

# 博士学位論文

**Induction of oviposition behavior  
of *Aphidoletes aphidimyza* by olfactory cues**

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## I. General introduction

The survival and reproduction of aphidophagous predators depend on their ability to locate suitable hosts, an ability that often relies on specific mechanisms for interpreting chemical cues derived from host – host plant complex. For instance, females of the aphidophagous parasitoid species *Aphidius colemani* (Viereck) respond to host plant and herbivore odors (Kalule and Wright, 2004). Both larvae and adults of *Chrysoperla carnea* (Stephens) are attracted to honeydew (Duelli 1987), whereas *Coccinella septempunctata* L. (Al Abassi et al., 2000; Ninkovic et al., 2001), *Adalia bipunctata* L. (Hemptinne et al., 2000; Francis et al., 2004), and *Episyrphus balteatus* DeGeer larva (Francis et al., 2005) all respond to the aphid alarm pheromone (*E*)- $\beta$ -farnesen. In addition, honeydew produced by *Sitobion avenae* (F.) is a strong contact stimulant for oviposition in *E. balteatus* (Budenberg and Powell 1992).

*Aphidoletes aphidimyza* (Rondani) (Diptera: Cecidomyiidae) is a common and widely distributed species that occurs in Europe, North America, the Middle East, and Japan. The predaceous larvae of this species attack over 80 species of aphids, including common pests such as *Myzus persicae* (Sulzer) and *Aphis gossypii* Glover (Harris 1973; Yukawa et al. 1998). Female *A. aphidimyza* lay eggs singly or in clusters of up to 40, usually placing them on plants near aphid colonies (Uygun 1971; Harris 1973; Nijveldt 1988). Upon hatching, larvae immediately search for aphids on which to prey.

*Aphidoletes aphidimyza* is an effective natural enemy of aphids that is used to control pests infesting greenhouse crops (Meadow et al. 1985; Morse and Croft 1987; Nijveldt 1988). However, the release of *A. aphidimyza* to control aphids often does not provide adequate control, as aphids multiply very quickly on greenhouse vegetables such as cucumbers or eggplants. A banker plant system has been developed to introduce natural enemies for pest control in protected cultures. This system allows natural enemies to be maintained in a greenhouse using alternative prey on banker plants. Populations of natural enemies remain stable on the banker plant system irrespective of the presence of pests on crops, providing a continuous source of predators in case of crop infestation. Typically, *Rhopalosiphum padi* (L.) is used as an alternative prey, and barley or wheat is used as a banker plant for *A. aphidimyza* (Bennison 1992; Nagasaka et al. 2010). This banker plant system has been used to control *A. gossypii* infestations on eggplant in greenhouses in Japan (Nagasaka et al. 2010). However, barley does not grow well in greenhouses when temperatures are high. Abe et al. (2011) estimated the intrinsic rates of increase of *Melanaphis sacchari* (Zehntner) and three other aphid species on sorghum plants at different temperatures, and found that the intrinsic rate of

increase of *M. sacchari* was highest at 35°C, and it was higher than those of other species at all temperatures. Therefore, a banker plant system using *M. sacchari* as an alternative prey and sorghum as a banker plant was developed to control *A. gossypii* infestations on eggplant during hot seasons (Yano et al. 2011).

There is a risk that *A. aphidimyza* females produced by the banker plant system may prefer the alternative prey aphids on banker plants to pest aphids on crops and will thus fail to attack the target species during infestations. It is therefore critical, in developing a banker plant system using *A. aphidimyza*, to ensure the correct oviposition selection. In banker plant systems, the natural enemy should move quickly from the banker plant to the food crop when pests occur on the crop. To ensure this behavior, predators should choose to oviposit among pest aphids even at a low population density. Jandricic et al. (2013), using *Aulacorthum solani* (Kaltenbach) on pansies, concluded that the oviposition preference of *A. aphidimyza* females is strongly affected by aphid densities in the aphid patch location. Mansour (1975) suggested that oviposition preference after emergence might be affected by conditioning during feeding in the larval stages. To ensure the efficacy of the banker plant system, it is necessary to study the oviposition preference of *A. aphidimyza* females when they are offered pest aphids on crops and non-pest aphids on banker plants. The effects of the density ratios of aphid species and conditioning by larval feeding experience on oviposition selection should be investigated.

When *A. aphidimyza* prefers alternative prey or is unable to locate pest aphids because the population density of aphid patches is too low, it is very important to intervene in order to attract *A. aphidimyza* females produced in the banker plant system to crop plants with pest aphids. Under laboratory conditions, volatiles of honeydew excreted by *M. persicae* on sweet pepper induced an attraction response and oviposition behavior in *A. aphidimyza* females (Choi et al. 2004). However, the chemical compounds responsible for this behavior have not been identified.

The first objective of the present study was to investigate oviposition selection by *A. aphidimyza* females offered the pest aphid *A. gossypii* on eggplants and an alternative prey species, either *R. padi* on barley plants or *M. sacchari* on sorghum plants. To meet this objective, experiments were carried out in the laboratory and in greenhouses. The second aim of this study was to investigate the volatile chemicals produced by interactions between *A. gossypii* and eggplants, which attract *A. aphidimyza* females and induce oviposition. We carried out olfactory experiments to study these chemical cues. Based on the results, GC-MS analyses of odor sources and further olfactory experiments were performed to identify the volatile compounds that induce attraction and

oviposition. These attractants and oviposition stimulants, once identified, may be useful in controlling the behavior of *A. aphidimyza*, and will contribute to improved performance of the banker plant system.



## **II. Oviposition selection by *Aphidoletes aphidimyza* (Diptera: Cecidomyiidae) on banker plants with alternative prey aphids or crop plants with pest aphids**

### **Introduction**

The Cecidomyiidae family includes numerous herbivorous species that form insect galls on plant leaves and stems. However, species that prey on whiteflies, spider mites, scale insects, and aphids are also often present (Harris 1968). *Aphidoletes aphidimyza* (Rondani) (Diptera: Cecidomyiidae) is an aphidophagous predator and widely distributed species (Yukawa et al. 1998). The females of this species oviposit in or near aphid colonies and the hatched larvae prey on aphids. Hatched larvae of *A. aphidimyza* starve to death if they are more than 63 mm from food (Wilbert 1972) and they usually stay in the aphid colony throughout their larval stages.

*Aphidoletes aphidimyza* is an effective natural enemy to control aphids in greenhouse crops worldwide (Meadow et al. 1985; Morse and Croft 1987; Nijveldt 1988). However, it can be difficult to establish and often does not provide adequate control because aphids multiply very quickly on greenhouse vegetables such as cucumber (*Cucumis sativus* L.) or eggplant (*Solanum melongena* L.). To overcome this risk, introductions could be made via a banker plant system, or an open-rearing system (Bennison 1992; Hansen 1983; Yano 2006). Prior to the occurrence of a pest, banker plants serve as a control agent reservoir in greenhouses by providing an alternative, non-pest food source. Typically, *Rhopalosiphum padi* (Linne) is used as an alternative prey, and barley (*Hordeum vulgare* L.) and wheat (*Triticum aestivum* L.) are used as banker plants for *A. aphidimyza* (Bennison 1992; Nagasaka et al. 2010). This banker plant system has been used to control *A. gossypii* infesting eggplant in greenhouses in Japan (Nagasaka et al. 2010).

From a practical standpoint, newly emerged females of *A. aphidimyza* from barley plants should move quickly to eggplant. In this case, *A. aphidimyza* should prefer *A. gossypii* on eggplants to *R. padi* on barley plants. Mansour (1975) suggested a role for the effects of conditioning, according to feeding experience in larval stages, on oviposition preference after emergence. Jandricic et al. (2013) concluded that *A. aphidimyza* females are affected strongly by aphid densities in the aphid patch location, using *Aulacorthum solani* (Kaltenbach) on pansies. The density ratio of *A. gossypii* to *R. padi* might affect the oviposition selection of the two aphid species due to the effect of density.

The objectives of this study were to investigate oviposition selection by *A.*

*aphidimyza* females that were offered the two combinations of aphids and plants was also studied in the laboratory and greenhouse. Moreover, the effects of conditioning, according to the feeding experience of *R. padi* and *A. gossypii*, and the density ratio of *A. gossypii* to *R. padi*, on oviposition selection by *A. aphidimyza*, were studied in the laboratory.

## **Materials and methods**

### **Insects and plants**

Larvae of *A. aphidimyza* attacking turnip aphids *Lipaphis erysimi* (Kaltenbach) were collected on potherb mustard (*Brassica rapa* L. var. *nipposinica*) in Nantan City, Kyoto Prefecture, on August 25, 2005. *Aphis gossypii* aphids were collected from strawberry (*Fragaria × ananassa* Duchesne) plants in fields in Ayabe City, Kyoto Prefecture, on April 21, 2006. *Rhopalosiphum padi* was collected from maize (*Zea mays* L.) in Nantan City, Kyoto Prefecture, on August 29, 2003. The descendants of one *A. gossypii* female and one *R. padi* female were reared for successive generations on eggplant (cv. Senryo Nigou) and barley (cv. Temairazu) plants, respectively, in rearing cages (30 cm wide, 25 cm long, 30 cm high) maintained at 25°C with a L16:D8 photoperiod. *Aphidoletes aphidimyza* were reared on *A. gossypii* for successive generations in rearing cages maintained at 25°C with a L16:D8 photoperiod. All plants used for rearing insects and experiments were grown in a glasshouse at the Nara campus of Kinki University.

### **Effect of the prey density ratio on the oviposition preference of female adults for the crop–pest or banker plant–alternative prey systems in the laboratory**

The oviposition preference of *A. aphidimyza* female adults, for *A. gossypii* on an eggplant seedling (the crop–pest system) or *R. padi* on barley plants (the banker plant–alternative prey system), was investigated in the laboratory. A potted eggplant seedling (ca. 20 cm high) and barley seedlings (ca. 20 cm high) were placed in a plastic cage (30 cm wide, 25 cm long, 30 cm high). The total leaf surface areas of both plants were adjusted to the same value (ca. 300 cm<sup>2</sup>). The bottom of the cage was covered with a wet paper towel for humidity. The numbers of second or third instar nymphs of *A. gossypii* and *R. padi* inoculated on the eggplant and barley seedlings were, respectively: (i) 250 vs. 250, (ii) 125 vs. 375, and (iii) 50 vs. 450. Five male and five female *A. aphidimyza* adults (< 24 h old) were released from the center of the bottom of the cage. Five males and five females were released to insure mating because the mating rate was often very low when only one female and one male were released. Interactions between

female adults have not been reported. *Aphidoletes aphidimyza* was reared on *R. padi* for one generation prior to the experiments. After 2 days, the number of eggs on each plant was checked under a stereomicroscope or by using a magnifying glass. To avoid position effects, we switched the position in which potted plants were placed for each replicate. The experiments were performed at 25°C under a L16:D8 photoperiod.

### **Effect of conditioning on the oviposition preference of female adults for the crop–pest or banker plant–alternative prey systems in the laboratory**

A potted eggplant seedling (ca. 20 cm high) and barley seedlings (ca. 20 cm high) were placed in a plastic cage (30 cm wide, 25 cm long, 30 cm high). A total of 150 second or third instar nymphs of *A. gossypii* and *R. padi* were inoculated on the eggplant seedling and the barley seedlings, respectively. The total leaf surface areas of both plants were adjusted to the same value (ca. 300 cm<sup>2</sup>). Five male and five female *A. aphidimyza* adults (< 24 h old) were released from the center of the bottom of the cage. The females had been reared on *A. gossypii* or *R. padi* prior to the experiments. After 2 days, the number of eggs on each plant was checked under a stereomicroscope or by using a magnifying glass. To avoid position effects, we switched the position in which potted plants were placed for each replicate. The experimental conditions were the same as those used for the experiments studying the effect of prey density ratio on oviposition preference.

### **Oviposition preference of female adults for the crop–pest or banker plant–alternative prey systems in a greenhouse**

The oviposition preference of *A. aphidimyza* female adults, for the crop–pest or banker plant–alternative prey systems, was investigated in a plastic greenhouse (5 m wide, 9 m long, 3 m high). Four potted barley (ca. 150 leaves, 20 cm high) and four potted eggplant seedlings (10–15 leaves, 50 cm high) were placed in each of the two rows in a cage (1.5 m wide, 2 m long, 1.5 m high) covered by fine nets constructed in the center of the greenhouse. The plant pots (18 cm diameter, 18 cm high) were placed at 0.5 m intervals in each row. The distance between rows was 0.5 m. One thousand *A. gossypii* nymphs were placed on each potted eggplant plant in one row; 1,000 *R. padi* nymphs were placed on each potted barley plant in the same manner in the adjacent row. Thirty male and 30 female *A. aphidimyza* adults (< 24 h old) were placed individually in small glass vials (0.5-cm diameter, 3 cm long), which were then plugged with cotton balls. The adult aphids were released from the center of the cage simultaneously by removing the cotton balls. The leaves of each plant in the two rows were cut after 48 h. Then, the

number of eggs laid on each plant was counted under a stereomicroscope in the laboratory. The first and second experiments were performed between 12–13 June 2012 and 27–28 September 2012, respectively. The positions of seedlings inoculated with the two aphid species were exchanged in the second experiment. The daily maximum and minimum temperatures were recorded on a thermometer during the experiments.

The number of eggs laid by *A. aphidimyza* females on eggplant plants infested with *A. gossypii*, and on barley plants infested with *R. padi*, in the oviposition preference experiments were analyzed by  $\chi^2$  tests. These were performed based on the assumption that *A. aphidimyza* females oviposit eggs in proportion to the density ratio of the two aphid species. *A. aphidimyza* females lay eggs at night; however, oviposition behavior has never been studied directly because of observational difficulties. Because oviposition of this species is undescribed, we assumed random oviposition against two populations of different aphid species. Accordingly, we could assume that the numbers of oviposited eggs would be proportional to the density ratio of the two aphid species. The  $\chi^2$  tests were performed using R2.70.

## Results

### **Effect of the prey density ratio on the oviposition preference of female adults for the crop–pest or banker plant– alternative prey systems in the laboratory**

The experimental results are summarized in Table 1. When 250 *A. gossypii* nymphs on eggplant seedlings and 250 *R. padi* nymphs on barley seedlings were provided, *A. aphidimyza* females laid significantly more eggs on eggplant plants with *A. gossypii* than on the barley plants with *R. padi*, in 9 of the 10 replicates. An oviposition preference for barley plants was significant in one replicate. When 125 *A. gossypii* nymphs on eggplant plants, and 375 *R. padi* nymphs on barley plants, were provided, *A. aphidimyza* females laid significantly more eggs on the eggplant plants than would be expected in random oviposition in 5 of the 11 replicates. Females tended to oviposit on the barley seedlings rather than on the eggplant plants in four replicates. No significant differences were found, to what would be expected with random oviposition, in the numbers of eggs laid on the eggplant and barley seedlings in two replicates. When 50 *A. gossypii* nymphs on eggplant plants and 450 *R. padi* nymphs on barley plants were provided, *A. aphidimyza* females laid significantly more eggs on the eggplant plants than would be expected in random oviposition in 4 of 11 replicates. Females tended to oviposit on the barley seedlings rather than on the eggplant seedlings in two replicates. No significant differences were found between the number of eggs laid on the eggplant

and barley seedlings in five replicates.

When the number of eggs laid on eggplant and barley plants by *A. aphidimyza* females in each replicate were pooled, *A. aphidimyza* females laid significantly more eggs on eggplant than on barley plants, irrespective of the prey density ratio. No significant effects of prey density ratio on oviposition preference were found from these results.

**Table 1** Numbers of eggs laid by five *A. aphidimyza* females on an eggplant with *A. gossypii* nymphs, and on barley plants with *R. padi* nymphs, of different numbers in a cage over 2 days at 25°C. The numbers of nymphs of *A. gossypii* and *R. padi* inoculated on eggplant and barley seedlings were, respectively: (a) 250 vs. 250, (b) 125 vs. 375, and (c) 50 vs. 450. *P*-values indicate probabilities of  $\chi^2$  values.

Trial number	Number of eggs laid on		$\chi^2$	$p$
	Eggplants with <i>A. gossypii</i>	Barley plants with <i>R. padi</i>		
(a) 250:250				
1	115	0	115.00	< 0.001
2	47	2	41.33	< 0.001
3	83	0	83.00	< 0.001
4	68	26	18.77	< 0.001
5	82	8	60.84	< 0.001
6	50	0	50.00	< 0.001
7	64	7	45.76	< 0.001
8	23	0	23.00	< 0.001
9	53	0	53.00	< 0.001
10	4	107	95.58	< 0.001
Total	589	150	260.79	< 0.001
(b) 125:375				
1	68	18	134.09	< 0.001
2	2	82	22.92	< 0.001
3	18	37	1.75	0.186
4	96	17	216.64	< 0.001
5	10	142	27.51	< 0.001
6	95	35	160.26	< 0.001
7	158	114	158.82	< 0.001
8	4	44	7.11	0.008
9	2	64	16.99	< 0.001
10	53	4	140.50	< 0.001
11	10	20	1.11	0.295
Total	516	577	287.54	< 0.001
(c) 50:450				
1	65	35	336.11	< 0.001
2	39	53	107.25	< 0.001
3	0	14	1.56	0.212
4	0	16	1.78	0.182
5	0	13	1.44	0.229
6	2	23	0.11	0.739
7	68	156	103.14	< 0.001
8	2	83	5.52	0.019
9	28	162	4.74	0.030
10	2	74	4.58	0.032
11	0	20	2.22	0.136
Total	206	649	188.70	< 0.001

### Effect of conditioning on the oviposition preference of female adults for the crop–pest or banker plant–alternative prey systems in the laboratory

When females conditioned by *A. gossypii* were used, they laid significantly more eggs on eggplant plants with *A. gossypii* than on barley plants with *R. padi* in 9 of the 11 replicates. When females were conditioned by *R. padi*, they laid significantly more eggs on eggplant plants with *A. gossypii* than on barley plants with *R. padi* in six of the nine replicates. When the number of eggs laid on eggplant and barley plants by *A. aphidimyza* females in each replicate were pooled, *A. aphidimyza* females laid significantly more eggs on eggplant plants than on barley plants in both conditions. Females tended to lay more eggs on eggplant plants than on barley plants in both cases (Table 2).

**Table 2** Numbers of eggs laid by five *A. aphidimyza* females on an eggplant with 150 *A. gossypii* nymphs, and on barley plants with 150 *R. padi* nymphs, in a cage over 2 days at 25°C. *Aphidoletes aphidimyza* were reared for one generation on *A. gossypii* (a) or *R. padi* (b) prior to the experiments. *p*-values are the same as for Table 1.

Trial number	Number of eggs laid on		$\chi^2$	$p$
	Eggplants with <i>A. gossypii</i>	Barley plants with <i>R. padi</i>		
(a) Conditioned by <i>A. gossypii</i>				
1	5	11	2.25	0.134
2	25	20	0.56	0.456
3	52	0	52.00	< 0.001
4	47	0	47.00	< 0.001
5	16	0	16.00	< 0.001
6	28	0	28.00	< 0.001
7	19	0	19.00	< 0.001
8	28	10	8.53	0.004
9	13	0	13.00	< 0.001
10	26	0	26.00	< 0.001
11	13	0	13.00	< 0.001
Total	272	41	170.48	< 0.001
(b) Conditioned by <i>R. padi</i>				
1	95	0	95.00	< 0.001
2	98	3	89.36	< 0.001
3	76	91	1.35	0.246
4	29	2	23.52	< 0.001
5	19	7	5.54	0.019
6	16	0	16.00	< 0.001
7	45	12	19.11	< 0.001
8	22	19	0.22	0.639
9	25	16	1.98	0.160
Total	425	150	131.52	< 0.001

### Oviposition preference of female adults for the crop–pest or banker plant–alternative prey systems in a greenhouse

In two replications, *A. aphidimyza* females laid significantly more eggs on eggplant plants with *A. gossypii* than on barley plants with *R. padi* (Table 3). In the first experiment, the daily maximum temperatures on 12 and 13 June were 35°C and 29°C, respectively, and the daily minimum temperatures were 19°C and 18°C, respectively. In the second experiment, the daily maximum temperature on 27 and 28 September was 39°C and the daily minimum temperature was 15°C.

**Table 3** Numbers of eggs laid by 30 *A. aphidimyza* females on four potted eggplants with 4,000 *A. gossypii* nymphs, and on four potted barley plants with 4,000 *R. padi* nymphs, in two greenhouse experiments. *p*-values are the same as for Table 1.

Trial number	Number of eggs laid on		$\chi^2$	<i>p</i>
	Eggplants with <i>A. gossypii</i>	Barley plants with <i>R. padi</i>		
1	55	1	52.07	<0.001
2	75	1	72.05	<0.001
Total	130	2	124.12	< 0.001

### Discussion

Our results showed that *A. aphidimyza* females laid significantly more eggs on the eggplant plants with *A. gossypii* than on the barley plants with *R. padi* in most replicates when there was an equal number of *A. gossypii* and *R. padi* nymphs. When the ratio of *A. gossypii* to *R. padi* changed from 1:1 to 1:3 or 1:9, females tended to oviposit more eggs on the eggplant plants with *A. gossypii* than on the barley plants with *R. padi*, if we assumed random oviposition. Five females and five males were released in each trial. The variable number of oviposited females in each trial resulted in high variability in the number of oviposition between trials.

Oviposition by *A. aphidimyza* females was affected by the host plants of aphids (Mansour 1975), the supply of honeydew (Mansour 1975), the height of the leaves infected by aphids (Jandricic et al. 2013; Mansour 1976), plant pubescence (Lucas and Brodeur 1999), light intensities (Mansour 1976), oviposition-detering pheromones secreted by *Aphidoletes* larvae (Ruzicka and Havelka 1998), aphid density (Guo et al. 2014; Jandricic et al. 2013; Lucas and Brodeur 1999), and the presence of eggs and larvae of conspecifics (Guo et al., 2014). Jandricic et al. (2013) concluded that *A. aphidimyza* females were affected strongly by aphid densities and not by aphid species



in the aphid patch location, using *M. persicae* and *A. solani* on pansies. On the other hand, Choi et al. (2004) reported that female adults of *A. aphidimyza* showed a positive response to honeydew volatiles produced by *M. persicae*. In the studies of *A. aphidimyza* by Choi et al. (2004) and Jandrinsic et al. (2013), there were significant linear positive relationships between oviposition and aphid density when the females were exposed to plants infested with different numbers of aphids. From a practical point of view, it is important to estimate the minimum aphid density above which *A. aphidimyza* females are able to oviposit. However, this threshold density has not been evaluated. The positive response in the oviposition of *A. aphidimyza* females, to increased aphid density, might be due to the accumulation of honeydew on leaves. In this study, both host plants and aphid species might have affected the oviposition preference of *A. aphidimyza* for the eggplant plants with *A. gossypii* versus the barley plants (Table 2, 3). This oviposition preference may reflect the preference of *A. aphidimyza* in natural habitats. The number of eggs laid per adult female was smaller in the experiments conducted in a greenhouse than in those conducted in the laboratory. This might have been due to differences in aphid densities, temperature conditions, and the spatial scales of the experiments.

Newly emerged adults of *A. aphidimyza* from barley plants with *R. padi* prefer to move to eggplants with *A. gossypii* irrespective of either conditioning by *R. padi* or a change in the density ratio of the two aphid species. This combination of crop–pest and banker plant–alternative prey system enables the dispersal of *A. aphidimyza*.

### III. Oviposition selection of *Aphidoletes aphidimyza* (Diptera: Cecidomyiidae) on a banker plant system of sorghum with *Melanaphis sacchari* (Hemiptera: Aphididae)

#### Introduction

*Aphidoletes aphidimyza* (Rondani) (Diptera: Cecidomyiidae) is an effective natural enemy used for controlling aphids that attack greenhouse crops worldwide (Meadow et al. 1985; Morse and Croft 1987; Nijveldt 1988). Females oviposit in or near aphid colonies, and the hatched larvae prey on aphids in the colony. The larvae usually stay in the same aphid colony throughout their larval stages. Oviposition by adults is thus crucial for effective predation by larvae to control aphids.

Release programs of *A. aphidimyza* to control aphids often do not provide adequate control because aphids multiply very quickly on greenhouse vegetables such as cucumber or eggplant. To overcome this risk, introductions can be made by means of a banker plant system or an open-rearing system (Hansen 1983; Bennison 1992; Yano 2006). Such systems provide the ability to maintain natural enemies within a greenhouse using alternative prey on banker plants.

Typically, *Rhopalosiphum padi* (Linnaeus) is used as an alternative prey, and barley or wheat is used as a banker plant for *A. aphidimyza* (Bennison 1992; Nagasaka et al. 2010). This banker plant system has been used to control *A. gossypii* that infest eggplant in greenhouses in Japan (Nagasaka et al. 2010). However, barley does not grow well during hot seasons in greenhouses. The main cultivation period for eggplants and peppers in unheated greenhouses is from planting in April to the end of the harvest in September, during which time the average temperature is about 25°C. The maximum temperature in summer rises to more than 35°C in the daytime, and barley does not grow well at these temperatures. Abe et al. (2011) estimated the intrinsic rates of increase in *Melanaphis sacchari* (Zehntner) and three other aphid species on sorghum plants at different temperatures, and found that the intrinsic rate of increase of *M. sacchari* was highest at 35°C and was always higher than that of other species at all temperatures. Therefore, a banker plant system using *M. sacchari* as an alternative prey and sorghum as a banker plant was developed to control *A. gossypii* infesting eggplant during hot seasons (Yano et al. 2011).

From a practical standpoint, newly emerged females of *A. aphidimyza* from sorghum plants should move quickly to eggplant. In this banker plant system, a low density of *A. gossypii* on eggplant should be chosen for oviposition. Examining *Aulacorthum solani* (Kaltenbach) on pansies, Jandricic et al. (2013) concluded that *A.*

*aphidimyza* females are affected strongly by aphid density in the aphid patch location. We showed that *A. aphidimyza* females laid more eggs on eggplants harboring *A. gossypii* than on barley plants harboring *R. padi* when offered the two combinations of aphids and plants, irrespective of the density ratio of the two aphid species (Higashida et al. 2016). However, if *A. aphidimyza* females require aphids to meet a threshold density for oviposition, extreme disparities in the densities of two aphid species might affect *A. aphidimyza*'s oviposition preference. However, ovipositional selection by *A. aphidimyza* on eggplant with *A. gossypii* or sorghum with *M. sacchari* has not been studied so far. The density ratio of *A. gossypii* to *M. sacchari* might affect the oviposition selection of *A. aphidimyza* to between the two aphid species.

The objectives of the present study are to investigate oviposition selection by *A. aphidimyza* females that were offered the two combinations of aphids and plants was also studied in the laboratory and greenhouse. Moreover, the effects of the density ratio of *A. gossypii* to *M. sacchari* upon oviposition selection by *A. aphidimyza* were studied in the laboratory. All oviposition experiments in the laboratory were conducted at 25°C, which is the average temperature during the main cultivation period for eggplants in unheated greenhouses.

## Materials and methods

### Insects and plants

Larvae of *A. aphidimyza* attacking turnip aphids *Lipaphis erysimi* (Kaltenbach) were collected on potherb mustard (*Brassica rapa* L. var. *nipposinica*) in Nantan City, Kyoto Prefecture, on August 25, 2005. *Aphis gossypii* individuals were collected from strawberry (*Fragaria × ananassa* Duchesne) plants in Ayabe City, Kyoto Prefecture, on April 21, 2006. *Melanaphis sacchari* individuals were collected from sorghum (*Sorghum bicolor* [L.] Moench) in Ayabe City, Kyoto Prefecture, on September 28, 2006. *Aphis gossypii* and *M. sacchari* were reared for successive generations on eggplant (cv. Senryo Nigou) and sorghum (cv. Kazetachi), respectively, in rearing cages (30 cm wide, 25 cm long, 30 cm high) at 25°C with a L16:D8 photoperiod. *Aphidoletes aphidimyza* was reared on *A. gossypii* for successive generations under the same environmental conditions as above. All plants used for rearing insects and experiments were grown in a glasshouse at the Nara campus of Kinki University.

### **Effect of prey density ratio on oviposition preference of female adults for the crop–pest or banker plant–alternative prey combinations in the laboratory**

The oviposition preference of *A. aphidimyza* adult females, for *A. gossypii* on eggplant seedlings (the crop–pest combination) or *M. sacchari* on sorghum seedlings (the banker plant–alternative prey combination), was investigated in the laboratory. A potted eggplant seedling (ca. 20 cm high) and sorghum seedlings (ca. 20 cm high) in a pot were placed in a plastic cage (30 cm wide, 25 cm long, 30 cm high). The total leaf surface areas of both plants were adjusted to the same value (ca. 600cm<sup>2</sup>). The bottom of the cage was covered with a wet paper towel to maintain humidity. The numbers of second or third instar nymphs of *A. gossypii* and *M. sacchari* inoculated on the eggplant and sorghum seedlings were (i) 250:250, (ii) 375:125, and (iii) 450:50, respectively. Five male and five female *A. aphidimyza* adults (< 24 h old) were released from the center of the bottom of the cage. Five males and five females were released to ensure mating, as the mating rate was often very low when only one female and one male were released. Interactions between adult females have not been reported. *Aphidoletes aphidimyza* was reared on *M. sacchari* for one generation prior to the experiments. After 2 days, the number of eggs on each plant was checked under a stereomicroscope or using a magnifying glass. To avoid position effects, we switched the position of the potted plants for each replicate. The experiments were performed 10 times at 25°C under a L16:D8 photoperiod.

### **Oviposition preference of female adults for the crop–pest or banker plant–alternative prey combinations in a greenhouse**

The oviposition preference of *A. aphidimyza* female adults for the crop–pest or banker plant–alternative prey combinations was investigated in a plastic greenhouse (5 m wide, 9 m long, 3 m high). Four potted sorghum (ca. 50 leaves, 70 cm long) and four potted eggplant (10–15 leaves, 50 cm high) seedlings were placed in each of the two rows in a cage (1.5 m wide, 2 m long, 1.5 m high) covered by fine nets constructed in the center of the greenhouse. The plant pots (18 cm in diameter, 18 cm high) were placed at 0.5 m intervals in each row, and the distance between rows was 0.5 m. One thousand *A. gossypii* nymphs were placed on each potted eggplant plant in one row, and 1,000 *M. sacchari* nymphs were placed on each potted sorghum plant in the same manner in the adjacent row. Thirty male and thirty female *A. aphidimyza* adults (< 24 h old) were placed individually in small glass vials (0.5 cm in diameter, 3 cm long), which were then plugged with cotton balls. The adults were released from the center of the cage simultaneously by removing the cotton balls. The leaves of each plant in the two rows

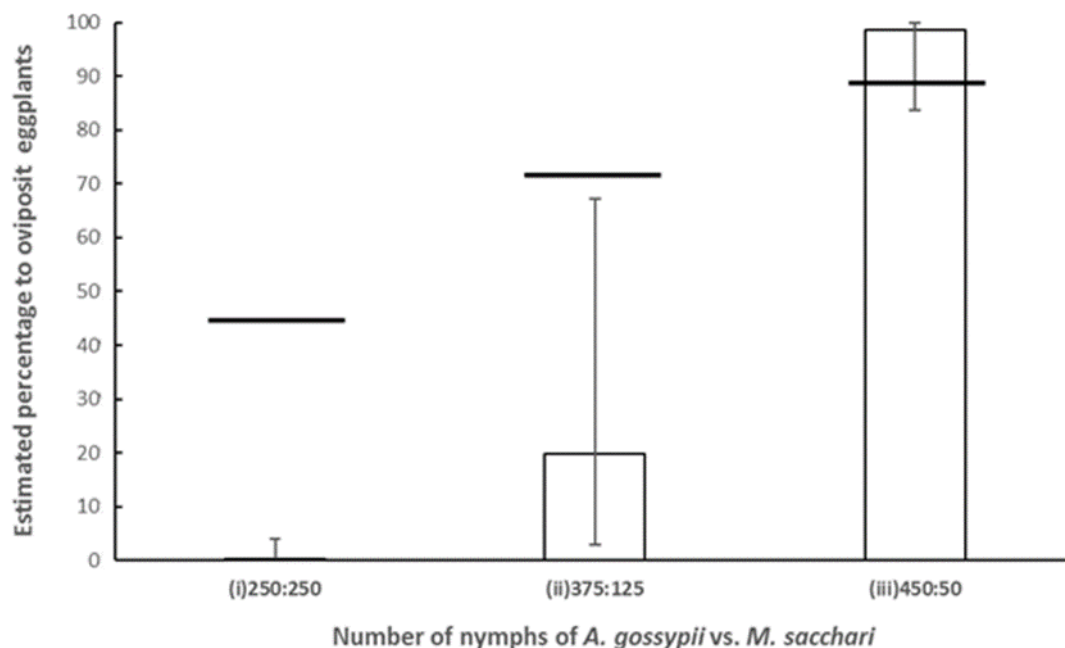
were cut after 48 h, and the number of eggs laid on each plant was counted under a stereomicroscope in the laboratory. The first and second experiments were performed on 19–20 June 2010 and 29–30 September 2010, respectively. The positions of potted plants inoculated with the two aphid species were exchanged in the second experiment. The daily maximum and minimum temperatures were recorded on a thermometer during the experiments.

The numbers of eggs laid by *A. aphidimyza* females on eggplant infested with *A. gossypii* and on sorghum infested with *M. sacchari* in the oviposition preference experiments were analyzed using a generalized linear mixed model (GLMM) with a binomial distribution and a logit link function. These tests were performed under the assumption that *A. aphidimyza* females oviposit in proportion to the density ratio of the two aphid species. *Aphidoletes aphidimyza* females lay eggs at night; however, oviposition behavior has never been studied directly because it is difficult to observe. Because oviposition in this species is undescribed, we assumed random oviposition by the two populations of different aphid species. Accordingly, we assumed that the numbers of oviposited eggs would be proportional to the density ratio of the two aphid species. Statistical analyses were performed using R3.0.1.

## Results

### **Effect of prey density ratio on oviposition preference of female adults for the crop–pest or banker plant–alternative prey combinations in the laboratory**

GLMM analyses were performed on the pooled data of the three aphid density ratio combinations. Fig. 1 shows the percentage of *A. aphidimyza* that oviposited on eggplant with *A. gossypii* and 95% confidence intervals estimated by GLMM analyses, and the hypothesized percentages that would oviposit on eggplants in proportion to three different density ratios of the two aphid species. When the ratios of nymphs of *A. gossypii* inoculated on eggplant seedlings and *M. sacchari* inoculated on sorghum seedlings were 250:250 and 375:125, *A. aphidimyza* females laid a significantly higher proportion of eggs on sorghum than predicted by proportional oviposition. When the numbers of nymphs of *A. gossypii* and *M. sacchari* inoculated on the eggplant and sorghum seedlings were 450 and 50, respectively, the proportions of eggs laid on each type of plant were not significantly different from predictions based on proportional oviposition.



**Fig. 1** Percentages with 95% confidence intervals of *A. aphidimyza* ovipositing on eggplant with *A. gossypii* estimated by GLMM analyses for three aphid density ratios. The numbers of nymphs of *A. gossypii* inoculated on eggplant seedlings and *M. sacchari* inoculated on sorghum seedlings were (i) 250:250, (ii) 375:125, and (iii) 450:50. The bold line shows the hypothetical percentages of oviposition on eggplants in proportion to the density ratio of the two aphid species.

### **Oviposition preference of adult females for the crop–pest or banker plant–alternative prey combinations in a greenhouse**

GLMM analysis was performed on the pooled data of the two replicates. *Aphidoletes aphidimyza* females laid significantly more eggs on eggplant with *A. gossypii* than on sorghum with *M. sacchari* ( $Z = 2.066$ ,  $P = 0.0388$ ). In the first experiment, the daily maximum temperatures on 19 and 20 June were 38°C and 32°C, respectively, and the daily minimum temperatures were 21°C and 20°C, respectively. In the second experiment, the daily maximum temperatures on 29 and 30 September were 37°C and 38°C, respectively, and the daily minimum temperature was 14°C in both cases.

### **Discussion**

Results of laboratory experiments examining oviposition preference showed that *A. aphidimyza* preferred to oviposit on sorghum harboring *M. sacchari* rather than eggplant

harboring *A. gossypii* when equal numbers of aphids of both species were provided. Our previous study showed that *A. aphidimyza* females laid significantly more eggs on eggplant harboring *A. gossypii* than on barley harboring *R. padi* (Higashida et al. 2016). These results may be due to differences in odor among honeydews. We identified phenyl acetaldehyde as an attractant of *A. aphidimyza* in the honeydew of *A. gossypii* (Watanabe et al. 2016). The oviposition preference of *A. aphidimyza* females may be affected by qualitative and quantitative differences in the honeydew attractants of different aphid species. Plant species used to rear *M. persicae* as prey for *A. aphidimyza* over many generations affected its ovipositional preference (Mansour 1975). However, such a shift of ovipositional preference was not observed in *A. aphidoletes* strains that had been reared on *A. gossypii* on eggplant or *R. padi* on barley (Higashida et al. 2016). No ovipositional preference was observed when 450 *A. gossypii* individuals and 50 *M. sacchari* individuals were provided. The observed shift in ovipositional preference may have been caused by changes in the density ratio of aphid species. *Aphidoletes aphidimyza* females are believed to be able to recognize small patches of *M. sacchari*, but this ability may have been inhibited by the odor of the honeydew from large patches of *A. gossypii*. *Aphidoletes aphidimyza* preferred *A. gossypii* to *M. sacchari* in the greenhouse experiment. The results of greenhouse experiments, which is favorable for the use of the banker plant system.

This discrepancy between the results of the greenhouse and the laboratory experiments may be due to differences in temperature, humidity, and plant architecture. In the greenhouse, temperature and humidity conditions are variable, and are not always suitable for the survival of *A. aphidimyza*. During the day, *A. aphidimyza* must avoid direct sunlight on plants. *Aphidoletes aphidimyza* adults are known to be active at night and prefer to lay eggs on the undersides of leaves (Mansour 1976). Eggplant leaves are wider than sorghum leaves, providing a suitably sheltered habitat during the day and an oviposition site at night.

In conclusion, the combination of sorghum and *M. sacchari* seem to be suitable for ovipositional preference of *A. aphidimyza*. It is therefore likely to be effective as a banker plant system under high temperature conditions.

#### IV. An attractant of the aphidophagous gall midge *Aphidoletes aphidimyza* from honeydew of *Aphis gossypii*

##### Introduction

Honeydew is a sugar-rich excretion produced by sap-feeding Sternorrhyncha that many natural insect enemies use for survival (Wäckers 2005). They also use it as a volatile cue to locate prey and hosts, as an oviposition stimulant, and as an arrestant for foraging (Lundgren 2009). Olfactometer studies suggest that many aphidophagous parasitoid species are attracted to volatiles of honeydew (Hågvar and Hofsvang 1989; Wickremasinghe and van Emden 1992). Aphidophagous predator species such as the Asian lady beetle *Harmonia axyridis* Pallas (Leroy et al. 2012) and larvae and adults of the lacewing *Chrysoperla carnea* (Stephens) are attracted by honeydew (Duelli 1987). Honeydew from *Sitobion avenae* (F.) is a strong contact oviposition stimulant for the syrphid *Episyrphus balteatus* (De Geer) (Budenberg and Powell 1992), whereas that from *Aphis fabae* Scop. has been shown to attract females of *E. balteatus* and elicit oviposition (Scholz and Poehling 2000). Moreover, many aphid predators and parasitoids alter their foraging behavior and explore more intensively when they are in contact with honeydew, including coccinellids (Carter and Dixon 1984; Ide et al. 2007; Purandare and Tenhumberg 2012), predatory mites (Fleschner 1950), parasitoids (Bouchard and Cloutier 1984; Budenberg et al. 1992), syrphid larvae (Leroy et al. 2014), and chrysopids (Downes 1974; Han and Chen 2002).

*Aphidoletes aphidimyza* (Rondani) (Diptera: Cecidomyiidae) is a gall midge species and widely distributed. Its larvae are predaceous and attack over 80 aphid species (Harris 1973; Yukawa et al. 1998). Female *A. aphidimyza* lay up to 40 eggs near aphid colonies, singly or in clusters (Harris 1973; Nijveldt 1988; Uygun 1971) and can locate an isolated infested *Brassica* plant among noninfested plants in a greenhouse (El-Titi 1972/1973, 1974).

Host selection is an important aspect of oviposition, as offspring are often restricted to feed on the host chosen by females during egg laying. The optimality theory predicts that females should choose oviposition sites associated with high expected fitness for their offspring (Mangel 1987). This is especially important for species that are relatively sessile and have limited mobility to forage immediately after hatching (Thompson 1988). Female *A. aphidimyza* lay eggs on foliage within or close to aphid colonies (Nijveldt 1988), as neonatal larvae starve to death if they hatch >63 mm away from food (Wilbert 1972), thus illustrating the importance of oviposition location for this



species. Furthermore, honeydew excreted by *M. persicae* on sweet pepper attracted *A. aphidimyza* females, and the volatiles extracted in pentane induced a similar response, although specific attractant compounds were not identified (Choi et al. 2004).

*Aphidoletes aphidimyza* can be an effective biological control agent against aphids, although establishing it to control rapidly multiplying aphid populations in greenhouses is difficult (Markkula and Tiittanen 1985; Meadow et al. 1985; Nijveldt 1988). To address this issue, banker plant or openrearing systems can effectively maintain the control agent (Bennison 1992; Hansen 1983; Yano 2006). Prior to pest occurrence, banker plants serve as a reservoir of control agents by providing such pests with a non-pest food source, but the control agent must move quickly from the banker plant to the food crop when pests occur. Therefore, controlling the behavior of the control agent is crucial to the banker plant system. The banker plant system currently is being used to develop *A. aphidimyza* as a control agent of *A. gossypii* on eggplants in Japan.

The objectives of this study were to investigate chemical cues that induce attraction of female *A. aphidimyza*. We used an olfactometer to observe behavioral responses of *A. aphidimyza* to volatile chemicals emitted from *A. gossypii* feeding on eggplants. Based on initial results, we analyzed honeydew with gas chromatography coupled with mass spectrometry (GC/MS), and then performed olfactometer experiments to identify attractant compounds. The results will help explain the response of *A. aphidimyza* to volatiles of *A. gossypii* and will provide useful information for the use of *A. aphidimyza* as a biological control agent in greenhouses.

## Methods and Materials

### Insects

We collected *A. aphidimyza* larvae attacking *Lipaphis erysimi* (Kaltenbach) on *Brassica rapa* (var. Nipponsinica) on August 25, 2005, in Nantan City, Kyoto, Japan, and *A. gossypii* from strawberry plants *Fragaria × ananassa* Duchesne ex Rozier in fields in Ayabe City, Kyoto, Japan, on April 21, 2006. We continually cultured descendants of one female *A. gossypii* on eggplants (cv. Senryo 2 gou) in plastic rearing cages (30 × 30 × 25 cm) at 25 °C with a L16:D8 photoperiod. *Aphidoletes aphidimyza* were reared on *A. gossypii* for successive generations under the same conditions.

### Handling *A. aphidimyza* females

We selected 20 female and 20 male newly emerged *A. aphidimyza* adults (< 24 hr old) and fed them a 20 % sucrose solution via tissue paper balls placed in 100-ml vials at the

four corners of a plastic cage (30 × 30 × 25 cm). To facilitate mating, we placed an artificial flower made from wires and filter paper at the center of the cage. After 48 hr, the mated female adults were used in olfactometer experiments.

### **Olfactometer**

A three-chamber olfactometer was constructed from three acrylic boxes (20 × 20 × 20 cm) attached side by side. We cut square holes (18 × 18 cm) in the walls between chambers to allow insect and air movement. A 3-mm-diam tube was fitted through the top of the middle chamber to remove air. A small acrylic box (4 × 3 × 3 cm) was attached to the outer wall of the outer two chambers, and a square hole (1.5 × 1.5 cm) was cut to connect the small boxes with the main chambers; holes were covered with a fine nylon filter (0.2-mm mesh) to diffuse air currents. The outer walls of the small boxes were fitted with 3-mm-diam tubes that were connected to 1-l glass containers holding different volatile sources. Air was drawn with a pump through the glass containers into the small boxes via the tubes at a constant flow rate of 0.5 l/min. Before reaching the glass containers, the air passed through an activated charcoal filter and a humidifier.

### **Honeydew collection**

We collected honeydew excreted by *A. gossypii* of unspecified ages feeding on eggplants by placing aluminum foil (9-cm diam) below a leaf holding about 1000 aphids for 48 hr at 25 °C with a L16:D8 photoperiod. We removed impurities (dead aphids or exuviae) from samples with forceps and performed bioassays within 24 hr of collection.

### **Behavioral responses to four odor sources**

Ten mated female *A. aphidimyza* were introduced into the middle olfactometer chamber through the top opening and left for 10 min before observations commenced. Experiments began immediately following the start of the scotophase, which generally coincides with oviposition. Chambers were placed in an environmental chamber maintained at 25 °C and illuminated with red light (which is not perceived by female adults) to facilitate the observation of midge behavior. The primary objective of this experiment was to evaluate the response of *A. aphidimyza* females to odor sources mentioned below. In preliminary experiments, we found that some of the released *A. aphidimyza* females laid eggs on the nylon filters covering the holes of the honeydew-treated olfactometer chamber. This suggested that the honeydew volatile may induce oviposition by the females. Therefore, we observed (1) the number of insects in

each chamber at 10, 20, and 30 min after the start of observations; (2) the number of eggs oviposited on the filters 30 min after the start of observations. After each assay, the main chambers were cleaned with pure ethanol, rinsed with distilled water, and dried. Two-way choice tests between controls and four odor sources were repeated 10 times per source. Our odor sources were the honeydew on aluminum foil that we previously collected, 200 aphids, an uninfested eggplant, and an eggplant infested with 200 aphids (fourth instar nymphs or adults) for 24 hr. A small amount of honeydew accumulated on leaves in the latter treatment during the 24 hr inoculation period. An empty jar was used as the control except in the honeydew trial, where a piece of clean aluminum foil was used. To avoid position effects, we switched the side where sources were placed for each replicate.

### **Analyses of honeydew volatiles**

Our results indicated that the honeydew attracted *A. aphidimyza* and induced oviposition. Therefore, we analyzed the chemical composition of the honeydew to identify crucial compounds inducing this behavior. Honeydew was collected on the surface of a glass Petri dish (9-cm diam, 1.5-cm height) over a period of 48 hr. The collection protocol was the same as previously described. Prior to the collection, we cleaned the Petri dish by carefully rinsing it with acetone and hexane. After the solvents fully evaporated, we kept the dish in a drier at 100 °C for 48 hr. To collect headspace volatiles directly from the honeydew in the Petri dish, we used solid-phase microextraction: five Twisters [polydimethylsiloxane (PDMS)-coated stir bar, film thickness 0.5 mm, 10-mm length; Gerstel GmbH & Co. KG, Mülheim an der Ruhr, Germany] were set on the inside surface of a Petri dish lid to prevent direct contact of the Twisters with the honeydew for 4 hr. As a control, we also set five Twisters in a glass Petri dish that had been located under an uninfested eggplant for 48 hr. The collected volatiles were directly desorbed in the injector with a thermal desorption-cold trap system (Gerstel GmbH & Co. KG, Mülheim an der Ruhr, Germany) and then analyzed by GC/MS with an HP-5MS capillary column (Agilent Technologies). The oven temperature was increased from 40 to 280 °C at 10 °C/min. Compounds detected in the headspace of honeydew and the control were identified by comparing their mass spectra to those in the Wiley7N database and their retention times to those of the following authentic compounds:  $\alpha$ -pinene, camphor, menthol (Tokyo Chemical Industry Co., Ltd., Tokyo, Japan), 6-methyl-5-hepten-2-one, limonene, 1,8-cineol, nonanal, dodecane decanal, tridecane, neryl acetone (Wako Pure Chemical Industries, Ltd., Osaka, Japan), isopulegol (Soda Aromatic Co., Ltd., Tokyo, Japan), and

phenylacetaldehyde (Sigma-Aldrich Inc. St Louis Mo, USA). Since thujone was not available, it was identified by comparing its mass spectrum to that in the database.

### **Behavioral responses to phenylacetaldehyde**

Since phenylacetaldehyde was found in significantly higher amounts in the honeydew than in the control (Table 6), its impact on behavior of *A. aphidimyza* was assayed by the same method as for the odor sources described above. Phenylacetaldehyde [100 ng/0.2 g in triethyl citrate (TEC)] was adsorbed by two pieces of filter paper (2 × 2 cm) in a glass Petri dish (9-cm diam, 1.5-cm high); this represented a similar quantity to that found in the honeydew. We used two pieces of filter paper to adsorb 0.2 g TEC as a control. Two-way choice tests between phenylacetaldehyde and controls were repeated 10 times.

### **Statistical analyses**

We compared the number of females choosing odor treatments and controls at 10-min intervals after the start of observations by using a generalized linear mixed model (GLMM) with a binomial distribution and a logit link function. We used a pairwise t-test to analyze the number of oviposited eggs during the experiment. We used a student *t*-test to compare the quantities of compounds detected in the honeydew and the control by GC/MS. All analyses were performed using R 2.15.2 (R Development Core Team 2012).

## **Results**

### **Behavioral responses to four odor sources**

Table 4 shows the number (mean ± SE) of *A. aphidimyza* females found in the treated and control chambers 10, 20, and 30 min after the start of observations, with the results of GLMM analysis. Females found in the middle chamber (no-choice individuals) were excluded from data analysis. Although we found no significant differences between the number of *A. aphidimyza* females in the honeydew treatment chamber and the number in the control chamber 10 min after observations began ( $Z=1.39$ ,  $P=0.165$ ), we found significantly more females in the treated chamber than in the control chamber 20 and 30 min after the start of the observation period (at 20 min:  $Z=2.02$ ,  $P < 0.05$ ; at 30 min:  $Z=2.46$ ,  $P < 0.05$ ). On the other hand, the number of adult midges in the chambers with the aphids, the uninfested plant, and the infested plant treatments did not differ from the control at any time after the start of observations (Table 4).

**Table 4** Female *Aphidoletes aphidimyza* responses to olfactory treatments and phenyl acetaldehyde ( $N= 10$ ). Ten mated females were introduced into the olfactometer. The number of females (mean  $\pm$  SE) found in treated and control chambers 10, 20, and 30 min after the start of observation are indicated. GLMM was used to test the differences between the numbers of females found in the treatment and control chambers

Comparison	after 10 min			after 20 min			after 30 min		
	Response	Z	P	Response	Z	P	Response	Z	P
Honeydew vs. Control	52 vs. 35	1.39	0.165	56 vs. 33	2.02	<0.05	61 vs. 28	2.46	<0.05
Aphids vs. Control	44 vs. 36	0.86	0.388	43 vs. 40	0.33	0.742	44 vs. 32	1.37	0.17
Infested plant vs. Control	44 vs. 43	0.1	0.923	47 vs. 38	0.77	0.441	44 vs. 40	0.42	0.672
Uninfested plant vs. Control	44 vs. 29	1.74	0.081	41 vs. 33	0.9	0.37	48 vs. 35	1.42	0.155
Phenyl acetaldehyde vs. Control	50 vs. 31	2.09	<0.05	59 vs. 26	3.48	<0.001	46 vs. 39	0.76	0.448

To estimate the effect of volatile chemicals on oviposition, we calculated the number of oviposited eggs per female attracted to the chamber as the total number of eggs on the filter in the treated and control chambers 30 min after the start of observations, divided by the average number of females found in the chamber in the observations at 10, 20, and 30 min. The number (mean  $\pm$ SE) of oviposited eggs per female attracted to the treated chamber at 30 min was significantly higher in the honeydew treatment than in the control ( $t = 3.32$ ,  $df=9$ ,  $P < 0.01$ ) (Table 5), whereas the aphid and infested plant treatments rarely induced oviposition (aphid:  $t=1.21$ ,  $df=9$ ,  $P = 0.258$ ; infested plant:  $t = -0.82$ ,  $df=9$ ,  $P= 0.435$ ), and uninfested plants never induced oviposition (Table 5).

**Table 5** Number of eggs laid per attracted female *Aphidoletes aphidimyza* (mean  $\pm$  SE) on the filters in treated and control chambers of the olfactometer over a 30-min period ( $N=10$ ). Ten mated females were introduced into the olfactometer

Comparison	Number of eggs laid per attracted female (mean $\pm$ SE) <sup>a</sup>		<i>t</i>	<i>P</i>
	Treatment	Control		
Honeydew vs. Control	2.20 $\pm$ 0.66	0	3.32	<0.01
Aphids vs. Control	0.31 $\pm$ 0.26	0	1.21	0.258
Infested plant vs. Control	0.03 $\pm$ 0.03	0.21 $\pm$ 0.21	0.82	0.435
Uninfested plant vs. Control	0	0	–	–

<sup>a</sup>Number of eggs laid per attracted female = total number of eggs laid over a 30-min period in each chamber / average number of females found in each chamber 10, 20, and 30 min after the start of observation

### Analysis of honeydew volatiles

We detected 14 compounds in the headspaces of both the Petri dish with honeydew and the control (Table 6). These included phenylacetaldehyde, which was present in significantly higher amounts in the honeydew Petri dish than in the control ( $t=5.24$ ,  $df=6$ ,  $P= 0.002$ ). The amounts of the other 13 compounds did not differ between the two samples, suggesting that they were plant volatiles adsorbed onto the surface of the Petri dishes.

**Table 6** Headspace volatiles of honeydew-containing Petri dishes and control Petri dishes

Compounds	Ion intensities (mean $\pm$ SE) <sup>a</sup>				<i>t</i>	<i>P</i>
	Honeydew		Control <sup>b</sup>			
Alpha-pinene	1,824,466 $\pm$	986,809	1,823,986 $\pm$	780,091	0.197	0.851
6-methyl-5-hepten-2-one	4,909,911 $\pm$	1,524,940	4,817,989 $\pm$	983,771	0.273	0.794
Limonene	3,397,674 $\pm$	1,243,918	4,085,574 $\pm$	1,763,925	-0.254	0.808
1,8-cineol	42,297,711 $\pm$	19,962,176	51,009,308 $\pm$	28,715,349	-0.169	0.872
Phenyl acetalddehyde	1,848,433 $\pm$	410,461	406,394 $\pm$	49,123	5.306	0.002
Nonanal	29,056,081 $\pm$	5,852,235	19,929,329 $\pm$	2,143,521	2.065	0.084
Thujone	11,918,240 $\pm$	7,096,702	22,267,758 $\pm$	12,436,347	-0.780	0.465
Camphor	22,919,886 $\pm$	18,212,896	38,673,579 $\pm$	26,734,703	-0.468	0.656
Isopulegol	6,277,348 $\pm$	5,135,756	10,751,036 $\pm$	7,884,496	-0.458	0.663
Menthol	1,363,349 $\pm$	818,773	1,717,820 $\pm$	1,266,995	-0.158	0.880
Dodecane	42,135,593 $\pm$	25,844,036	269,127,355 $\pm$	317,059,720	-0.872	0.417
Decanal	34,455,315 $\pm$	5,444,153	25,483,921 $\pm$	3,766,720	1.900	0.106
Tridecane	75,666,099 $\pm$	47,546,035	68,560,674 $\pm$	50,150,005	0.301	0.774
Neryl acetone	3,758,129 $\pm$	3,577,004	2,985,083 $\pm$	2,324,303	0.436	0.678

<sup>a</sup> We performed four replicates of volatile collection for each type of Petri dish

<sup>b</sup> Petri dishes were placed under uninfested eggplants as a control

### Behavioral responses to phenylacetaldehyde

Table 4 shows the number (mean  $\pm$  SE) of *A. aphidimyza* females found in the phenylacetaldehyde chamber treatment and control chamber 10, 20, and 30 min after the start of observations, with the results of GLMM analysis. Significantly more *A. aphidimyza* were found in the chamber with the phenylacetaldehyde treatment than in the control chamber at 10 and 20 min after the start of the observation period (at 10 min:  $Z = 2.09$ ,  $P < 0.05$ ; at 20 min:  $Z = 3.48$ ,  $P < 0.001$ ) (Table 4), whereas *A. aphidimyza* were not attracted to phenylacetaldehyde at 30 min ( $Z = 0.76$ ,  $P = 0.448$ ) (Table 4). Oviposition was not observed in any of the chambers at any time.

### Discussion

We found that *A. aphidimyza* females were attracted to volatiles emitted from honeydew produced by *A. gossypii*, but neither to the aphids nor to uninfested plants, or infested plants (Table 4). The small amount of accumulated aphid honeydew on the leaves of infested plants did not induce attraction. This suggests that volatiles in honeydew act as cues to help *A. aphidimyza* locate prey. Similarly, Choi et al. (2004) reported that adult *A. aphidimyza* was attracted to honeydew excreted from *M. persicae*, but did not respond to aphids, sweet pepper plants, or the combination of aphids and sweet pepper

plants. Thus, honeydew from both *M. persicae* and *A. gossypii* attracts *A. aphidimyza*, and honeydew of other aphid species should be investigated for such properties. The chemical cues contained in honeydew attract the green lacewing, *C. carnea* (Duelli 1987). Choi et al. (2004) suggested that it would be profitable to use a more general indicator of prey presence such as honeydew to attract aphidophagous natural enemies with a wide prey range, such as *A. aphidimyza* and lacewings rather than using herbivore-induced plant volatiles (HIPV).

We detected phenylacetaldehyde in honeydew produced by *A. gossypii* (Table 6), which attracted *A. aphidimyza*. Phenylacetaldehyde is a common floral scent and attracts many lepidopterans, especially noctuid moths (Cantelo and Jacobson 1979; Creighton et al. 1973; El-Sayed et al. 2008; Honda et al. 1998). Tóth et al. (2009) found that phenylacetaldehyde attracted aphidophagous green lacewings (*C. carnea*) in field tests, indicating that aphid predators may use phenylacetaldehyde emitted from honeydew to locate prey.

Leroy et al. (2012) found that lady beetles, *H. axyridis*, are attracted by honeydew of *Megoura viciae* Buckton and identified four attractive compounds released from the aphid honeydew, of which limonene was the most effective. Takemoto and Takabayashi (2015) reported that HIPV blends produced by broad bean plants infested by two aphid species were more attractive to *Aphidius ervi* than single volatiles. Based on statistical comparison, single behaviorally active components other than phenylacetaldehyde were not detected in the *A. gossypii* honeydew, however, it cannot be ruled out that some of the components are active only in a mixture, contributing to an attractive bouquet.

We found no significant differences between the number of *A. aphidimyza* females in the honeydew treatment chamber and the number in the control 10 min after the start of observations. Neither were *A. aphidimyza* attracted to the phenylacetaldehyde treatment chamber at 30 min (Table 4). Concentration of attractants or effects of attractant components other than phenylacetaldehyde might have caused the slow attractive response of *A. aphidimyza* to honeydew volatiles. After 30 min, the concentration of synthetic phenylacetaldehyde in air might be lower than the level of attraction threshold 30 min.

We showed that *A. aphidimyza* deposited eggs more frequently on the filters attached to the honeydew treatment chamber than on the control filters (Table 5). Moreover, previous studies have reported that numbers of *A. aphidimyza* females are nearly proportional to aphid densities (Choi et al. 2004). Mansour (1976) reported that *A. aphidimyza* laid eggs on artificial substrates, such as muslin, parafilm, and filter paper, supplemented with aphids and honeydew. These previous results corroborate our



finding that aphid honeydew stimulates *A. aphidimyza* oviposition. Similarly, aphid honeydew stimulates oviposition by syrphid flies, and artificially applied honeydew elicited oviposition and increased landing by *E. balteatus* on wheat (Budenberg and Powell 1992). Finally, *E. balteatus* females also were able to evaluate aphid numbers and adjust oviposition rates (Bargen et al. 1998).

We showed that the volatiles emitted from honeydew produced by aphids not only attracted *A. aphidimyza* but, in contrast to phenylacetaldehyde also induced oviposition. Other volatile compounds contained in honeydew might cause oviposition behavior and also have an effect as mentioned above. Further detailed analyses of headspace volatiles of honeydew are needed. As described in the introduction, the primary objective of this study was to identify the attractant of *A. aphidimyza* derived from *A. gossypii* honeydew. Since the olfactometer was developed for detecting the behavioral response of *A. aphidimyza*, the oviposition data from the olfactometer filter should be viewed with caution. The filter is too small for oviposition by ten females, and it is unclear, whether the filter is a good substrate for oviposition at all. Consequently, results are not suitable for any quantitative evaluation of oviposition stimulants. Our oviposition data suggest that volatile chemicals derived from *A. gossypii* honeydew do affect oviposition, and a critical next step is to identify the crucial compounds. However, a diagnostically more conclusive bioassay must be developed to tackle this problem. Phenylacetaldehyde, the attractant for *A. aphidimyza* females, and oviposition stimulants may be useful to control gall midges in greenhouses.

## **V. Volatiles from eggplants infested by *Aphis gossypii* induce oviposition behavior in the aphidophagous gall midge *Aphidoletes aphidimyza***

### **Introduction**

Host location and oviposition by female aphidophagous predators are crucial for offspring performance. The choice of habitat, host plant, aphid species, aphid colony size, visual and chemical stimuli, and oviposition site must be considered during the searching and egg-laying behavior of syrphid predators (Almohamad *et al.* 2009). Factors that determine such choice include compounds that stimulate oviposition by aphidophagous insects. Honeydew from *Sitobion avenae* (F.) is a strong contact oviposition stimulant for the syrphid *Episyrphus balteatus* (De Geer) (Budenberg and Powell 1992). Field observations and laboratory studies indicate that both *Adalia bipunctata* L. and *Coccinella septempunctata* L. respond to aphid cues and tend to oviposit close to aphid colonies (Dimetry and Mansour 1976; Evans and Dixon 1986).

*Aphidoletes aphidimyza* (Rondani) (Diptera: Cecidomyiidae) is a common, widely distributed pest species. Female *A. aphidimyza* lay their eggs on the undersides of plant leaves with aphid colonies (Makkula and Tittanen 1985; Lucas and Brodeur 1999; Choi *et al.* 2004). The hatched larvae immediately look for aphids on which to prey.

*Aphidoletes aphidimyza* is an effective natural enemy used to control aphid pests infesting greenhouse crops (Meadow *et al.* 1985; Morse and Croft 1987; Nijveldt 1988). However, the release of *A. aphidimyza* in greenhouses often does not control aphids adequately because the aphids multiply very quickly on greenhouse vegetables, such as cucumbers or eggplants. To overcome this problem, predators can be introduced using banker plant or open-rearing systems (Hansen 1983; Bennison 1992; Yano 2006). These are systems used to maintain the natural enemy in a greenhouse using alternative prey and a banker plant. In a banker plant system, *A. aphidimyza* females produced by this system are expected to move to eggplants immediately after infestation by aphids.

In a banker plant system, the natural enemy should move quickly from the banker plant to the food crop to oviposit when pests establish themselves in the crop. When *A. aphidimyza* prefers an alternative prey, it is necessary to attract this species to the crop plants to oviposit using behavioral control cues. *A. aphidimyza* females are attracted to volatile chemicals from honeydew excreted by *Myzus persicae* (Sulzer) on sweet pepper under laboratory conditions, but the chemical compounds in this attractant have not been identified (Choi *et al.* 2004). We showed that volatile chemicals emitted from honeydew produced by *Aphis gossypii* Glover attracted *A. aphidimyza* and induced

oviposition on filters in an olfactometer (Watanabe *et al.* 2016). We identified phenyl acetaldehyde from honeydew excreted by *A. gossypii* as an *A. aphidimyza* attractant (Watanabe *et al.* 2016). However, this compound did not induce oviposition by *A. aphidimyza* females. Since the olfactometer used by Watanabe *et al.* (2016) was designed for detecting the attractive response of *A. aphidimyza*, bioassay methods for detecting oviposition stimulants need to be developed for the quantitative evaluation of oviposition stimulants.

Therefore, this study investigated the chemical cues produced by the interaction between the eggplant (*Solanum melongena* L.) and *A. gossypii* to identify chemicals that induce oviposition by *A. aphidimyza* females. As the candidates are volatile chemicals produced by the interaction between the eggplant and *A. gossypii*, then both *A. gossypii*-induced volatiles and honeydew volatiles have to be considered. Here, gas chromatography coupled with mass spectrometry (GC/MS) analyses and bioassays using a newly designed chamber were used to identify volatile compounds inducing ovipositional behavior. The identified compounds could be used to control the ovipositional behavior of *A. aphidimyza*, and such behavioral control should contribute to the biological control of this pest.

## Material and methods

### Insects and plants

Larvae of *A. aphidimyza* attacking turnip aphids, *Lipaphis erysimi* (Kaltenbach), were collected on potherb mustard (*Brassica rapa* L. var. *nipposinica*) in Nantan, Kyoto Prefecture, on August 25, 2005. *Aphis gossypii* were collected from strawberry (*Fragaria × ananassa* Duchesne) plants in Ayabe, Kyoto Prefecture, on April 21, 2006. The *A. gossypii* were reared for successive generations on eggplant plants (cv. Senryo Nigou) in a plastic cage [30 × 25 × 30 cm (width × length × height)] at 25°C with an L16:D8 photoperiod. *Aphidoletes aphidimyza* was reared on *A. gossypii* for successive generations under the same environmental conditions. All plants used for rearing insects and for the experiments were grown in a glasshouse at the Nara campus of Kinki University.

### Ovipositional responses to natural odor sources

An eggplant plant subject to one of the following treatments was placed in the center of a 30 × 25 × 30 cm plastic cage as an odor source: 1) an uninfested eggplant (7 cm high); 2) an eggplant infested with 200 aphids (fourth-instar nymphs or adults) for 48 h

(eggplant-aphid complex); 3) an infested eggplant without aphids and their associated products (infested eggplants); and 4) an uninfested eggplant with honeydew on a Petri dish (honeydew). The infested eggplants without aphids and their associated products were prepared by infesting a plant with 200 aphids for 48 h and then flushing the plant with water to remove the aphids and honeydew. We collected honeydew excreted by *A. gossypii* of unspecified ages feeding on eggplants in a glass Petri dish (9 cm diameter, 1.5 cm high) placed below a leaf holding about 1000 aphids for 48 h at 25°C with a L16:D8 photoperiod. We removed any impurities (dead aphids or exuviate) from the samples with forceps and performed bioassays within 24 h of collection. The Petri dish with honey was placed in a plastic box [13.5 × 10 × 3.5 cm (width × length × height)] with a lid that had two square 3 × 3 cm holes covered with a fine nylon filter (0.4-mm mesh). We put the plastic box under an uninfested eggplant. Then, we released five mated female *A. aphidimyza*. After 48 h, we counted the number of eggs laid on the eggplant and at other places in the cage. Mated female adults were obtained as described by Watanabe *et al.* (2016). To feed the *A. aphidimyza* females, 20% sucrose solution in tissue paper balls was placed in 100-mL vials at the four corners of the plastic cage. We replicated each treatment 10 times. Experiments were carried out in an environmental chamber maintained at 25°C with a L16:D8 photoperiod. The numbers of oviposited eggs were analyzed by analysis of variance after the Box–Cox transformation. Means were separated using Tukey’s honestly significant difference (HSD) test. The difference in the numbers of eggs laid on the eggplant versus other places in cages was analyzed using a paired *t*-test. Statistical analyses were performed using R3.0.1.

### **Analyses of volatiles from infested eggplants**

We analyzed the chemical composition of the volatiles from infested eggplants to identify compounds inducing oviposition. Volatiles were collected in an environmental chamber (25°C, without light). A piece of filter paper infiltrated with a 1 µL solution of tridecane in hexane (0.1 mg/mL) was used as an internal standard. We placed a potted eggplant and filter paper in a glass bottle (1 L) that had two holes connected to two nozzles. One nozzle was connected to an air cylinder and the other nozzle was connected to a glass tube (4.0 mm i.d., 177 mm length) filled with Tenax TA (60/80 mesh; Gerstel GmbH & Co. KG). Pure air gas from the cylinder was drawn into the glass bottle, and volatiles from the bottle headspace were collected at a flow rate of 100 mL/min for 2 h. We collected volatiles from an uninfested eggplant as a control. The volatile collection was repeated 7 and 11 times for uninfested and infested eggplants,

respectively, using different odor samples.

### **Chemical analyses of volatiles from infested eggplants**

The collected volatile compounds were analyzed by GC-MS [Agilent Technologies GC 6890 with an HP-5MS capillary column (30 m long, 0.25 mm I.D. and 0.25  $\mu$ m film thickness) and Agilent Technologies MS 5973 mass selective detector, 70 eV] equipped with a thermal desorption system (TDS), cooled injection system (CIS), and cold trap system (CTS) (Gerstel GmbH & Co. KG). The collected volatiles were released from the Tenax TA by heating in the TDS at 250°C for 4 min, within a He flow. The desorbed compounds were collected in the CIS at -150°C, and then the collected compounds were released from the CIS by heating. The desorbed compounds were collected again in the CTS at -50°C, and then flash heating of the CTS resulted in sharp injection of the compounds into the capillary column of the gas chromatograph to which the CTS was connected. The oven temperature of the GC was programmed to rise from 40°C (9 min hold) to 280°C at 10°C/min. The compounds were identified by comparing their spectra with mass spectra in a database (Wiley7), and the retention times and mass spectra with those of synthetic compounds.

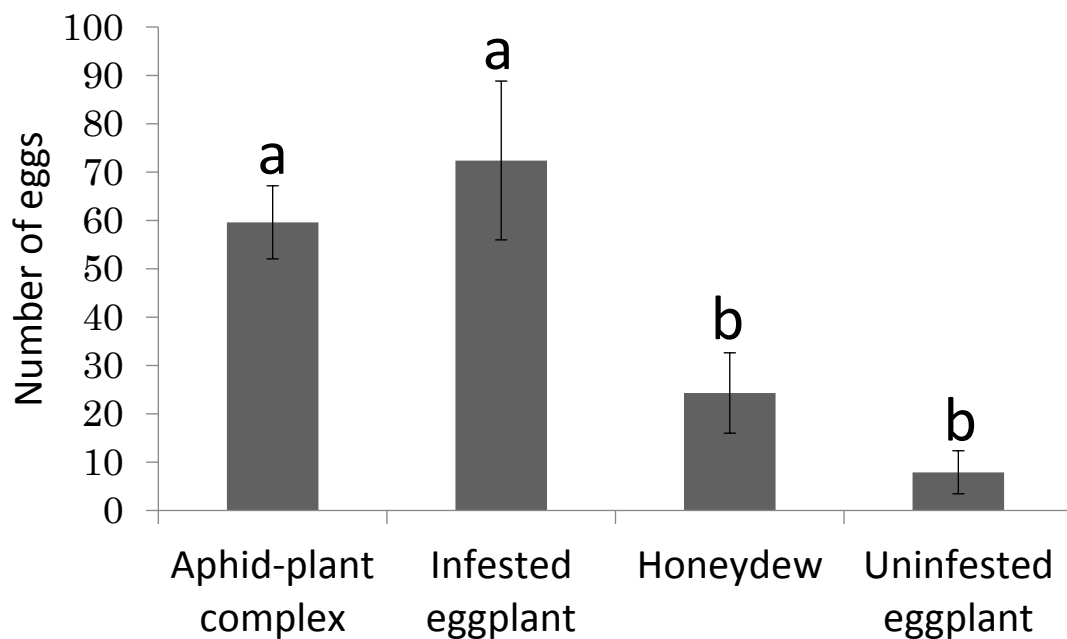
### **Ovipositional responses to synthetic compounds**

Since the amounts of three compounds (6-methyl-5-hepten-2-one, nonanal, and decanal) were found to be significantly higher in the headspace of infested plants than in the headspace of uninfested plants, these three compounds were used as the odor source for bioassays. Their effects on inducing oviposition by *A. aphidimyza* were tested using the same method as for the odor sources described above. The three compounds were dissolved together in triethyl citrate (TEC) [0.2 g, concentration of each compound 0.01  $\mu$ g/g] and absorbed by two pieces of filter paper (2  $\times$  2 cm) attached to the tip of a metal rod (7 cm high), which was put on the soil beside the eggplant. The amount was similar to the quantity found in the headspace of infested eggplants. We used two pieces of filter paper to adsorb 0.2 g of TEC as a control. We replicated each treatment 10 times. The experiments were conducted in an environmental chamber maintained at 25°C with a L16:D8 photoperiod. We used Student's *t*-test to compare the numbers of oviposited eggs. Differences in the numbers of eggs laid on the eggplant compared with other places were analyzed using paired *t*-tests. The statistical analyses were performed using R3.0.1.

## Results

### Ovipositional responses to natural odor sources

Since the assumption of homoscedasticity of the data for the numbers of oviposited eggs was not satisfied, the data were transformed using the Box–Cox transformation. The differences in the numbers of oviposited eggs among the four treatments were significant ( $F_{3,36} = 15.34$ ,  $P < 0.01$ ). Significantly more eggs were laid in the eggplant-aphid complex and infested eggplant treatments than in the honeydew and uninfested eggplant treatments (Fig. 2, Table 7). In the infested eggplant treatment, significantly more eggs were laid on the eggplant than at other places in the cage (Table 7). In the honeydew treatment, all eggs were laid at other places in the cage and no eggs were laid on the eggplant (Table 7). In the other treatments, the number of eggs laid on the eggplant did not differ significantly from the number laid at other places in the cage (Table 7).



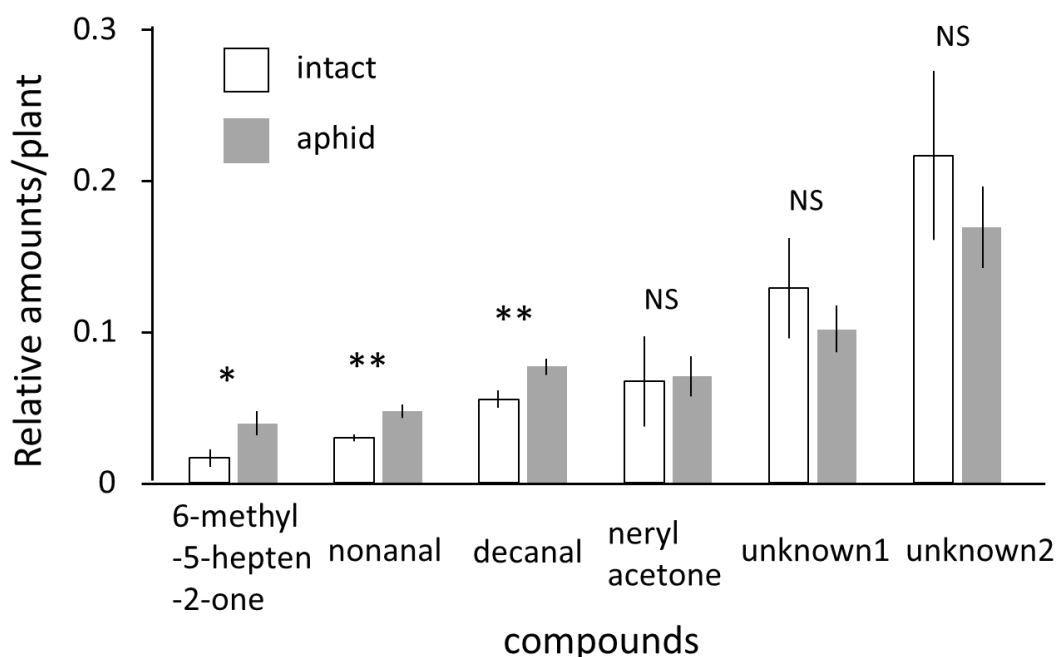
**Fig. 2** Number of oviposited *A. aphidimyza* eggs induced by each treatment. Data are the mean  $\pm$  SE ( $n = 10$ ). Mean numbers of eggs followed by the same letter for different treatments are not significantly different (Tukey's HSD test,  $P > 0.05$ ).

**Table 7** Mean ( $\pm$  SE) number of eggs of *A. aphidimyza* laid on eggplant and other places in four treatments.

Treatment	Number of eggs laid on		Total number of eggs	n	t	P
	Eggplant	Other places				
Aphid-plant complex	54.7 $\pm$ 7.9	2.2 $\pm$ 0.7	56.9 $\pm$ 7.6	10	2.13	0.062
Infested eggplant	60.5 $\pm$ 19.4	11.9 $\pm$ 4.5	72.4 $\pm$ 16.4	10	6.27	< 0.001
Honeydew	0.0	24.3 $\pm$ 8.3	24.3 $\pm$ 8.3	10	-2.92	0.009
Uninfested eggplant	4.7 $\pm$ 4.5	3.2 $\pm$ 1.7	7.9 $\pm$ 4.5	10	0.29	0.775

### Analyses of volatiles from infested eggplants

We detected six compounds (6-methyl-5-hepten-2-one, nonanal, decanal, neryl acetone, and two unidentified compounds) in the headspace volatiles from eggplants. Of these, significantly more of the first three compounds were emitted from aphid-infested eggplants than from uninfested control plants ( $t = -2.05095$ ,  $df = 16$ ,  $P < 0.05$  for 6-methyl-5-hepten-2-one;  $t = -3.17213$ ,  $df = 16$ ,  $P < 0.01$  for nonanal; and  $t = -2.63771$ ,  $df = 16$ ,  $P < 0.01$  for decanal; Fig. 3).



**Fig. 3** Headspace volatiles emitted from eggplants infested with *Aphis gossypii* for 2 days and uninfested plants as controls. Relative emission levels were determined with tridecane as an internal standard. Data represent the mean  $\pm$  standard error ( $n = 7-11$ ). \*  $P < 0.05$ , \*\*  $P < 0.01$ , NS, not significantly different.

### Ovipositional responses to synthetic compounds

The mixture of the three compounds induced the oviposition significantly more eggs than did the control ( $t = -6.0213$ ,  $df = 18$ ,  $P < 0.001$ ; Table 8). In treatment with the mixture of the three compounds, no eggs were oviposited on the eggplant (Table 8). In the control, although only a few eggs were laid; the number of eggs laid on the eggplant was not significantly different from that at other places in the cage (Table 8).

**Table 8** Mean ( $\pm$  SE) number of eggs of *A. aphidimyza* laid on eggplant and other places in treatment of mixture of three compounds and control (TEC).

Treatment	Number of eggs laid on		Total number of eggs	n	t	P
	Eggplant	Other places				
Mixture of three compounds	0.0	31.0 $\pm$ 3.7	31.0 $\pm$ 3.7	10	8.38	< 0.001
Control (TEC)	2.9 $\pm$ 2.3	1.5 $\pm$ 1.2	4.4 $\pm$ 2.4	10	0.50	0.627

### Discussion

This study found that volatiles from the aphid-plant complex and infested eggplants induced the oviposition of *A. aphidimyza* females. Odors from the aphid-plant complex are derived from the odors of honeydew and the infested eggplant themselves. In the experiment, the honeydew treatment induced oviposition, but the number of eggs laid was not significantly different from that in the control treatment with an uninfested eggplant. Therefore, the oviposition stimulants are thought to be derived mainly from *A. gossypii*-infested eggplant volatiles. As mentioned above, several oviposition stimulants of aphidophagous insects are known. However, no other studies have identified both attractants and oviposition stimulants. In addition, this is the first finding that aphid-induced plant volatiles stimulate oviposition by an aphidophagous predator.

Three compounds (6-methyl-5-hepten-2-one, nonanal, and decanal) were detected from infested eggplants. The mixture of these compounds induced oviposition by *A. aphidimyza* females. Therefore, one or more of these compounds stimulate *A. aphidimyza* oviposition. 6-Methyl-5-hepten-2-one is a floral scent that attracts lepidopterans (Andersson *et al.* 2002). Nonanal and decanal are also floral scents or foliar volatiles. Nonanal and decanal contained in the floral scents of *Jatropha curcas* (L.) attracted *Apis cerana* (F.) (Luo *et al.* 2013). The foliar volatiles of *Cinnamomum camphora*, which contain both, attracted *Graphium sarpedon nipponum* (Li *et al.* 2010). Nonanal and decanal were also found in volatiles of honeydew excreted by *A. gossypii*.



(Watanabe *et al.* 2016). They might have caused the oviposition behavior of *A. aphidimyza* females in the oviposition induction experiments conducted in the current study. The differences in the effects of oviposition induction in these experiments might be due to quantitative effects of honeydew volatiles. From our results and those of Watanabe *et al.* (2016), we concluded that phenylacetaldehyde in honeydew is used as an attractant to locate aphid colonies, and that 6-methyl-5-hepten-2-one, nonanal, and decanal in infested plant volatiles induce oviposition. Since both herbivore-induced plant volatiles and honeydew are produced by the interaction between plants and aphids, both volatiles can be used in oviposition site selection by *A. aphidimyza*.

When oviposition stimulants such as honeydew or a mixture of the three synthetic compounds were introduced separately from the plant, *A. aphidimyza* eggs were not oviposited on the treated plant, but were oviposited at other places in the cage. The concentration gradients of these oviposition stimulants might have been different from the natural gradients with their release from plants. The oviposition stimulants in the small closed space in the cage might have caused irregular oviposition site selection by *A. aphidimyza*. Moreover, it is possible that the oviposition stimulants lack attractant effects.

If oviposition stimulants are introduced near aphid colonies in a greenhouse with attractants, the attracted *A. aphidimyza* might oviposit more eggs than under natural conditions because of the effects of the oviposition stimulants.

## VI. General discussion

The first objective of this study was to investigate oviposition selection by *A. aphidimyza* females that were offered *A. gossypii* on eggplants and either *R. padi* on barley or *M. sacchari* on sorghum plants. The second objective of this study was to investigate the chemical cues that induce attraction and oviposition of female *A. aphidimyza*.

*Aphidoletes aphidimyza* females laid more eggs on eggplant with *A. gossypii* than on barley plants with *R. padi* when offered a choice between the two. These results indicate that *R. padi* on barley is a suitable combination to use as a banker plant system. Oviposition by *A. aphidimyza* females is known to be affected by aphid density (Lucas and Brodeur 1999; Jandricic et al. 2013; Guo et al. 2014) and conditioning by feeding experience in larval stages (Mansour 1975). In our study, however, *A. aphidimyza* showed an apparently innate oviposition preference for *A. gossypii* over *R. padi* regardless of density ratio or larval conditioning.

In a laboratory-based experiment using *M. sacchari* as an alternative prey and sorghum as a banker plant, *A. aphidimyza* always laid more eggs on sorghum with *M. sacchari* than on eggplant with *A. gossypii*, except at the highest density ratio of *A. gossypii* to *M. sacchari*. However, in greenhouse experiments where equal numbers of both aphid species were provided, *A. aphidimyza* laid more eggs on eggplant with *A. gossypii* than on sorghum with *M. sacchari*. This discrepancy between the results of greenhouse and laboratory experiments may be due to differences in temperature, humidity, and plant architecture. In the greenhouse, temperature and humidity conditions are variable, and are not always suitable for the survival of *A. aphidimyza*. During the day, *A. aphidimyza* must avoid direct sunlight on plants. *Aphidoletes aphidimyza* adults are known to be active at night and to prefer to lay eggs on the undersides of leaves (Mansour 1976). Eggplant leaves are wider than sorghum leaves, providing suitable shelter during the day and oviposition sites at night. The results of greenhouse experiments using *M. sacchari* and sorghum indicate that this combination would be suitable for use as a banker plant system during the hot season. However, *A. aphidimyza* may miss small colonies of the pest aphid, which are less attractive than large colonies of the alternative prey.

We showed that the volatile chemicals emitted from honeydew produced by *A. gossypii* attracted *A. aphidimyza* and induced females to oviposit on the filters in the olfactometer. We identified phenylacetaldehyde as an *A. aphidimyza* attractant in honeydew excreted by *A. gossypii*. However, this compound did not induce oviposition

in *A. aphidimyza* females. The olfactometer used to search for attractants was designed to detect *A. aphidimyza*'s attraction response; new bioassay methods that are tailored to detecting an oviposition response should be developed for quantitative evaluation of oviposition stimulants. When using an olfactometer to detect attractants, we observed the behavioral response to odors using air flow. In contrast, the experimental system used to detect oviposition stimulants allows us to observe behavioral responses only to natural diffusion of odors.

We found that oviposition behavior was induced by eggplants infested with *A. gossypii*. Moreover, using gas chromatography–mass spectrometry (GC/MS), we identified 6-methyl-5-hepten-2-one, nonanal, and decanal as compounds involved in stimulating oviposition. In laboratory experiments, mixtures of these compounds induced oviposition in *A. aphidimyza*. One of these compounds, or a mixture of two or more, is likely to be the oviposition stimulant of *A. aphidimyza*. 6-methyl-5-hepten-2-one is a floral scent and is known to attract lepidopterans (Andersson et al. 2002). Nonanal and decanal are floral scents or foliar volatiles. Nonanal and decanal in the floral scent of *Jatropha curcas* (L.) attract *Apis cerana* (F.) (Luo et al. 2013); foliar volatiles of *Cinnamomum camphora* containing these compounds attract *Graphium sarpedon nipponum* (Li et al. 2010). Nonanal and decanal were also found in volatiles of honeydew excreted by *A. gossypii*. These compounds might be responsible for the oviposition behavior observed in *A. aphidimyza* females in the olfactometer experiments and oviposition induction experiments. The observed differences in the effects of oviposition induction might be due to quantitative effects of honeydew volatiles. From the results of these experiments, it can be concluded that *A. aphidimyza* uses phenylacetaldehyde in honeydew as an attractant to locate aphid colonies, and 6-methyl-5-hepten-2-one, nonanal, and decanal from *A. gossypii* honeydew and eggplants infested with this species to induce oviposition.

Oviposition selection by *A. aphidimyza* females among different aphid species on host plants may be affected by differences in the quality and quantity of the attractants and oviposition stimulants of *A. aphidimyza*, which are derived from volatiles produced by aphid–plant complexes. Honeydews from different aphid species have different sugar components (Watanabe et al. 2014); similarly, the quality and quantity of attractants and oviposition stimulants of *A. aphidimyza* may vary among aphid species.

Through our research, we were able to clarify the mechanisms that determine oviposition behavior in *A. aphidimyza*. For practical implementation of the *A. aphidimyza* attractants and oviposition stimulants discovered here, it will be necessary to determine the best method for introducing these compounds into greenhouse systems.

The methods used in this study to detect the attractants and oviposition stimulants of *A. aphidimyza* can be applied to identify chemicals that can be implemented for behavioral control of other natural enemies of crop pests. In the future, research on behavioral control of natural enemies is likely to be important in promoting integrated pest management.

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## Summary

*Aphidoletes aphidimyza* (Rondani) (Diptera: Cecidomyiidae) has predaceous larval stages and is used to control aphid pests infesting greenhouse crops. However, the release of *A. aphidimyza* to control aphids often does not provide adequate control because aphids multiply very quickly on greenhouse vegetables such as cucumbers or eggplants. A banker plant system has been developed to introduce natural enemies for pest control in protected cultures. This system allows natural enemies to be maintained in a greenhouse using alternative prey on banker plants. Populations of natural enemies remain stable on the banker plant system irrespective of presence of pests on crops, providing a continuous source of predators in case of crop infestation. For these systems to function, the natural enemy should move quickly from the banker plant to the food crop when pests occur. Therefore, oviposition selection is a critical factor for the effectiveness of banker plant systems using *A. aphidimyza*. The first objective of this study was to investigate oviposition selection by *A. aphidimyza* females that were offered the pest aphid *A. gossypii* on eggplants and the alternative prey aphid *R. padi* on a banker plant, barley and *M. sacchari* on a banker plant, sorghum.

When *A. aphidimyza* prefers the alternative prey, or the pest aphid occurs at too low a density to be found by *A. aphidimyza*, the system can be made effective by controlling the behavior of *A. aphidimyza* females produced in the banker plant system to attract them to crops with pest aphids. Aphidophagous predators must be able to locate hosts in order to survive and reproduce. Different species have individual strategies for finding hosts using chemical cues. However, the chemical compounds of attractants and oviposition stimulants of *A. aphidimyza* have not been identified. The second objective of this study was to investigate the volatile chemicals produced by the interaction between *A. gossypii* and eggplants that induce attraction and oviposition of females of *A. aphidimyza*.

### **Oviposition selection by *Aphidoletes aphidimyza* (Diptera: Cecidomyiidae) on banker plants with alternative prey aphids or crop plants with pest aphids**

A banker plant system, using *A. aphidimyza* maintained on barley plants infested with *R. padi*, has been developed to control *A. gossypii* Glover infestations of eggplants in greenhouses. For this system to be successful, recently emerged *A. aphidimyza* females should move quickly from barley to eggplants. *Aphidoletes aphidimyza* females laid more eggs on eggplants with *A. gossypii* than on barley plants with *R. padi* when offered both aphid–plant combinations both in the laboratory and greenhouse

experiments. Neither conditioning of *A. aphidimyza* to *A. gossypii* or *R. padi* by larval feeding experience nor the density ratio between the two aphid species affected the oviposition preference of *A. aphidimyza* females for *A. gossypii*. This combination of crop–pest and banker plant–alternative prey system enables effective dispersal of *A. aphidimyza*.

### **Oviposition selection of *Aphidoletes aphidimyza* (Diptera: Cecidomyiidae) on a banker plant system of sorghum with *Melanaphis sacchari* (Hemiptera: Aphididae)**

To control *A. gossypii* infestations on eggplants during the hot season in greenhouses, a banker plant system for *A. aphidimyza* was developed using *Melanaphis sacchari* (Zehntner) as an alternative prey on sorghum as a banker plant. We examined the effects of the density ratio of *A. gossypii* to *M. sacchari* on oviposition selection by *A. aphidimyza* between *A. gossypii* on eggplant and *M. sacchari* on sorghum in the laboratory. *A. aphidimyza* always laid more eggs on sorghum with *M. sacchari* than on eggplant with *A. gossypii*, except when the density ratio of *A. gossypii* to *M. sacchari* was extremely high. In greenhouse experiments where equal numbers of both aphid species were provided, *A. aphidimyza* laid more eggs on eggplant with *A. gossypii* than on sorghum with *M. sacchari*. The results of greenhouse experiments using the combination of *M. sacchari* and sorghum seem to indicate that this combination is suitable for use as a banker plant system during the hot season.

### **An attractant of the aphidophagous gall midge *Aphidoletes aphidimyza* in the honeydew of *Aphis gossypii***

Many natural enemies of aphids use honeydew as a volatile cue to locate a host or prey, as an oviposition stimulant, and as a foraging arrestant. Previous studies have shown that honeydew excreted by the aphid *M. persicae* attracts female *A. aphidimyza*, but the specific attractants have not been identified. We used an olfactometer to study the responses of female *A. aphidimyza* to volatiles emitted from honeydew excreted by the aphid *Aphis gossypii* on eggplants. The volatiles attracted female midges and induced oviposition. Moreover, using gas chromatography–mass spectrometry (GC/MS), we identified phenylacetaldehyde as the attractant compound in the honeydew, although it did not induce oviposition in olfactometer experiments.

### **Volatiles from eggplants infested by *Aphis gossypii* induce oviposition behavior in the aphidophagous gall midge *Aphidoletes aphidimyza***

Host location and oviposition by female aphidophagous predators are crucial steps in

reproduction. Previously, we identified phenylacetaldehyde from honeydew excreted by *Aphis gossypii* Glover as an attractant of *A. aphidimyza*. However, this compound did not induce oviposition by *A. aphidimyza* females. Therefore, we studied the effects of volatile chemicals produced by the interaction between the eggplant and *A. gossypii* on the oviposition behavior of *A. aphidimyza* in a bioassay chamber. We found that volatiles from *A. gossypii*-infested eggplants and from infested eggplants from which aphids and their associated products had been removed induced oviposition behavior. We determined that this behavior was induced by herbivore-induced plant volatiles from eggplants. Moreover, using gas chromatography–mass spectrometry (GC/MS), we identified 6-methyl-5-hepten-2-one, nonanal, and decanal as the herbivore-induced plant volatiles from eggplants; a mixture of these three synthetic compounds induced oviposition by *A. aphidimyza* under laboratory conditions.