

# Varying the spatial arrangement of synthetic herbivore-induced plant volatiles and companion plants to improve conservation biological control

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## Abstract

1. Conservation biological control aims to control pests by promoting wild populations of natural enemies. One challenge is to attract and retain efficient natural enemies in crop fields, which often are a suboptimal environment. Towards this goal, the *attract-and-reward* strategy relies on combining attractive synthetically produced herbivore-induced plant volatiles (HIPVs) with companion plants (non-crop plants which provide alternative resources to the targeted natural enemies). Although severely overlooked, the spatial arrangement of HIPV dispensers and rewards inside crop fields may strongly influence the foraging behaviour and persistence of natural enemies and thus the success of this pest management strategy.
2. We tested the impact of two contrasting spatial arrangements of HIPV dispensers and rewards, alternatively inside and around a block of target apple trees, on the efficacy of the biological control of *Aphis citricola* populations by the common predatory ladybird *Propylea japonica* in apple orchards in northern China. We used synthetic methyl salicylate (MeSA) as an attractant and the companion plant *Calendula officinalis* as a reward. To better understand how the spatial arrangement of MeSA dispensers and companion plants affected the attraction and foraging behaviour of adult ladybirds, we conducted indoor experiments in a flight mill, an olfactometer and a wind-tunnel.
3. Blocks of target trees treated with MeSA dispensers inside and companion plants around provided the most efficient pest control in orchards, compared with the opposite spatial arrangement.
4. The synthetic MeSA dispenser and the companion plant synergistically attracted ladybirds in the olfactometer and enhanced their flight activity in the flight mill. In the wind-tunnel, MeSA served as a spatial cue for ladybirds to find nearby prey, while companion plants were sought in the absence of prey.
5. *Synthesis and applications*. This study aims to further improve aphid control in apple orchards through a careful spatial arrangement of herbivore-induced plant volatiles dispensers (HIPVs) and rewards (companion plants) in optimized

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*attract-and-reward* strategies. Without such assessment, these strategies may be hazardous even with well-identified targeted natural enemies. Associated lab experiments highlight that interactions between HIPVs and companion plants influence ladybird foraging pattern, and that their spatial arrangement can modulate the ability of such key predators to find their prey.

#### KEYWORDS

*attract-and-reward*, foraging behaviour, herbivore-induced plant volatile, integrated pest management, methyl salicylate, natural enemies, semiochemicals, synomone

## 1 | INTRODUCTION

Conservation biological control (CBC) has drawn increasing attention in recent decades, along with important development and implementation (Gurr, Wratten, Landis, & You, 2017; Michaud, 2018; Tschumi et al., 2016). It relies on environmental manipulation to preserve pests' natural enemies and promote their effectiveness (Fiedler, Landis, & Wratten, 2008; Gurr et al., 2017). This approach thus avoids the detrimental side effects associated with classical augmentative biological control programs, such as the overgrowth of introduced natural enemies (Louda, Pemberton, Johnson, & Follett, 2003; Simberloff & Stiling, 1996).

A promising method in CBC is the *attract-and-reward* strategy, in which the colonization of crop fields by natural enemies is enhanced via attraction from surrounding habitats and their persistence and population growth is enhanced via the reward (Simpson et al., 2011). The attractants are synthetically-produced molecules similar to natural herbivore-induced plant volatiles (HIPVs), such as methyl salicylate (MeSA) and methyl jasmonate. Plants naturally emit large amounts of HIPVs in response to herbivorous attacks, and these chemicals are long-distance attractants of herbivorous arthropods' natural enemies (Turlings & Erb, 2018). The effect of synthetic MeSA is comparable to that of naturally emitted MeSA, and it has been widely implemented in CBC targeting natural enemies such as hemipteran bugs, coccinellids and hoverflies (Gadino, Walton, & Lee, 2012; Rodriguez-Saona, Kaplan, Braasch, Chinnasamy, & Williams, 2011; Zhu & Park, 2005). In particular, the MeSA plant-defence pathway is induced by phloem-sucking insects such as aphids, while the methyl jasmonate pathway is induced by leaf chewers (Soler et al., 2012). Therefore MeSA is likely more relevant to coccinellids, which essentially depend on aphids as a food resource (Ali, Desneux, Lu, & Wu, 2018; Vandereycken et al., 2013).

Rewards are provided by companion plants artificially incorporated within cropping systems. Companion plants have specific functional traits which may enhance the settlement and population growth of pests' natural enemies (Gurr et al., 2015, 2017; Perović et al., 2018). They are also known as ecosystem service providers (Kremen, 2005) or secondary plants (Parolin et al., 2012). They may provide complementary food resources, such as pollen and nectar, for targeted natural enemies (Baggen, Gurr, & Meats, 1999; Li et al.,

2015), or resources for alternative hosts or prey of natural enemies (banker plants: Bugg & Waddington, 1994; Huang et al., 2011). They may also provide shelter or reproductive sites for natural enemies and attract them through visual and olfactory cues, drawing them to suboptimal or disturbed environments such as crop fields (Beane & Bugg, 1998; Gurr et al., 2017; Perović et al., 2018; Zhao et al., 2017). Frequently used companion plants include marigold (*Calendula officinalis*; Martínez-Uña, Martín, Fernández-Quintanilla, & Dorado, 2013; Nemeč, Beckendorf, Hesler, Riedell, & Lundgren, 2016; Zhao et al., 2017) and sweet alyssum (*Lobularia maritima*; Brennan, 2016; Gontijo, Beers, & Snyder, 2013). In those studies, these companion plants were considered as abundant pollen and nectar providers, and they mostly attracted hoverflies and hemipteran bugs, resulting in a successful control of arthropod pests. In a preliminary experiment, we fed adults of *Propylea japonica* (Coccinellidae) with *C. officinalis* flowers along with *ad libitum* *Mizus persicae* aphid nymphs in a laboratory experiment, and we measured a 1.43- and 1.34-fold increase in their longevity and fecundity, respectively (Figure S1). This highlights the potential for *C. officinalis* to act as a reward to coccinellids in crop fields.

Simpson et al. (2011) implemented the *attract-and-reward* strategy in various crops (sweetcorn, broccoli and grapevine) which were treated with synthetic HIPVs (including MeSA and methyl jasmonate) and surrounded by buckwheat (*Fagopyrum esculentum*) as a companion plant providing nectar resource. They found that natural enemies (parasitoids and predators) were more abundant near plants treated with synthetic HIPVs than near non-treated plants, which resulted in efficient top-down regulation of pests and reduced crop damage. Other implementations of this strategy yielded mixed success (Salamanca, Souza, & Rodriguez-Saona, 2018; Simpson et al., 2011), highlighting the importance of optimizing the provision of companion plants and synthetic HIPVs in crop fields. Both companion plants and synthetic HIPVs may affect non-target species. For instance, they may attract other pests as well as non-target natural enemies and higher trophic levels (Orre, Wratten, Jonsson, & Hale, 2010; Orre-Gordon, Wratten, Jonsson, Hale, & Simpson, 2013; Parolin et al., 2012; Wäckers, Romeis, & van Rijn, 2007), which may in turn increase intra-guild predation between natural enemies (Jonsson, Wratten, Landis, & Gurr, 2008) and fail to increase crop productivity (Salamanca et al., 2018).

Much work remains to understand the influence of landscape composition on the densities of pests and their natural enemies at various spatial scales (Gurr et al., 2017; Karp et al., 2018). Floral resource provisioning such as flower strips within and around fields has been shown to increase the abundance of natural enemies and reduce pest pressure in neighbouring fields (Balzan, Bocci, & Moonen, 2016; Tschumi et al., 2016). In practical implementations of *attract-and-reward* strategies, both floral resources and HIPVs are manipulated within fields, and how companion plants with respect to synthetic HIPV dispensers are spatially arranged may influence the movement of natural enemies, because they signal for different resources and elicit different foraging behaviours (Landis, Wratten, & Gurr, 2000). Once inside crop fields, natural enemies use HIPVs to locate prey nearby, but may be unable to detect them if prey-specific signals are too weak and/or prey too distant from HIPVs emitters (Vet & Dicke, 1992). They then might turn to alternative resources, such as companion plants. On the contrary, too high concentrations of synthetic HIPVs may repel natural enemies (Van Wijk, De Bruijn, & Sabelis, 2008), and also induce neighbouring plants' own production of volatiles (Delaney, Wawrzyniak, Lemańczyk, Wrzesińska, & Piesik, 2013), resulting in confusing air signals to targeted natural enemies (Kaplan & Lewis, 2015). Finally, they might become habituated to HIPVs (i.e. learn to dissociate HIPVs from the presence of prey), and disperse away (Kaplan, 2012; Kaplan & Lewis, 2015; Lucchi et al., 2017). The overlooked spatial arrangement of companion plants and synthetic HIPVs within fields might be key to improve the success of *attract-and-reward* strategies (Kaplan & Lewis, 2015).

In this study, we investigated how the spatial arrangement of synthetic MeSA dispensers and companion plants *C. officinalis* within apple orchards affected the attraction and persistence of ladybirds *P. japonica*, and the subsequent impact on aphid *Aphis citricola* populations. By sucking leaf phloem and producing honeydew, aphids drastically reduce apple tree photosynthesis and yields (Yin, Qiu, Yan, Sun, & Zhang, 2013). *Propylea japonica* is among the most abundant species of naturally occurring ladybirds in Northern China, and is able to efficiently control aphids in cotton fields (Ali, Desneux, Lu, Liu, & Wu, 2016; Lu, Wu, Jiang, Guo, & Desneux, 2012; Wu & Guo, 2005). In preliminary experiments, we showed that *C. officinalis* companion plants alone did not significantly improve aphid control in orchards, while MeSA alone yielded a 30% reduction in aphid densities relative to control in the long term (Figure S2). However, the initial aphid population growth was not prevented. We also found that clustering MeSA dispensers and companion plants did not improve aphid control compared with an untreated orchard (Figure S3). In this study we tested how two contrasting spatial arrangements of MeSA dispensers and companion plants – MeSA dispensers inside a block of target apple trees and companion plants around it, or vice versa – affected the density of ladybird and aphid populations inside the block. To gain insights into the mechanisms underlying observed differences in insect densities, we conducted three indoor experiments to measure the long-distance flight capacities (in a flight mill), the short distance attraction (in an olfactometer), and the short-distance foraging behaviour of *P. japonica* adults (in a wind-tunnel) in response to the presence of MeSA dispensers and companion plants.

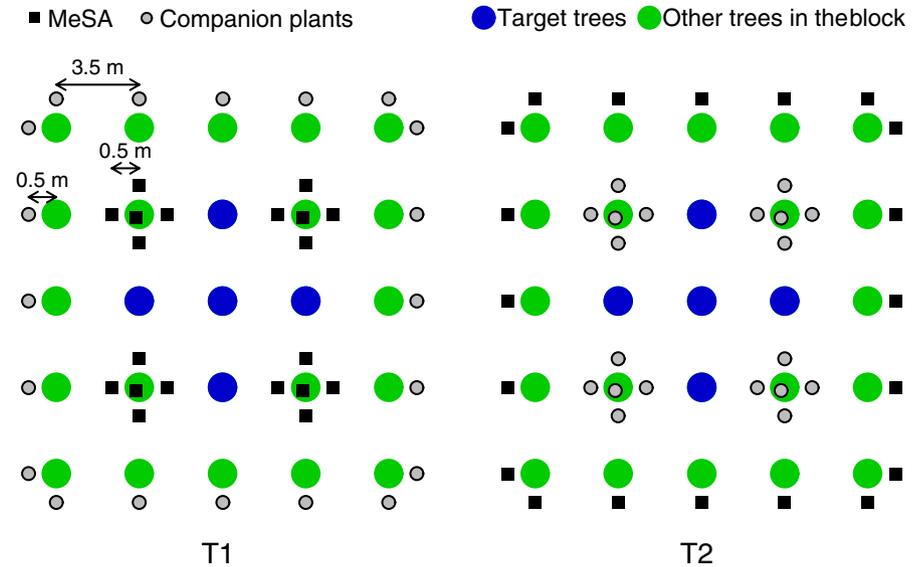
## 2 | MATERIALS AND METHODS

### 2.1 | MeSA dispensers and companion plants

Methyl salicylate (99% purity, ZZStandard, Shanghai, China) was diluted in *n*-hexane (97% purity; Yufeng, Liaoyang, China) at a low (0.2 µg MeSA dissolved in 0.1 mL hexane) and a high concentration (200 µg in 0.1 mL hexane), following the protocol of Petterson et al. (1994). In field experiments, we used 1.5-mL polyethylene bottles of high-concentration MeSA and with a 1.0-mm-diameter hole in the cover to allow evaporation in the ambient air. In indoor experiments, we used either 1.5-mL bottles of low-concentration MeSA (flight mill and wind-tunnel) or filter paper cards (1.0 × 4.0 cm) loaded with 2 mL of high-concentration MeSA and evaporated for 5 min to remove solvent (olfactometer; De Boer & Dicke, 2004). Companion plants *C. officinalis* var. Kablouna were obtained from the SinicHorticulture and Lower Co. Ltd, Beijing, China, from seeds collected during the previous season. Seedlings were started in plastic trays and individually transplanted in plastic pots (height 20 cm, diameter 13 cm) when they had three to four true leaves. After 20–25 days, the plants were ~20 cm tall and the terminal bud was removed to ensure flower production. Two weeks later, they were blooming and could be used in our experiments starting in April 2016. They were maintained in a greenhouse to ensure constant flowering throughout the season.

### 2.2 | Orchard experiment: spatial arrangement of HIPV dispensers and rewards

Nine organic apple orchards were surveyed in the Changping County, Beijing, China in 2016 (Figure S4 and Table S2). Each orchard contained more than 1,000 mature apple trees (*Malus pumila* cv. Fuji) planted in 2006. Two different treatments (T1 or T2) were applied on a 5 × 5 block of 25 apple trees of similar age at the centre of each orchard (Figure 1). In T1, MeSA dispensers and companion plants were placed inside and around the block of trees, respectively (spacing is shown in Figure 1). In T2, the opposite spatial arrangement was used. T1 was applied in orchards #1, #2 and #3, while T2 was applied in orchards #4, #5 and #6 and orchards #7, #8, #9 were controls (no treatment; Figure S4). In all orchards, agricultural practices were conducted according to the USDA-Organic standard (#7-CFR-Part-205, certified by ECOCERT®), which specifies tree fertilization with organic botanical/micro-organism formulations, no pesticide application and weed removal twice a month. Each MeSA dispenser was placed in a plastic box (3 × 3 × 3 cm) covered with a 50-mesh plastic fabric net to block rainfall but not ventilation. The MeSA boxes were placed at each site in early May and partially buried, leaving 3 cm above the ground surface. The MeSA dispensers inside the boxes were replaced with new ones weekly. Potted companion plants with four to six open flowers each were placed in each site at the same time. They were renewed every 10 days, during which they were watered every 2 days and had at least four open flowers.



**FIGURE 1** Treated block of 25 trees in orchards: spatial arrangement of MeSA dispensers and *Calendula officinalis* companion plants in T1- and T2-treated orchards. MeSA dispensers and companion plants overlaid on trees were placed 10 cm from the base of the tree

From mid April (10 days before the treatment application) to mid August, *P. japonica* ladybird and *A. citricola* aphid populations were surveyed once a week on five target trees at the centre of each treated block (Figure 1). Each sampling day, four randomly selected young branches per target tree (length 65–70 cm) were visually inspected and all ladybirds (adults and larvae) and aphids (adults and juveniles) were counted. Visual inspection is the preferred method in Chinese orchards, as it provides repeatable and non-destructive insect population monitoring at affordable costs (S. Wang, personal obs.). At the time of harvest, commercially acceptable apples from the treated block of 25 trees in each orchard were weighed, as a measure of yield.

We analysed the short-term and long-term influences of the contrasting spatial arrangements of MeSA dispensers and companion plants on the ladybird and aphid population dynamics in Weeks 3–5 and Weeks 9–18 of the experimental period, respectively. Weeks 1–2 corresponded to the seasonal immigration of ladybirds from wild habitats into orchards (2008–2014 historic records from S. Wang). Weeks 3–5 corresponded to the short-term response of insect population dynamics to the treatment, applied 5 days before the sampling of Week 3. Weeks 9–18 corresponded to the stabilizing phase, and coincided with two to three generation cycles of *P. japonica* ladybirds, which have a life cycle very similar to that of *Harmonia axyridis* (S. Wang personal obs.; Wang, Michaud, Tan, Zhang, & Guo, 2011). To account for correlation among samples taken repeatedly from the same five trees per orchard, a random subject effect of tree number nested in the orchard was first included in Generalized Linear Mixed models, with a negative binomial distribution to account for the non-normality of count data. This distribution was chosen against a Poisson distribution based on AIC/BIC criteria. The random effects were systematically estimated to zero in the models, meaning that residual deviance completely accounted for the variation in data. Therefore, we simply used GLMs. Since the treatment significantly affected population dynamics, multiple comparisons of means were performed by Tukey's HSD (function "glht" from R

library "multcomp"). Yield data were analysed by performing a one-way ANOVA on the treatment, and residuals were normally distributed. All statistical analyses were performed using R version 3.2.3 (R Core Team, 2015).

## 2.3 | Indoor experiments: olfactometer, flightmill and wind-tunnel

### 2.3.1 | Insects

Five hundred pairs of *P. japonica* adults were collected from an alfalfa field at Beijing Academy of Agriculture and Forestry Sciences (BAAFS, Beijing, China) on April 2016. The ladybirds were maintained in the Entomology Research Laboratory of the Institute of Plant and Environment Protection, BAAFS at a density of 30–40 pairs per cage (40.0 × 40.0 × 55.0 cm, made of 40-mesh plastic net and aluminium alloy frames). They were fed *ad libitum* with reared *A. citricola* (Hemiptera: Aphididae) as well as young horse bean shoots. Ten white paper sheets per cage (10.0 × 3.0 cm) were provided as oviposition substrate. Paper sheets were transferred daily into another cage where hatched juveniles were maintained until adulthood with the same diet as described above. Newly emerged adults were transferred daily into new cages at the same density as above and fed *ad libitum*. Ten-day-old, mature adult ladybirds were selected for the tests. Environmental rearing conditions were  $T = 23^{\circ}\text{C}$ ,  $\text{RH} = 65\%$ , photoperiod = 16 L:8D at 1,000 lux.

### 2.3.2 | Flight mill test: Long-distance flying capacities

We tested how the presence and spatial arrangement of MeSA dispensers and companion plants affected the flight capacity of *P. japonica* adults, using a flight mill device in sunny, controlled greenhouse conditions. Insects flying a longer distance in these experiments are likely to disperse a greater distance in natural conditions (Bradley &

Altizer, 2005; Maes, Massart, Grégoire, & De Clercq, 2014). Flight mill experiments are also used to test the impact of environmental cues on insects' flying capacities (Rouyar et al., 2015; Salom & McLean, 1991). The flight mill was composed of 16 independent channels, allowing us to test 16 individuals simultaneously (SY2-16A, Jiaduo Equip., Anyang, China). The channels were 15 m apart from each other to avoid visual and olfactory interference. Each channel was composed of two titanium arms (upper arm length: 40 cm; lower arm length: 5 cm) fixed to a common axis forcing a synchronous rotation with standardized perturbations (Ribak, Barkan, & Soroker, 2017; Taylor, Bauer, Poland, & Windell, 2010). The tested insect was attached to one end of the upper arm with a nylon fabric thread (diameter: 0.2 mm, length: 2.5 cm), and a counterweight was attached to the other end. We chose ladybirds of very similar body size, so that the counterweight was adjusted only once at the beginning of the experiment, at the average ladybirds' weight. The number of revolutions, the flight distance (km), the flight duration (min) and the average flight speed (m/min) were recorded and calculated automatically in each channel. Each test lasted 12 hr.

Five treatments were applied, with a varying combination of olfactory cues: (a) two companion plants; (b) two MeSA dispensers; (c) one companion plant and one MeSA dispenser placed together (<5 cm apart); (d) one companion plant and one MeSA dispenser separated; and (e) control (no environmental cue). Each plant had exactly three open flowers. The MeSA dispensers were positioned at a height comparable to plants, ensuring that chemical cues were emitted from similar positions. Cues were placed at 75 cm from the flight mill's rotating axis at opposite positions (except in Treatment 3). Each treatment was replicated 20 times with a new ladybird individual, for a total of 100 ladybirds. Companion plants and MeSA dispensers were renewed each time.

We analysed the flight distance, the flight duration and the square root-transformed average flight speed using a MANOVA with a Pillai test with treatment (presence of different environmental cues) as a fixed effect. We then performed Tukey's HSD tests to compare means for each variable.

### 2.3.3 | Olfactometer test: short-distance attraction

We tested whether adult ladybirds were attracted by MeSA and by the odour of companion plant *C. officinalis*, isolated or together, in a four-arm olfactometer (CT10 Camsonar; Kunming, China), following Vet, Van Lenteren, Heymans, and Meelis (1983) and Li et al. (2015). Ambient air was vacuumed from the bottom of the olfactometer's central arena at 1.5 mL/min. Each arm was connected using Teflon tubing to a series of four glass vials (height 18 cm, diameter 9.5 cm; except second vial: height 35 cm, diameter 18 cm). The first vial collected the ladybird choosing that arm. The second contained the odour source, the third contained activated charcoal to purify air, and the last vial contained distilled water to humidify air. Air flowed at 1 mL/min inwards from the last vial to the central arena. The odour sources in the four arms were: (a) MeSA dispenser; (b) potted companion plant with exactly three open flowers carefully placed in the vial; (c) MeSA

dispenser and plant; or (d) no MeSA dispenser or plant (control). The central arena of the olfactometer was kept in a dark chamber with a 24-W lamp placed above to provide uniform light and avoid visual interference. Behavioural observations were conducted at 22–25°C. The experiment consisted of 20 replicates of 60 ladybirds, starved for 8 hr prior to the experiment. They were introduced one at a time in the walking arena. A new piece of filter paper was placed on the arena floor after each 60-ladybird replicate. The olfactometer was also cleaned with soap-water and entirely rinsed with hexane, and odour sources were replaced with fresh ones (new MeSA dispensers and new plants). After 5 min to allow air flow to stabilize, a new ladybird was placed in the walking arena. The position of the four different odour sources was randomized in the four arms between each replicate. As soon as the ladybird entered one arm, it fell in the sampling chamber where it was collected and the chosen arm was recorded, before introducing a new ladybird into the olfactometer.

The relative attractiveness of the different olfactory stimuli in the four-arm olfactometer was estimated using the "prop.multinom.test" function of the R library RVAideMemoire. This function builds multinomial log-linear models on the number of ladybirds choosing each arm (Davison & Ricard, 2011; Ricard & Davison, 2007), and performs Wald tests with the Benjamini and Hochberg (1995) correction method for comparing multiple *p*-values (*p.adjust* = "fdr"). The mean proportions and SEs were calculated with the "prop.multinom" function which relies on the same methodology.

### 2.3.4 | Wind-tunnel: short-distance foraging behaviour

We measured the short-distance foraging behaviour of adult ladybirds in a wind-tunnel in the presence of MeSA dispensers, companion plants, and *A. citricola* prey. The wind-tunnel had three contiguous chambers (Figure S5). The position of the MeSA chamber and the companion plant chamber (right vs. middle), and the presence or absence of prey in the left chamber were varied among treatments in a full factorial design, with 15 replicates per treatment. Five MeSA dispensers and three companion plants each with five fully open flowers were placed in boxes under their respective main chambers and connected to them with a fine dark mesh masking plants' visual cues (Figure S5). In treatments with prey, 1,000 *A. citricola* second- or third-instar nymphs were placed in the prey chamber (left), in a plastic box covered with a dark fine mesh to prevent them from being accessible or visible to ladybirds; however, they could smell each other. The number of MeSA dispensers, companion plants and aphids was established based on maximum sensitivity of ladybirds as tested in a preliminary experiment. Thirty randomly selected 10-day-old *P. japonica* adults were introduced into the releasing chamber for each replicate. A 2.0 L/min airflow was then established from the prey chamber towards the release chamber to spread olfactory cues and avoid their accumulation and degradation in the wind-tunnel. The walls separating the chambers were removed after the airflow had stabilized (about 30 s) to allow the ladybirds to freely disperse in the three contiguous chambers. The number of ladybirds staying in each chamber was recorded

20, 40 and 60 min after the walls' removal. At the end of each 60-min sequence, all insects, the MeSA dispensers and the companion plants were removed from the wind-tunnel, and all sections of the tunnel were cleaned with distilled water and hexane (95%, v/v).

We examined how the proportions of ladybirds in each of the three chambers of the wind-tunnel varied through time, depending on whether they first entered the MeSA chamber or the plant chamber, and the prey was present or absent. Based on the olfactometer and flight mill experiments, we hypothesized that MeSA was more attractive than companion plants and ladybirds would thus always focus on MeSA dispensers first. Then, they would try to find prey if present, and otherwise find alternative food resource. Therefore, the proportion of ladybirds in the MeSA chamber should always decrease through time, while the proportions in the prey and plant chambers should increase when prey are present versus absent, respectively. We also hypothesized that ladybirds used the strong MeSA signal to locate aphid prey, which typically cause emissions of HIPVs (including MeSA) in attacked plants. Therefore, we expected that ladybirds would find aphids faster if they are located close to the MeSA chamber (i.e. in treatments with the MeSA dispenser in the middle chamber), but the spatial arrangement of the MeSA dispenser and the companion plant should have no effect on the distribution of ladybirds in the absence of prey. We performed a repeated measures analysis to account for the correlation between observations made at 20, 40 and 60 min in the same replicate, and a random subject effect (replicate) was included. We performed two sets of Generalized Linear Mixed Models (function "glmer" in the library "lme4") with a binomial distribution and a logit link function. First, we analysed the proportion of ladybirds in the MeSA chamber versus the other two (total proportion of ladybirds in prey chamber plus plant chamber), and second the proportion of ladybirds in the plant chamber versus the other two (total proportion of ladybirds in prey chamber plus MeSA chamber). In each set of tests, the fixed effects were the presence/absence of prey, the spatial arrangement of MeSA and companion plants in the middle versus right chamber, and time (implemented as a fraction of hour), as well as all second-order interactions between fixed effects. Generally multinomial rather than binomial tests are expected for this kind of experiment; however, they are intrinsically difficult to interpret and the implementation of pseudo-replication worsens this problem, leading us to use GLMMs. The significance of second-order interactions was estimated by a type-II model comparison based on chi-squared tests. The most likely model was obtained by removal of the non significant interaction, and we verified that residuals were normally distributed using Shapiro–Wilk tests. Since we conducted two sets of tests on the same dataset, we adjusted the paired  $p$ -values from the same factors in the two best models using the Benjamini and Hochberg (1995) to reduce the risk of type-I errors (false detection).

### 3 | RESULTS

We tested the impact of the spatial arrangement of MeSA dispensers and companion plants in orchards on the efficacy of aphid control

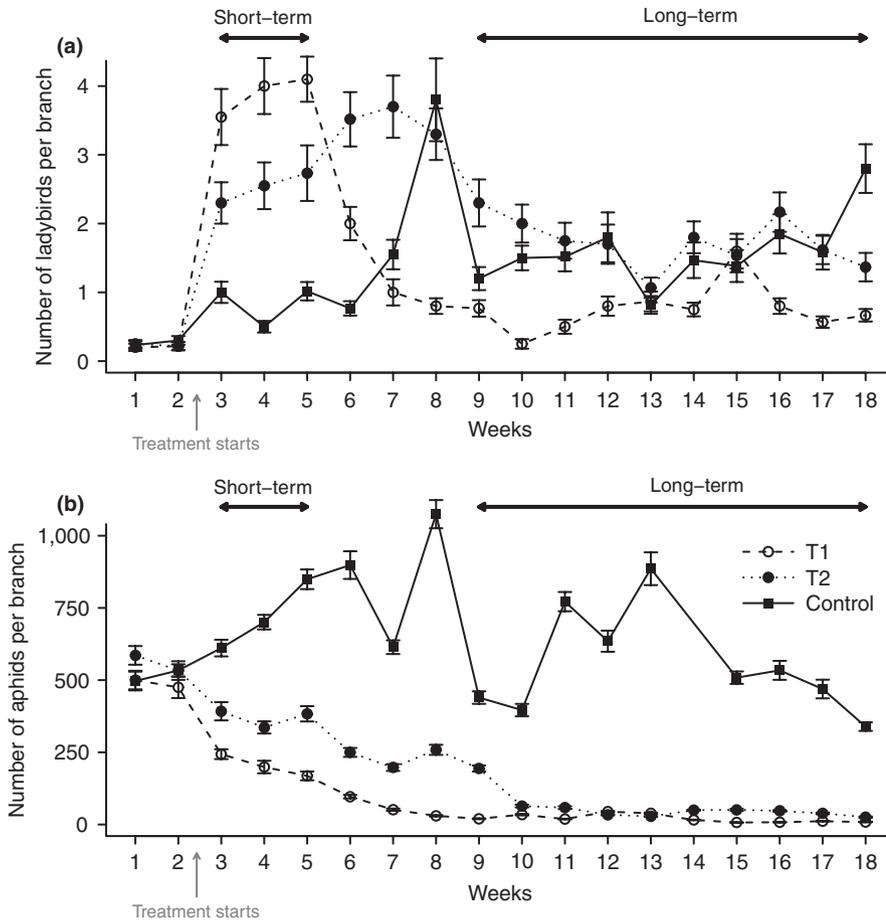
by ladybirds. The treatment significantly affected population dynamics of ladybirds and aphids both on the short-term and on the long-term (Figure 2; Table 1 and Table S3). In the short-term, treatment T1 (MeSA dispensers inside the block of 25 trees and companion plants around it) resulted in a 1.5-fold increase in ladybirds counts compared with T2 (MeSA dispensers around the block and companion plants inside it), and a 4.6-fold increase compared to control orchards. However, the ladybird population rapidly decreased in T1-treated orchards and stabilized around 0.75 ladybirds per branch after Week 8, compared with 1.73 and 1.59 ladybirds per branch in T2-treated orchards and control orchards, respectively. Aphid populations were regulated faster in T1, resulting in a twofold and a fourfold decrease in aphid counts in T1 compared with T2 and control, respectively over Weeks 3–5. After Week 9, aphid counts stabilized around 20 and 60 per branch in T1 and T2, respectively, while they fluctuated between 300 and 700 per branch in control orchards. The treatment significantly affected yields ( $F_{2,6} = 52.75$ ,  $p < 0.001$ ; Figure S6; Table S3), with yields 1.07 and 1.16 times higher in T1-treated blocks than in T2-treated and control blocks, respectively.

We then conducted indoor experiments to gain insights on the mechanisms underlying the observed differences between treatments. Specifically, we measured the long-distance flying capacities of adult ladybirds in a flight mill, the short-distance attraction in an olfactometer, and the short-distance foraging behaviour in a wind-tunnel.

We found that the presence, nature and position of MeSA dispensers and companion plants in the flight mill experiment significantly affected ladybirds' flight capacities (Pillai = 0.74,  $df = 4,95$ ,  $p < 0.001$ ). Flight capacities were significantly improved when the MeSA dispenser and the companion plant were provided as compared to the control or treatments with two identical olfactory sources. This response increased when the dispenser and the plant were placed opposite to one another rather than together in the flight mill experiment (Figure 3, Tables S5–S7).

In the olfactometer experiment, we found that both MeSA and *C. officinalis* volatiles attracted adult ladybirds. The combination of the two was the most attractive, followed by a MeSA dispenser alone, then the companion plant and finally the control (no scented cue; Figure 4, Table S8).

In the wind-tunnel experiment, we found that the interaction between the presence/absence of prey and time significantly affected both the proportion of ladybirds in the MeSA chamber (MeSA vs. prey + plant) and the proportion of ladybirds in the plant chamber (plant vs. MeSA + prey; Figure 5; Table 2). The proportion of ladybirds in the MeSA chamber (vs. prey + plant) was also affected by the interaction between position and prey, while the proportion of ladybirds in the plant chamber (vs. MeSA + prey) was affected by the interaction between position and time. Basically, as shown by the coefficients of the best models for each set of tests (Table 2), ladybirds first went to the MeSA chamber (positive intercept for MeSA vs. prey + plant and negative intercept for plant vs. MeSA + prey). In the absence of prey, they left the MeSA chamber for the plant chamber. In the presence of prey, they left the MeSA chamber for the prey chamber, and the position of the MeSA versus plant chamber affected how quickly they found prey: they tended to search for prey close



**FIGURE 2** Impact of the spatial arrangement of MeSA dispensers and *Calendula officinalis* companion plants on the population dynamics of (a) *Propylea japonica* predatory ladybirds (adults and larvae) and (b) *Aphis citricola* aphids (adults and juveniles). Mean numbers ( $\pm$ SE);  $N = 60$  (three orchards per treatment, five target trees per orchard, four branches per target tree). T1: MeSA dispensers inside the block and companion plants around it; T2: opposite arrangement; Control: no treatment

**TABLE 1** Impact of the spatial arrangement of MeSA dispensers and companion plants in orchards on the population dynamics of ladybirds and aphids

Fixed effects	Deviance, residual deviance	df, Residual df	p-value
<b>Short-term</b>			
Ladybirds	174, 606	2, 537	<0.001
Aphids	445, 570	2, 537	<0.001
<b>Long-term</b>			
Ladybirds	126, 1,858	2, 1,797	<0.001
Aphids	5,965, 1,885	2, 1,737	<0.001

Note. Generalized Linear Models were used, with a negative binomial distribution and the spatial arrangement of MeSA dispensers and companion plants as a fixed effect.

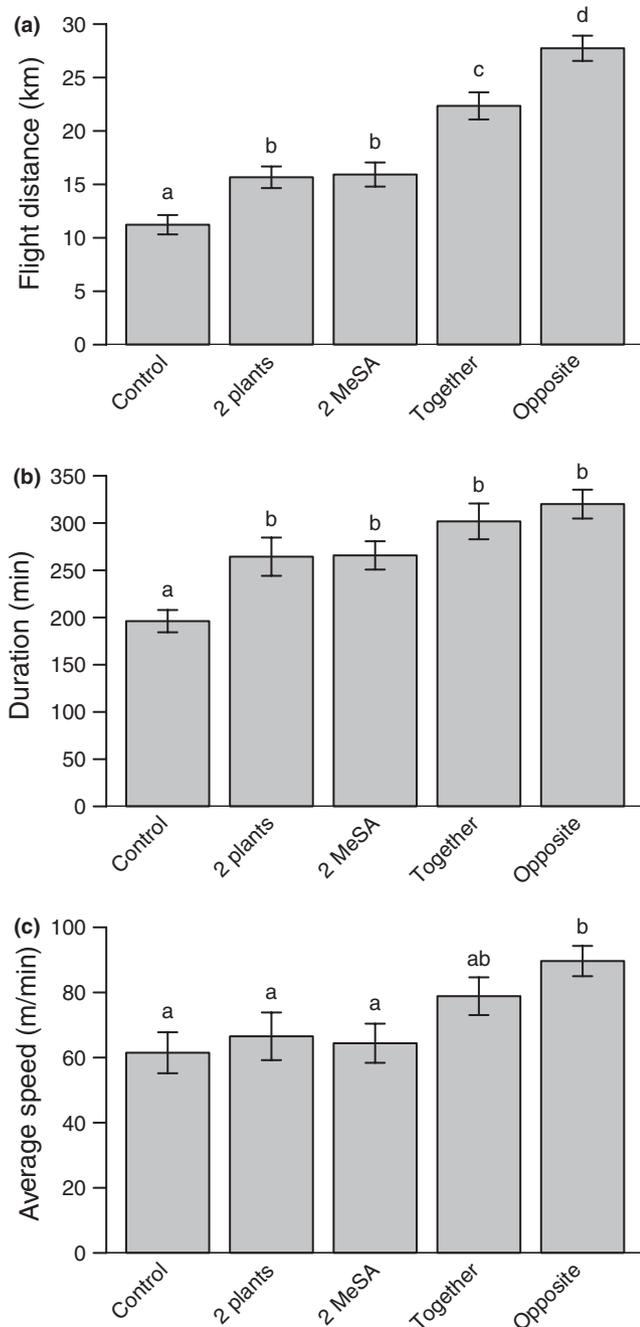
to MeSA dispensers, and hence spent more time in the MeSA chamber when it was in the right position (i.e. distant from the prey chamber).

#### 4 | DISCUSSION

In this study, we applied an *attract-and-reward* strategy and investigated how the spatial arrangement of MeSA dispensers and companion plants affected the densities of ladybird and aphid populations in

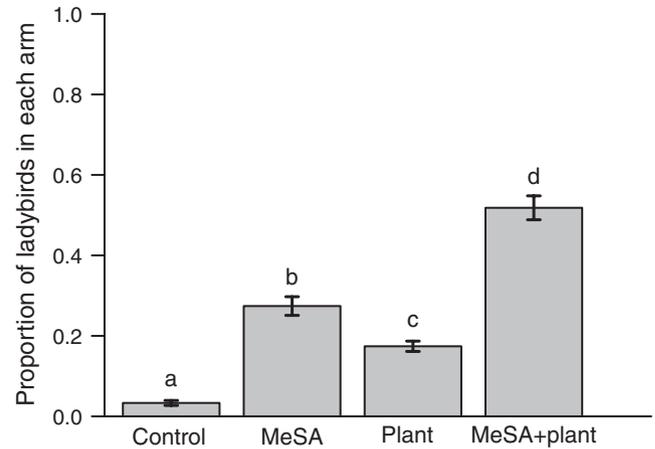
apple orchards. When MeSA dispensers were placed inside a block of trees and companion plants around it (T1), aphid populations were more strongly controlled inside the block, resulting in higher yields, compared with non-treated orchards. The opposite spatial arrangement (T2: MeSA around the block and companion plants inside it) resulted in intermediate control of aphid populations due to lower densities of ladybirds (Figure 2). To gain insights on the mechanisms underlying such difference, we conducted three indoor experiments. The flight mill and olfactometer experiments showed that MeSA and companion plants synergistically attracted ladybirds (Figures 3 and 4). The wind-tunnel results suggested that ladybirds used MeSA as an indicator of nearby prey, and that the presence of prey negatively affected their attraction to companion plants (Figure 5). This possibly explains why the control of aphid populations was faster in T1-treated orchards.

The *attract-and-reward* strategy resulted in a rapid regulation of aphid populations, preventing exponential growth observed in control orchards (Figure 2), and contrary to our preliminary experiment relying on MeSA-only treatment (Figure S2). Spatially separating MeSA dispensers and companion plants allowed a stronger control of aphid populations compared with MeSA and companion plants being clustered in treated orchards (Figure S3). One reason might be a stimulation of ladybirds' flight activity (Figure 3) and foraging behaviour (as compared to clustered cues), increasing the probability that ladybirds would find aphids. We measured an



**FIGURE 3** Flight distance (a), duration of flight (b) and average speed (c) of ladybirds in the flight mill experiment, exposed to different environmental cues (means  $\pm$  SE). Control: no cue; two plants: two companion plants at opposite positions; 2 MeSA: two MeSA dispensers at opposite positions; together: one companion plant and one MeSA dispenser at the same position; opposite: one companion plant and one MeSA dispenser at opposite positions. Different letters above bars represent significantly different means as estimated by Tukey's HSD tests (Tables S5–S7)

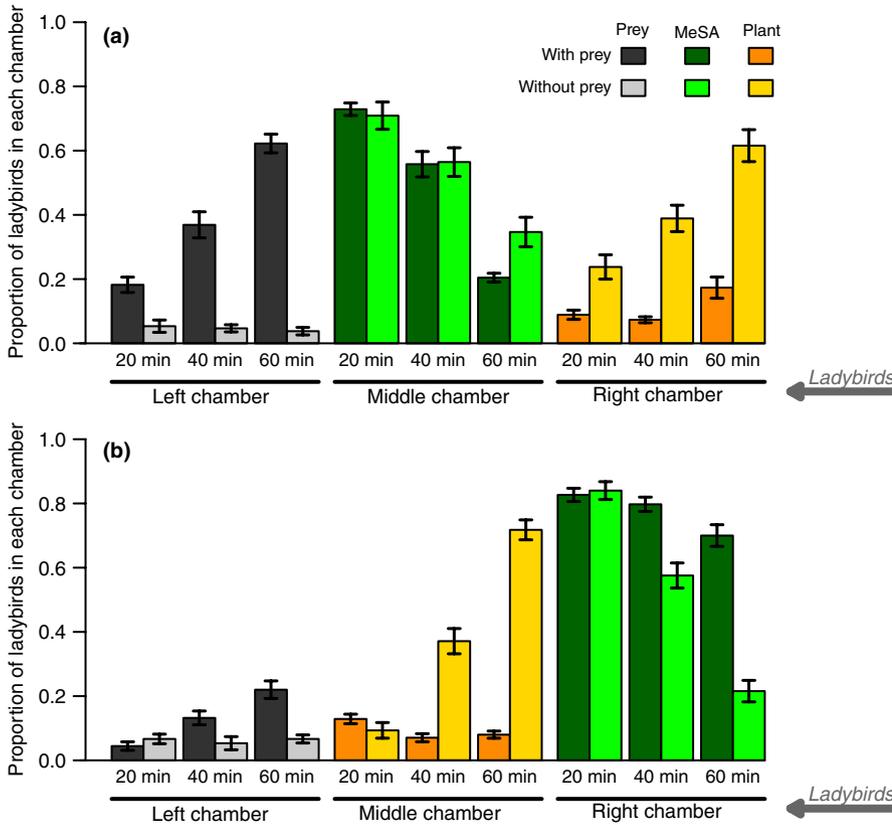
immediate positive impact of a large number of ladybirds being attracted to orchards on the regulation of aphid populations in the short term. The timing of natural enemies' movement into crop fields relative to pests' population dynamics and outbreaks is indeed crucial (Brewer et al., 2017; Kean, Wratten, Tylanakis,



**FIGURE 4** Proportion of ladybirds in each arm of the olfactometer connected to different olfactory stimuli. The different letters above bars represent significantly different proportions as estimated by the Wald tests (Table S8)

& Barlow, 2003). In the short term, higher densities of ladybirds were measured in T1 compared with T2-treated orchards, resulting in a faster decrease in aphid densities. In T1-treated orchards, MeSA likely served as a reliable cue for finding prey nearby target trees, as shown in the wind-tunnel experiment (Figure 5). The subsequent faster decrease in ladybird densities in T1 compared with T2 might have been due to food resource shortage inside the treated block, partly because of the faster decrease in aphid population densities, and partly because of the absence of plant resources from companion plants. On the long-term aphid populations stabilized below 100 aphids per branch in both T1 and T2 compared with 500 aphids per branch in control (Figure 2), showing the consistent long-term regulation of aphid populations in *attract-and-reward* strategies with a spatial separation of MeSA dispensers and companion plants. This emphasizes the potential of such strategies. In comparison our preliminary experiments with a clustered spatial arrangement of MeSA dispensers and companion plants did not improve aphid control (Figure S3). When we used MeSA alone or companion plants alone, aphid populations stabilized to only 300 and 400 aphids per branch, respectively, in the long term (Figure S2).

The T1 spatial arrangement is therefore a more promising bio-control strategy for several reasons. First, it attracted high densities of ladybirds in the short term, leading to a faster regulation of aphid populations and higher apple yields. Brewer et al. (2017) found that yield loss was increased when populations of the sugarcane aphid *Melanaphis sacchari* grew rapidly in sorghum fields, emphasizing the importance of pest short-term regulation of pests. Second, since ladybirds drawn to orchards by MeSA may have difficulty locating aphids, the companion plants around the treated block in T1 may have provided a "belt" of alternative resources until they find alternative prey in target trees. Third, ladybirds remained at lower densities in target trees in T1 compared with T2 in the long term. This outcome is especially important in the context of CBC for avoiding



**FIGURE 5** Proportion of ladybirds in the three chambers of the wind-tunnel through time. The bottom-right arrows indicates in which side the ladybirds were introduced. The MeSA dispenser and the companion plants were alternatively placed in connexion with the middle versus right chambers (a and b)

the negative impacts of attracting natural enemies after target pest populations are controlled. If predators remain in the treated area too long, they may destabilize the ecosystem by attacking alternative non-target prey (Louda et al., 2003; Ricciardi & Simberloff, 2009; Simberloff & Stiling, 1996). Stephens, France, Wratten, and Frampton (1998) demonstrated that sowing buckwheat in orchards as shelter plants increased the biodiversity of arthropods but not that of beneficial insects. Finally, T1 allowed a treatment by blocks at the scale of the orchard without reducing pest control inside blocks, as opposed to T2.

Both MeSA and *C. officinalis* scent attracted *P. japonica* in our olfactometer experiment, with MeSA being more attractive than *C. officinalis* (Figure 4). The HIPV MeSA has been shown to attract natural enemies of herbivorous arthropods, including predatory mites, hoverflies, lacewings, mirid bugs, ladybirds and parasitoid wasps, under laboratory and field conditions (Braasch, Wimp, & Kaplan, 2012; Gadino et al., 2012; Gençer, Kumral, Seidi, & Pehlevan, 2017; James, 2003; Rodríguez-Saona et al., 2011). The literature on the attractiveness of semiochemicals from *C. officinalis* to ladybirds or other natural enemies is limited, although semiochemicals from other plant species have been shown to attract ladybirds (Francis, Lognay, & Haubruge, 2004; Ninkovic & Pettersson, 2003; Qi, Jiang, Qin, Han, & Wang, 2008). Interestingly, olfactory cues from both sources provided together in the olfactometer synergistically attracted ladybirds (Figure 4), enhancing the potential of their combined use in an attract-and-reward strategy for *P. japonica*.

The synergistic attraction of MeSA and companion plants was also clear in the flight mill experiment: the flight capacities of adult

**TABLE 2** Effects of the presence of prey, the position of MeSA dispensers and companion plants, and time on the proportions of ladybirds in the three chambers of the wind-tunnel. GLMMs were used with a binomial distribution and the replicate number as random effect

Fixed effects	$\chi^2$	df	p-value
MeSA versus Prey+Plant			
Position:Time	0.078	1	0.78
Position:Prey	52.06	1	<0.001
Prey:Time	12.79	1	0.00035
Plant versus MeSA+Prey			
Position:Time	6.73	1	0.019
Position:Prey	0.14	1	0.71
Prey:Time	121.13	1	<0.001

Note. Best model's coefficients for MeSA versus Prey+Plant: Intercept: 2.39; Prey[Yes]: -0.77; Position[Plant\_middle]: 0.02; Time: -3.29 × Time; Prey[Yes]: Position[Plant\_middle]: 1.36; Prey[Yes]: Time: 0.84 × Time. Best model's coefficients for Plant versus MeSA + Prey: Intercept: -2.52; Prey[Yes]: 0.43; Position[Plant\_middle]: -0.65; Time: 3.19 × Time; Position[Plant\_middle]: Time: 0.72 × Time; Prey[Yes]: Time: -3.23 × Time.

ladybirds were enhanced, possibly through stimulation of their foraging behaviour (Figure 3). In comparison with parasitoid wasps or moths, the flight behaviour of ladybirds has been less investigated, with the exception of long distance movement, such as overwinter migration (Ricci, Ponti, & Pires, 2005; Wang et al., 2011). Prey semiochemicals and flower volatiles have been shown to trigger the long-range flight behaviour of predatory arthropods (Rouyar et al.,

2015; Salom & McLean, 1991). In the field, the stimulation of ladybirds' foraging behaviour by multiple environmental cues may increase their dispersal capacity, and thus attract them from further away or enhance their flying foraging behaviour. Ladybirds' flight capacities were further increased when both MeSA and companion plants were positioned separately in the flight mill. This may have *in situ* applications: if ladybirds spend more time flying in response to spatial cues, they may have a greater probability of finding aphid populations in the field. Note that in the flight mill and in orchards, ladybirds may also have been attracted and stimulated by visual cues from companion plants; yellow flowers have been demonstrated to be particularly attractive to insects (Gumbert, 2000; Lunau & Wacht, 1994).

The wind-tunnel experiment provided insights on the contextual, short-distance foraging behaviour of *P. japonica* ladybirds. Although used in other ladybird species (Leroy et al., 2012), this design with three contiguous chambers and ladybirds being introduced at one end of the tunnel has some limitations regarding the statistical analysis because not all permutations of the three chambers were tested. However, the spatial separation versus the proximity of environmental cues (MeSA, companion plant and prey) affected ladybirds' foraging behaviour, and a symmetrical design with ladybirds introduced at equal distances from all tested cues would not have captured this result (Dötterl, Jürgens, Wolfe, & Biere, 2009; Du, Poppy, & Powell, 1996).

MeSA was the most attractive cue in the wind-tunnel, but ladybirds most likely used it as a means to find prey because they later moved to the prey chamber or the plant chamber if no prey was present (Figure. 5) They therefore adjusted their foraging behaviour to the presence or absence of prey. We also showed that ladybirds used MeSA as a precise cue for the spatial location of prey: they found prey faster when the MeSA chamber was close to the prey chamber, and otherwise kept searching for prey close to MeSA dispensers. This finding is in agreement with the literature on HIPVs, used by herbivores' natural enemies to find plants hosting prey (Braasch et al., 2012; Gadino et al., 2012; Gençer et al., 2017; Ode, 2006; Turlings & Erb, 2018). Aphids were the preferred food resource over floral resources, likely because of their higher nutritional value for ladybirds (Lundgren, 2009). Because aphids were not visible to ladybirds in the wind-tunnel, the specific foraging behaviour elicited by their presence was likely mediated by semiochemicals, such as the alarm pheromone (E)- $\beta$ -farnesene. This semiochemical is produced by aphids in response to a danger, such as predators, and attracts predatory ladybirds into crop fields (Al Abassi et al., 2000; Francis et al., 2004); however, it may not always be a short-distance attractant for ladybirds (Joachim, Vosteen, & Weisser, 2015). Compared with arthropods' semiochemicals that are mostly species-specific, more generalist HIPVs may result in a more sustained attraction of natural enemies and hence enhance pest management (Kaplan, 2012; Rodriguez-Saona & Stelinski, 2009).

In our study, we did not quantify the impact of MeSA dispensers and companion plants application within orchards on non-targeted arthropods. Additional food resources from companion plants may also enhance pest populations (Parolin et al., 2012; Wäckers et al.,

2007). Although *A. citricola* is among the most abundant pest in apple orchards in north China (Yin et al., 2013) and was efficiently regulated in our study, other minor pests in apple orchards may be differentially affected by the treatment, and even benefit from reduced competition by *A. citricola* in the long term. Outbreaks of non-target pests due to the regulation of major pests have been shown in other systems (Lu et al., 2012). In addition, the presence of synthetic HIPVs and companion plants may affect pollinators (Kearns & Inouye, 1997) and non-entomophagous arthropod predators (Tabata, De Moraes, & Mescher, 2011) but also non-targeted natural enemies (Orre et al., 2010; Orre-Gordon et al., 2013). Therefore it will be important to measure to what extent the application of synthetic HIPVs and companion plants in apple orchards affect the arthropod community in an integrated pest management perspective.

Another aspect which will deserve further attention is the role of visual cues from *C. officinalis* as well as the number and concentration of MeSA dispensers, since it may affect the efficacy of different spatial arrangements in attracting targeted natural enemies (Kaplan & Lewis, 2015; Van Wijk et al., 2008). In our orchard experiment, the concentration of synthetic MeSA may have varied locally with microclimatic conditions. We minimized these differences as much as possible by placing them into partially buried boxes covered with a fine mesh to allow volatile diffusion, and similarly exposed to sun across orchards. MeSA concentration may have also varied temporally according with meteorological conditions, and this may have affected insects' population dynamics. However, the temporal variations were likely equivalent across orchards because they were in the same geographic area.

Conservation biological control, by managing ecosystem services, includes the consideration of functional habitat, the temporary application of specific companion plants and synthetic HIPVs to enhance the control of target pests and the overall biodiversity in agricultural systems (Gurr et al., 2017). Enhancing biodiversity to promote biocontrol services may be more complex in orchards than in open, homogenous crop fields (Gurr et al., 2015), and must be carefully investigated. Our study reports a significant improvement of the practical implementation of an *attract-and-reward* strategy combining synthetic MeSA and *C. officinalis* companion plants in apple orchards to suppress aphid populations. We showed the importance of the spatial arrangement of MeSA dispensers and companion plants for improved pest management. Future work is necessary to better disentangle the link between the spatial arrangement of synthetic HIPVs and rewards and insects' population dynamics in both organic and conventional orchards to understand the long-term consequences of this strategy on the local ecosystem. Still our study on the implementation of an *attract-and-reward* strategy provides useful information for optimizing integrated pest management on apple orchards.

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## AUTHORS' CONTRIBUTIONS

N.D. and S.W. designed the study; D.X., F.Z. and S.W. conducted the experiments; C.C.J., Q.X., X.G. and R.R. analysed the data; and C.C.J., D.X., Q.X., S.W. and N.D. wrote the manuscript. All authors approved the final version of this manuscript for publication.

## DATA ACCESSIBILITY

Data available via the Dryad Digital Repository <https://doi.org/10.5061/dryad.dt1h407> (Jaworski et al., 2019).

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## SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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