A. hygrophila* 2nd-instar larvae (LP). Leaves were scanned to show that the mechanical damage treatment caused a similar degree of damage as beetle feeding (Table S1). Plants were then used either for volatile collection or for behavioural assays.

## Field



## Y-tube



**Fig. 1.** Preferences of *Agasicles hygrophila* females in the field trials and in Y-tube olfactometers. (a) Schematic diagram of a field plot experiment. The 13 plant species were arranged randomly in the field to form a small plot of circle (about 16 m<sup>2</sup>, up), where three plants of same species were planted in a group and the distance between adjacent groups was 0.2 m. This random plot arrangement was repeated six times alongside each other in a 2 m distance (low). The females were released in the center of the circle. (b) Hierarchical cluster analysis of homogeneity based on the first choice of *A. hygrophila* females to host *Alternanthera philoxeroides* (AP) plants and 12 non-host plants, including relatives of AP, in the field trials. (c) The female feeding areas (mm<sup>2</sup>) were measured 24 h after the releasing of female beetles in the field. In (b) and (c), the X axis is the relative Euclidean distance between plants (b, left), the choice probability (b, right) and the feeding amount (c) on the 13 plant species. CS, *Cucumis sativus*; LE, *Lycopersicon esculentum*; AP, *Alternanthera philoxeroides*; VF, *Vicia faba*; PO, *Portulaca oleracea*; AM, *Amaranthus mangostanus*; AR, *Amaranthus retroflexus*; BVC, *Beta vulgaris* variety *cicla*; AS, *Alternanthera sessilis*; BP, *Brassica pekinensis*; SM, *Solanum melongena*; BVS, *Beta vulgaris* variety *saccharifera*; CA, *Chenopodium album*. The preference results were compared by analysis of Kruskal–Wallis test (spss, version 17.0). The experiment was done on 30 different days and with about 1800 females in total. (d) The number of eggs (top), hatching rate (middle) and survivorship of 2nd-instar larvae (bottom) per plant in the field trial. This experiment was also repeated six times on 15 different days and in total, about 180 females completed the experiment. The results were compared by analysis of Mann–Whitney test. \**P* < 0.05. (e) Choice of *A. hygrophila* females between plant odours emitted from undamaged AP, AS, BVS or AM plants vs. controls in Y-tube olfactometers respectively. (f) Choice of *A. hygrophila* between paired odours of *A. philoxeroides* (AP) with different treatments in Y-tube olfactometers: mechanically damaged plants (CLP), plants with female feeding (FP), plants that were fed upon by *A. hygrophila* 2nd-instar larvae (LP) (black bars) vs. undamaged plants (UP; white bars) respectively. In (e) and (f), bars represent the percentages of *A. hygrophila* choosing either each odour source in the Y-tube olfactometer. Numbers in bars are the numbers choosing the corresponded odour sources; \**P* < 0.05; \*\**P* < 0.01; \*\*\**P* < 0.001; ns, not significant. In total, each of 50 females made a choice in a period of 5 min. The frequencies of females choosing each olfactometer arm were analysed by a  $\chi^2$  test. [Colour figure can be viewed at [wileyonlinelibrary.com](http://wileyonlinelibrary.com)]

## PLANT VOLATILE COLLECTIONS

A headspace sampling method was used to collect volatiles from treated plants based on the method described by Wei *et al.* (2007) with small modifications. For each potted plant, the potting medium was covered with aluminium foil (Xindan Co., Shanghai, China) to avoid collecting soil volatiles, and the plant and pot were enclosed in a glass jar (40 cm high  $\times$  17 cm internal diameter). For insect-damaged plants, the insects were removed prior to collection of volatiles. For an additional background control, the aluminium foil was

put into a clean glass jar. Compressed air (Beijing Gas Main Plant, Beijing, China) was purified and humidified through a series of three glass jars (500 mL) filled with molecular sieve (0.5 nm; Beijing Chemical Company, Beijing, China), freshly activated charcoal (Beijing Chemical Company) and distilled water respectively. The air was pushed into the jars at a rate of 500 mL min<sup>-1</sup> and drawn from the jars via a collector (a glass tube with an internal diameter of 3 mm) containing 100 mg of Porapak Q (80–100 mesh; Supelco, Bellefonte, PA, USA) on the outlet of each jar at a rate of 450 mL min<sup>-1</sup> using a membrane pump (Beijing Institute of Labour



Instruments, Beijing, China) for 12 h (from 08.00 h until 20.00 h). Overpressure in the jar prevented contamination from ambient air. Each collection was replicated five to seven times. Volatile compounds were dissolved from the collectors with 600  $\mu\text{L}$  HPLC-grade dichloromethane (Tedia Company, Fairfield, OH, USA) and the extracts were stored at  $-20^\circ\text{C}$  until analysis. An internal standard was not used in order to avoid artefactual responses in electrophysiological assays. The weight of the shoot was measured immediately after headspace sampling.

#### CHEMICAL IDENTIFICATION AND QUANTIFICATION

Volatile extracts were analysed on an Agilent gas chromatographer (GC) (6890N) coupled to a mass spectrometry (MS) system (5973 MSD) as described by Wei *et al.* (2007) with small modifications. The GC was equipped with a DB-WAX column (30 m  $\times$  0.25 mm  $\times$  0.15  $\mu\text{m}$ ; Agilent Technologies, Palo Alto, CA, USA) and the initial oven temperature was kept at  $40^\circ\text{C}$  for 4 min and then ramped up at a rate of  $5^\circ\text{C min}^{-1}$  to  $180^\circ\text{C}$ , followed by a rate of  $10^\circ\text{C min}^{-1}$  to  $230^\circ\text{C}$  and held for 3 min. The injector temperature was set at  $250^\circ\text{C}$  with a constant flow rate of  $1.0\text{ mL min}^{-1}$ . The inlet was operated in the splitless injection mode. The GC-MS electron impact source was operated in the scan mode with the MS source temperature at  $240^\circ\text{C}$  and the MS quad temperature at  $150^\circ\text{C}$ . Volatile compounds were identified by comparing their retention times and mass spectra to synthetic standards run on the same column, and to reference mass spectra from the NIST09 library (Scientific Instrument Services, Inc., Ringoes, NJ, USA).

Mixed samples consisting of hexenal, (*E*)- $\beta$ -Ocimene, (3*E*)-4,8-dimethyl-1,3,7-nonatriene (DMNT), (*Z*)-3-hexenol, (*Z*)-3-hexenyl acetate,  $\beta$ -Caryophyllene and (3*E*,7*E*)-4,8,12-trimethyl-1,3,7,11-tridecatetraene (TMTT) in different dosages (1, 5, 10, 20, 40 and 80 ng) were used as external standards for quantification. The same program as described above was used to analyse standards.

#### Y-TUBE OLFACTOMETER BEHAVIOURAL EXPERIMENTS

A Y-tube olfactometer [stem 5 cm; arms 25 cm at  $60^\circ$  angles, internal diameter (ID) 2.5 cm] was used to test the responses of *A. hygrophila* females to odours from its *A. philoxeroides*, non-host plants, *A. philoxeroides* with various treatments versus control, electrophysiologically active compounds vs. control or DMNT and (*Z*)-3-hexenol in different combinations. The Y-tube olfactometer was placed in an observation chamber (95  $\times$  60  $\times$  45 cm). Four 22 W white fluorescent tubes above the device provided uniform lighting. The temperature in the chamber was maintained at  $25 \pm 1^\circ\text{C}$  at all times using an air conditioner. A pump (Beijing Institute of Labour Instruments) was used to draw the air through a water jar and activated charcoal so that it was humidified and purified of any contaminating odours before entering the Y-tube. Airflow through each of the olfactometer arms was maintained at  $250\text{ mL min}^{-1}$  using a flow meter. One naïve adult *A. hygrophila* female, which was deprived of food but supplied with water by wet cotton balls for 12 h, was put into the Y-tube stem per trial. Odour sources from host or non-host plants with different treatments were prepared by putting a potted plant into a glass jar (25 cm high  $\times$  17 cm internal diameter) from which a Teflon tube (ID 0.7 cm) connected to one arm of the Y-tube. Another glass jar containing a pot with a culture mixture of peat and vermiculite served as a control attached to the other arm. Pots were covered with aluminium foil as for volatile collection. Synthetic compounds were taken up into glass capillary tubes (10  $\mu\text{L}$ ; Sigma-Aldrich, Steinheim,

Germany) at several dosages (1, 10, 20, 40 and 80 ng/10  $\mu\text{L}$  hexane) and placed in one of the olfactometer arms while the hexane control (equal volume of HPLC-grade hexane) was set in the other arm. For chemical complementation experiments in Y-tubes, the non-host *A. mangostanus* (AM), supplemented with synthetic DMNT (40 ng/10  $\mu\text{L}$  hexane) by placing a capillary tube in the same arm as the plant odour source, was compared to hexane-supplemented AM, and the host plant AP supplemented in the same manner with (*Z*)-3-hexenol (40 ng/10  $\mu\text{L}$  hexane) was compared with hexane-supplemented AP. The compound ratios of DMNT (D) vs. (*Z*)-3-hexenol (Z) were prepared and compared by mixing the two compounds in a series of combinations (D : Z = 1 : 1, 9 : 1, 99 : 1; Z : D = 1 : 1, 9 : 1, 99 : 1; in a total amount of 10 ng/10  $\mu\text{L}$ ). Each flea beetle was given 5 min to choose in the olfactometer. Flea beetles that remained inactive or did not choose after more than 5 min (c. 4%) were not recorded. The position of the arms containing the treatment and control odours was reversed after every five individuals had been tested to avoid position bias. The Y-tube was replaced with a clean tube after 10 individuals had been tested.

#### HOST SELECTION AND OVIPOSITION PREFERENCES OF FEMALES TO HOST AND NON-HOST PLANTS WITH ADDITION OF EITHER DMNT OR (*Z*)-3-HEXENOL IN FIELD TRIALS

To test the third prediction, which is that we can alter the headspace content of the plant volatiles DMNT and (*Z*)-3-hexenol of host and non-host plants to change the host preference of *A. hygrophila* flea beetles in the field, we conducted field experiments with the beetles to determine their feeding and oviposition preferences for volatile-supplemented host and non-host plants. Based on the result of the attractiveness of 13 plant species to beetles in the field trial (Fig. 1), the host *A. philoxeroides* and five non-host plant species: the relatives of *A. philoxeroides*: *A. sessilis* and *A. mangostanus*, as well as *B. vulgaris* variety *saccharifera*, *B. vulgaris* var. *ciela* and *C. sativus*, were used to test the effects of different dosages of DMNT or (*Z*)-3-hexenol applied to host or non-host plants on host selection and oviposition preferences of *A. hygrophila* adults. These six plant species were arranged randomly in the field to form a circular plot (about 4 m<sup>2</sup>) where three plants of the same species were arranged in a group and the distance between adjacent groups was 0.2 m, as for the 13-plant species experiments. This randomly arranged plot was repeated six times, with the plots alongside each other, again at a distance of 2 m (Fig. 4a). The six field plots were divided randomly into four treated plots and two control plots. The total area of this experiment was about 252 m<sup>2</sup> (including six plots and the adjacent empty areas).

Treatments of intact *A. philoxeroides* host plants with the repellent compound (*Z*)-3-hexenol or *A. mangostanus* non-host plants with the attractive compound DMNT at different dosages in hexane (1, 10, 100 and 1000  $\mu\text{g mL}^{-1}$ ) were prepared by taking up the dilutions in a glass capillary tube (20  $\mu\text{L}$ ) and fixing these capillaries on the plant leaves using a small piece of double-sided tape. For the controls, the same volume of hexane was used. The solutions were replenished every 30 min during the monitoring of beetle host selection with naïve female *A. hygrophila*. We monitored the host preference every 12 h between 07.00 and 09.00 h and between 17.00 and 19.00 h over 24 h post-release. This experiment was repeated six times on three different days, three times in the morning and three times in the evening, and plants were replaced after each repetition. In total, 120 adult females were released into the field at each dosage of each volatile.

To test our fourth prediction of the effect of volatile supplementation on oviposition and reproduction of flea beetles, mated females were used in the same set-up for oviposition and egg hatching responses to (*Z*)-3-hexenol addition to host plants among six plant species. Treatments of intact *A. philoxeroides* host plants with (*Z*)-3-hexenol at three dosages in hexane (0, 1 and 10  $\mu\text{g } \mu\text{L}^{-1}$ ) were prepared by taking up the dilutions in a glass capillary tube (20  $\mu\text{L}$ ). The solutions were replenished every 30 min during the monitoring of beetle oviposition preferences among six plant species (Table S5). We monitored the oviposition preference and the number of egg masses every 30 min over 2 h post-release. Plants with egg masses were then monitored for hatching rate as described above. This experiment was repeated six times on three different days and plants were replaced after each repetition. In total, 120 adult females were released into the field at each dosage of (*Z*)-3-hexenol.

#### STATISTICAL ANALYSES

Data were analysed using spss 17.0 (SPSS Inc., Chicago, IL, USA) statistical analysis software except where noted. For data of multiple-choice tests in an open field trial, the categories of the first-choice plant of *A. hygrophila* female beetles among 13 plants were determined by hierarchical cluster analysis using a between-group-linkage method and square Euclidean distances; the females' preferences (the choice probability %) and the female feeding areas among the 13 plant species were compared using Kruskal–Wallis tests and Mann–Whitney tests for further mean separation; the number of eggs, hatching rate and survival rate of 2nd-instar larvae of *A. hygrophila* female beetles between host *A. philoxeroides* and non-host *A. sessilis* were compared by Mann–Whitney tests. For all Y-tube assays, pairwise choices of *A. hygrophila* females were compared using chi-square ( $\chi^2$ ) tests (Hoballah, Tamo & Turlings 2002; Wei & Kang 2006; Wei *et al.* 2007). To assess whether volatile profiles could distinguish plant species and treatments, we measured the volatiles and then analyzed the volatile profiles using Projection to Latent Structures Discriminant Analysis (PLS-DA). The PLS-DA extension of the SIMCA P + 12.0 software program (Umetrics AB, Umeå, Sweden) revealed which volatile compounds were important for the separation of volatile blends emitted by differently treated plants (Wei *et al.* 2014). In addition, the variable importance in the projection (VIP) was calculated. Variables with VIP values >1 are most influential for the model. Because there were no differences in plant volatiles induced by feeding of adult male and female beetles, and no differences in antennal or behavioural responses to the different volatile sources between adult males and females, the data were pooled together as treatment with adults. The levels of volatiles released from the treatment plants were normalized to ng per hour per 10 g of plant fresh weight. For volatile comparisons among undamaged, mechanically damaged and flea beetle-damaged *A. philoxeroides* plants, data were log-transformed and statistically analysed by ANOVA following Tukey's honestly significant difference test. For field trial analyses with volatile supplementation, Pearson's correlations were used to analyse the correlation between the preference curve of the beetles to the volatile-supplemented plants at different dosages and the choice curves in response to the other five plants at the corresponding dosages. In addition, the preferences of naïve female *A. hygrophila* beetles for host or non-host plants with DMNT or (*Z*)-3-hexenol at 10  $\mu\text{g}$  dosage and without them at 0  $\mu\text{g}$  dosage were compared using Mann–Whitney tests. Mean numbers of eggs and hatching rates of mated female *A. hygrophila* on *A. philoxeroides* with (*Z*)-3-hexenol at 0 and 10  $\mu\text{g}$  dosages or on *A. sessilis* plants at two dosages were also compared by Mann–Whitney tests.

## Results

### OLFACTORY CUES CONDITION THE HOST FEEDING AND OVIPOSITION PREFERENCES OF ADULT *A. HYGROPHILA*

To test our first prediction, we conducted a multiple-choice preference test in an open field (Fig. 1a). Hierarchical cluster analysis of the first-choice plant revealed three main categories of preference (Fig. 1b left). First, *A. hygrophila* significantly preferred the host plant AP over non-host plant species ( $\chi^2 = 54.284$ ,  $P < 0.001$ , d.f. = 12). Second, within the non-host plant species, *A. sessilis* (AS) (a relative of *A. philoxeroides*) was the second most-preferred plant. Third, the beetles preferred *B. vulgaris* variety *saccharifera* (BVS), *B. vulgaris* variety *cicla* (BVC) and *C. sativus* (CS) significantly less than they preferred AS. Overall, the non-host plants BP, AR, PO, LE, SM and VF were all roughly half as likely, relative to the preference for the host plant, to be chosen by the beetles. The non-host plants CA and AM were the least preferred, around 5% relative to the preference for the host plant (Fig. 1b).

Remarkably, we found that although the females preferred to feed on the leaves of *A. philoxeroides* (feeding areas:  $59.50 \pm 4.62 \text{ mm}^2$ , measured 24 h post-release in the field) they also consumed non-host plants, with the largest feeding areas ( $46.50 \pm 3.69 \text{ mm}^2$ ) on AS plants (Fig. 1c, Table S2). Similarly, we found that mated females laid eggs on the leaves of AS plants ( $12.33 \pm 1.20$  per plant), although fewer than on AP leaves ( $194.00 \pm 10.58$  per plant). All other plants were free from egg laying by females in the field (Fig. 1d). Moreover, the egg hatching rate on the non-host AS was about 50% and the survivorship of 2nd-instar larvae was over 75% (Fig. 1d).

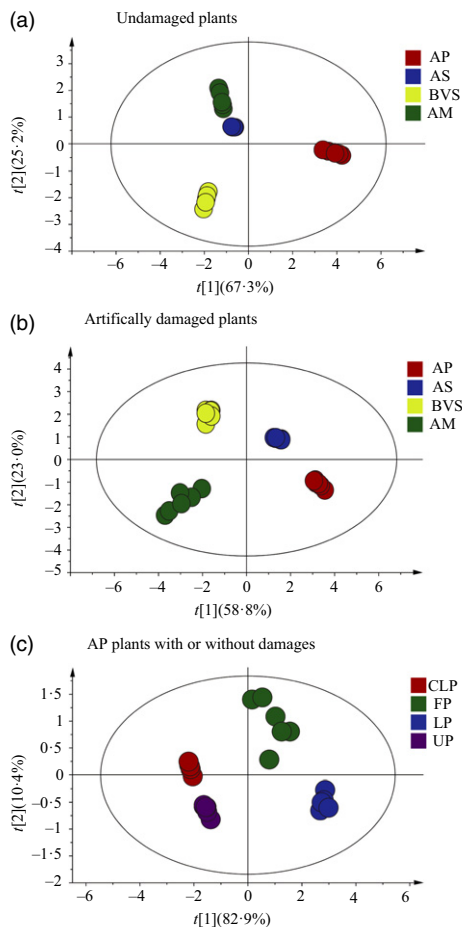
In the behavioural studies in the Y-tube olfactometers, adult *A. hygrophila* females significantly preferred the volatile compounds produced by AP ( $\chi^2 = 28.88$ ,  $P < 0.001$ ,  $n = 50$ ), AS ( $\chi^2 = 13.52$ ,  $P < 0.001$ ,  $n = 50$ ) or BVS plants ( $\chi^2 = 6.48$ ,  $P < 0.05$ ,  $n = 50$ ) over the controls, whereas they did not prefer the volatile compounds of the AM plants to the control ( $\chi^2 = 1.28$ ,  $P > 0.05$ ,  $n = 50$ ) (Fig. 1e).

To understand the preferences of adult *A. hygrophila* for damage-induced cues from host plants, we conducted dual-choice behavioural tests in the laboratory. Individual female beetles did not prefer the volatiles of AP plants damaged by mechanical means (CLP) over the volatiles of undamaged AP plants (UP) (CLP vs. UP,  $\chi^2 = 0.32$ ,  $P > 0.05$ ,  $n = 50$ ), but the females preferred the volatiles of AP plants damaged by feeding (FP and LP) over the volatiles of UP (FP vs. UP,  $\chi^2 = 6.48$ ,  $P < 0.05$ ,  $n = 50$ ; 2nd-instar larvae-damaged plants, LP vs. UP,  $\chi^2 = 18.000$ ,  $P < 0.001$ ,  $n = 50$ ; Fig. 1f).

### THE HOST AND NON-HOST PLANTS OF *A. HYGROPHILA* RELEASE DISTINCT VOLATILE COMPOUNDS

Projection to latent structures discriminant analysis of volatile profiles produced a model with two significant principal

components (PCs; model statistics:  $R^2X = 0.99$ ,  $R^2Y = 0.95$  and  $Q^2 = 0.95$ ) of which the first two explained 95% of the variance and clearly separated the data points for undamaged AP, AS, BVS and AM plants (Fig. 2a). Similarly, PLS-DA clearly separated the data points for the mechanically damaged AP, AS, BVS and AM plants (the first two PCs explained 97% of the variance; model statistics:  $R^2X = 0.97$ ,  $R^2Y = 0.98$  and  $Q^2 = 0.97$ ; Fig. 2b) and also clearly separated the data for the undamaged, mechanically damaged and



**Fig. 2.** Projection to latent structures discriminant analysis (PLSDA) of headspace volatile collections from different treatments of the host plant *Alternanthera philoxeroides* (AP), and non-host plants *Alternanthera sessilis* (AS), *Beta vulgaris* variety *saccharifera* (BVS) and *Amaranthus mangostanus* (AM). Score scatter plots are shown for intact plants (a) and artificially damaged plants (b) of AP (red solid circles in quadrant IV), AS [(in (a) blue solid circles in quadrant II; in (b) blue solid circles in quadrant I), BVS [in (a) yellow solid circles in quadrant III; in (b) yellow solid circles in quadrant II] and AM [in (a) green solid circles in quadrant II; in (b) green solid circles in quadrant III], and for different treatments of AP (c). Undamaged plants [UP (in (c) dark blue solid circles in quadrant III)], mechanically damaged plants [CLP (in (c) red solid circles in quadrant II)], plants with female feeding [FP (in (c) green solid circles in quadrant D)], plants that were fed upon by *Agasicles hygrophila* 2nd-instar larvae [LP (in (c) blue circles in quadrant IV)]. The ellipse defines the Hotelling's T<sup>2</sup> confidence region (95%). [Colour figure can be viewed at [wileyonlinelibrary.com](http://wileyonlinelibrary.com)]

insect-damaged AP plants (the first two PCs explained 41% of the variance; model statistics:  $R^2X = 0.93$ ,  $R^2Y = 0.51$  and  $Q^2 = 0.41$ ; Fig. 2c).

For undamaged plants, six compounds had a VIP >1 (see Table 1 for details on their VIPs) and DMNT, with a VIP of 1.39, was the most important compound for group separation. For artificially damaged plants, five compounds had a VIP >1 (see Table 1 for details on their VIPs) and the most important compound for separation was (Z)-3-hexenol (1.25). The green leaf volatiles, such as hexanal, 2-hexanol, (Z)-3-hexenyl acetate and (Z)-3-hexenol predominated in the artificially damaged non-host plants BVS and AM, but they were not detected in the volatile blend of the host plant AP and the non-host AS (Table 1). For insect-damaged host plants, DMNT was released in the highest amount in the various treatments (ANOVA,  $F = 1866.83$ , d.f. = 23,  $P < 0.001$ ) and had the highest VIP, 1.56, for separation of groups (see Table 1 for details on VIPs for the other compounds). Absolute amounts of volatiles released from the insect-damaged plants also were much higher than those from undamaged or artificially damaged plants (ANOVA,  $F = 1909.21$ , d.f. = 35,  $P < 0.001$ ).

#### OLFACTORY RESPONSES OF ADULT *A. HYGROPHILA* TO THE VOLATILE COMPOUNDS DMNT AND (Z)-3-HEXENOL AT VARIOUS DOSAGES AND COMBINATIONS

Our GC coupled with electroantennographic detection (EAD) studies revealed that female *A. hygrophila* antennae responded strongly to the homoterpenes DMNT and TMTT in undamaged plants (UP), mechanically damaged plants (CLP), insect-damaged plants (FP and LP) and to a mixture of the six green leaf volatiles, with the greatest responses to (Z)-3-hexenyl acetate, (E)-3-hexenol and (Z)-3-hexenol (Fig. S2, Tables S3 and S4). Therefore, the capability of the adult flea beetles to detect to several volatiles produced by host and non-host plants provides a basis for plant volatile-mediated host discrimination.

In Y-tube olfactometer, female *A. hygrophila* adults significantly preferred DMNT over the hexane control at or above 20 ng (20 ng,  $\chi^2 = 28.88$ ,  $P < 0.001$ ,  $n = 50$ ; Fig. 3a). In contrast, (Z)-3-hexenol was repellent at or above 10 ng (10 ng,  $\chi^2 = 6.48$ ,  $P < 0.01$ ,  $n = 50$ ; Fig. 3a). (E)- $\beta$ -Ocimene was significantly preferred only at dosages of 20 and 40 ng, and  $\beta$ -caryophyllene was only preferred at 20 ng (Fig. 3a). The *A. hygrophila* females showed no preferences for TMTT or (Z)-3-hexenyl acetate over the solvent control at any of the dosages tested (Fig. 3a). Therefore, among the compounds eliciting a behavioural response, DMNT was the most attractive and (Z)-3-hexenol was the most repellent.

The host plant AP was more attractive to *A. hygrophila* females than the non-host plant AM in the Y-tube olfactometer ( $\chi^2 = 38.72$ ,  $P < 0.001$ ,  $n = 60$ ; Fig. 3b). However, when synthetic (Z)-3-hexenol (40 ng/10  $\mu$ L hexane) was added to the arm with the AP odour, the female beetles showed a significant preference for the other arm, in which hexane was added to the AP odour ( $\chi^2 = 18.00$ ,  $P < 0.001$ ,  $n = 60$ ; Fig. 3b). For the non-host plant AM, the addition of synthetic

**Table 1.** Volatile compounds detected in the headspace of the undamaged and mechanically damaged *Alternanthera philoxeroides* (AP), *Alternanthera sessilis* (AS), *Beta vulgaris* variety *saccharifera* (BVS), and *Amaranthus mangostanus* (AM) plants, and different treatments of *A. philoxeroides* (AP) plants

Compound	Mean ( $\pm$ SE) units ng h <sup>-1</sup> 10 g <sup>-1</sup> FW <sup>-1</sup>				VIP*	
	AP	AS	BVS	AM		
<i>Undamaged</i>						
1	DMNT <sup>†</sup>	11.48 $\pm$ 0.44	1.13 $\pm$ 0.19	n.d.	n.d.	1.39
2	( <i>E</i> )- $\beta$ -Ocimene	1.15 $\pm$ 0.04	0.19 $\pm$ 0.02	n.d.	n.d.	1.13
3	Hexanal	n.d.	n.d.	n.d.	2.03 $\pm$ 0.25	1.09
4	TMTT <sup>‡</sup>	0.65 $\pm$ 0.03	n.d.	n.d.	n.d.	1.05
5	$\beta$ -Caryophyllene	9.89 $\pm$ 0.35	n.d.	n.d.	n.d.	1.05
6	Copaene	0.68 $\pm$ 0.04	n.d.	n.d.	n.d.	1.04
7	2-Hexanol	n.d.	n.d.	86.22 $\pm$ 3.01	n.d.	0.39
8	3-Hexen-1-ol,acetate	n.d.	n.d.	18.71 $\pm$ 0.75	n.d.	0.39
<i>Mechanically damaged</i>						
1	( <i>Z</i> )-3-hexenol	n.d.	n.d.	57.82 $\pm$ 1.36	368.77 $\pm$ 22.47	1.25
2	2-Hexenal	n.d.	n.d.	n.d.	98.54 $\pm$ 7.09	1.18
3	3-Hexenal	n.d.	n.d.	n.d.	11.07 $\pm$ 0.87	1.17
4	( <i>E</i> )- $\beta$ -Ocimene	0.51 $\pm$ 0.00	0.39 $\pm$ 0.04	n.d.	n.d.	1.15
5	3-Hexen-1-ol,acetate	n.d.	n.d.	115.90 $\pm$ 4.90	127.62 $\pm$ 13.68	1.13
6	Copaene	0.48 $\pm$ 0.00	n.d.	n.d.	n.d.	0.91
7	TMTT	0.55 $\pm$ 0.01	n.d.	n.d.	n.d.	0.91
8	DMNT	13.57 $\pm$ 0.41	1.19 $\pm$ 0.17	19.11 $\pm$ 1.74	25.75 $\pm$ 2.59	0.88
9	$\beta$ -Caryophyllene	2.73 $\pm$ 0.41	n.d.	n.d.	n.d.	0.85
10	2-Hexanol	n.d.	n.d.	164.49 $\pm$ 3.94	68.16 $\pm$ 5.90	0.72
11	Hexanal	n.d.	n.d.	905.65 $\pm$ 47.72	287.52 $\pm$ 24.67	0.63
<i>Different AP treatments</i>						
		UP <sup>§</sup>	FP <sup>¶</sup>	LP <sup>**</sup>	CLP <sup>††</sup>	
1	DMNT	11.48 $\pm$ 0.44	240.67 $\pm$ 6.51	483.42 $\pm$ 8.09	13.57 $\pm$ 0.41	1.56
2	$\beta$ -Caryophyllene	9.89 $\pm$ 0.35	10.76 $\pm$ 0.81	20.67 $\pm$ 0.45	2.73 $\pm$ 0.41	1.13
3	( <i>E</i> )- $\beta$ -Ocimene	1.15 $\pm$ 0.04	34.56 $\pm$ 3.71	53.67 $\pm$ 0.69	0.51 $\pm$ 0.00	0.70
4	TMTT	0.65 $\pm$ 0.03	9.77 $\pm$ 0.56	7.39 $\pm$ 0.12	0.55 $\pm$ 0.01	0.64
5	Copaene	0.68 $\pm$ 0.04	0.93 $\pm$ 0.28	1.83 $\pm$ 0.01	0.49 $\pm$ 0.00	0.62

n.d., not detected.

\*VIP, variable importance in the projection for PLS-DA. VIP values >1 are most influential for separation of the treatments.

<sup>†</sup>DMNT, (*E*)-4,8-dimethyl-1,3,7-nonatriene.

<sup>‡</sup>TMTT, (*3E,7E*)-4,8,12-trimethyl-1,3,7,11-tridecatetraene.

<sup>§</sup>UP: undamaged plants.

<sup>¶</sup>FP: plants on which females had fed.

<sup>\*\*</sup>LP: plants on which 2nd-instar larvae had fed.

<sup>††</sup>CLP: plants with cut leaves.

DMNT (40 ng/10  $\mu$ L) increased the preferences of *A. hygrophila* vs. the control plant odour with hexane ( $\chi^2 = 8.00$ ,  $P < 0.01$ ,  $n = 60$ ; Fig. 3b). We, therefore, hypothesized that this beetle might use a ratio of these repellent and attractive volatiles to locate its host plant and avoid non-host plants.

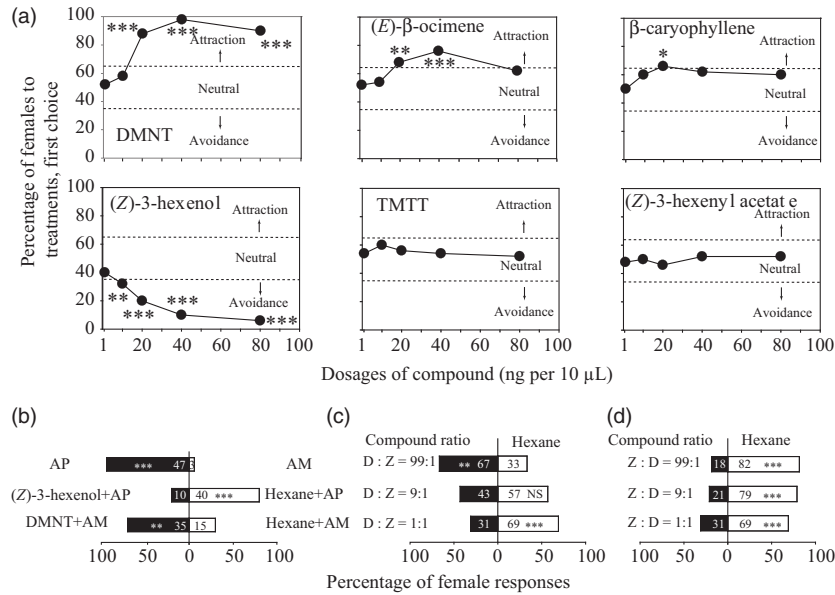
In two-compound mixtures consisting of DMNT and (*Z*)-3-hexenol, when DMNT was the variable fraction, adult female *A. hygrophila* significantly preferred a high proportion of DMNT (D:Z = 99 : 1) over the control ( $\chi^2 = 11.56$ ,  $P < 0.01$ ,  $n = 50$ ). A 9 : 1 ratio was no longer preferred ( $\chi^2 = 1.96$ ,  $P > 0.05$ ,  $n = 50$ ), and a ratio of D : Z = 1 : 1 became repellent for female beetles (Fig. 3c). When we switched (*Z*)-3-hexenol as the variable fraction, the mixtures were repellent to the female beetles in all ratios tested (Fig. 3d). Therefore, the ratio of DMNT and (*Z*)-3-hexenol determined the selectivity of females and the repellent effect of (*Z*)-3-hexenol was greater than the attractive effect of DMNT.

#### SUPPLEMENTATION EXPERIMENTS WITH SYNTHETIC DMNT OR (*Z*)-3-HEXENOL ALTER *A. HYGROPHILA* PREFERENCES TO HOST AND NON-HOST PLANTS IN THE FIELD

Our supplementation experiments in the field showed that the addition of synthetic (*Z*)-3-hexenol at different dosages (1, 10, 100 and 1000  $\mu$ g  $\mu$ L<sup>-1</sup> in a 20- $\mu$ L capillary) on intact *A. philoxeroides* host plant negatively correlated with the selection of non-host plants (Figs 4a and S3a), especially AS (Pearson correlation coefficient:  $R = -0.857$ ), BVS ( $R = -0.958$ ), BVC ( $R = -0.958$ ), CS ( $R = -0.911$ ) and AM plants ( $R = -0.779$ ; Table 2).

When the addition of synthetic (*Z*)-3-hexenol at 10  $\mu$ g dosage, the preferences of naïve female *A. hygrophila* beetles for AP, AS, BVS, BVC and CS were significantly different from the control (0  $\mu$ g dosage) (Fig. 4b). The choices of females for AP were decreased with increasing dosage of (*Z*)-





**Fig. 3.** Olfactory responses of *Agasicles hygrophila* females to the synthetic volatile compounds at various dosages and combinations. (a) First choice of naïve female *A. hygrophila* offered individual synthetic compounds at five different dosages (filled symbols, 1, 10, 20, 40, 80 ng/10  $\mu$ L hexane) vs. a solvent control (10  $\mu$ L hexane). For individual compounds at every dosage, in total each of 60 females chose within a period of 5 min. Data points located in areas above or below dotted lines indicated by arrows designated 'attraction' or 'avoidance' indicate a choice distribution significantly different from 50 : 50 ( $\alpha = 0.05$ ,  $\chi^2$  test; \* $P < 0.05$ ; \*\* $P < 0.01$ ; \*\*\* $P < 0.001$ ). DMNT: (3*E*, 7*E*)-4,8,12-trimethyl-1,3,7,11-tridecatetraene; ns, not significant. (b) Responses of naïve female *A. hygrophila* in Y-olfactometer to the odours of host plant *Alternanthera philoxeroides* vs. non-host plant AM, or host plant AP supplemented with (Z)-3-hexenol (40 ng/10  $\mu$ L hexane) vs. AP with hexane control or non-host AM with DMNT (40 ng/10  $\mu$ L hexane) vs. AM with hexane control; AP: *Alternanthera philoxeroides*; AM: *Amaranthus mangostanus*;  $\chi^2$  test for significant differences between numbers of females in each arm: \*\* $P < 0.01$ ; \*\*\* $P < 0.001$ . Each of 50 females chose within a period of 5 min for each pair. (c) Responses of female beetles to different compound ratios of DMNT (D) and (Z)-3-hexenol (Z) vs. hexane. The ratios containing D : Z = 1 : 1, 9 : 1, 99 : 1 (10 ng of D and Z in mixtures totally). (d), responses of female beetles to different compound ratios of (Z)-3-hexenol and DMNT vs. hexane. The ratios containing Z : D = 1 : 1, 9 : 1, 99 : 1 (10 ng of D and Z in mixtures totally).  $\chi^2$  test for significant differences between numbers of females in each arm. \*\* $P < 0.01$ ; \*\*\* $P < 0.001$ . One hundred females have made a choice in each experiment. ns, not significant.

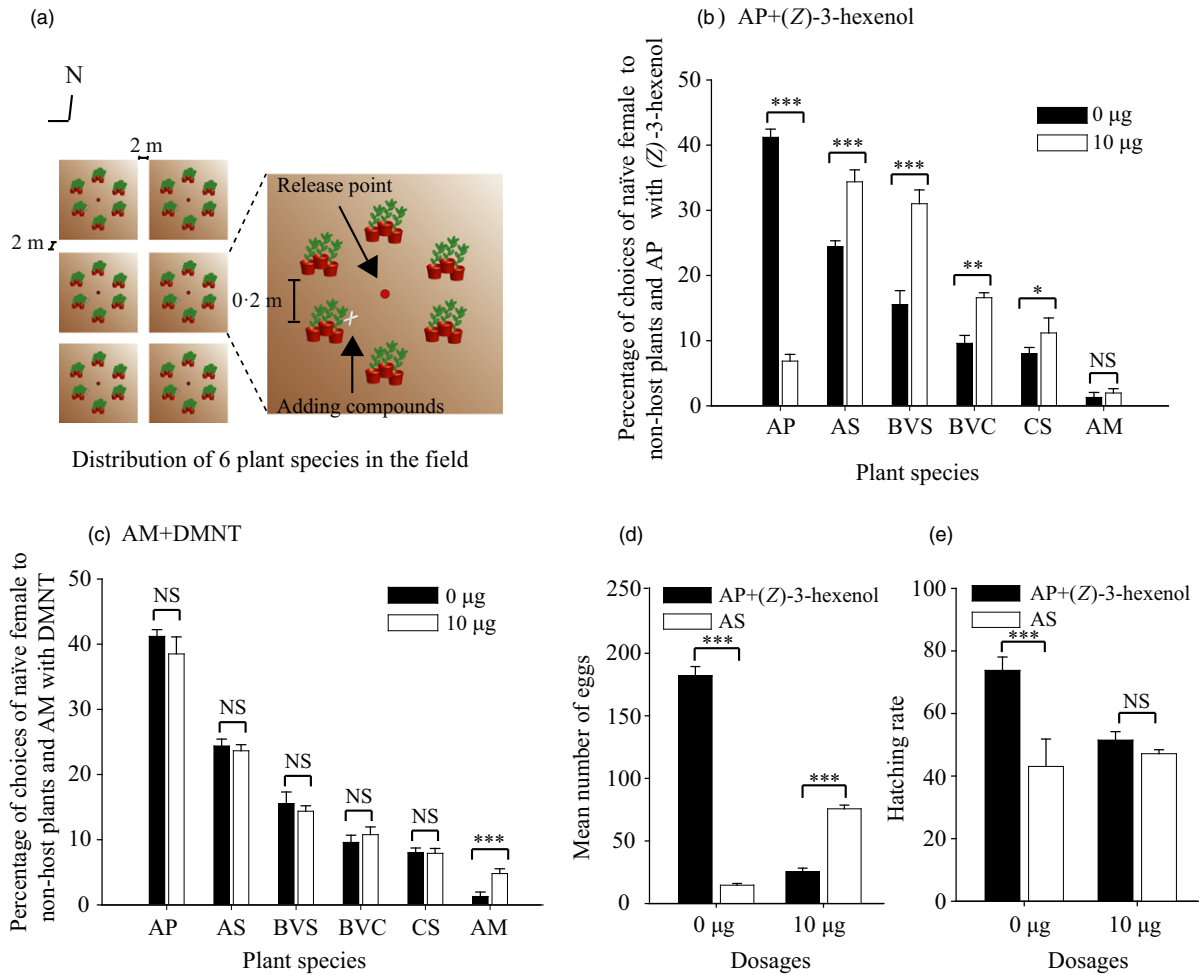
3-hexenol (Fig. S3a). Conversely, the choices of females for AM increased significantly with the addition of DMNT from 0 to 10  $\mu$ g dosage (Fig. 4c). The preferences of naïve females for non-host AM plants with synthetic supplements of DMNT gradually increased with increasing dosages (1, 10, 100 and 1000  $\mu$ g), and was negatively associated with selection of other non-host plants (Fig. S3b) (Pearson correlation between AM+DMNT and AP:  $R = -0.958$ ; AM + DMNT vs. AS,  $R = -0.825$ ; Table 3).

Moreover, with increasing (Z)-3-hexenol concentration (0 and 10  $\mu$ g) added to the headspace of the host plant AP, the oviposition preference of mated female beetles for *A. sessilis* gradually increased while oviposition preference for *A. philoxeroides* gradually fell (Fig. 4d, Table S5) (Pearson correlation between (Z)-3-hexenol + AP and AS:  $R = -0.739$ . Table S6). The trend of hatching rate was consistent with the oviposition preference (Pearson correlation between (Z)-3-hexenol + AP and AS:  $R = -0.937$  (Fig. 4e, Tables S5 and S6).

## Discussion

Our results demonstrate that instead of using species-specific infochemicals to locate their host plant *A. philoxeroides* (AP),

adults of the specialist beetle *A. hygrophila* use two common plant volatiles for host discrimination: DMNT emitted in large quantity by its host plant upon flea beetle attack, and (Z)-3-hexenol detected from damaged non-hosts plants. Among the 12 electrophysiologically active compounds from the host and non-host plants tested, female beetles were attracted to DMNT and repelled by (Z)-3-hexenol in comparison to solvent controls in dose–response bioassays conducted in laboratory Y-tube choice experiments. These two olfactory cues are the most ubiquitous chemicals present in the volatile bouquets of many plant species studies (Hatanaka 1993; Hoballah, Tamo & Turlings 2002; Wei *et al.* 2007; Kappers *et al.* 2010; Wei & Kang 2011; Karban *et al.* 2014). For instance, under undamaged conditions, 30% of investigated plants, such as kidney bean (*Phaseolus vulgaris*), bell pepper (*Capsicum annuum*) and cucumber (*C. sativus*), release DMNT in small amounts, whereas upon mechanical damages or insect damages, 70% of plants studied, from Fabaceae, Solanaceae, Cucurbitaceae, Apiaceae, Asteraceae, Rosaceae and Vitaceae families, release this compound in higher concentrations (Wei *et al.* 2007). DMNT was shown to be an attractant for natural enemies, such as the predatory mite *Phytoseiulus persimilis* feeding on the spider mite *Tetranychus urticae* (Kappers *et al.* 2010), and a parasitic wasp *Opius dissitus* of the pea



**Fig. 4.** Host selection and oviposition preferences of females to host and non-host plants with addition of either (Z)-3-hexenol or DMNT in field trials. (a) Schematic diagram of distribution of six plant species in the field. For detailed experimental procedures, please see Materials and methods under ‘Host selection and oviposition preferences by manipulation of chemicals in field trials’. Percentage of choices of naïve female *Agasicles hygrophila* in field trials to non-host plants BVS (BVS: *Beta vulgaris* variety *saccharifera*), CS (*Cucumis sativus*), BVC (*Beta vulgaris* variety *cicla*), AS (*Alternanthera sessilis*), AM (*Amaranthus mangostanus*) and host plant AP (*Alternanthera philoxeroides*) with (Z)-3-hexenol standard compound (b), or AM adding the DMNT standard compound (c) respectively, at 0 or 10 µg dosages (taken up into a 20-µL glass capillary). (d) Mean number of eggs and (e) hatching rate of mated female *A. hygrophila* on (Z)-3-hexenol+AP and AS plants in the field trial at 0 and 10 µg dosages. The results were analysed by Mann–Whitney test (SPSS, version 17.0). \* $P < 0.05$ ; \*\* $P < 0.01$ ; \*\*\* $P < 0.001$ . ns, not significant. [Colour figure can be viewed at [wileyonlinelibrary.com](http://wileyonlinelibrary.com)]

**Table 2.** Pearson correlation of first choice of naïve female *Agasicles hygrophila* in field trials to five non-host plants (BVS, CS, BVC, AS, and AM) and host plant AP with (Z)-3-hexenol standard compound at different dosages (0, 1, 10, 100, 1000 µg µL<sup>-1</sup>)

Treatment	(Z)-3-hexenol + AP	AS	BVS	BVC	CS	AM
(Z)-3-hexenol + AP	1	-0.857	-0.958*	-0.958*	-0.911*	-0.779
AS		1	0.746	0.837	0.732	0.646
BVS			1	0.905*	0.787	0.711
BVC				1	0.967*	0.811
CS					1	0.786
AM						1

AP, *A. philoxeroides*; AS, *Alternanthera sessilis*; BVS, *Beta vulgaris* variety *saccharifera*; BVC, *Beta vulgaris* variety *cicla*; CS, *Cucumis sativus*; AM, *Amaranthus mangostanus*.

The experiment was done on three different days and in total, about 120 females made their choices for each dosage.

\* $P < 0.05$ . Correlation is significant at the 0.05 level (two-tailed).

leafminer *Liriomyza huidobresis* (Wei & Kang 2006). For common green leaf volatiles, (Z)-3-hexenol was shown not only the most common inducible chemical from various plant

species when they are wounded by herbivorous insects or physical damage (Hoballah, Tamo & Turlings 2002; Wei *et al.* 2007) but also among the most important infochemicals

**Table 3.** Pearson correlation of first choice of naïve female *Agasicles hygrophila* in field trials to five plant species (AP, BVS, CS, BVC, and AS) and AM (*Amaranthus mangostanus*) with the DMNT standard compound at different dosages (0, 1, 10, 100, 1000 µg µL<sup>-1</sup>)

Treatment	DMNT+AM	AP	AS	BVS	BVC	CS
DMNT + AM	1	-0.958*	-0.825	0.846	0.072	0.844
AP		1	0.766	-0.918*	-0.017	-0.867
AS			1	-0.815	-0.165	-0.818
BVS				1	-0.225	0.972*
BVC					1	-0.352
CS						1

AM, *Amaranthus mangostanus*; AP, *A. philoxeroides*; AS, *Alternanthera sessilis*; BVS, *Beta vulgaris* variety *saccharifera*; BVC, *Beta vulgaris* variety *cicla*; CS, *Cucumis sativus*.

The experiment was done on three different days and a total of about 120 females made their choices for each dosage.

\* $P < 0.05$ . The correlation is significant at the 0.05 level (two-tailed).

for the attraction of parasitoids and predators (Kessler & Baldwin 2001; Wei & Kang 2006). In contrast, a few studies also showed that (*Z*)-3-hexenol can significantly reduce the preference and performance of aphids (*Myzus nicotianae* Blackman) (Hildebrand *et al.* 1993), and repel the neonate larvae of Asian corn borer *Ostrinia furnacalis* (Huang *et al.* 2009) and mated males of the *Ectropis oblique* moth (Lepidoptera: Geometridae) (Sun *et al.* 2014). Clearly, in our study, *A. hygrophila* appears to aggregate and to use con-specific beetle-induced DMNT to recognize infested plants.

We inferred that the feeding and oviposition preferences of beetles for hosts over non-hosts in field trials might be due to interspecific divergence in the emission of these volatiles among plant species. For instance, it is unusual that we did not detect the most common inducible green leaf volatiles, such as (*Z*)-3-hexenol, in host *A. philoxeroides* and non-host *A. sessilis* upon physical damage. When one of two key volatiles, DMNT or (*Z*)-3-hexenol, was added to host or non-host plants, the beetles altered their visitation frequencies depending on the dosage of the synthetic compounds. These results suggested that *A. hygrophila* beetles select plants according to the ratio of two key volatiles belonging to host and non-host volatile compound profiles, and thus, a host shift could occur in environments where this ratio of plant volatiles is no longer indicative of only the host plant.

In fact, recent field surveys in the geographically overlapping ranges of *A. philoxeroides* and *A. sessilis* demonstrated a host switch by flea beetles from the host-plant AP to non-host plant AS (Lu J *et al.* 2010; Lu X *et al.* 2015). In line with our chemical analysis of the headspace volatiles of *A. philoxeroides* and *A. sessilis* plants, these data indicate that host range expansion can occur among plants with qualitatively similar volatile bouquets, supporting the hypothesis that host shifts occur between chemically analogous plants (Futuyma & McCafferty 1990; Köpf *et al.* 1998). Moreover, the previous post-release evaluations of host specificity in laboratory no-choice feeding experiments by our group and other researchers demonstrated that adult flea beetles can feed on *P. oleracea* and *B. vulgaris* variety *saccharifera* plants, although their performance is significantly poorer than that on the host plant *A. philoxeroides* (Wang *et al.* 1988; Wu *et al.* 1994; Lu J *et al.* 2010). A further behavioural assessment in multiple-plant-choice tests showed

that host preference and performance of adult flea beetles largely rely on the presence or absence of *A. philoxeroides* plants, which implies a possible tendency of this biological control agent to use non-target plants when the target is not available (Lu J *et al.* 2012). In this study, we also found that the selection ranks and correlations of non-host BVS and BVC plants were only slightly lower than that of AS in field trials (Fig. 1 and Table 2). Thus, we predict that these native, non-target plants could become hosts of this flea beetle in matched phenological conditions and geographical ranges, which merits further investigation in the field.

Push-pull strategies using herbivore-induced plant volatiles (HIPVs) rely on the fact that certain HIPVs are attractive or repellent to herbivores and their natural enemies (Turlings & Wäckers 2004; Cook, Khan & Pickett 2007; Gurr & Kvedaras 2010; Mumm & Dicke 2010; Hare 2011; Zakir *et al.* 2013; Veyrat *et al.* 2016). For example, *Manduca sexta* larvae-induced emission of (*Z*)-3-hexenol, linalool and *trans*- $\alpha$ -bergamotene from tobacco plants functions as an indirect defence leading to more than 90% herbivore mortality by predators in the field (Kessler & Baldwin 2001; Schuman *et al.* 2009). In another example, as an important volatile cue, (*Z*)-3-hexenol emitted from herbivore-infested tomato plants is used by receiver plants to form a glycoside with defensive function against the common cutworm (*Spodoptera litura*) (Sugimoto *et al.* 2014). In this study, we demonstrated that two key volatiles, DMNT and (*Z*)-3-hexenol, can be used to stimulate increased feeding and oviposition on non-host plants and upon which survivorship of larvae is 75% in the field, indicating that the effect of the ratios of these compounds on host selection preferences could be effectively exploited in biological control. However, we also noticed that *A. hygrophila* larvae survive less well on the headspace-supplemented *A. philoxeroides* plants. The observed changes in survival may be caused by the priming effect of the volatiles. For instance, corn plants pre-exposed to (*Z*)-3-hexenol released significantly higher levels of jasmonic acid and volatile sesquiterpenes than the plants treated with solvent control after mechanical damage or caterpillar regurgitation treatments (Engelberth *et al.* 2004). Transcriptional analysis of *Arabidopsis thaliana* defensive response to priming volatiles, such as (*Z*)-3-hexenol and DMNT, after 24- or 48-h treatments showed that the pre-

treatments can activate expression of some defence genes in the jasmonic acid and salicylic acid pathways (Zhang, Wei & Kang 2012). The priming effect of volatiles could negatively affect any attempt to increase the efficiency of biological control. Therefore, the conflicting functions of the priming volatiles should be taken into account and evaluated under more natural conditions in developing novel weed control strategies.

In conclusion, this study showed that the use by important biocontrol agent, *A. hygrophila* flea beetles, of two common plant volatiles to identify its host plants can readily leads to a host shift. We suggest that olfactory cue-mediated interactions between biocontrol agents and native plants should be monitored and evaluated for the discovery of the potential risks of host shifting in biocontrol agents. In addition, comparative approaches in experimental studies on introduced biocontrol agents in a broad geographical area and under phenological conditions would facilitate the practices of ecological conservation and the management of biological invasions.

## Authors' contributions

J.W., R.M. and N.L. conceived of the experiment and designed the study. N.L., J.G. and S.L. were involved in the experiments and data collection. J.W., N.L., R.M. and M.C.S. performed the statistical analyses and wrote the paper. All authors discussed the results and commented on the manuscript.

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## Data accessibility

Data available from the Dryad Digital Repository: <https://doi.org/10.5061/dryad.md310> (Li *et al.* 2017).

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## Supporting Information

Details of electronic Supporting Information are provided below.

**Data S1.** Methods: GC–EAD recording.

**Fig. S1.** Monitoring data for temperature (a) and humidity (b) in the field trial.

**Fig. S2.** Simultaneously recorded antennal responses of naïve female *Agasicles hygrophila* to different volatile blends of *Alternanthera philoxeroides* with or without damage, and a mixture of six standard compounds to mimic the volatile blend of a non-host plant.

**Fig. S3.** Percentage of choices of naïve female *Agasicles hygrophila* in field trials to non-host plants and AP (*Alternanthera philoxeroides*) with (Z)-3-hexenol standard compound (a); AM adding the DMNT standard compound (b), respectively, at different dosages.

**Table S1.** Information about degrees of damage for different treated *Alternanthera philoxeroides* plants for volatile collections.

**Table S2.** The amount of feeding of females on 13 plant species in the field trial.

**Table S3.** Gas chromatography (GC) coupled with electroantennographic detection (EAD) responses of flea beetle antennae to head-space extracts.

**Table S4.** Gas chromatography (GC) coupled with electroantennographic detection (EAD) responses of flea beetle antennae to a mixture of standards.

**Table S5.** Mean number of eggs and hatching rate of mated female *Agasicles hygrophila* beetles on (Z)-3-hexenol + AP and five non-host plants in the field trial.

**Table S6.** Pearson correlation of mean number of eggs and hatching rate on (Z)-3-hexenol+AP and AS plants in the field trial.