Oviposition Experience by the Larval Parasitoid, *Cotesia kariyai*, on Nonhost, *Spodoptera litura*, Can Deter Subsequent Attacks

Fumika K. Aikawa · Kazumu Kuramitsu ⬤ ·
Yooichi Kainoh ⬤

Abstract Some parasitoids oviposit in nonhosts. Parasitization of nonhosts potentially wastes gametes, risks the death of offspring, and reduces fitness. Associative learning, a strategy for efficient reproduction, has been observed in various parasitoid species. We conducted two types of experiments to reveal whether larval parasitoid wasps, *Cotesia kariyai* (Hymenoptera: Braconidae), learn associatively by ovipositing in nonhosts. In dissection experiments, we found wasp eggs in both host [*Mythimna separata* (Lepidoptera: Noctuidae)] and nonhost [*Spodoptera litura* (Lepidoptera: Noctuidae)] caterpillars. However, the mean number of eggs in nonhosts was significantly smaller than in hosts. In oviposition experiments, most naive *C. kariyai* females attacked both hosts and nonhosts. On the other hand, wasps that had previously attacked nonhosts tended to avoid them thereafter. We conclude that *C. kariyai* females may be able to detect and identify nonhost metabolites and/or cuticular hydrocarbons. Negative associative learning enhances *C. kariyai* reproductive success when hunting in complex host habitats.

Keywords Host searching · oviposition behavior · associative learning · Hymenoptera · Braconidae

F. K. Aikawa
Laboratory of Applied Entomology and Zoology, Graduate School of Life and Environmental Sciences, University of Tsukuba, 1-1-1 Tennodai, Tsukuba, Ibaraki 305-8572, Japan

K. Kuramitsu · Y. Kainoh (✉)
Faculty of Life and Environmental Sciences, University of Tsukuba, 1-1-1 Tennodai, Tsukuba, Ibaraki 305-8572, Japan
e-mail: kainoh.yooichi.gf@u.tsukuba.ac.jp

Present Address:
F. K. Aikawa
Fukushima Prefectural Soso Agriculture and Forestry Office, 1-30, Nishiki-cho, Haramachi-ku, Minamisoma, Fukushima 975-0031, Japan

Introduction

In the field, insect parasitoids are exposed to various volatile compounds from hosts, nonhosts, healthy plants, and plants infested by hosts and/or nonhosts, and they must locate their hosts in this complicated environment. Recently, a number of studies on host searching behavior of parasitoids in complicated environments have been conducted (reviewed in De Rijk et al. 2013; Takabayashi 2014). In natural habitats, plants are often infested by several herbivore species. Parasitoids sometimes encounter nonhosts on such plants (De Rijk et al. 2013), and mistakenly oviposit in them (Takabayashi and Takahashi 1990; Obonyo et al. 2008). Not only do these wasps waste reproductive effort and energy, but they also risk death due to counterattacks from nonhosts (Potting et al. 1999; Gentry and Dyer 2002).

In order to locate hosts efficiently, parasitoids have developed a variety of tactics, including associative learning. Vet et al. (1990) defined associative learning

as "the establishment through experience of an association between two stimuli or between a stimulus and a response." Various studies have revealed that some parasitoids learn by associating ovipositing experience on their hosts with certain stimuli (Wäckers and Lewis 1994; Takasu and Lewis 2003; Costa et al. 2010; Feng et al. 2015; Giunti et al. 2015).

Some studies have suggested that parasitic wasps recognize host suitability by inserting their ovipositors (Obonyo et al. 2008; McDonald et al. 2015). Therefore, when parasitoids attack nonhosts, they may be able to learn by negative association. However, no studies about associative learning on nonhost by parasitoids have been performed.

Cotesia kariyai (Hymenoptera: Braconidae) is a specialized larval endoparasitoid of the common armyworm, *Mythimna separata* (Lepidoptera: Noctuidae). When a female *C. kariyai* finds a common armyworm, she lands on and inserts her ovipositor into it. However, we have observed that female wasps also insert their ovipositors into larvae of *Spodoptera litura* (Lepidoptera: Noctuidae), a nonhost for *C. kariyai*. In a preliminary experiment, we dissected *S. litura* larvae that had been attacked by *C. kariyai* females and found wasp eggs in their hemolymph. *C. kariyai* eggs likely cannot develop properly due to an immune response (Hayakawa and Yazaki 1997), since *S. litura* is a nonhost. Therefore, it is unclear whether *C. kariyai* females recognize *S. litura* as a nonhost and reduce the number of eggs deposited. Both *M. separata* and *S. litura* are distributed in Southeast and East Asia, and they share some host plant species, such as grasses (Poaceae) (Zaidi et al. 2009; Koyama and Matsumura 2019). Therefore, it is possible that *C. kariyai* encounters *S. litura* in the field.

In this study, we dissected larvae attacked by *C. kariyai* females and observed their 'attack' behavior. Herein, we discuss how *C. kariyai* females can recognize nonhosts using associative learning, so as to minimize waste of reproductive output by reducing inappropriate oviposition or by avoiding nonhosts.

Materials and Methods

Insects

Cotesia kariyai, *Mythimna separata*, and *Spodoptera litura* were from colonies that have been maintained for many years in the Laboratory of Applied Entomology and Zoology, University of Tsukuba, Japan. *Mythimna separata* and *S. litura* were reared on an artificial diet (Silkmate® 2 M, Nosan Corporation) in the rearing room of the laboratory (25 ± 1 °C, 16 L: 8D photoperiod, 700 lx., and $60 \pm 10\%$ RH). Third and fourth instars were used for experiments.

Cotesia kariyai was reared under the same conditions as *M. separata*. Third to early 6th instar larvae of *M. separata* were offered to *C. kariyai* females for parasitization. After wasp larvae spun cocoons, they were put into plastic cages ($15 \times 17 \times 24$ cm). Honey and wet cotton were provided for food and water. When adult wasps emerged from their cocoons, they mated immediately in the plastic cages. Mated females were kept in the cages and 3- to 4-day-old females were used for experiments.

Dissection

In this study, we define "attack behavior" as riding on the dorsum of a larva, bending the abdomen, inserting the ovipositor into the larva, and remaining stationary for more than 0.5 s. Duration of oviposition insertion was recorded using a stopwatch.

To determine whether *C. kariyai* females oviposit into *S. litura*, we dissected larvae that had been attacked by *C. kariyai*. In Petri dishes (9 cm × 1.5 cm), one 3rd instar larva of *M. separata* or *S. litura* was exposed to a 3–4-day-old *C. kariyai* female for a single attack. We measured the time that a wasp spent attacking a caterpillar. Soon after oviposition behavior, we transferred larvae to dissection plates and dissected them with micro-scissors and fine forceps, adding a small amount of distilled water in order to separate wasp eggs from body fluid contaminants. Under a stereomicroscope (SZH, Olympus, Tokyo, Japan), we counted the number of wasp eggs.

Attacking Experiment

In order to clarify whether *C. kariyai* females use associative learning to distinguish hosts and nonhosts, we exposed nonhost *S. litura* to *C. kariyai* that had attacked nonhosts previously and observed wasp behavior in a Petri dish, as before. We put a single 4-day-old *C. kariyai* female and a single 4th instar *S. litura* larva into a Petri dish together and the wasp was allowed to attack the larva once. Next, the wasp was transferred to

an empty Petri dish and kept with honey and wet cotton for 3 h. Then, the wasp was allowed to contact a 4th instar *M. separata* or *S. litura* larva once. We observed wasp behavior and recorded whether wasps attacked the larvae. The same experiments with host-experienced *C. kariyai* were also performed using the method described above.

Statistics

An exact Wilcoxon rank sum test was used to analyze egg numbers and attack time of wasps. We used Fisher's exact test to analyze the attack rate by wasps in the attacking experiment. All data were analyzed with R version 3.0.2 (R Development Core Team 2013).

Results

Dissection

Parasitoid eggs were readily removed from *S. litura* larvae and counted. The mean number of wasp eggs found in nonhost, *S. litura* (9.2 ± 2.0, mean $\pm$ S.E., $n = 27$) was significantly smaller than counts from host, *M. separata* (17.8 ± 2.2, $n = 24$) (Exact Wilcoxon rank sum test, $p < 0.01$) (Fig. 1a, Supplementary Material 1). The mean attack duration of wasps on nonhost, *S. litura* (2.08 ± 0.23 s, mean $\pm$ S.E., $n = 27$) was significantly shorter than that on host, *M. separata* (2.91 ± 0.16 s, $n = 24$) (Exact Wilcoxon rank sum test, $p < 0.01$) (Fig. 1b).

Attacking Experiment

The attack rate on nonhost *S. litura* by *C. kariyai* females that had previously attacked *S. litura* (6.9%, $n = 29$) was significantly lower than that on *M. separata* (30%, $n = 30$) (Fisher's exact test, $p < 0.05$, Fig. 2). On the other hand, the attack rates on *M. separata* and on nonhost, *S. litura* by naive *C. kariyai* females were 95% ($n = 20$) and 85% (n = 20) respectively. There was no significant difference between these rates (Fisher's exact test, $p < 0.05$, Fig. 2). Host-experienced *C. kariyai* females showed low (10.0–13.3%) attack rates and there was no significant difference between host and nonhost (Fisher's exact test, p < 0.05, Fig. 2). *C. kariyai* females that did not attack an *S. litura* larva immediately retreated from the larva after the first contact and avoided it thereafter.

Discussion

From the dissection experiment, *C. kariyai* females clearly oviposited in *S. litura* larvae, despite the fact that these are nonhosts for the wasp. However, the number of eggs deposited in nonhosts was significantly smaller than in *M. separata,* the normal host (Fig. 1a). In addition, we also found that *C. kariyai* females spent less time when they attacked nonhost caterpillars than when they attacked appropriate hosts (Fig. 1b). From these results, we conclude that *C. kariyai* females recognize nonhost *S. litura* and that they reduce the number of eggs laid. These results suggest that *C. kariyai* females that commence oviposition in *S. litura* recognize the inappropriate choice after inserting the ovipositor and stop oviposition. *C. kariyai* females may recognize nonhosts by detecting metabolites and/or cuticular hydrocarbons in the larva with the ovipositor. In fact, some hymenopteran parasitoids have chemosensory sensilla on their ovipositors (van Lenteren et al. 2007; Quicke 2015). According to the results of rearing experiments, *Cotesia kariyai* females normally lay 27–56 eggs in 3rd instar larvae of *M. separata* (Sato and Tanaka 1984). The number of eggs that we found in 3rd instars was smaller than in the previous study. Therefore, we hesitate to claim that using a stereomicroscope, we were able to find all eggs laid by the wasps; however, we are confident that our dissection technique was at least consistent, and that numbers of eggs laid in the two caterpillar species were significantly different.

There was a tendency for *C. kariyai* females to avoid *S. litura* when they had previously attempted to oviposit in them. In this study, 85% and 95% of naive wasps attacked nonhost and host wasps, respectively (Fig. 2). On the other hand, the attack rate on hosts decreased from 95% to 30% if *C. kariyai* females had previously attacked nonhosts and to 13.3% if they had attacked hosts previously. This decrease may be caused by lower oviposition activity of wasps due to a previous attack, as parasitoids need time after oviposition to produce additional mature eggs (Ueno 1999; Islam and Copland 2000). Therefore, attack rate on nonhosts by host-experienced females was also low (10.0%), even when the females had never experienced the nonhost before. However, even taking that into consideration, experienced wasps had a tendency to avoid nonhosts more than hosts. It appears that female wasps that had attacked nonhosts learned to recognize certain cue(s), enabling them to avoid nonhosts in subsequent

(a)

(b)
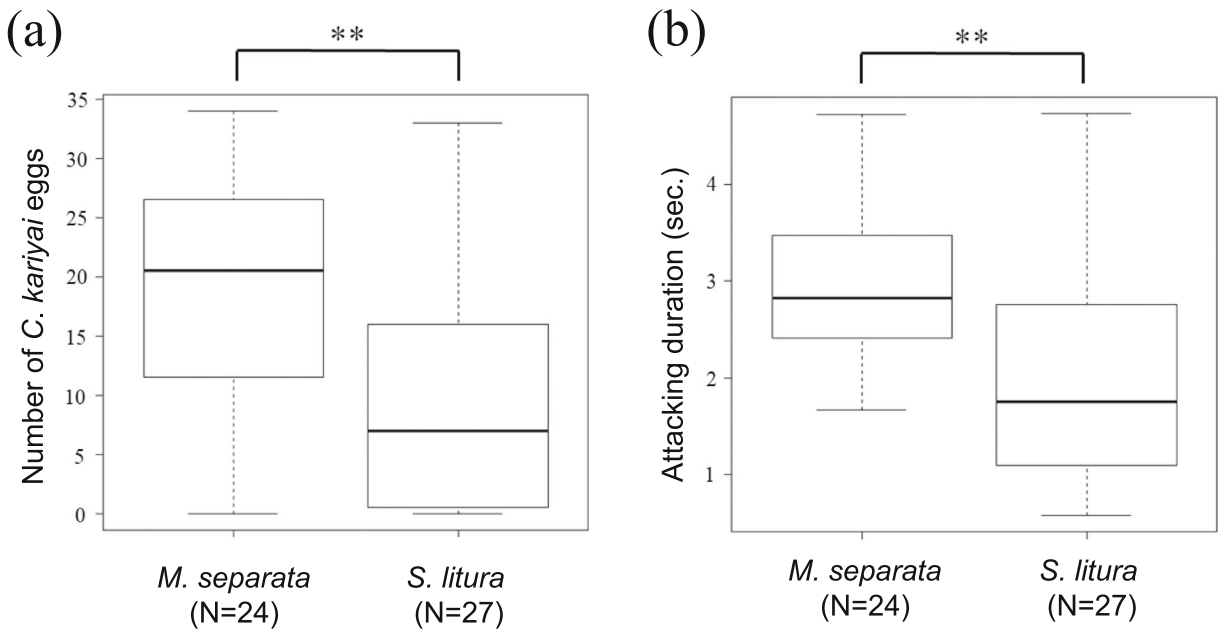
Fig. 1 Attack of *C. kariyai* females on *M. separata* (host) and *S. litura* (nonhost) larvae. **a** Numbers of wasp eggs found by dissecting *M. separata* and *S. litura* larvae that had been attacked once by a single female *C. kariyai* and **b** attack duration of *C. kariyai* females on *M. separata* and *S. litura* larvae. Whiskers show maximum and the minimum values. The bold dark line shows the median, and box top and bottom edges represent the first and third quartiles (**: $p < 0.01$ by Exact Wilcoxon rank sum test)

encounters. It is probable that *C. kariyai* females recognize *S. litura* larvae as nonhosts by inserting their ovipositors. Because *C. kariyai* larvae cannot complete development in *S. litura* larvae (Hayakawa and Yazaki 1997), this behavior minimizes the cost of inappropriate

oviposition. Thus, early experience with *S. litura* inhibits *C. kariyai* females subsequent attempts to parasitize this nonhost species. Since we put only one *C. kariyai* female and one *S. litura* larva together in a Petri dish, it is clear that *C. kariyai* females were
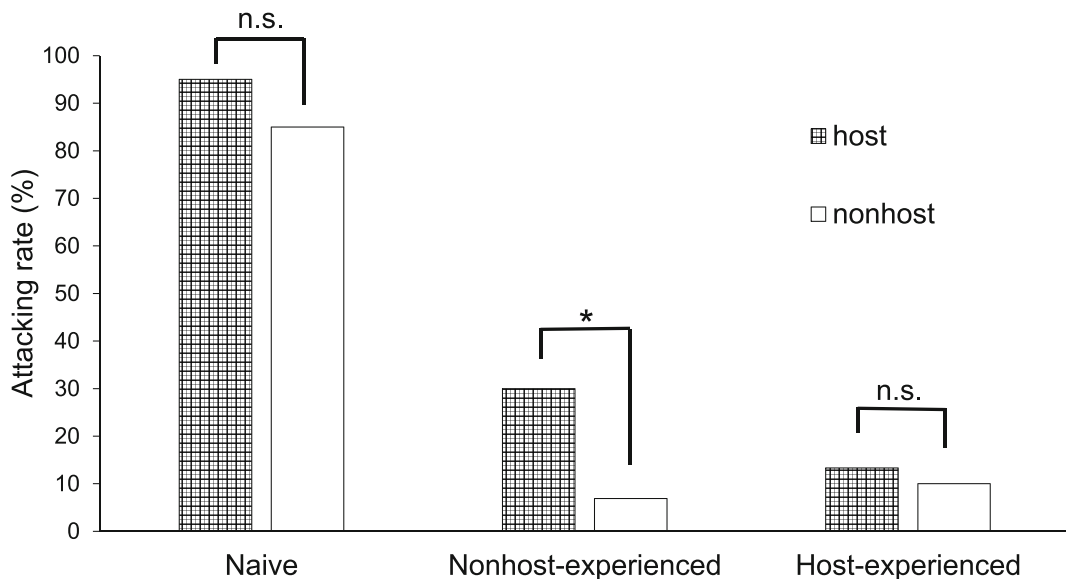
Fig. 2 Attack rates on host and nonhost larvae by naïve, nonhost-experienced and host-experienced *Cotesia kariyai* females. Numbers in bars indicate numbers of wasps (n.s.: no significant differences, *: $p < 0.05$ by Fisher's exact test)

responding only to compounds from *S. litura*, and that *C. kariyai* females had learned from their prior experience with nonhosts.

From these results, we still do not know how *C. kariyai* females recognize *S. litura* as a nonhost. Previous studies revealed that some parasitic wasps have sensilla on their ovipositors that detect gustatory stimuli (van Lenteren et al. 2007; Goubault et al. 2011; Ruschioni et al. 2015). *Cotesia kariyai* females may also have ovipositor sensilla to detect metabolites specific to *S. litura* or the absence of compound(s) from *M. separata* when they insert their ovipositors. SEM evaluation of *C. kariyai* ovipositors may reveal useful morphological information, and electro-physiological techniques may also be applied.

When *C. kariyai* females contact host or nonhost larvae, they drum on the larvae with their antennae. We observed that many wasps that declined to oviposit in nonhost larvae first drummed on them briefly before aborting an attack. Studies on other parasitic wasps suggest that wasps can detect compounds on host body surfaces (Obonyo et al. 2010). Ohara et al. (1996) showed that when presented with a brown glass rod coated with host body wax, *C. kariyai* females examined the rod using their antennae and tried to insert their ovipositors into the rod. *C. kariyai* females may likewise detect body wax of nonhost caterpillars. It is important to emphasize that we did not put any other materials, e.g., host feces or diet, in the Petri dishes beside a single wasp and a larva, so wasps were exposed only to compounds from larvae in these experiments. Behavioral observations and/or experiments on *C. kariyai* responses to isolated chemical(s) from body wax and fluids of nonhosts are needed.

These results show that *C. kariyai* females detect chemical(s) in *S. litura* larvae and lay fewer eggs, recognizing them as nonhosts. They also show that *C. kariyai* females avoid laying eggs in *S. litura* larvae after prior experience attacking them. Thanikkul et al. (2017) suggested that *C. kariyai* females can distinguish host-related and nonhost-related herbivore-induced plant volatiles (HIPVs). From these results, *C. kariyai* females apparently minimize egg waste caused by oviposition into nonhost species. They accomplish this by recognizing host habitat using HIPVs and by associative learning from experience with nonhosts.

Compliance with Ethical Standards

Conflict of Interest There are no conflicts of interest to declare.

Ethics Approval Not applicable.

Consent to Participate Not applicable.

Consent for Publication Not applicable.

Code Availability Not applicable.

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