Olfactory behavior of convergent lady beetles (Coleoptera: Coccinellidae) to alarm pheromone of green peach aphid (Hemiptera: Aphididae)

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Abstract—A previous investigation from our laboratory showed that the odor of live green peach aphids, *Myzus persicae* (Sulzer), highly attracts the convergent lady beetle, *Hippodamia convergens* Guérin-Méneville. In this study, we isolated the odor and identified it as (*E*)-β-farnesene (an aphid alarm pheromone) using gas chromatography – mass spectrometry. The olfactory response of the convergent lady beetle to (*E*)-β-farnesene was investigated using an eight-arm air-flow olfactometer and tracing the pathways of the lady beetles. The results clearly indicate that *H. convergens* can perceive and orient to (*E*)-β-farnesene released by green peach aphids. These findings suggest that this species of lady beetle has evolved a sensory system for detecting the green peach aphid alarm pheromone as a means of finding its prey.

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Résumé—Une recherche antérieure dans notre laboratoire avait démontré que l'odeur de Pucerons verts du pêcher, *Myzus persicae* (Sulzer), vivants attire fortement la Coccinelle convergente, *Hippodamia convergens* Guérin-Méneville. Nous avons isolé l'odeur et l'avons identifiée par chromatographie en phase gazeuse et spectrométrie de masse. Il s'agit de l'(*E*)-β-farnésène (phéromone d'alerte des pucerons). La réponse olfactive de la Coccinelle convergente a été évaluée au moyen d'un olfactomètre à air, à huit bras, et par l'étude des parcours empruntés par les coccinelles. Les résultats montrent clairement que la coccinelle est capable de percevoir la phéromone émise par les pucerons et de s'orienter en conséquence. Il semble donc que cette espèce de coccinelle ait développé un système sensoriel propre à détecter la phéromone d'alerte du Puceron vert du pêcher et ainsi de repérer ses proies.

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Introduction

The biological control of insects with predators and parasitoids became a crucial factor in pest management after the 1890 introduction of the Australian lady beetle,

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Rodolia cardinalis (Mulsant) (Coleoptera: Coccinellidae), into California to destroy the cottony cushion scale, *Icerya purchasi* (Maskell) (Hemiptera: Margarodidae) (Metcalf and Metcalf 1993; Dixon 1998). Since then, lady beetles continue to be one of the key natural enemies that controls aphid, mite, and coccid damage to commercially important plants (Dixon 1998; Hardie and Minks 1999).

Coccinellidae respond to various cues when they seek food, shelter, or oviposition sites (Udayagiri *et al.* 1997). It has been proposed that lady beetles find their prey using visual stimuli (Nakamuta 1984; Colett 1988; Ferran and Dixon 1993) and that adults and larvae of lady beetles can perceive color contrasts (Khalil *et al.* 1985). Nakamuta (1985) proposed that coccinellids can visually identify their prey by their size and shape. Other studies suggest that lady beetles use olfactory stimuli to find their prey (Stubbs 1980; Obata 1986; Liu and Şengonca 1994; Şengonca and Liu 1994). Obata (1986) and Heidari and Copland (1992) showed that *Harmonia axyridis* (Pallas) (Coleoptera: Coccinellidae) and *Cryptolaemus montrouzieri* Mulsant (Coleoptera: Coccinellidae) can detect prey by odor. The convergent lady beetle, *Hippodamia convergens* Guérin-Méneville (Coleoptera: Coccinellidae), uses sensillae at the tip of the antennae to detect olfactory stimuli released by aphids and their host plant (Hamilton *et al.* 1999).

Some volatile and nonvolatile chemicals in plants and herbivores may also affect plant herbivore or natural enemy – prey interactions. For example, aphids developed a unique system to protect their colonies from natural enemies by secreting the alarm pheromone (*E*)-β-farnesene from their cornicles (Bowers *et al.* 1972; Edwards *et al.* 1973; Bowers *et al.* 1977; Rice *et al.* 1983). Aphids in the colony detect the pheromone through antennae and respond by dispersing rapidly (Nault *et al.* 1976; Phelan *et al.* 1976). Nault *et al.* (1976) observed that aphids do not disperse as rapidly when ants tend the colony and that the colony depends on ants for protection.

Previous olfactometer studies provided evidence that lady beetles perceive prey-related odors (Hamilton *et al.* 1999). Thus, one of our objectives was to isolate and positively identify the odor emitted by the green peach aphid, *Myzus persicae* (Sulzer) (Hemiptera: Aphididae), using gas chromatography – mass spectrometry (GC–MS) analysis. In addition, we conducted behavioral tests with an eight-arm air-flow olfactometer and ran a tracing experiment to determine whether lady beetles were attracted to alarm pheromone.

Materials and methods

Insects

Field-collected lady beetles (*H. convergens*) were purchased from Rincon-Vitova Insectaries, Inc (Ventura, California). They were maintained according to the procedure explained by Hamilton *et al.* (1999). Green peach aphids were reared on Scarlet White Tip radishes, *Raphanus sativus* L. (Brassicaceae) (The Chas Lily Co, Portland, Oregon), growing in a green house at 23°C and 70% RH.

GC and GC–MS analysis

Small quantities of pheromones are best analyzed with GC–MS (Pickett 1989). In a typical experiment, about 1000 green peach aphids were placed in a vial and covered with 1 mL of hexane. Two vials of aphids were prepared: one with winged and the other with wingless aphids. The vials were left in a freezer for 2 h; 1 µL of extract was analyzed using a Hewlett-Packard 5890 GC (Hewlett-Packard, Little Falls, Pennsylvania) equipped with a flame ionization detector (FID) and a PTE-5 column

[30 m × 250 μm i.d., 0.25 μm d_f (stationary phase film thickness); Supelco, Bellefonte, Pennsylvania]. The temperature program was 40°C for 4 min, then 4°C/min to 280°C. Helium was used as the carrier gas, and the injector and detector ports were both maintained at 280°C. Quantitation of the amount of (*E*)-β-farnesene extracted from the aphids was carried out for 30 min, using the external-standard method. To prepare a calibration curve, three different concentrations of (*E*)-β-farnesene dissolved in CH_2Cl_2 were analyzed by gas chromatography with FID detection. The correlation coefficient of the calibration line was $r^2 = 0.9996$, showing a good linear detector response over the concentration range used for quantitation.

For the GC–MS analysis, a 0.5-μL sample was injected into a Hewlett-Packard 5890 series II gas chromatograph (Little Falls, Pennsylvania) and, after separation, the sample was introduced into the mass spectrometer (mass scan range 50–100 *m/z*). The instrument was a Jeol JMS-SX102A (Jeol, Peabody, Massachusetts) double-focusing reverse-geometry magnetic-sector mass spectrometer in the standard electron impact mode (70 eV). (*E*)-β-Farnesene was positively differentiated from α-farnesene by analyzing standards (from Bedoukian, Inc, Danbury, Connecticut) with a grade of purity greater than 90%. The grade of purity of the standards was high enough (a minimum of 90%) to allow us to obtain a mass spectrum for comparison and identification purposes.

Olfactometer experiments

Olfactometer experiments were conducted to determine whether the lady beetles could detect various odor stimuli. An eight-arm air-flow olfactometer based on the design of Liu and Şengonca (1994) was used. The olfactometer design, as explained in detail in Hamilton *et al.* (1999), consisted of an exposure chamber, eight arms extending radially at 45°, and an acrylic lid. A sample chamber that contained lady beetles prior to the experiment was in the center of the exposure chamber. An aluminum piston that would introduce the beetles into the exposure chamber at the beginning of each experiment was attached to the sample chamber. A vacuum pump and an oxygen flow meter that drew air through the olfactometer provided the air outlet. Beetles to be used in the experiment were randomly selected from mature adults, regardless of gender. Six active convergent lady beetles that had been starved for at least 24 h were placed in the sample chamber. The odor source was placed in the designated odor chamber, defined as the active chamber, and the active chamber attached to the randomly selected arm, defined as the active arm. Seven empty chambers were attached to the remaining arms, defined as nonactive arms. After the acrylic lid was placed on the olfactometer, the piston was pushed up to release the beetles in the center of the exposure chamber. Beetles moved freely in the exposure chamber, sampled air from the various arms, and entered any of them. During the experiment, the number of entrances of beetles into each arm was recorded. Each beetle was used only once to eliminate a possible learning bias. Experiments consisted of five treatments, and each treatment had 16 runs that lasted 45 min ($n = 96$ per treatment) (Table 1). For every run, the active arm was selected randomly and each active arm was used twice.

The treatments consisted of a 1-μL droplet of (*i*) neat (*E*)-β-farnesene, (*ii*) water, or (*iii*) hexane at the center of a filter paper placed in the active arm, (*iv*) 25–30 live green peach aphids, or (*v*) an untreated clean filter paper put in the assigned active chamber. For every run of the olfactometer, lady beetles were exposed simultaneously to air plumes from one active chamber (one of the five treatments) and seven nonactive chambers (air alone).

An approximate amount of alarm pheromone to be used in the olfactometer and tracing experiments was determined *via* quantification of the amount of alarm pheromone released per aphid. It was observed that small aphid clusters on radishes

TABLE 1. Response of *Hippodamia convergens* exposed to five treatments using an eight-arm olfactometer (n = 96).

Treatment	Mean ± SD no. of lady beetle entrances into active arms[†]	95% CI for mean no. of entrances into active arms		Mean ± SD no. of lady beetle entrances into nonactive arms[‡]	95% CI for mean no. of entrances into nonactive arms	
		Lower limit	Upper limit		Lower limit	Upper limit
Live aphids (about 25 aphids)	21.75±1.50[§]	18.54	24.95	12.84±0.81	11.12	14.57
(*E*)-β-Farnesene (1 μL) on filter paper	14.62±1.18[§]	12.11	17.14	7.58±0.78	5.90	9.25
Water (1 μL) on filter paper	7.75±1.37	5.32	10.54	9.11±1.08	6.79	11.41
Hexane (1 μL) on filter paper	7.62±1.15	4.70	10.19	8.25±1.27	5.50	10.96
Untreated filter paper	5.87±0.78	4.19	7.55	5.72±0.75	4.11	7.33

[†] Active arm refers to the olfactometer arm in which the stimulus was placed.

[‡] Nonactive arm refers to the olfactometer arms that were left empty during each treatment.

§ Mixed model multivariate test ($P < 0.005$).

consisted of approximately 25–40 individuals, and the amount of (*E*)-β-farnesene used in the behavior experiments was optimized accordingly. The approximate amount of (*E*)-β-farnesene that 30 aphids would excrete was calculated as 1 μL, using

$$V = m/d$$

where V is the volume of (*E*)-β-farnesene excreted by 30 aphids, m is the total weight of the (*E*)-β-farnesene extracted from 30 aphids, and d is the density of (*E*)-β-farnesene, *i.e.*, 0.86 g/cm^3.

All experiments were conducted in a climate controlled growth chamber at 23°C and 70% RH and between 09:30 and 17:30, the period during which lady beetles were observed to be most active.

Tracing experiments

Tracing experiments consisted of recording the search pattern of lady beetles and the amount of time spent to come into contact with an odor source. One 9-cm glass Petri dish was placed on a flat surface. A round filter paper (9-cm diameter) was placed in the Petri dish and the stimulus placed at the center of the filter paper. If a live aphid was used as a stimulus, the aphid was fixed in place with a tiny amount of Vaseline® to prevent its movement. For stimuli such as (*E*)-β-farnesene, water, or hexane, a 1-μL droplet was placed at the center of the filter paper. Once the stimulus was in place, one randomly selected adult lady beetle that had been starved for 24 h was released into the Petri dish from the right-hand side at the perimeter of the dish. The dish was covered immediately and transparent paper placed over the top. The path that the lady beetle followed until it found the stimulus or until 5 min had elapsed was traced on the transparent paper. The amount of time lady beetles spent to come into contact with an odor source was recorded. Each beetle was used only once, to eliminate a possible learning bias. The complete experiment consisted of six treatments: a 1-μL droplet of (*E*)-β-farnesene, water, or hexane or one live, frozen, or crushed aphid placed in the center of a filter paper as a stimulus (Table 2). Each treatment was replicated 100 times.

Statistical analysis

The results of GC and GC–MS analyses demonstrated that the compound extracted from green peach aphids was (*E*)-β-farnesene. A simple linear regression was used to statistically analyze the quantification of peak area against different concentrations of (*E*)-β-farnesene.

The entrance data from the olfactometer study was analyzed using a mixed-model multivariate analysis, with runs as whole units and odor chambers as subunits (SPSS 1997). The number of entrances into each nonactive arm was pooled together after equalizing the variances by logarithmic transformation. A mixed-model multivariate analysis was used to test null hypotheses about the effect of various odor sources on the mean value of several correlated dependent variables.

Differences among treatments for the tracing experiments were tested with ANOVA. Tukey's multiple comparison test was used to compare means of the time spent to find each stimulus: treatment with (*E*)-β-farnesene was paired with live-aphid and hexane treatments, as well as with water and crushed- and frozen-aphid treatments. The live-aphid treatment was also paired with crushed- and frozen-aphid treatments (SPSS 1997). As 1 μL of (*E*)-β-farnesene corresponded to 25–30 aphids, by comparing 1 μL of alarm pheromone to one individual aphid it was possible to measure the time spent to find one colony *versus* one individual.

TABLE 2. Proportions of *Hippodamia convergens* that located a stimulus and mean searching times.

Treatment	Proportion of beetles that found stimulus (%)[†]	Mean ± SE time to find stimulus (min)
One live aphid	66	02:21.80±0:00.09
One frozen aphid	51	02:22.01±0:00.11
One crushed aphid	76	02:16.41±0:00.09
(*E*)-β-Farnesene (1 μL)	87	01:49.93±0:00.09[‡]
Water (1 μL)	45	02:18.10±0:00.08
Hexane (1 μL)	0	na

[†] For each treatment, $n = 100$.

[‡] Time spent to locate (*E*)-β-farnesene was significantly different from time spent locating other stimuli (Tukey's multiple comparison test, $P < 0.05$).

Results and discussion

The results of GC and GC–MS analyses positively identified the compound extracted from aphids as (*E*)-β-farnesene, and the correlation coefficient of the calibration line showed a good linear detector response over the concentration range used for quantification ($y = 0.0043x + 6.504$, $r^2 = 0.9996$, $F_{1,3} = 8330$, $P < 0.0005$). The amount of (*E*)-β-farnesene extracted from each aphid was determined by GC and GC–MS to be 19 ng for winged aphids and 28.9 ng for wingless aphids. These values are similar to those reported for honeysuckle aphids, *Hyadaphis tataricae* (Aizenberg) (Hemiptera: Aphididae) (Hedin *et al.* 1991).

The olfactometer experiments were conducted to determine whether lady beetles were attracted *via* olfaction when exposed to (*E*)-β-farnesene and various other odor sources. The differences among treatments were significant (Wilk's lambda, $F_{28,600} = 1.969$, $P = 0.005$) (SPSS 1997). Lady beetles responded to live aphids ($F_{4,600} = 0.842$, $P < 0.01$) and (*E*)-β-farnesene ($F_{4,600} = 3.342$, $P = 0.02$) but not to water ($F_{4,600} = 0.788$, $P > 0.5$), hexane ($F_{4,600} = 1.85$, $P > 0.2$), or untreated filter paper ($F_{4,600} = 1.092$ $P > 0.5$) (Table 1).

The tracing experiments revealed that no specific pattern was followed by the lady beetles in finding the stimulus. The beetles moved along the periphery of the experimental arena before "prey capture" (Fig. 1). Nakamuta (1982) also noted that *Coccinella septempunctata* L. (Coleoptera: Coccinellidae) moved similarly when searching. Vibration of the hand during tracing did not appear to interfere with the lady beetles' movements. When search times were compared, it was found that lady beetles favored filter paper treated with (*E*)-β-farnesene over live, crushed, and frozen aphids ($F_{5,594} = 53.83$, $P < 0.001$) (SPSS 1997) (Table 2). It was observed that, once lady beetles oriented towards (*E*)-β-farnesene, they immediately walked towards the stimulus; however, when they touched the center where the stimulus was placed, they moved slightly away from it. There was no difference in search time among live-, crushed-, or frozen-aphid treatments (Table 2). There was also no difference among the three aphid treatments *versus* water as a stimulus (Table 2). It was also observed that the average time spent to find water was similar to the time spent to locate live aphids (Table 2). In nature, lady beetles drink water droplets on the leaves. When placed on the filter paper, hexane evaporates within a few seconds, allowing us to test the effect of hexane initially and of a blank control later. None of the lady beetles responded to hexane-treated filter paper or to the filter paper after the hexane evaporated (Table 2).

The results of both the olfactometer and tracing experiments clearly suggest that convergent lady beetles use the aphid alarm pheromone to find their prey. The results of

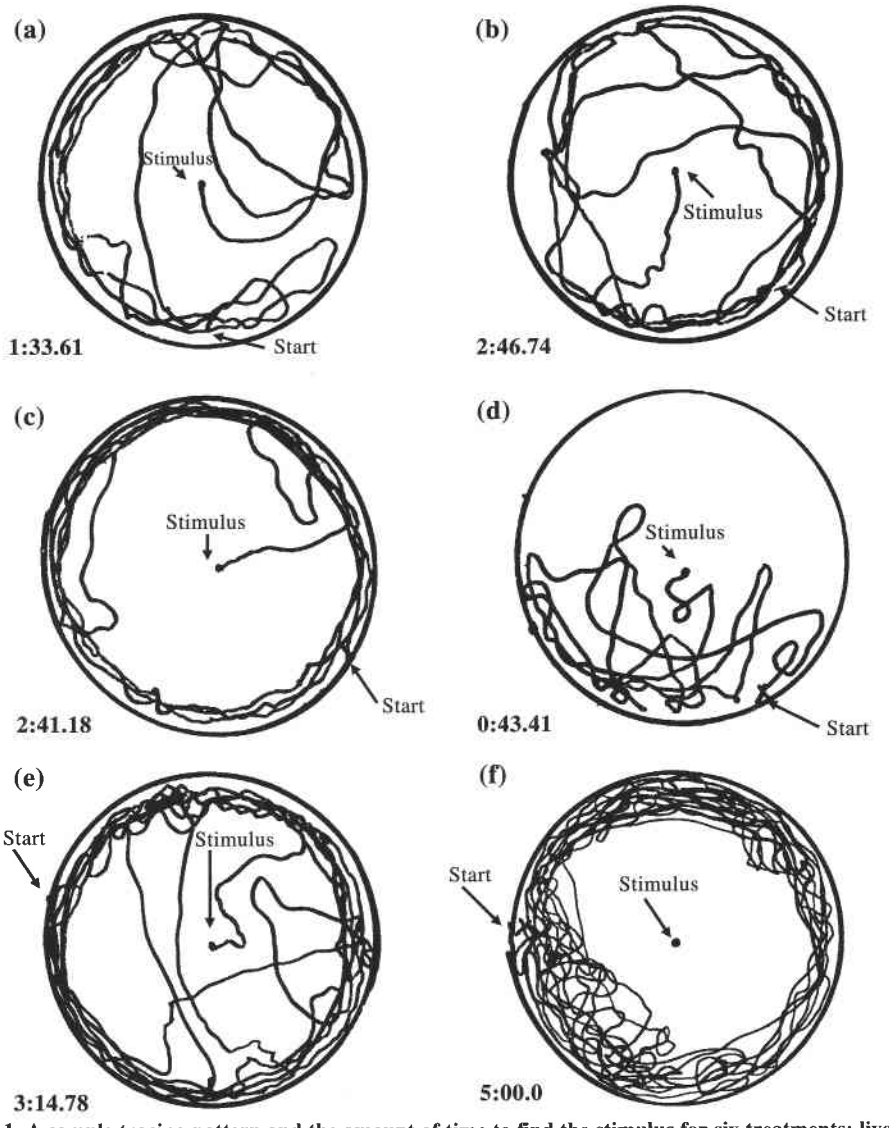

FIGURE 1. A sample tracing pattern and the amount of time to find the stimulus for six treatments: live *Myzus persicae* (*a*), frozen *M. persicae* (*b*), crushed *M. persicae* (*c*), 1 μL (*E*)-β-farnesene (*d*), 1 μL water (*e*), or 1 μL hexane (*f*).

this study differ from the findings of Nakamuta (1991), who suggested that *C. septempunctata* were not attracted to synthetic (*E*)-β-farnesene, and in addition, do not support Nakamuta's (1991) proposal that lady beetles use visual not olfactory cues to find their prey. In a preliminary (unpublished) study, no difference was found between blind (acrylic paint over the eyes to induce temporary blindness) and untreated lady beetles in the time spent to find stimuli (on average, 2:18.8 and 2:21.8 min, respectively; $t_{0.05(2),198} = 0.223$, $P = 0.082$). In addition, Hamilton *et al.* (1999) showed that convergent lady beetles use olfactory sensillae at the tip of their antennae to locate prey.

Detailed analyses of chemical components of the prey, which stimulate changes in movement of coccinellids, are still unknown. Most coccinellids feed on a large number of aphid species and, thus, chemicals common to many aphid species might stimulate these changes in prey-seeking behavior. For example, Carter and Dixon (1984) showed that the presence of aphid honeydew increased the time spent by *C. septempunctata* in searching for prey on plants, suggesting that chemicals excreted by aphids could stimulate intensive search behavior.

It has been observed that aphids exposed to alarm pheromone leave the proximity or drop from the leaves (Edwards *et al.* 1973; Nault *et al.* 1976; Phelan *et al.* 1976). Nakamuta (1991) suggested that spending too much time in areas contaminated with the alarm pheromone would not be advantageous to coccinellids. Since the pheromone causes aphids to disperse from such areas, the author suggested that coccinellids should move away to increase their chances of prey finding. Calabrese and Sorensen (1978) reported that, after exposure to alarm pheromone, usually one or two of the three aphids that left the plant by dropping from the leaf returned to the base of the stem, with the remaining aphids often moving away from the plant. Most of the aphids that moved a short distance from the plant stem returned to the plant within 1 h. Together with lady beetles' attraction to (*E*)-β-farnesene, this aphid behavior might be advantageous for convergent lady beetles, increasing their search and chances of finding prey.

In summary, these data clearly show that convergent lady beetles can detect (*E*)-β-farnesene. The beetles apparently use this aphid alarm pheromone as an olfactory method of locating its prey. This is one of the first instances in invertebrate predator–prey studies in which the predator used a prey alarm pheromone to aid in detection and capture of its food. Further physiological and behavioral studies with different species of lady beetles and using lower concentrations of (*E*)-β-farnesene are needed to determine how widespread this behavior is among coccinellids.

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