

## Orientation of *Hippodamia variegata* (Coleoptera: Coccinellidae) to healthy and *Beauveria bassiana*-infected *Aphis fabae* (Hemiptera: Aphididae) in an olfactometer system

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Received: 20.10.2013 • Accepted: 08.05.2014 • Published Online: 02.01.2015 • Printed: 30.01.2015

**Abstract:** Determination of attractive and avoidance behavior of *Hippodamia variegata* (Goeze) (Coleoptera: Coccinellidae) is very important in the concomitant use of *Beauveria bassiana* for control of *Aphis fabae* (Hemiptera: Aphididae). We investigated the olfactory response of the predatory insect *H. variegata* when receiving odors pertaining to *Aphis fabae* infected by *B. bassiana* EUT116 at 0-, 24-, 48-, and 72-h intervals in a Y-tube olfactometer set-up. Behavioral assays were conducted to assess the ability of adult *H. variegata* to avoid *A. fabae* infected with *B. bassiana* conidia on plants. In these experiments, *H. variegata* exhibited significant attraction towards the control when the predator alternative option was *B. bassiana* with 24-h intervals ( $P = 0.04$ ) and 48- and 72-h intervals ( $P < 0.01$  in both cases). We suggest that under laboratory conditions *H. variegata* could detect and avoid *B. bassiana* conidia; thus, *B. bassiana* would be a negligible threat to this predator.

**Key words:** Olfactory responses, *Beauveria bassiana*, *Aphis fabae*, Aphididae, *Hippodamia variegata*, Coccinellidae

### 1. Introduction

Predators encounter a diverse array of natural enemies that include other predators, parasitoids, parasites, and pathogens (Mburu et al., 2009; Alma et al., 2010). Recognition and response to such dangers is an important trait for the survival of any species (Mburu et al., 2009). Most studies on interactions between arthropod natural enemies and entomopathogenic fungi have involved parasitoids, while intraguild interactions involving entomopathogenic fungi and predators have not been extensively investigated (e.g., Rosenheim et al., 1995; Roy and Pell, 2000; Jaronski et al., 2003; Seiedy et al., 2012a, 2012b). Entomopathogenic fungi have negative effects on natural enemies after direct exposure (Roy et al., 2008). Interaction between aphid enemies may have positive, neutral, or negative effects on pest control (Rosenheim et al., 1995; Straub et al., 2008). It is difficult to predict the outcome of intraguild interactions on pest suppression (Baverstock et al., 2009). When 2 or more biological control agents are used simultaneously, intraguild interactions may limit their impact on the target (Rosenheim et al., 1995; Polis et al., 1989).

Detection and avoidance of pathogens are important for predator survival, longevity, and ultimately fitness (Simelane et al., 2008; Seiedy et al., 2012a).

*Beauveria bassiana* (Balsamo) Vuillemin is a generalist entomopathogen that has been found infecting many

arthropod species (Baverstock et al., 2006). The variegated lady beetle, *Hippodamia variegata* (Goeze) (Coleoptera: Coccinellidae), has been reported as an important natural enemy to at least 12 different aphid species, including the cotton aphid and the pea aphid (Fan and Zhao, 1988; Franzmann, 2002; Kontodimas and Stathas, 2005).

The entomopathogenic fungus *B. bassiana* and the predator *H. variegata* are both potential biological control agents for *A. fabae*. There is one critical question regarding the simultaneous use of *B. bassiana* and *H. variegata*: can the predatory insect identify the presence of the entomopathogen fungus without coming into physical contact with it?

### 2. Materials and methods

#### 2.1. Rearing and preparation of plants and predator

Broad bean plants (*Faba vulgaris* Moench) were used in this study. The broad bean plants were planted in pots filled with sawdust and fertilized with HortiGrow (Hortiland, the Netherlands), a quality fertilizer to which microelements (N: K: P: Mg = 20: 20: 20: 2 percent; 1–2 kg per 1000 L water) were added twice per week.

Plants were approximately 20 days old when used for experiments. Aphids were reared on the broad bean plants at  $21 \pm 1$  °C and  $70 \pm 10\%$  relative humidity (RH), with

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a photoperiod of 16:8 h (L:D). Lady beetles, *H. variegata*, were collected by sweep-net from an alfalfa field in the Karaj region of Alborz Province, Iran. The stock colony of *H. variegata* was propagated on *A. fabae* and maintained in a room with a controlled environment ( $25 \pm 1$  °C,  $70 \pm 10\%$  RH, 16 L:8 D).

## 2.2. Preparation of fungus conidia

*Beauveria bassiana* isolate EUT116 has been observed to cause nonsignificant mortalities to *H. variegata* (mortality of this isolate was 24.37% with discriminatory single concentration [ $1 \times 10^5$  conidia/mL], which produced  $LC_{50}$  for *A. fabae*) (Seiedy, unpublished data). Conidia were cultured on Sabouraud dextrose agar plus 1% yeast extract (SDAY) at  $25 \pm 1$  °C and 60%–70% RH, for 10–15 days in darkness. Cultures were scrapped after sporulation and conidia were obtained (Seiedy et al., 2012a, 2012b). Dry conidia were suspended in 0.02% Tween 80 in water and diluted to  $1 \times 10^5$  conidia/mL. The conidial suspension used for the experiment was less than 4 h old and was refrigerated. The viability of the conidia and germination rates were obtained according to Rashki et al. (2009).

## 2.3. Y-tube olfactometer set-up and odor sources and experiments

The olfactometer consisted of a Y-shaped Pyrex tube (diameter: 4 cm) with an entry arm (13 cm in length) and 2 side arms (13 cm in length) (Sabelis and van de Baan, 1983), which was used to test the response of *H. variegata* to broad bean plants infested by 0.02% Tween 80-treated *A. fabae* and *A. fabae* infected with *B. bassiana* EUT116 at 4 time intervals: 0, 24, 48, and 72 h after infection. The air passed through activated charcoal before reaching the cylinders. Air was blown through both arms at 5 m/s by a small electrical motor. Plexiglas boxes ( $50 \times 50 \times 50$  cm) containing the plants with *A. fabae* infected with *B. bassiana* for 0, 24, 48, and 72 h were connected to the end of each arm. Same-aged female predators were starved for 24 h by keeping them in separate petri dishes (9 cm in diameter). Subsequently, these females were individually placed at the basal end of the Y-tube to initiate upwind movement. The predator was observed until it passed the junction and moved into one of the arms of the Y-tube. Predators that did not choose a side arm within 5 min were recorded as “no choice”. For each treatment, tests were performed in 5 independent replicates; each replicate was done with 10 predators, and each with a new set of odor sources. To cancel out any unforeseen asymmetry in environmental factors (e.g., light, temperature), odor sources were swapped each time after 4 predators had been tested.

For experiments, the infested material was produced by infesting broad bean plants with 300 third-instar nymphs of *A. fabae* 3 days prior to the experiment; they were then sprayed with *B. bassiana* EUT116 with a

discriminatory single concentration ( $1 \times 10^5$  conidia/mL) at 4 time intervals (0, 24, 48, and 72 h) and were placed in the Plexiglas olfactometer box. Other broad bean plants were also infested by 300 third-instar nymphs of *A. fabae* 3 days prior to the experiment and then were sprayed with 0.02% Tween 80. During the 3-day period, the plants were maintained in a climate-controlled growth chamber in the aforementioned conditions. The fungal suspension of *B. bassiana* conidia/mL was sprayed once (1 mL per spray application) onto the aphids using a fine mist held above the aphids at a 90° angle (Groszek, Kwazar Corporation, Poland; <http://www.galactic-sprayers.com/KWAZAR/presentation.html>).

Three experiments were conducted to examine the response of *H. variegata* to treatments as follows:

Experiment 1: Response of *H. variegata* to broad bean plants infested with *A. fabae* infected with *B. bassiana* EUT116 at intervals of 0, 24, 48, and 72 h after infection ( $T_{0P}$ ,  $T_{24P}$ ,  $T_{48P}$ , and  $T_{72P}$ ) versus broad bean plants infested with 0.02% Tween 80-treated *A. fabae* ( $T_{CP}$ ) (P and C are abbreviations for plant and control). In the control treatment, *A. fabae* was treated only with Tween 80.

Experiment 2: Response of *H. variegata* to broad bean plants infested with *A. fabae* infected with *B. bassiana* EUT116 24 h after infection ( $T_{24P}$ ) versus broad bean plants infested with *A. fabae* infected with *B. bassiana* EUT116 at intervals of 48 and 72 h after infection ( $T_{48P}$  and  $T_{72P}$ ).

Experiment 3: Response of *H. variegata* to broad bean plants infested with *A. fabae* infected with *B. bassiana* EUT116 48 h after infection ( $T_{48P}$ ) versus broad bean plants infested with *A. fabae* infected with *B. bassiana* EUT116 72 h after infection ( $T_{72P}$ ).

## 2.4. Statistics

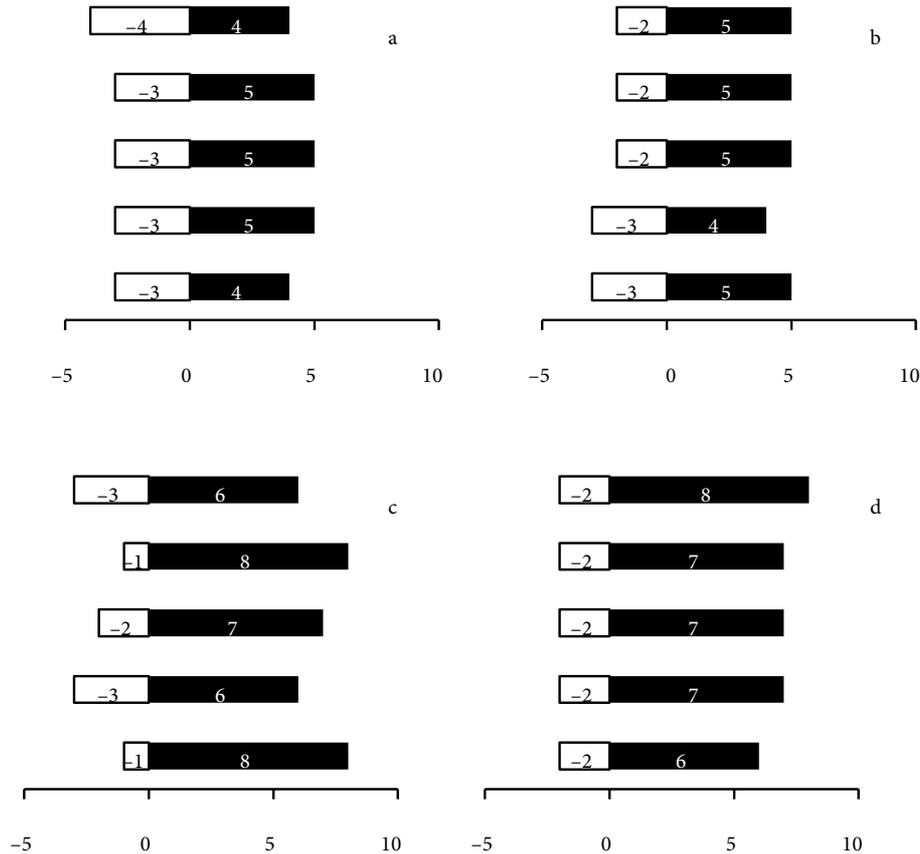
Data were analyzed with a replicated goodness-of-fit test (G statistic), which included a test for heterogeneity among replicate experiments (Sokal and Rohlf, 1995). Figures were drawn using Excel 2007.

## 3. Results

Orientation of *H. variegata* toward one of the arms between odors from broad bean plants infested with 0.02% Tween 80-treated *A. fabae* ( $T_{CP}$ ) versus broad bean plants infested with *A. fabae* infected with *B. bassiana* is shown in Figures 1a–1d.

In these experiments, predatory insects in the first hour after infection showed no significant preference for broad bean plants infested by 0.02% Tween 80-treated *A. fabae* ( $T_{CP}$ ) ( $G = 0.39$ ,  $df = 4$ ,  $P = 0.26$ ), while predatory insects 24 h after infection had a significant preference for broad bean plants infested by 0.02% Tween 80-treated *A. fabae* ( $T_{CP}$ ) ( $G = 0.55$ ,  $df = 4$ ,  $P = 0.04$ ).

Predatory insects 48 h ( $G = 2.66$ ,  $df = 4$ ,  $P = 0.0001$ ) after infection and 72 h ( $G = 0.06$ ,  $df = 4$ ,  $P = 0.0001$ ) after



**Figure 1.** Response of *Hippodamia variegata* to broad bean plants infested by *Aphis fabae* infected by *Beauveria bassiana* at intervals of 0 (a), 24 (b), 48 (c), and 72 (d) h after infection versus broad bean plants infested by *A. fabae* infected by 0.02% Tween 80. The white bars indicate the numbers of insects choosing broad bean plants infested by *A. fabae* infected by *B. bassiana*, whereas the black bars indicate the numbers of insects that chose broad bean plants infested by *A. fabae* infected by 0.02% Tween 80.

infection had a significant preference for broad bean plants infested by 0.02% Tween 80-treated *A. fabae* ( $T_{CP}$ ).

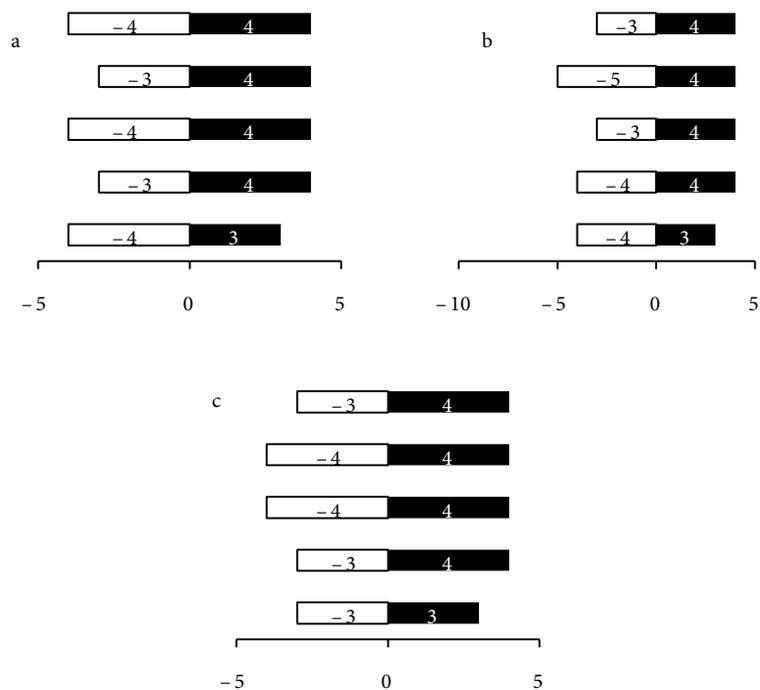
When *H. variegata* was offered a choice between odors from  $T_{OP}$  versus  $T_{24P}$  ( $G = 0.40$ ,  $df = 4$ ,  $P = 0.86$ ),  $T_{OP}$  versus  $T_{48P}$  ( $G = 0.54$ ,  $df = 4$ ,  $P = 1$ ),  $T_{OP}$  versus  $T_{72P}$  ( $G = 0.17$ ,  $df = 4$ ,  $P = 0.73$ ),  $T_{24P}$  versus  $T_{48P}$  ( $G = 0.17$ ,  $df = 4$ ,  $P = 0.61$ ),  $T_{24P}$  versus  $T_{72P}$  ( $G = 0.35$ ,  $df = 4$ ,  $P = 0.36$ ), and  $T_{48P}$  versus  $T_{72P}$  ( $G = 0.58$ ,  $df = 4$ ,  $P = 0.20$ ), predators did not exhibit a preference for either arm.

Orientation of *H. variegata* toward one of the arms upon the olfactory response of *H. variegata* between  $T_{OP}$  and  $T_{24P}$ ,  $T_{48P}$  and  $T_{72P}$ ;  $T_{24P}$  and  $T_{48P}$  and  $T_{72P}$ ; and  $T_{48P}$  and  $T_{72P}$  is shown in Figures 2a–2c, Figures 3a and 3b, and Figure 4, respectively.

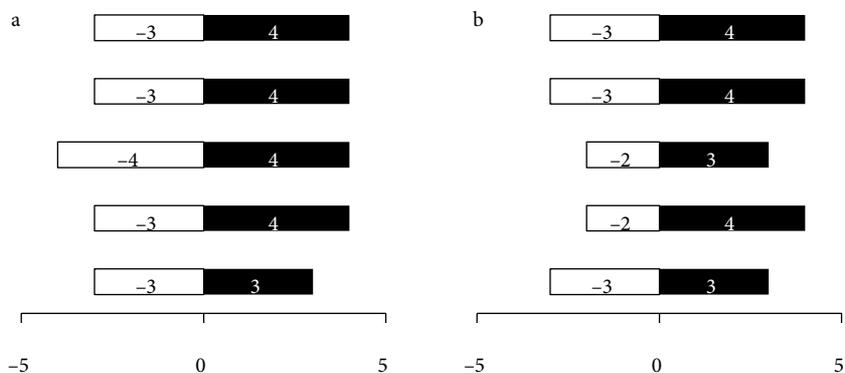
#### 4. Discussion

Our results indicate that the presence of *B. bassiana* affects the behavioral responses of *H. variegata* and affects their foraging behavior at intervals of 24, 48, and 72 h.

Nonpreference of *H. variegata* to *A. fabae* infected with *B. bassiana* EUT116 at intervals of 24 to 72 h after infection versus 0.02% Tween 80-treated *A. fabae* in the presence of broad bean plant will result in a reduced encounter between the predator insect and fungal cadavers; thus, it would reduce the impact of intraguild interactions between the predator and the fungus and allow for coexistence in laboratory conditions. There have also been several studies about the effect of fungi-mediated interactions on insect or mite behavior (Rath, 2000; Staples and Milner, 2000; Myles, 2002; Seiedy et al., 2013). Seiedy et al. (2013) showed that in plant-present experiments, *Phytoseiulus persimilis* was not attracted to control plants when tested against plants with spider mites infected with *B. bassiana* for 0 or 24 h, but it was attracted to control plants when tested against plants with spider mites infected with *B. bassiana* for 48 or 72 h. This suggests that the predator was able to detect the presence of *B. bassiana* in the 48- and 72-h treatments.



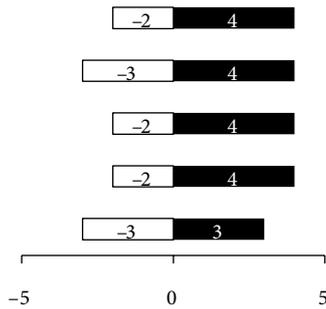
**Figure 2.** Response of *Hippodamia variegata* to broad bean plants infested by *A. fabae* infected by *Beauveria bassiana* at the interval of 0 h after infection versus broad bean plants infested by *A. fabae* infected by *B. bassiana* at intervals of 24 (a), 48 (b), and 72 (c) h after infection. The white bars indicate the numbers of insects choosing broad bean plants infested by *A. fabae* infected by *B. bassiana* at intervals of 24, 48, and 72 h after infection, whereas the black bars indicate the numbers of insects that chose broad bean plants infested by *A. fabae* infected by *B. bassiana* at the interval of 0 h after infection.



**Figure 3.** Response of *Hippodamia variegata* to broad bean plants infested by *A. fabae* infected by *Beauveria bassiana* at the interval of 24 h after infection versus broad bean plants infested by *A. fabae* infected by *B. bassiana* at intervals of 48 (a) and 72 (b) h after infection. The white bars indicate the numbers of insects choosing broad bean plants infested by *A. fabae* infected by *B. bassiana* at intervals 48 and 72 h after infection, whereas the black bars indicate the numbers of insects that chose broad bean plants infested by *A. fabae* infected by *B. bassiana* at the interval of 24 h after infection.

Villani et al. (1994) showed that the larvae of Japanese beetles, *Popilia japonica* Newman, avoided soil containing *Metarhizium anisopliae* for up to 20 days after applications.

Sun et al. (2008) found that organic mulches supplemented with *M. anisopliae* significantly repelled the foraging behavior of *Coptotermes formosanus* (Shiraki) and reduced



**Figure 4.** Response of *Hippodamia variegata* to broad bean plants infested by *A. fabae* infected by *Beauveria bassiana* at the interval of 48 h after infection versus broad bean plants infested by *A. fabae* infected by *B. bassiana* at the interval of 72 h after infection. The white bars indicate the numbers of insects choosing broad bean plants infested by *A. fabae* infected by *B. bassiana* at the interval of 72 h after infection, whereas the black bars indicate the numbers of insects that chose broad bean plants infested by *A. fabae* infected by *B. bassiana* at the interval of 48 h after infection.

mulch consumption by up to 71%. Furthermore, Mburu et al. (2009) showed that the termite *Macrotermes michaelseni* can detect and avoid *B. bassiana* and *M. anisopliae*.

There have also been several studies about the effects of fungi on the behavioral responses of predator beetles, which mirror our results. Ormond (2007, 2011) found that *Coccinella septempunctata* (L.) detected and avoided *B. bassiana* on leaves and in the soil; Pell and Vandenberg (2002) detected nonfeeding of the predator *H. convergens* Guerin-Meneville, 1842 on aphids treated with *Pacilomyces*

*fumosoroseus* (Wize, 1904); Meyling and Pell (2006) detected an intensive decrease in the feeding of the predator *Anthocoris nemorum* on aphids treated with *B. bassiana*; Pell et al. (1997) and Roy et al. (1998) revealed reduction in the feeding activity of *C. septempunctata* on *A. pisum* treated with *Erynia neoaphidis* Remaudiere & Hennebert, 1980.

Currently, it is only possible to speculate whether or not the herbivore-induced plant volatiles are the origin of the attractive compounds, but the ability of an insect to detect entomopathogenic fungi may not only be dependent on the species and isolate of the fungus, but also on the substrate on which the fungus is deposited (Baverstock et al., 2009). Therefore, it is possible to assume that the fungus or the infected prey could produce avoiding compounds. Baverstock et al. (2005) found that entomopathogenic fungi have a range of chemical volatiles, so it is possible to assume that the fungus produces some compounds that predatory insects can detect; as a result, the insect has avoidance behavior to  $T_{24P}$ ,  $T_{48P}$  and  $T_{72P}$ . An insect gains selective advantage if it is able to detect entomopathogenic fungi from a distance and respond via behavioral avoidance (Chouvenc et al., 2008). These findings improve our understanding of tritrophic interactions by revealing a novel association between *H. variegata* and *B. bassiana*. Although the avoidance behavior in predatory insects from the plant–fungus system is evident, it needs to be clarified which compounds are responsible for such avoidance.

#### Acknowledgment

The project was supported by a grant from the University of Tehran, which is greatly appreciated.

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