



Oviposition Experience of Parasitoid Wasps with Nonhost Larvae Affects their Olfactory and Contact-Behavioral Responses toward Host- and Nonhost-Infested Plants

著者 (英)	Saw Steven, Masayoshi Uefune, Rika Ozawa, Junji Takabayashi, Yooichi KAINOH
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1 OVIPOSITION EXPERIENCE OF PARASITOID WASPS WITH NONHOST
2 LARVAE AFFECTS THEIR OLFACTORY AND CONTACT-BEHAVIORAL
3 RESPONSES TOWARD HOST- AND NONHOST-INFESTED PLANTS
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5 SAW STEVEN¹, MASAYOSHI UEFUNE², RIKA OZAWA³, JUNJI TAKABAYASHI³,
6 YOOICHI KAINOH^{4*}
7

8 ¹ *Master's Program in Education, University of Tsukuba, Tsukuba, Ibaraki, 305-8572,*
9 *Japan*

10 ² *Department Agrobiological Resources, Faculty of Agriculture, Meijo University,*
11 *Nagoya, Aichi 468-8502, Japan*

12 ³ *Center for Ecological Research, Kyoto University, Otsu, Shiga, 520-2113, Japan*

13 ⁴ *Faculty of Life and Environmental Sciences, University of Tsukuba, Tsukuba, Ibaraki,*
14 *305-8572, Japan*
15

16 * Corresponding author.

17 Yooichi Kainoh, Faculty of Life and Environmental Sciences, University of Tsukuba,
18 Tsukuba, Ibaraki, 305-8572, Japan, E-mail: kainoh.yooichi.gf@u.tsukuba.ac.jp
19

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28 **Abstract** - In nature, parasitoid wasps encounter and sometimes show oviposition
29 behavior to nonhost species. However, little is known about the effect of such negative
30 incidences on their subsequent host-searching behavior. We tested this effect in a
31 tritrophic system of maize plants (*Zea mays*), common armyworms (hosts), tobacco
32 cutworms (nonhosts), and parasitoid wasps, *Cotesia kariyai*. We used oviposition
33 inexperienced *C. kariyai* and *negative-experienced* individuals that had expressed
34 oviposition behavior toward nonhosts on nonhost-infested maize leaves. We first
35 observed the olfactory behavior of *C. kariyai* to volatiles from host-infested plants or
36 nonhost-infested plants in a wind tunnel. Negative-experienced wasps showed
37 significantly lower rates of taking-off behavior (Step-1), significantly longer duration
38 until landing (Step-2), and lower rates of landing behavior (Step-3) toward nonhost-
39 infested plants than inexperienced wasps, however, the negative-experience did not
40 affect these three steps toward host-infested plants. We concluded that the negative
41 experience reduced the selectivity of the olfactory response in wasps to nonhost-infested
42 plants. The chemical analyses suggested that the wasps associated (*Z*)-3-hexenyl
43 acetate, a compound that was emitted more in nonhost-infested plants, with the negative
44 experience, and reduced their response to nonhost-infested plants. Furthermore, we
45 observed that the searching duration of wasps on either nonhost- or host-infested plants
46 (Step-4) was reduced on both plant types after the negative experiences. Therefore, the
47 negative experience in Step-4 would be nonadaptive for wasps on host-infested plants.
48 Our study indicated that the density (i.e., possible encounters) of nonhost species as
49 well as that of host species in the field should be considered when assessing the host-
50 searching behavior of parasitoid wasps.

51

52 **Key Words** - Tritrophic interaction, negative experience, host-finding behavior, (*Z*)-3-
53 hexenyl acetate.

54

INTRODUCTION

55

56

57 In nature, nonhosts and hosts of parasitoid wasp species coexist in a plant community or
58 even on the same plant. Thus, it is likely that wasps encounter nonhosts, and
59 consequently, may show oviposition behavior to nonhosts (e.g., Takabayashi and
60 Takahashi 1990). However, the effects of such “misunderstandings” on the subsequent
61 host-searching behavior of parasitoid wasps is not well understood. Although the
62 conditions were unnatural, Takasu and Lewis (2003) demonstrated that the flight
63 response of parasitoid wasps *Microplitis croceipes* to vanilla odor was positively
64 affected by their previous oviposition experience in a host (*Helicoverpa zea* larva) in the
65 presence of vanilla odor, but their response was not affected by their previous
66 oviposition experience in a nonhost (*Spodoptera exigua* larva) in the presence of vanilla
67 odor.

68 Carnivorous natural enemies such as parasitoid wasps and other carnivorous
69 species respond to “herbivory-induced plant volatiles” (HIPVs) that are emitted from
70 plants infested with their hosts/prey (Arimura et al. 2009; Hare 2011; McCormick et al.
71 2012; Turlings and Erb 2018). The responses by natural enemies to HIPVs are either
72 due to their innate olfactory preferences (e.g., Geervliet et al. 1996; Shiojiri et al. 2000)
73 or their development of appropriate responses to HIPVs via olfactory learning (e.g.,
74 Takemoto et al. 2012), or both (van Oudenhove 2017 for review; Yoneya et al. 2018).
75 Herein, we studied whether the oviposition behavior of parasitoid wasps to nonhosts on
76 nonhost-infested plants affected their subsequent response to host-HIPVs and nonhost-
77 HIPVs.

78 In the present study, we used a tritrophic system of maize plants (*Zea mays*:
79 Poaceae), hosts (*Mythimna separata*, Lepidoptera: Noctuidae) (common armyworms:
80 CAWs), nonhosts (*Spodoptera litura*, Lepidoptera: Noctuidae) (tobacco cutworms:
81 TCWs), and specialist parasitoid wasps (*Cotesia kariyai*, Hymenoptera: Braconidae).
82 CAWs use plants from the family Poaceae as food sources, while TCWs use a wider
83 range of plant families, including Poaceae, as food sources. Plants infested with either
84 CAWs or TCWs have been shown to emit respective HIPVs (Choh et al. 2013;
85 Takabayashi et al. 1995). *Cotesia kariyai* females have been shown to be attracted to
86 host (CAWs)-HIPVs in their search for host-infested plants (Takabayashi et al. 1995).
87 When encountering nonhosts (e.g., TCWs) in a petri dish, *C. kariyai* females show

88 oviposition behavior towards them and lay fewer eggs than when encountering CAWs
89 (Y. Kainoh, unpublished data).

90 In this study, we aimed to answer the following two questions: (1) are *C.*
91 *kariyai* females attracted to HIPVs emitted from maize plants infested with nonhosts
92 (TCWs), and (2) if so, how does the experience of oviposition in nonhosts affect their
93 subsequent olfactory responses to host- and nonhost-HIPVs, and their antennal host
94 searching behavior on host- and nonhost-infested plants? We also conducted chemical
95 analyses of CAW-HIPVs and TCW-HIPVs to assess the possible volatile cue(s) related
96 to the nonhost-experience that affected the olfactory responses of *C. kariyai*.

97

98

METHODS AND MATERIALS

99

100 *Insects and Plants.* The laboratory CAW and TCW larval colonies were reared on an
101 artificial diet [Silkmate[®] 2(s), Nosan Corporation, Yokohama, Japan] in a climate-
102 controlled room [25 ± 1 °C, $60 \pm 10\%$ relative humidity (RH), 16 light (L):8 dark (D)].
103 To collect eggs, ~30 adult moths of one species were housed per container and a piece
104 of paraffin paper, folded in an accordion manner, was placed in each container (35×21
105 $\times 23$ cm, length \times depth \times height) as an oviposition substrate. Paraffin papers with
106 deposited eggs were collected daily. Newly emerged larvae of CAWs and TCWs were
107 maintained in plastic Petri dishes (14.5×2.5 cm, diameter \times height) in early stages and
108 in plastic containers ($28 \times 21 \times 10$ cm, L \times D \times H) in later stages until pupation.

109 The laboratory colony of *C. kariyai* was maintained under the same
110 conditions used for rearing herbivores. Fourth stadium CAW individuals were offered to
111 2~3-day-old female *C. kariyai* individuals in small plastic Petri dishes (5.5×2.0 cm, D
112 \times H) for parasitization. Soon after the attack, the host larvae were collected and reared
113 with Silkmate[®] in plastic Petri dishes (14.5×2.5 cm, D \times H). The containers were
114 checked daily until egression, which occurred ~10 d after the incidence of parasitism. A
115 cluster of cocoons of *C. kariyai* was collected and placed in a transparent plastic
116 container ($29 \times 18 \times 17$ cm, L \times D \times H) until emergence of the adults. Cotton wool
117 moistened with honey droplets was provided as food for the parasitic wasps. Mating
118 occurred immediately after emergence. All experimental parasitoid females were 3-day-
119 old, mated, and well-fed individuals.

120

Maize seedlings (*Z. mays* L., Honey-Bantam Peter 619; Sakata Seed Co.,

121 Kanagawa, Japan) were grown separately in plastic pots (5.5 × 7.0 cm, D × H) in an
122 incubator (25 ± 1 °C, 60 ± 10% RH, 1500 lx, 16 L:8 D photoperiod). Nine-day-old
123 seedlings were then transplanted to bigger plastic pots (11 × 9 cm, D × H) and reared in
124 a greenhouse (25 ± 1 °C, 14 L:10 D, 60 ± 10% RH). Plants were watered daily and 15-
125 day-old maize plants (~30 cm in height) were used for all experiments.

126 For the bioassays, CAWs, TCWs, *C. kariyai*, and maize plants were reared in
127 the Laboratory of Applied Entomology and Zoology, University of Tsukuba, Japan. For
128 the chemical analyses, CAWs and TCWs were reared in the Center for Ecological
129 Research, Kyoto University.

130

131 *Wind Tunnel Bioassay.* Behavioral responses of *C. kariyai* females were observed in a
132 wind tunnel (30 × 30 × 150 cm) (25–30 cm/s wind speed, 25 ± 1 °C, 60 ± 10% RH, 16
133 L:8 D, ~900 lx light intensity) from 13:00 to 16:00 on three or four different days. In
134 each experiment, an individual *C. kariyai* female was released from a platform (15 cm
135 in height), which was placed 30 cm downwind from the odor source. *Cotesia kariyai*
136 females were given 5 min to fly to the target plants. If *C. kariyai* females did not reach
137 the plants within the 5 min period, the wasps were considered as nonresponsive
138 individuals and were not included in the statistical analyses.

139 Infested plants were prepared in a mesh cage (50 × 50 × 90 cm). The plastic
140 pot (11 × 9 cm, D × H) and the soil surface were covered with aluminum foil. We
141 prepared host-infested plants (damaged by five CAW larvae) and nonhost-infested
142 plants (damaged by five TCW larvae). Damage area of each treatment was adjusted to
143 be ~30% of the total leaf area. The duration of feeding was 19 h (from 18:00 to 13:00).
144 Feces on the surface of aluminum foil were carefully removed with a fine brush prior to
145 the experiments.

146 *Cotesia kariyai* is a diurnal wasp species (Sato et al. 1983) and, therefore,
147 oviposits into hosts or nonhosts during the daytime. Based on this, the preparation of
148 experienced *C. kariyai* females were as follows. Three detached maize leaves were
149 placed in a Petri dish (14.5 × 2.5 cm, D × H) and 20 fourth-instar TCW larvae were
150 allowed to feed on the leaves for 1 h under light conditions. An individual *C. kariyai*
151 female was then released into the dish and allowed to search for a nonhost that was
152 feeding on one of the maize leaves. Once a wasp encountered a TCW larva, it expressed
153 oviposition behavior towards the larva. Attacked larvae were removed, and the wasps

154 were collected as experienced wasps. Groups of experienced wasps were prepared ~3 h
155 before the experiments.

156 The following steps were measured in each experiment: Step-1: the relative
157 ratio (%) of taking-off; Step-2: the duration from taking-off to landing; Step-3: the
158 relative ratio (%) of landing, and Step-4: the duration of searching on the maize plants.
159 The experiments were repeated 10 times per day using same odor sources. In total, 30
160 (the response of inexperienced female to host-infested plants) and 40 individuals
161 (others) were tested in 3 or 4 different experimental days.

162

163 *Chemical Analyses.* We analyzed the headspace volatiles of potted maize plants that had
164 been infested with five fourth-stadium larvae (either CAW or TCW) for 19 h. Plants
165 were the same size as those used for the wind tunnel experiments. During the volatile
166 collection, the plant-growing cups and the soil surface were covered with aluminum foil
167 to prevent the collection of non-target volatiles from these sources. Infested plants were
168 used after the removal of feces with a fine brush. We also analyzed volatiles from three
169 detached maize leaves that had been infested with 20 fourth-instar TCW larvae for 1 h.

170 Volatile collections were performed in a climate-controlled room (25 ± 2 °C,
171 50–70% RH). A piece of filter paper infiltrated with 1 μ L of hexane solution of
172 tridecane (0.1 μ g/ μ L) was used as an internal standard. We placed an odor source and
173 the internal standard in a glass bottle (2 L) that had two holes connected to two nozzles.
174 One nozzle was connected to a pure air cylinder and the other nozzle was connected to a
175 glass tube (6 mm outer diameter) filled with Tenax TA 60/80 (Gerstel GmbH & Co. KG,
176 Mülheim an der Ruhr, Germany). Air from the cylinder was sent to the glass bottle
177 containing the odor source plant sample, and then to the Tenax tube at a flow rate of 100
178 mL/min. Sampling of the headspace volatiles was conducted for 1 h and volatile
179 collection was repeated 5–9 times for each treatment.

180 The collected volatile compounds were analyzed using a gas chromatograph-
181 mass spectrometer (GC-MS) (GC: Agilent Technologies, Inc., Santa Clara, CA, USA;
182 6890 with HP-5MS capillary column: 30 m long, 0.25 mm I.D., and 0.25 μ m film
183 thickness; MS: Agilent Technologies, Inc., 5973 mass selective detector, 70 eV)
184 equipped with a separate desorption system, a cooled injection system, and a cold trap
185 system (Gerstel GmbH & Co. KG). The headspace volatiles were tentatively identified
186 by comparing their mass spectra with those from the Wiley databases (Wiley7N and

187 Wiley275). The mass spectra and retention times of detected volatile compounds were
188 further compared with those of standard compounds [β -myrcene, (*Z*)-3-hexenyl acetate,
189 linalool, methyl salicylate, indole, geranyl acetone, and (*E*)- β -farnesene; Wako Pure
190 Chemical Industries, Ltd, Osaka, Japan, α -copaene; Fluka Chemie GmbH, Buchs,
191 Germany, *cis*-jasmone; Tokyo Chemical Industries, Tokyo, Japan]. (*E*)- β -Ocimene, (*E*)-
192 4,8-dimethyl-1,3,7-nonatriene (DMNT), and (*E*)-4,8,12-trimethyl-1,3,7,11-
193 tridecatetraene (TMTT) were obtained from Dr. W. Boland. Compounds for which no
194 standards were available were regarded as tentatively identified when more than a 90%
195 of their spectra matched those of the databases.

196

197 *Statistics.* Comparisons were made between the following four pairs: (1) inexperienced
198 females to nonhost-infested plants versus inexperienced females to host-infested plants,
199 (2) inexperienced females to nonhost-infested plants versus experienced females to
200 nonhost-infested plants, (3) experienced females to nonhost-infested plants versus
201 experienced females to host-infested plants, and (4) inexperienced females to host-
202 infested plants versus experienced females to host-infested plants. The effects of the
203 treatment (combination of female experience and infested plants) on the ratios of
204 taking-off and landing behavior of *C. kariyai* in the wind tunnel were analyzed by
205 generalized linear mixed models (GLMMs) with a binomial distribution and logit-link
206 using the function `glmer` in the package `lme4` version 1.17 (Bates et al. 2015) in R
207 version 3.3.3 (R Core Team, 2017). The effects of the treatment on the duration of time
208 from the taking-off to landing and that of host-searching behavior were analyzed using
209 GLMMs with a normal distribution and identity-link using `lmer` in the package `lme4`.
210 All duration data were Box-Cox transformed using JMP version 11.2.1 (SAS Institute,
211 2013) before the GLMMs. The replication was a random effect in all models.
212 Significance was evaluated with the likelihood ratio test using the function `anova` and
213 by comparing models with or without the effect of the treatment. Pairwise comparisons
214 between nonhost-infested plants and host-infested plants, and between inexperienced
215 females and experienced females were performed with Holm's sequentially rejective
216 Bonferroni test (Holm, 1979) to adjust for the effects of multiple comparisons. We
217 conducted a Wilcoxon rank sum test using the function `wilcox.test` in R to determine if
218 there were differences in the relative amounts of volatile compounds emitted from
219 maize leaves infested with host (CAW) and nonhost (TCW) larvae for *C. kariyai*.

220

221

RESULTS

222

223 *Behavioral Responses of C. kariyai to Host-, and Nonhost-infested Maize Plants.* Step-
224 1: the relative ratio of taking-off (Fig. 1A). The ratio (%) that the inexperienced wasps
225 showed toward nonhost-infested plants and that toward host-infested plants were not
226 significantly different (the lowercase letters on the first versus the second bar)
227 (GLMMs; $P = 0.8178$, $\alpha = 0.05$). The ratio that the experienced wasps showed toward
228 nonhost-infested plants and that toward host-infested plants were significantly different
229 (the lowercase letters on the third vs the fourth bar) (GLMMs; $P = 0.0127$, $\alpha = 0.0167$).
230 Experience decreased the ratio toward nonhost-infested plants (the uppercase letters on
231 the first versus the third bar) (GLMMs; $P = 0.0051$, $\alpha = 0.0125$) but not toward host-
232 infested plants (the uppercase letters on the second versus the fourth bar) (GLMMs; $P =$
233 0.7522 , $\alpha = 0.025$) (Fig. 1A and Table 1A).

234 Step-2: the duration from taking-off to landing (Fig. 1B). The flight duration
235 of inexperienced wasps in experiments with nonhost-infested plants was not
236 significantly shorter than that of experiments with host-infested plants (the lowercase
237 letters on the first versus the second bar) (GLMMs; $P = 0.0679$, $\alpha = 0.0167$). The flight
238 duration of experienced wasps was not significantly different in experiments with
239 nonhost-infested and host-infested plants (the lowercase letters on the third versus the
240 fourth bar) (GLMMs; $P = 0.3337$, $\alpha = 0.05$). Experience increased the duration
241 significantly in experiments with nonhost-infested plants (the uppercase letters on the
242 first versus the third bar) (GLMMs; $P = 0.0033$, $\alpha = 0.0125$), but not in those with host-
243 infested plants (the uppercase letters on the second versus the fourth bar) (GLMMs; $P =$
244 0.2861 , $\alpha = 0.025$). (Fig. 1B and Table 1B).

245 Step-3: the relative ratio of landing (Fig. 1C). The ratios (%) of landing that
246 the inexperienced wasps showed toward nonhost-infested and host-infested plants were
247 not significantly different (the lowercase letters on the first versus the second bar)
248 (GLMMs; $P = 0.2852$, $\alpha = 0.025$). The ratios that the experienced wasps showed toward
249 nonhost-infested and host-infested plants were significantly different (the lowercase
250 letters on the third versus the fourth bar) (GLMMs; $P = 0.0011$, $\alpha = 0.0125$). Experience
251 significantly decreased the ratios of landing toward nonhost-infested plants (the
252 uppercase letters on the first versus the third bar) (GLMMs; $P = 0.0123$, $\alpha = 0.0167$) but

253 not toward host-infested plants (the uppercase letters on the second versus the fourth
254 bar) (GLMMs; $P = 0.7025$, $\alpha = 0.05$) (Fig. 1C and Table 1C).

255 Step-4: the duration of searching on maize plants. The duration that the
256 inexperienced wasps spent searching on nonhost-infested plants was significantly
257 shorter than that on host infested plants (the lowercase letters on the first versus the
258 second bar) (GLMMs; $P = 0.0075$, $\alpha = 0.025$). The experienced wasps spent
259 significantly different durations searching on nonhost-infested and on host-infested
260 plants (the lowercase letters on the third versus the fourth bar) (GLMMs; $P = 0.0024$, α
261 $= 0.0125$). Experience significantly decreased the searching durations on both nonhost-
262 infested plants (the upper cases on the first versus the third bar) (GLMMs; $P = 0.0020$,
263 $\alpha = 0.05$) and on host-infested plants (the upper cases on the second versus the fourth
264 bar) (GLMMs; $P = 0.0062$, $\alpha = 0.0167$) (Fig. 1D and Table 1D).

265

266 *Chemical Analyses.* A total of 22 volatile compounds were detected in the headspace of
267 maize plants infested with either host larvae or nonhost larvae (Table 2). Among them,
268 the amounts of (*Z*)-3-hexenyl acetate ($P = 0.0496$: Wilcoxon rank sum test) and α -
269 copaene ($P = 0.0450$: Wilcoxon rank sum test) were significantly higher in the nonhost-
270 HIPVs than in host-HIPVs. We detected 10 compounds in the headspace volatiles from
271 three detached maize leaves infested with 20 fourth-stadium nonhost larvae during
272 sampling for 1 h (Table 2). Among them, (*Z*)-3-hexenyl acetate was the major
273 compound, while α -copaene was not detected.

274

275 DISCUSSION

276 *Are C. kariyai females attracted to HIPVs emitted from maize plants infested with*
277 *nonhosts (TCWs)?*

278 We showed that inexperienced *C. kariyai* females were attracted to nonhost
279 (TCW)-HIPVs under the single-target experimental conditions, which indicated that the
280 wasps would find nonhost-infested plants and consequently may show oviposition
281 behavior towards nonhosts in field conditions. Although not significantly different
282 (GLMMs; $P = 0.0679$, $\alpha = 0.0167$), the inexperienced wasps exhibited less flight time
283 in Step-2 (the duration from taking-off to landing) to nonhost-infested plants than to
284 host-infested plants (Fig. 1.B). This suggests that HIPVs from nonhost-infested plants
285 are more attractive to *C. kariyai* than those from host-infested plants.

286 No qualitative differences were detected between the HIPVs from plants
287 infested with hosts and nonhosts for 19 h. However, quantitative differences between
288 the HIPVs from plants infested with hosts and nonhosts were detected, whereby there
289 were significantly higher amounts of (Z)-3-hexenyl acetate and α -copaene in the
290 nonhost-HIPVs than in the host-HIPVs. The shorter duration in Step-2 by inexperienced
291 wasps to HIPVs from nonhost-infested plants than to those from host-infested plants
292 may have been due to the higher amounts of (Z)-3-hexenyl acetate and/or α -copaene in
293 nonhost-HIPVs. In a Y-tube olfactometer, inexperienced *C. kariyai* females are attracted
294 to (Z)-3-hexenol but not to (Z)-3-hexenyl acetate (Takabayashi et al. 1991). It is
295 noteworthy that the mode of the olfactory response of an insect in a Y-tube olfactometer
296 and in a wind tunnel differs. Further studies are needed to assess if and how (Z)-3-
297 hexenyl acetate and/or α -copaene are involved in the attraction of the inexperienced *C.*
298 *kariyai*.

299

300 *How does the experience of oviposition in nonhosts affect subsequent olfactory*
301 *responses of C. kariyai females to host- and nonhost-HIPVs, and their antennal host*
302 *searching behavior on host- and nonhost-infested plants?*

303 We found that the negative experience (oviposition behavior to nonhosts) on a
304 freshly nonhost-infested leaf negatively affected their subsequent olfactory response
305 intensities to nonhost-HIPVs, but not to host-HIPVs in Step-1 to Step-3 (Fig. 1ABC).
306 We hypothesized that the wasps associated the negative experience with the freshly
307 produced nonhost-infested leaf volatiles, and exhibited reduced responses only to the
308 nonhost-HIPVs. In the headspace of leaves infested with nonhost larvae for 1 h, (Z)-3-
309 hexenyl acetate was the major compound while most of the other HIPVs (i.e., volatile
310 terpenoids) from host- and nonhost-infested plants, including α -copaene, were not
311 detected (Table 2). This absence can be explained by the fact that most of the herbivory-
312 induced volatile terpenoids are synthesized *de novo* in plants and are emitted ~1 day
313 after herbivory (Arimura et al. 2008). In contrast, (Z)-3-hexenyl acetate is one of the
314 green leaf volatiles that are emitted immediately after mechanical wounding or
315 herbivory (Matsui 2006).

316 The relative amount of (Z)-3-hexenyl acetate in nonhost-HIPVs was
317 significantly higher than in host-HIPVs, and (Z)-3-hexenyl acetate was also found in
318 volatiles from freshly nonhost-infested leaves. Collectively, the chemical and biological

319 data supported our hypotheses: *C. kariyai* would have associated the negative
320 experience with the (*Z*)-3-hexenyl acetate emitted from freshly nonhost-infested leaves,
321 and this experience ultimately reduced their responses to the nonhost-HIPVs, in which
322 the higher amount of (*Z*)-3-hexenyl acetate was involved. The question of why *C.*
323 *kariyai* did not show negative responses to host-HIPVs, even though the smaller amount
324 of (*Z*)-3-hexenyl acetate was also in the host-HIPVs, still remains unanswered.

325 In Step-4, the longer that the *C. kariyai* females search on a plant, the higher
326 the possibility that they would find a host or nonhost. Interestingly, after negative
327 experience, the wasps spent significantly less time searching on both host- and nonhost-
328 infested plants (Fig.1 D). On host-infested plants, *C. kariyai* females were seen to
329 exhibit antennal contact with the host-infested edges of a leaf and host feces, and this
330 caused the longer duration of time on the host-infested plants than on uninfested plants
331 (Sato et al. 1983; Takabayashi et al. 1985). One of the factors that elicit the antennal
332 searching behavior of *C. kariyai* females on host-infested edges and host feces is a
333 series of 2,5-dialkyltetrahydrofuran homologs (mainly from C27 to C35) (Takabayashi
334 and Takahashi 1986ab). During negative experiences on nonhost-infested leaves, *C.*
335 *kariyai* may have perceived nonhost-derived cues that elicited the antennal searching of
336 the wasps on the leaves. It still remains to be elucidated whether *C. kariyai* females
337 associate negative experiences with such cues and, therefore, explain the reduced
338 searching duration in Step-4.

339 This study showed, for the first time, that the oviposition behavior to nonhosts
340 (negative experience) on a nonhost-infested leaf negatively affected the subsequent
341 responses to nonhost-HIPVs. To date, most studies have focused on positive experience
342 (e.g., oviposition success and host-finding cues) with host-HIPVs (e.g., Costa et al.
343 2010; Fukushima et al. 2002). A comparative study on the effects of positive and
344 negative experiences with synthetic host-HIPVs in attracting *Cotesia vestalis*, a
345 specialist parasitoid of diamondback moth larvae, has been reported (Uefune et al.
346 2013). It is adaptive for wasps to become choosier in their responses to detected host-
347 HIPVs after a negative experience. However, wasps spent less time not only on
348 nonhost-infested plants but also on host-infested plants after a negative oviposition
349 experience, and the reduction of time spent on host-infested plants is not considered to
350 adaptive for the wasps. Our study indicated that the density (i.e., possible encounters) of

351 nonhost species as well as that of host species should be considered when assessing the
352 host-searching behavior of parasitoid wasps.

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438 Figure legend

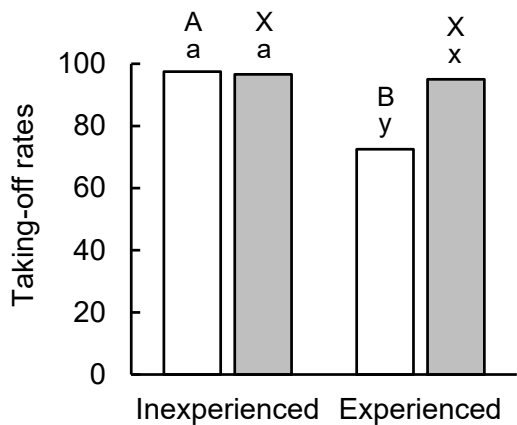
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440 **Fig. 1** The flight responses to infested plants (Step-1 to Step-3) and the host searching
441 response on infested plants (Step-4) of *Cotesia kariyai* in the wind tunnel. The different
442 lowercase letters for the data of the same wasp condition (either inexperienced or
443 experienced) indicate significant differences by GLMM based on each Holm-adjusted
444 significance level between nonhost-infested and host-infested plants. The different
445 uppercase letters for the data of the same plant condition [either nonhost-infested (white
446 bars) or host-infested (gray bars)] indicate significant differences by GLMM based on
447 each Holm-adjusted significance level between inexperienced and experienced females.
448 Thirty (the response of inexperienced female to host-infested plants) and 40 individuals
449 (others) were tested.

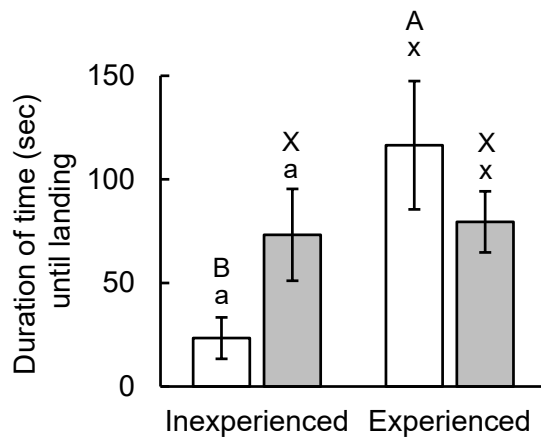
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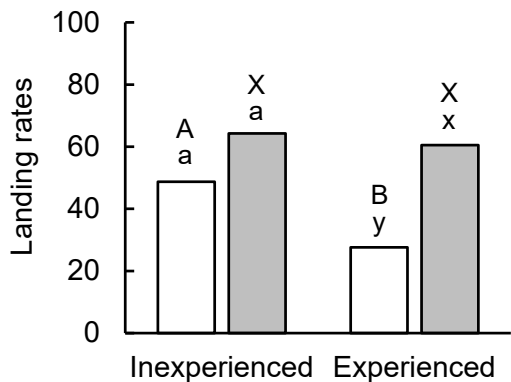
(A) Step-1: the relative ratio (%) of taking-off



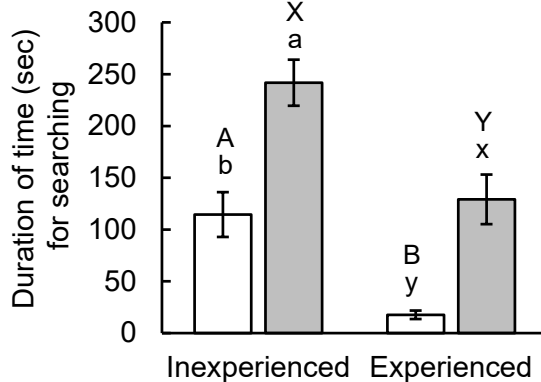
(B) Step-2: the durations of time from the taking-off to landing



(C) Step-3: the relative ratio (%) of landing



(D) Step-4: : the duration of searching on plants



□ Nonhost-infested plant

■ Host-infested plant

Table 1 Summary table showing degrees of freedom (df), chi-squared values (χ^2), *P*-values (*P*), Holm-adjusted significance level (α) after generalized linear mixed models (GLMMs) for the differences of each step in treatment in combination with experience and infested plant.

(A) Step-1: the relative ratio (%) of taking-off

Treatment	df	χ^2	<i>P</i>	α
IF to NIP vs IF to HIP	1	0.053	0.8178	0.05
IF to NIP vs EF to NIP	1	7.856	0.0051 *	0.0125
EF to NIP vs EF to HIP	1	6.1888	0.0127 *	0.0167
IF to HIP vs EF to HIP	1	0.0997	0.7522	0.025

(B) Step-2: the durations of time from the taking-off to landing

Treatment	df	χ^2	<i>P</i>	α
IF to NIP vs IF to HIP	1	3.3339	0.0679	0.0167
IF to NIP vs EF to NIP	1	8.6596	0.0033 *	0.0125
EF to NIP vs EF to HIP	1	0.9346	0.3337	0.05
IF to HIP vs EF to HIP	1	1.1377	0.2861	0.025

(C) Step-3: the relative ratio (%) of landing

Treatment	df	χ^2	<i>P</i>	α
IF to NIP vs IF to HIP	1	1.1422	0.2852	0.025
IF to NIP vs EF to NIP	1	6.2631	0.0123 *	0.0167
EF to NIP vs EF to HIP	1	10.624	0.0011 *	0.0125
IF to HIP vs EF to HIP	1	0.1459	0.7025	0.05

(D) Step-4: : the duration of searching on plants

Treatment	df	χ^2	<i>P</i>	α
IF to NIP vs IF to HIP	1	7.1515	0.0075 *	0.025
IF to NIP vs EF to NIP	1	9.5365	0.0020 *	0.05
EF to NIP vs EF to HIP	1	9.2325	0.0024 *	0.0125
IF to HIP vs EF to HIP	1	7.5053	0.0062 *	0.0167

IF: Inexperienced females, EF: experienced females, NIP: nonhost infested plants. HIP: host infested plants. The asterisk indicates significant *P*-value based on Holm-adjusted significance level.

Table2. Volatile compounds emitted from maize leaves infested with host (*Mythimna separate*) and nonhost (*Spodoptera litura*) caterpillars of *Cotesia kariyai* for different durations of time (1 h and 19 h)

Compound	Relative amounts / gFW			
	1 h Nonhost	19 h		
	Median (1st–3rd quartiles)	Median (1st–3rd quartiles)		
		Nonhost	Host	
β -myrcene	ND	0.009 (0.000 - 0.027)	0.006 (0.000 - 0.019)	
(Z)-3-hexenyl acetate	0.128 (0.078 - 0.338)	0.276 (0.172 - 0.994)	0.062 * (0.017 - 0.253)	
(E)- β -ocimene	ND	0.048 (0.003 - 0.080)	0.030 (0.008 - 0.118)	
linalool	0.044 (0.031 - 0.071)	0.468 (0.103 - 0.907)	0.450 (0.149 - 2.470)	
DMNT	0.020 (0.017 - 0.084)	0.394 (0.233 - 1.401)	0.645 (0.206 - 1.763)	
methyl salicylate	0.014 (0.004 - 0.030)	0.000 (0.000 - 0.011)	0.000 (0.000 - 0.010)	
indole	ND	0.553 (0.262 - 2.749)	1.337 (0.128 - 4.509)	
unidentified compound	0.044 (0.037 - 0.069)	0.121 (0.092 - 0.150)	0.079 (0.062 - 0.134)	
ylangene†	0.037 (0.028 - 0.059)	0.113 (0.090 - 0.141)	0.071 (0.043 - 0.117)	
α -copaene	0.015 (0.011 - 0.021)	0.019 (0.007 - 0.034)	0.003 * (0.000 - 0.010)	
cis-jasmone	ND	0.000 (0.000 - 0.018)	0.013 (0.000 - 0.022)	
unidentified sesquiterpene 1	ND	0.046 (0.026 - 0.053)	0.026 (0.020 - 0.049)	
unidentified sesquiterpene 2	ND	0.166 (0.073 - 0.264)	0.081 (0.036 - 0.274)	
β -sesquiphellandrene†	ND	0.000 (0.000 - 0.000)	0.000 (0.000 - 0.020)	
geranyl acetone	0.005 (0.000 - 0.008)	0.025 (0.000 - 0.163)	0.039 (0.012 - 0.083)	
(E)- β -farnesene	ND	0.748 (0.314 - 0.997)	0.292 (0.119 - 1.056)	
unidentified sesquiterpene 3	ND	0.015 (0.000 - 0.044)	0.015 (0.000 - 0.024)	
unidentified sesquiterpene 4	ND	0.000 (0.000 - 0.024)	0.008 (0.000 - 0.020)	
α -amorphene†	0.021 (0.014 - 0.031)	0.062 (0.050 - 0.095)	0.052 (0.027 - 0.103)	
δ -cadinene†	0.015 (0.011 - 0.027)	0.053 (0.051 - 0.095)	0.033 (0.026 - 0.070)	
unidentified sesquiterpene 5	ND	0.022 (0.000 - 0.026)	0.017 (0.011 - 0.026)	
TMTT	ND	0.109 (0.061 - 0.197)	0.037 (0.024 - 0.159)	

ND, not detected.

Data represent the mean \pm SE of 5-9 replications. Values with asterisks are significantly different ($P < 0.05$, Wilcoxon rank sum test) between nonhost-infested plant and host-infested plant. Compounds with † are tentatively identified.

DMNT; (*E*)-4,8-dimethyl-1,3,7-nonatriene , TMTT; (*E,E*)-4,8,12-trimethyl-1,3,7,11-tridecatetraene