Occurrence of diamondback moths *Plutella xylostella* and their parasitoid wasps *Cotesia vestalis* in mizuna greenhouses and their surrounding areas

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Author Contribution

JA, MU and JT conceived research. A, MU, YK and KS conducted experiments. JA and MU analysed field data and conducted statistical analyses. JT wrote the manuscript. JT secured funding. All authors read and approved the manuscript.

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Short title: Seasonal variations in pest insects and parasitoid wasps

Abstract

Pest insects invade commercial greenhouses from the surrounding areas. We aimed to clarify whether (1) the seasonal population dynamics of local pest insects affects their greenhouse invasions and (2) carnivorous natural enemies of the pests mirror this invasion. We compared the occurrence of diamondback moth [DBM: *Plutella xylostella* (Lepidoptera: Plutellidae)] larvae and their native parasitoid wasps Cotesia vestalis (Hymenoptera: Braconidae) on mizuna plants [*Brassica rapa* var. *laciniifolia* (Brassicales: Brassicaceae)] in commercial greenhouses, with their population dynamics on a wild cruciferous weed *Rorippa indica* (Brassicales: Brassicaceae) in satoyama in Kyoto, Japan. *C. vestalis* followed the occurrence of DBM larvae on both mizuna and *R. indica*; no *C. vestalis* were recorded in greenhouses free from DBM larvae. *C. vestalis* females were more attracted to volatiles emitted from DBM-infested than from uninfested mizuna. However, the presence of DBM in greenhouses could not always be explained by its seasonal population dynamics in the surroundings.

Keywords

Plutella xylostella (Lepidoptera: Plutellidae), *Cotesia vestalis* (Hymenoptera: Braconidae), invasion, plant volatiles, attractants, seasonal variation

Introduction

The rural "satoyama" forest and village landscape in Japan consists of areas of small-scale wet rice paddy fields, crop fields, and greenhouses (Kobori et al. 2003). Similar agricultural landscapes are also found in other countries (Takeuchi et al. 2003). One of the ecological characteristics of satoyama environments is that populations of several pest insects are harbored in the surrounding natural areas (Katoh et al. 2009), and invasions of these pest insects in greenhouses from the surroundings are common. Here, an intriguing question is whether the seasonal population dynamics of pest insects in the surrounding natural environment affects the incidence of pest invasions in greenhouses.

In satoyama areas, carnivorous natural enemies of pest insects also live in the natural environment (Kagawa and Maeto 2009). Several studies have shown that, in response to damage caused by herbivorous arthropods, plants start emitting so-called "herbivory-induced plant volatiles (HIPVs)" that attract the carnivorous natural enemies of the currently infesting herbivores (Arimura et al. 2009; Dicke et al. 1990; Hare 2011; McCormick et al. 2012; Takabayashi and Dicke 1996). The attraction capability of some of these HIPVs has been confirmed under field conditions (James 2003; James and Grasswitz 2005; James and Price 2004; Rodriguez-Saona et al. 2011; Uefune et al. 2012). Most of the crops infested by pest insects in greenhouses start emitting carnivore-attractive HIPVs. However, the question of whether such HIPVs attract natural enemies from the surroundings into the greenhouses remained unanswered.

To clarify the above two questions, we conducted field experiments in greenhouses in the "Miyama" satoyama area in the Kyoto Prefecture of Japan (35.3°N, 135.5°E), where a cruciferous crop, "mizuna" [*Brassica rapa* var. *laciniifolia*

83 (Brassicales: Brassicaceae)] was produced; pesticides were not routinely applied in
84 these greenhouses, but only to control observed outbreaks of pest insects as part of an
85 integrated pest management strategy. Populations of diamondback moth (DBM)
86 [*Plutella xylostella* (Lepidoptera: Plutellidae)] live in the surrounding area, and DBM
87 larvae are one of the most important pests of mizuna plants in greenhouses in Miyama
88 (J. Abe, personal observation). Populations of *Cotesia vestalis* (Hymenoptera:
89 Braconidae), a native parasitoid wasp of DBM larvae (Furlong et al. 2013; Talekar and
90 Shelton 1993), are also harbored in the Miyama area (J. Abe, personal observation). We
91 have previously reported that HIPVs emitted from komatsuna plants (*B. rapa*
92 var. *perviridis*, i.e., the same species as mizuna but a different cultivar) infested by
93 DBM larvae attract *C. vestalis* under both laboratory and greenhouse conditions (Ohara
94 et al. 2017; 2018; Uefune et al. 2012; Yoneya et al. 2018).
95 We observed the occurrence of DBM and *C. vestalis* on mizuna plants in four
96 commercial greenhouses and on *Rorippa indica* (Brassicales: Brassicaceae) plants in the
97 surrounding area in Miyama. Further, we tested whether mizuna plants infested by
98 DBM larvae attracted *C. vestalis*. Based on these data, together with our previously
99 reported results on the olfactory response of *C. vestalis* to DBM larvae-infested crucifer
100 plants, we discuss the relationship between the occurrence of DBM larvae and *C.*
101 *vestalis* in greenhouses and the surrounding areas.
102
103 **Materials and Methods**
104 **Field observation**
105 We used four greenhouses owned by one farmer, set in a "dice four" arrangement with
106 2–3 m distance between each. They were surrounded by open agricultural fields and a

107 thicket with a river, and DBM and *C. vestalis* were believed to inhabit these areas
108 (Supplemental Figure 1). Mizuna plants were grown in these greenhouses, but since the
109 growth stages in the four greenhouses differed, we treated the data from each
110 greenhouse independently.

111 The occurrence of DBM larvae and *C. vestalis* cocoons on mizuna plants in
112 the greenhouses and their surrounding areas were observed. Observations were made
113 approximately every 7–14 days during the observation period. In greenhouses, when
114 plants had fewer than 10 leaves, 11 to 30 leaves, or more than 30 leaves, we assessed
115 100, 50, and 20 plants, respectively. We also counted the numbers of DBM larvae and *C.*
116 *vestalis* cocoons on a wild cruciferous species, *Rorippa indica*, which was growing in
117 the surrounding area, up to around 3 m away from the tested greenhouses. DBM larvae
118 found on *R. indica* plants and mizuna plants were reared in a climate-controlled room in
119 the laboratory (25 ± 2 °C, 50–60% RH, 16L:8D), to check the incidence of parasitism.
120

121 **Laboratory experiments**

122 *Insects and plants*

123 *Plutella xylostella* larvae were collected from fields in Ayabe, Kyoto, Japan (35°N,
124 135°E) in 2001, and were reared with potted komatsuna plants [*Brassica rapa*
125 var. *perviridis* (Brassicales: Brassicaceae)] in a climate-controlled room (25 ± 3°C, 60 ±
126 10% RH, 16L:8D). The laboratory colony of DBM was reared on potted komatsuna
127 plants in a climate-controlled room (25 ± 3 °C, 60 ± 10% RH, 16L:8D) to obtain eggs.
128 Newly emerged adults of DBM were maintained in acrylic cages (35 cm × 25 cm × 30
129 cm high) in a climate-controlled room (25 ± 3 °C, 60 ± 10% RH, 16L:8D) and provided
130 with a 50% (v/v) honey solution as food and potted komatsuna plants, to ensure mating.

131 Komatsuna plants with eggs were collected daily, and hatched larvae were reared on cut
132 komatsuna plants in small cages (25 cm × 15 cm × 10 cm high).
133 *C. vestalis* were obtained from parasitized DBM larvae collected in the same
134 field. Adults of the parasitoid species were maintained separately in plastic cages (30
135 cm × 20 cm × 13 cm high) with a 50% (v/v) honey solution as food in a
136 climate-controlled room (18 ± 3 °C, 60 ± 10% RH, 16L:8D) for 3 days to ensure mating.
137 The second stadium DBM larvae that were newly parasitized by *C. vestalis* were put in
138 a polypropylene box (25 cm × 15 cm × 10 cm high) with detached komatsuna leaves for
139 food; the leaves were replaced by fresh ones every other day until the egression of *C.*
140 *vestalis* larvae from DBM larvae. After egression, *C. vestalis* formed cocoons in the
141 polypropylene box. Cocoons were collected and kept in closed end glass tubes until
142 emergence. To ensure mating, emerged females were kept together with males in a
143 plastic cage for 3 days. Thereafter, they were maintained in glass tubes at 18 °C to
144 prolong their lifespan, and in continuous darkness to suppress flight. They were a
145 maximum of 10 days old since emergence from the host and were acclimatized for 1–2
146 h in the climate room before the experiments were started.
147 Mizuna (*Brassica rapa* var. *nipposinica* 'Jounan-Sensuji') and komatsuna (*B.*
148 *rapa* var. *perviridis* L. 'Rakuten') plants were cultivated in a greenhouse (25 ± 3 °C, 60
149 ± 10% relative humidity, 16L:8D). Four plants were cultivated in a plastic pot
150 (diameter: 72 mm, depth: 65 mm) for 4–5 weeks; these potted plants were used as the
151 odor sources in the laboratory experiments.
152
153 *Response of C. vestalis to DBM-infested plants*

154 Prior to each experiment, the potted mizuna or komatsuna plants had either remained
155 uninfested or had received damage from one second stadium DBM larva per plant
156 which had been allowed to feed for 24 hours to produce plants with at least one infested
157 edge/leaf. Prior to the tests, the larvae, their silk, and their feces were removed from the
158 infested plants with the aid of a fine brush.

159 *C. vestalis* females were tested for their flight responses towards a pot of
160 DBM-infested mizuna plants versus a pot of uninfested mizuna plants in an acrylic cage
161 (25 × 30 × 35 cm; 3 nylon gauze-covered windows and one door) under fluorescent
162 light (20 W, 3000 lux) in a climate-controlled room (25 ± 3 °C, 60 ± 10% RH, 16L:8D).
163 In the cage, there was no wind. The results of Shiojiri et al. (2000, 2010) show that
164 visual cues are not involved in the flight responses of *C. vestalis* in the choice chamber.

165 Females were released individually from a glass tube (25 mm inner diameter,
166 120 mm length) positioned halfway between two plant pots. Upon their first visit to one
167 of the tested plants (defined as landing), they were removed with an insect aspirator.
168 Ten wasps were tested using the same set of two potted seedlings. Each wasp was only
169 tested once; the experiments were repeated on three or four experimental days with new
170 sets of parasitoids and plants. We also compared the flight response of the parasitoid
171 wasps toward a pot of DBM-infested mizuna plants versus a pot of DBM-infested
172 komatsuna plants in the same manner. Two-choice data under laboratory conditions
173 were analyzed using the replicated *G*-test (Sokal and Rohlf 1995); parasitoids that made
174 no choice for either plant were discarded from this analysis.

175

176 **Results**

177 **Field observations**

178 In the surrounding areas, *R. indica* plants were detected throughout the observation
179 period (Figure 1a: surroundings), with the highest number on August 17. We observed
180 seasonal variation in the occurrence of DBM larvae in the surroundings, with two major
181 peaks (May 9: 246 plants, 0.13 DBM/plant; June 6: 243 plants, 0.20 DBM/plant).
182 Seasonal variation in the occurrence of *C. vestalis* was also observed, with two peaks
183 that were synchronous with those of the DBM (Figure 1: surroundings).
184 In the four greenhouses (Figure 1b-e), most of the plants were mizuna, with a
185 few *R. indica* plants found solely around the edges of the greenhouses. The cyclical
186 changes in the numbers of mizuna plants in the greenhouses represented the planting
187 and harvesting of the mizuna plants. The control threshold (level of infestation at which
188 pesticides were applied to the crop) of DBM in a mizuna greenhouse was set at 0.05
189 DBM/plant (after Abe et al. 2007); hereafter, we define the occurrence of more than
190 0.05 DBM/plant as a peak.
191 In greenhouse 1 (Figure 1b), the occurrence of DBM larvae had five peaks.
192 The dominant occurrence was at the second peak (June 21: 9451 plants; 0.51
193 DBM/plant) and was detected after the dominant peak for DBM had been recorded in
194 the surroundings. At each peak, the occurrence of *C. vestalis* was also detected.
195 In greenhouse 2 (Figure 1c), the occurrence of DBM larvae had three peaks.
196 The dominant occurrence was again at the second peak (June 26: 12331 plants; 0.64
197 DBM/plant). *C. vestalis* was detected at each peak.
198 In greenhouse 3 (Figure 1d), the occurrence of DBM larvae had four peaks.
199 This time, the dominant occurrence was the third peak (July 4: 10962 plants; 0.36
200 DBM/plant); however, the second most dominant peak (June 13: 3053 plants; 0.18

201 DBM/plant) was detected after the dominant peak for DBM had been recorded in the
202 surroundings. *C. vestalis* was detected at each peak.
203 In greenhouse 4 (Figure 1e), there were five peaks in the emergence of DBM
204 larvae, and mizuna production was stopped between May 16 to June 6 and July 19 to
205 October 14, due to outbreaks of DBM. As the occurrence of September 20 consisted of
206 one DBM on an *R. indica* weed when no mizuna plants were being grown in the
207 greenhouse, this was not classified as a peak. The third peak was observed when *C.*
208 *vestalis* was recorded in the surroundings (July 12: 2859 plants; 3.2 DBM/plant). *C.*
209 *vestalis* was detected in the greenhouse at each peak.
210
211 **Olfactory responses of *C. vestalis* to DBM larvae-infested plants**
212 We offered DBM-infested mizuna plants versus uninfested mizuna plants to *C.*
213 *vestalis* females in a choice chamber. *C. vestalis* females preferred infested mizuna
214 plants over uninfested mizuna plants (G_P = 7.1034, df = 1, P = 0.0077; G_H = 0.5977, df
215 = 2, P = 0.7417; G_T = 7.7011, df = 3, P = 0.0526, replicated G-test) (Figure 2: upper
216 bar). We then offered DBM-infested mizuna plants versus DBM-infested komatsuna
217 plants to *C. vestalis* females in a choice chamber. *C. vestalis* females showed an equal
218 distribution between the two odor sources (G_P = 1.6108, df = 1, P = 0.2044; G_H =
219 0.8402, df = 2, P = 0.8398; G_T = 2.4511, df = 3, P = 0.6534, replicated G-test) (Figure
220 2: lower bar).
221
222 **Discussion**
223 Throughout the observation period, DBM larvae were followed by *C. vestalis* on both
224 mizuna plants and *R. indica* plants. This synchronized occurrence was observed in all

225 four greenhouses when DBM occurred at a density of more than 0.05 DBM/plant.
226 Though the seasonal occurrences of DBM larvae and *C. vestalis* in the greenhouses
227 were similar to their seasonal population dynamics in the surrounding areas, some could
228 not be explained by their population dynamics. Thus, while the incidence of the
229 invasion was affected by the surroundings, some took place irrespective of their density
230 in the surrounding areas. Further, there were some differences in the occurrence patterns
231 of DBM even within the four adjacent greenhouses with a dice four arrangement. This
232 could be explained by the differences in the growth stages of the mizuna plants in the
233 four greenhouses. Studies on the time series observations of the invasions of pest insects
234 and their natural enemies in greenhouses, coupled with their seasonal population
235 dynamics in the surrounding satoyama areas, as shown in this study, are not
236 accumulating to date. However, further studies are needed to evaluate the seasonal
237 invasions of pests and the efficacy of native natural enemies that are harbored in the
238 surroundings. Shimomoto (2002) reported that the invasion of five native parasitoid
239 species was observed in eggplant greenhouses in which leafminers (*Liriomyza trifolii*)
240 occurred on eggplants.
241 Notably, native *C. vestalis* were recorded only in the presence of DBM larvae
242 in greenhouses. This specific shadowing by *C. vestalis* would in part be explained by
243 their response to plant volatiles. *C. vestalis* are attracted to volatiles emitted from
244 various crucifer plants infested by DBM larvae under both laboratory (Reddy et al.
245 2002; Shiojiri et al. 2000, 2010; Yoneya et al. 2018) and field conditions (Uefune et al.
246 2012). In the present study, we showed that mizuna plants started attracting *C. vestalis*
247 after being damaged by DBM larvae, and that the attractiveness was equal to that of
248 DBM-infested komatsuna plants (the same species as mizuna). Under experimental field

249 conditions, Uefune et al. (2012) reported that *C. vestalis* females were attracted to
250 HIPVs emitted from DBM-infested cabbage plants. Taken together, volatiles from
251 DBM-infested mizuna plants that are attractive to *C. vestalis* would be one of the factors
252 causing the co-occurrence of DBM larvae and *C. vestalis* in greenhouses.
253 It was concluded that the synchronized invasion of *C. vestalis* did not
254 effectively suppress the DBM larvae in the greenhouses in our study because the DBM
255 density in the greenhouses was more than 0.1 larva/plant at each occurrence. However,
256 Abe et al. (2007) reported that the single release of five *C. vestalis* in experimental
257 greenhouses with 0.05 DBM per komatsuna plant successfully suppressed two
258 successive generations of DBM (i.e., for ca 40 days); in their experiments, there were
259 outbreaks of DBM in the control greenhouses. Based on their study, we hypothesized
260 that, in the present study, the number of *C. vestalis* invading from the surroundings was
261 not high enough to meet the conditions for successful suppression, and that the artificial
262 recruitment of additional *C. vestalis* from the surroundings into greenhouses would be
263 one way to control the DBM. Four volatile compounds [(*Z*)-3-hexenyl acetate, α-pinene,
264 sabinene, and *n*-heptanal] emitted from DBM-infested cabbage plants have been shown
265 to attract *C. vestalis* under both experimental greenhouse and field conditions (Ohara et
266 al. 2017; 2018; Uefune et al. 2012). Whether the use of the volatiles that recruit native *C.*
267 *vestalis* into greenhouses will successfully suppress DBM is the subject of a subsequent
268 study.
269
270 **Conflicts of Interest**
271 The authors declare no conflicts of interest.
272

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354

355 **Figure Captions**

356

357 **Fig. 1** Seasonal changes in the occurrence of diamondback moth (larva and pupal
358 stages) and its larval parasitoid wasp *Cotesia vestalis* in greenhouses and in their
359 surroundings. The numbers of host plants (mizuna and *R. indica*) in both areas are
360 also shown. There were no plants on October 24 (Greenhouse 1), July 19 and
361 September 14-20 (Greenhouse 3), and May 30 (Greenhouse 4). In Greenhouse 4,
362 mizuna production was stopped from May 16 to June 6, and July 19 to October 14,
363 due to outbreaks of DBM

364

365 **Fig. 2** Olfactory responses of *C. vestalis* females to komatsuna crop plants with
366 different treatments. The experiment was repeated on 3 or 4 experimental days
367 (upper and lower bars, respectively); the data were pooled and subjected to a G-test.
368 Numbers next to bars indicate the numbers of *C. vestalis* that responded to the
369 volatiles. ** $0.01 > P > 0.001$; ns: not significantly different

370

371 **Supplemental Fig 1** Map of the four greenhouses. This map was revised from an
372 original one by Google Maps (Google, Menlo Park, California, USA)

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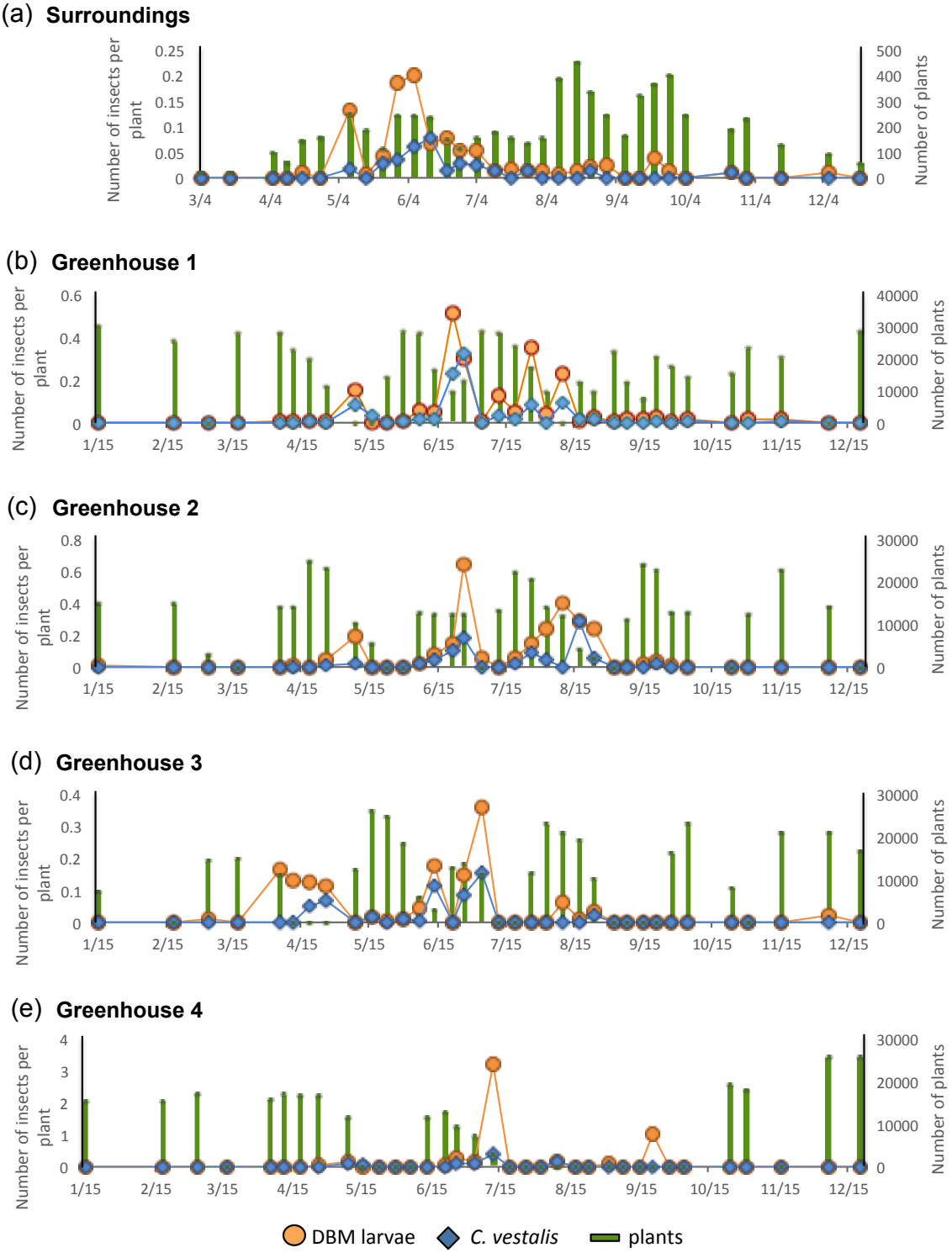
(a) Surroundings
Number of insects per plant
Number of plants
0.25
0.2
0.15
0.1
0.05
0
500
400
300
200
100
0
3/4
4/4
5/4
6/4
7/4
8/4
9/4
10/4
11/4
12/4
(b) Greenhouse 1
Number of insects per plant
Number of plants
0.6
0.4
0.2
0
40000
30000
20000
10000
0
1/15
2/15
3/15
4/15
5/15
6/15
7/15
8/15
9/15
10/15
11/15
12/15
(c) Greenhouse 2
Number of insects per plant
Number of plants
0.8
0.6
0.4
0.2
0
30000
20000
10000
0
1/15
2/15
3/15
4/15
5/15
6/15
7/15
8/15
9/15
10/15
11/15
12/15
(d) Greenhouse 3
Number of insects per plant
Number of plants
0.4
0.3
0.2
0.1
0
30000
20000
10000
0
1/15
2/15
3/15
4/15
5/15
6/15
7/15
8/15
9/15
10/15
11/15
12/15
(e) Greenhouse 4
Number of insects per plant
Number of plants
4
3
2
1
0
30000
20000
10000
0
1/15
2/15
3/15
4/15
5/15
6/15
7/15
8/15
9/15
10/15
11/15
12/15
DBM larvae
C. vestalis
plants

Figure 1

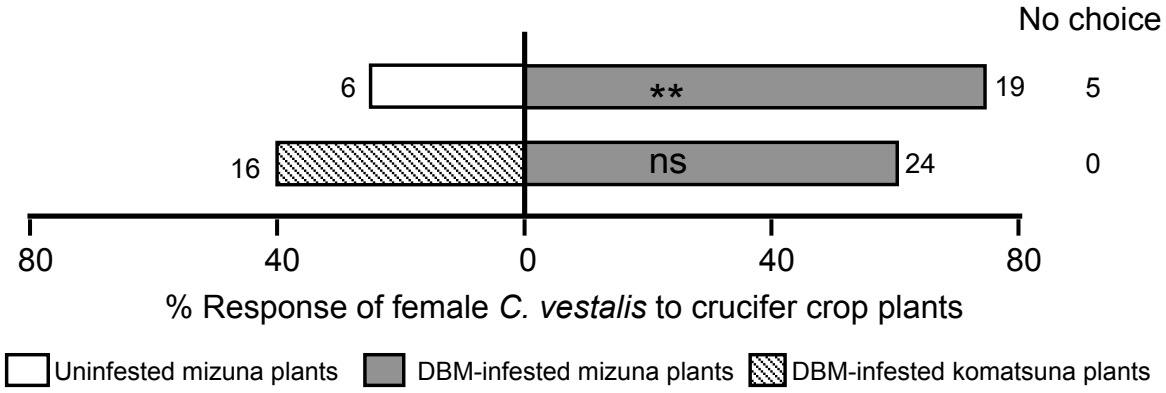
No choice
6
**
19
5
16
ns
24
0
80
40
0
40
80
% Response of female C. vestalis to crucifer crop plants
Uninfested mizuna plants
DBM-infested mizuna plants
DBM-infested komatsuna plants

Figure 2