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Changing Leaf Geometry Provides a Refuge from a Parasitoid for a Leaf Miner

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The use of physical barriers is a common defensive strategy in small-sized endophagous arthropods, but this feeding mode often results in tracks being left on host organisms, thus increasing predation risk. Mechanisms of escape from tracking predators are thus particularly important for endophagous arthropods. Leaf miners are herbivorous insects that inhabit the interiors of leaves and produce various forms of tracks on their host plants. Such tracks are called “mines,” and parasitoid wasps, which are the primary enemy of leaf miners, use mines as cues to find host larvae. In the present study, we use the leaf-mining moth *Acrocercops transecta* (Insecta: Lepidoptera: Gracillariidae), which changes mine forms during larval growth, and its primary parasitoid *Aneurobracon philippinensis* (Hymenoptera: Braconidae). Larvae of *A. transecta* make narrow linear mines in the first and second instars, the third instars expand the mines to flat blotch mines, and the fourth and fifth instars construct three-dimensional tentiform blotch mines. A laboratory parasitization experiment showed that successful oviposition rates were significantly lower on tentiform blotch mines than on other mine types. In contrast, all fifth instars that were transplanted into flat blotch mines were oviposited, suggesting that older instars did not deter ovipositing parasitoids and that the lower rates of successful oviposition on tentiform blotch mines were attributable to refuges inside such mines provided by their three-dimensional structure. Field data demonstrated a plateau in parasitism rates in fourth instar larvae, confirming the results of the laboratory experiment. These results indicate that different mine forms affect the viability of endophagous larvae.

Key words: Braconidae, defensive strategy, endophagous, Gracillariidae, host-parasite interaction, prey-predator interaction

INTRODUCTION

Prey–predator interactions have driven the evolution and diversification of defensive strategies in prey organisms. Various defensive strategies are used by animals, from weapons such as allomonal chemicals (Brodie and Smatresk, 1990; Krall et al., 1999), to body armor or armor-like structures (Strankowich and Campbell, 2016; Schoeller et al., 2018), to crypsis (Edmunds, 1990). Another common defensive strategy in animals is the use of physical barriers (Lenderhouse, 1990), and this antipredator adaptation is categorized as a primary defense mechanism (Robinson, 1969). The merit of primary defense mechanisms is that prey avoid the energetic expenditures arising from predator encounters (Lenderhouse, 1990). Small-sized arthropods, particularly insects, often feed within plant bodies, and this feeding mode in turn provides barriers (i.e., plant tissues) that defend the interior prey insects from attack by predators. However, endophagous prey insects often leave tracks and/or signals on their host plants, increasing the risk of predation by leading predators to the prey (Sabelis and van de Baan, 1983; Salvo and Valladares, 2004; Hatano et al.,

2008). Thus, mechanisms of escape from tracking predators are of particular importance for such endophagous insect herbivores.

Leaf miners are tiny insect herbivores that inhabit and feed within leaves. Leaf miners exhibit a unique defensive strategy, the modification of habitat geometry to “puzzle” predators. They produce various forms of tracks (from lines to blotches, and from flat to three dimensional) on leaves; these tracks are called “mines.” The primary enemy of leaf miners are parasitoid wasps (Askew and Shaw, 1986; Hespeneheide, 1991; Hawkins et al., 1997), which use mines as cues to find target host larvae (Sugimoto, 1977; Casas, 1989; Salvo and Valladares, 2004). However, it takes parasitoids a longer time to find larvae when they encounter mines with complex structures (Djemai et al., 2000; Ayabe et al., 2008). This implies that complex mine forms are in part the result of prey–predator interactions between leaf miners and parasitoids. Both field observations (Sato, 1995; Ayabe and Ueno, 2012) and theoretical studies (Kato, 1985; Ayabe et al., 2008) have demonstrated decreased parasitism rates with an increase in mine complexity, and this hypothesis has also been supported by laboratory experiments. The eulophid parasitoid *Hemiptarsenus varicornis* spends more time locating its dipteran host *Liriomyza trifolii* in linear mines with more than one crossing (Ayabe et al., 2008). Additionally,

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the gracillariid leaf miner *Phyllonorycter malella* creates a mine with uneaten tissues in its central area, which acts as a protective shield and greatly diminishes the risk of parasitization by *Sympiesis sericeicornis* (Europhidae) (Djemai et al., 2000). However, we know very little about the effects of mine-form differences (between linear and blotch or between flat and three dimensional) on the risk of parasitization.

Since each leaf-miner species makes a species-specific mine form on particular host plants, in order to assess the significance of differences in mine form on the ease of parasitization we must evaluate different leaf-miner species. However, comparisons among different species make it difficult to separate the effect of mine-form differences from those of other factors, such as host-plant and insect-species differences, and this has hampered the validation of the effect of mine-form differences on the risk of parasitization.

In this study, we used a leaf-mining insect species that changes its mine form according to larval growth. Larvae of *Acrocercops transecta* (Lepidoptera: Gracillariidae) make narrow, linear mines during the first and second instars (Fig. 1A, D), third instar larvae expand their mines to make flat blotch mines (Fig. 1B, E), and, finally, fourth and fifth instar larvae gather the upper surface of the mines by spinning silk and construct deep, three-dimensional tentiform blotch mines (Fig. 1C, F). First to third instar larvae are flattened in body shape, with no thoracic legs or prolegs. These larvae mine into the epidermis of host plants and are called sap feeders, while fourth and fifth instar larvae have cylindrical bodies with both thoracic legs and prolegs, similar to other lepidopteran larvae, and are called tissue feeders. Thus, we can easily distinguish the various mine forms and instars,

providing a valuable opportunity to evaluate the effect of mine-form differences using the same insect species mining on the same host plant species.

In the wild, larvae of *A. transecta* are heavily parasitized by a braconid wasp, *Aneurobracon philippinensis* (Agathidiinae), and *A. philippinensis* is a primary parasitoid of *A. transecta* (Ohshima et al., 2015). Females of *A. philippinensis* lay their eggs in larvae of *A. transecta* that are mining within host plants. After *A. transecta* larvae finish making their cocoons outside mines, parasitoid larvae immediately egress from host larvae, then feed on the host inside the cocoons and pupate in the cocoons that were made by the host insects (Ohshima et al., 2015). In the present study, we conducted a laboratory parasitization experiment using the *A. transecta* and *A. philippinensis* system and compared rates of successful oviposition and lengths of host searching time by *A. philippinensis* among different mine forms. We also investigate the parasitism rates by *A. philippinensis* for each host instar in the wild, and the significance of mine form differences is discussed in light of prey-predator interactions.

MATERIALS AND METHODS

General procedure of insect collection and rearing

We collected larvae of the host insect (*Acrocercops transecta*) from three sites; Kyoto (35°0' N, 135°46' E; Kyoto Prefecture, western Honshu), Niimi (34°59' N, 133°25' E; Okayama Prefecture, western Honshu) and Kiso (35°50' N, 137°41' E; Nagano Prefecture, central Honshu) (Fig. 2). Although *A. transecta* consists of two host races that are associated with either Juglandaceae or *Lyonia ovalifolia* (Ericaceae) (Ohshima, 2008), we used only the Juglandaceae race in the present study because larvae of the *Lyonia* race

are very rarely parasitized by *A. philippinensis* (Ohshima et al., 2015). The moth larvae were collected from Juglandaceae plants, along with the leaves they were mining, and reared in the same plant leaves in the laboratory at Kyoto Prefectural University following the method described by Ohshima (2005). Emerged *A. transecta* adults were sexed and transferred to clear centrifuge tubes (118 mm long, 28 mm diameter) containing a rolled wiping paper soaked with a 2% sucrose solution for maintenance. Adult *A. philippinensis* that emerged from the host cocoons were sexed and transferred to clear cell culture dishes (33.9 mm diameter, 10 mm high). Each dish contained layered wiping papers soaked with a 30% honey solution for maintenance. All rearing and experiments were conducted at $25 \pm 1^\circ\text{C}$ with photoperiodic conditions of 16 h light:8 h dark (16L:8D) and 40–60% relative humidity, except for the emerged adult wasps, which were kept at 20°C until the experiment was conducted.

Preparation of prey mines for the parasitization experiment

To prepare mined leaves for the parasitization experiment, a single pair of virgin moths was transferred to a centrifuge tube (118 mm length, 28 mm diameter) with an aspirator. After mating, each

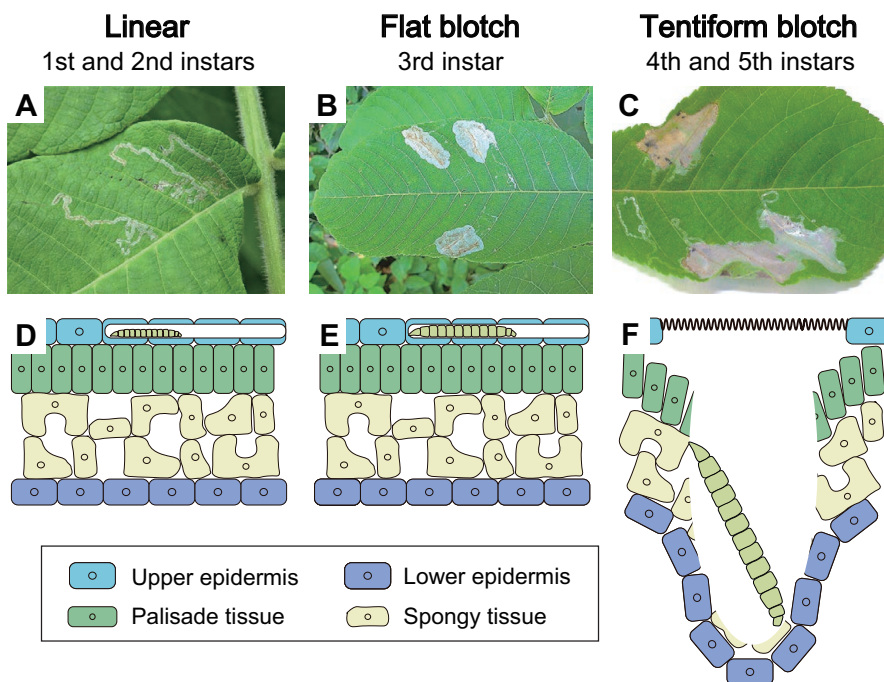


Fig. 1. Mine forms of *A. transecta*. Photos of three different mine forms (white-colored area) on *Juglans* leaves (A–C) and illustrations of the cross-sections of each form (D–F). First and second instars make linear mines and feed on the upper epidermis (A, D), and third instars still mine within the upper epidermis and make the mining surface larger to form flat blotch mines (B, E). In the fourth to fifth instars, larvae feed on palisade and spongy tissues, making the mines deeper and tentiform (C, F).

female that had begun oviposition in the centrifuge tube was introduced to a clear plastic container ($125 \times 205 \times 50$ mm). Two or three fresh young leaves of *Juglans regia* were placed in the container before the lights were turned off, and females of *A. transecta* laid their eggs after the dark period began. After the lights were turned on, we evaluated the oviposited leaves and removed some eggs from the leaves to adjust the number of eggs per leaf. Leaves with eggs were individually transferred to a clear plastic bag (195×177 mm) to maintain the *A. transecta* eggs and hatched larvae. When the larvae reached the final instar, they were transferred to a plastic container ($85 \times 125 \times 30$ mm). Adults of *A. transecta* collected at the three sites (Kyoto, Niimi, and Kiso) were randomly used for the preparation of mines. We used leaves with linear, flat blotch, or tentiform blotch mines (Fig. 1) for the subsequent parasitization experiments involving *A. philippinensis*. Assessed female wasps often failed oviposition against tentiform blotch mines (Fig. 4, see Results), but this experimental design cannot separate the

effects of mine structures from larval instars because each mine structure contains a specific instar larva. Thus, we further prepared flat blotch mines containing transplanted fifth instar larvae following the method described by Ohshima (2008) in order to test whether the relatively free mobility in tissue-feeding instars and/or parasitoids' dispreference for older host instars affect reduced oviposition success towards older instars.

Parasitization experiment

Adult females of *A. philippinensis* track the mines of *A. transecta* by tapping with their antennae and ovipositor to find host larvae (Fig. 3A). When a female wasp finds a host larva inside a mine, the female stops at the position of the mining larva. Then, the female wasp immediately inserts her ovipositor into the mine and injects the larva with her eggs. Females raise their antennae at this moment (Fig. 3B). In this study, we defined the tapping behavior as "searching behavior" and the raising-antennae behavior as "oviposition behavior". The female wasps sometimes gave up searching for a host and left a mine. In the parasitization experiment, we recorded females that successfully found host larvae as "succeeded" and females that gave up as "failed". Unmated female wasps were individually introduced to clear plastic containers ($125 \times 205 \times 50$ mm) containing a *J. regia* leaf with either a linear, flat blotch, tentiform blotch mine (Fig. 1), or flat blotch mine containing transplanted fifth instar larva. When a female wasp successfully completed oviposition or gave up searching for a host, we recorded the time, and we defined the "searching time" as the length of time that a female wasp spent searching for a host larva (from the beginning of mine tapping to the insertion of the ovipositor or giving up). We also defined the "successful oviposition rate" as the ratio of the number of female wasps that successfully oviposited to the number of all females showing searching behavior (the sum of succeeded and failed females). In the transplantation experiment, in order to assess the suitability of older host instar for parasitoid development we maintained parasitized host larvae and recorded the number of *A. philippinensis* adult individuals that successfully emerged. Adults of *A. philippinensis* collected from the three sites (Kyoto, Niimi, and Kiso) were used at random in this experiment, but each wasp was used only once in the parasitization experiment.

Rate of parasitism by *A. philippinensis* in the wild

We recorded the instar of each *A. transecta* larva collected in the wild and reared in the laboratory to assess whether the larvae were parasitized. Since *A. transecta* larvae change their morphology and mining patterns according to their growth, we can precisely identify the instars of collected larvae based on mine and larval characters. We only collected living larvae to avoid collecting larvae

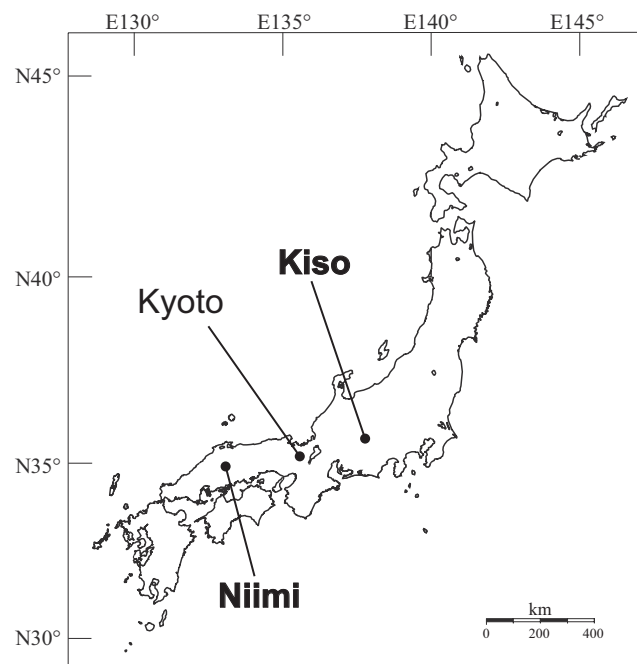


Fig. 2. Map showing sampling sites of *A. transecta* and *A. philippinensis* that parasitized *A. transecta*. Bold letters indicate sites where wild parasitism rates by *A. philippinensis* were recorded.

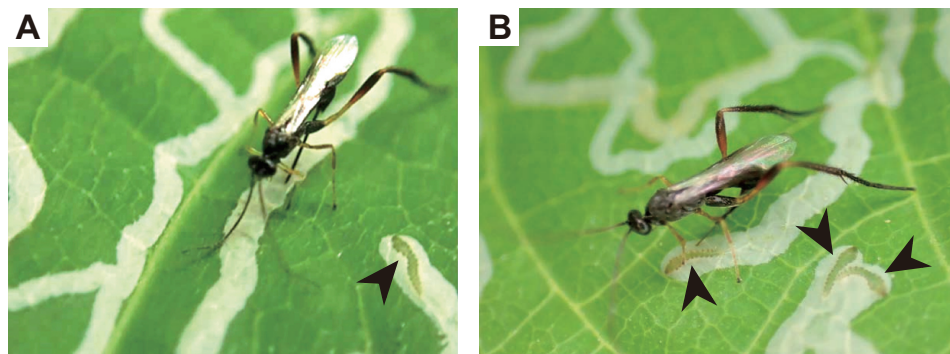


Fig. 3. Searching and ovipositing behavior of *A. philippinensis* females. **(A)** Searching behavior. A female parasitoid tracks mines by tapping their antennae and ovipositor to find host larvae. **(B)** Ovipositing behavior. A female stops at the position of a found larva and immediately inserts her ovipositor into the mine and stings the larva. The arrows indicate the location of host larvae within mines.

that had already been parasitized by idiobiont parasitoids. Because the larvae of *A. philippinensis* rapidly begin their development after *A. transecta* larvae finish making their cocoons (Ohshima et al., 2015), *A. transecta* larvae that died before making their cocoons were removed from the dataset. We calculated the parasitism rates as the ratio of the number of emerged *A. philippinensis* adults to the number of host larvae that successfully made their cocoons. The Niimi and Kiso populations were used for the assessing parasitism rates in the wild, and sampling was conducted in mid-June, 2018 in Niimi and in late July, 2017 in

Kiso.

Statistical analysis

The parasitism rates in the parasitization experiment and in the wild-collected samples were compared among the four treatments (the three mine forms and the transplanted treatment) and among the collected instars, respectively. We used Fisher's exact test, and the sequential Bonferroni correction (Rice, 1989) was applied to adjust the *P*-values to maintain the significance level at 0.05 throughout the multiple comparisons. The corrected *P*-values were indicated as *P* (adjusted *P*-value). We identified significant differences in searching times among the four treatments (the three mine forms and the transplanted treatment) using the Mann-Whitney *U* test. All statistical tests were carried out using the R package version 3.2.1 (R development Core Team, 2015).

RESULTS

Successful oviposition rates for different mining forms

The rate of successful oviposition for tentiform-blotch mines (27.3%, $n = 11$) was significantly lower than those for linear (88.9%, $n = 9$, $p = 0.0098 < P(0.0125)$), flat-blotch mines (100%, $n = 8$, $p = 0.0034 < P(0.0083)$) and the transplantation treatment (100%, $n = 7$, $p = 0.0040 < P(0.01)$, Fisher's exact test) (Fig. 4). However, the differences in successful oviposition rates among the remaining treatments were not significant (linear and flat blotch, $p = 1 > P(0.0167)$; linear and transplantation, $p = 1 > P(0.0167)$; flat-blotch and transplantation $p = 1 > P(0.0167)$; Fisher's exact test) (Fig. 4). During the observation of the parasitization process, larvae in linear mines could not escape from wasps because they cannot move actively in the narrow linear mines (Fig. 1), so wasps very easily achieve oviposition once they find a host larva. Similarly, the wasps searching on flat blotch mines press the larvae using their long hind legs to hold the larvae in place and sting the larvae at a

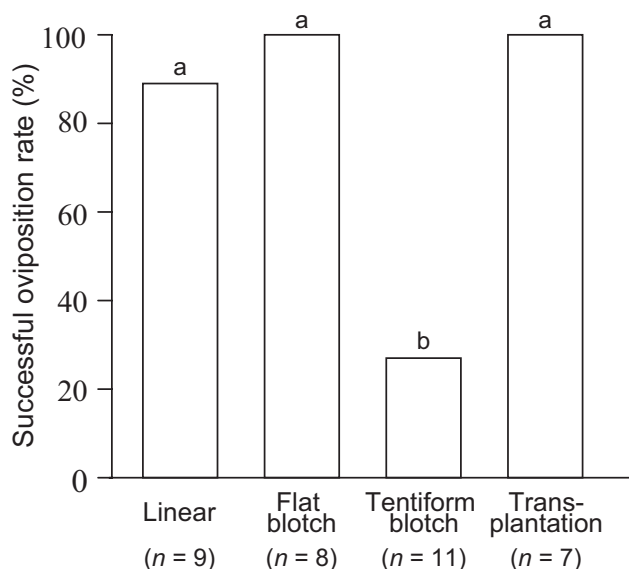


Fig. 4. Successful oviposition rates for three mine forms and the transplantation treatment in the laboratory experiment. The oviposition rate for the tentiform blotch mine was significantly lower than those for the linear and flat blotch mines and the transplantation treatment. The different letters associated with each bar indicate significant differences in oviposition rates among treatments.

highly successful rate, although the host larvae can move more actively than those in linear mines. Of the seven parasitized fifth instar host larvae in the transplantation treatment, five survived until cocooning, and parasitoid adults successfully emerged from all five cocoons, whereas the other two host larvae died before making cocoons.

Searching time for different mining forms

The searching time of parasitoids tended to be longer on tentiform-blotch mines (Fig. 5). Although there were no significant differences in mean length among the four treatments (the three mine forms and the transplanted treatment) (Table S1), the median searching time on the tentiform blotch mine was larger than the highest values in the 95% confidence intervals of the median searching times for the other three treatments (Fig. 5).

Parasitism rates in the wild

The parasitism rates by *A. philippinensis* in the Niimi and Kiso populations are shown in Fig. 6. In the Niimi population, the parasitism rate increased according to the larval instar and was highest in the fourth instar (92.0%, $n = 100$). There was significant difference in parasitism rates between third and fourth instars. (Fig. 6, Table S2). In the Kiso population the parasitism rates increased until the fourth instar, reached the highest value (59.4%, $n = 197$) in the fourth instar. There was no significant difference in parasitism rates between the third and fourth instars (Fig. 6, Table S2), but the fifth instar showed a significantly lower parasitism

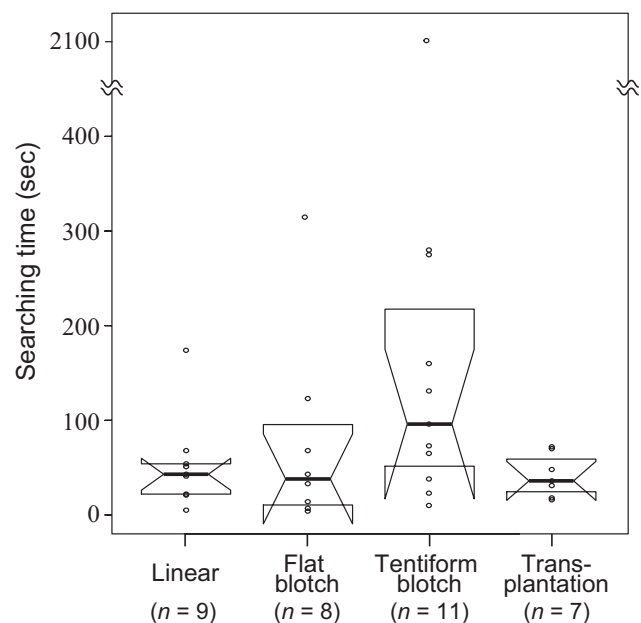


Fig. 5. Searching time lengths are shown in box plots for each of the three mine forms and the transplantation treatment. Upper and lower margins of boxes indicate upper and lower quartiles, respectively, and notched areas show 95% confidence intervals of the medians (thick horizontal bar) for respective treatments. Dots indicate raw data points for each treatment. The median searching time on the tentiform blotch mine was greater than the highest values of the 95% confidence intervals of the median searching times for other treatments.

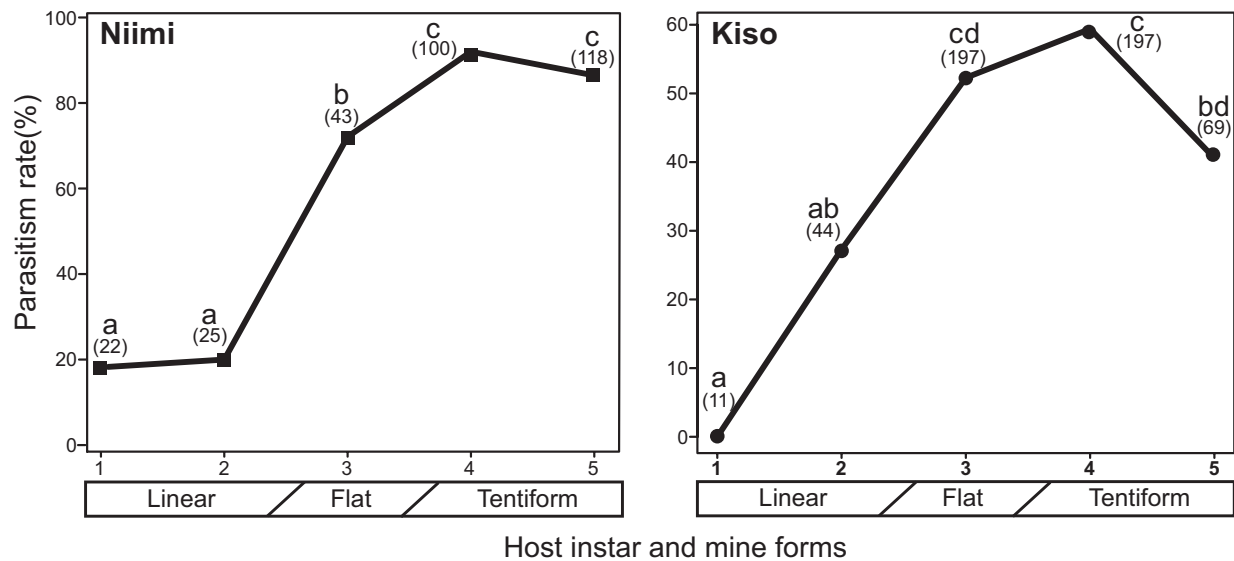


Fig. 6. Parasitism rates by *A. philippinensis* in the wild for *A. transecta* instars. The numbers associated with each plot show the total number of samples used for the calculation of parasitism rates for the different instars. The different letters associated with each plot indicate significant differences in parasitism rates among instars in each population.

rate than that of fourth instar (Fig. 6, Table S2).

DISCUSSION

The present laboratory experiment shows the significantly lowered successful oviposition rate of *A. philippinensis* for the tentiform blotch mine than those for the two other mine forms, indicating that differences in mine form indeed change the viability of *A. transecta* larvae. Females of *A. philippinensis* can recognize the presence of *A. transecta* larvae even when the larvae are in tentiform blotch mines, but the larvae inside quickly escape from the wasps, using the depth of the mine to maintain their distance from the enemy (Fig. 1C, F). This means that tentiform blotch mines can provide an evacuation space for leaf miners. Such an evacuation space, coupled with the escape behavior of the hosts, leads to an increase in the searching time of parasitoid wasps on tentiform blotch mines (Fig. 5). Although no significant differences were detected among the four treatments in terms of the mean searching time based on the Mann-Whitney *U* test, the fact that the median searching time on the tentiform blotch mine was greater than the highest value in the 95% confidence intervals of the median searching times for linear, flat blotch mines and the transplantation treatment (Fig. 5) suggests that the tentiform structure increases searching time in parasitoid wasps. Given that ovipositing parasitoid wasps follow optimal foraging theory (Charnov, 1976), we can expect that parasitoid females should search for hosts in a manner that maximizes the number of successful oviposition events per unit time (Cook and Hubbard, 1977; Hubbard and Cook, 1978). This implies that parasitoids have evolved to avoid hosts that require a long time for successful oviposition to occur, and thus larvae inside the tentiform blotch mines are able to escape from the parasitoids.

Other possible reasons for the decreased successful oviposition rate on the tentiform blotch mine are age of host larvae within the mines. As is ordinary in other lepidopteran

larvae, tissue feeding instars have legs. It is possible that their comparatively greater mobility compared with sap feeding instars contributes to helping older larvae escape from parasitoids. Also, older instar hosts often show higher resistance against oviposited parasitoid offspring than younger instars (e.g. Harvey et al., 1994), thus such stronger resistance may result in parasitoids' dispreference for older host instars. However, the fact that all fifth instar larvae that had been transplanted into flat blotch mines were successfully oviposited by *A. philippinensis* females indicates that no or very weak effects of host ages on the decreased successful oviposition rate for the tentiform blotch mine. Thus, the decreased successful oviposition rate on the tentiform blotch mine is attributed to the difficulty of laying eggs in larvae within three-dimensional mines and highly successful oviposition rates for linear and flat blotch mines are ascribed to the ease of oviposition against larvae within flat mines.

The above results from the laboratory oviposition experiment are largely consistent with the observations of parasitism rates by *A. philippinensis* in the wild (Fig. 6); these did not increase between fourth and fifth instars, which make tentiform blotch mines. These results, coupled with the fact that *A. philippinensis* can develop into adult even when they were oviposited in fifth instar larvae of *A. transecta*, support the hypothesis that the change in mine form from flat to three-dimensional is effective for escaping from parasitoid attack.

However, our present results of wild parasitization rates also provide an unexpected finding: a significant decrease in the parasitization rate in the fifth instar in the Kiso population (Fig. 6). A possible explanation for this phenomenon is that frequent superparasitism of *A. transecta* larvae by *A. philippinensis* results in high mortality in later-stage host larvae. Several studies have reported that superparasitism often causes higher mortality in the host insect (Salt, 1936; Kuno, 1962; Devescovi et al., 2015). In the Kiso population, the collected *A. transecta* larvae were occurred in

high-density conditions (Fig. 6). Some studies of leaf miner parasitoids conducted in the field have found that parasitoids disproportionately visited leaves with higher miner densities (Casas, 1989; Connor and Cargain, 1994). Therefore, we can postulate that high-density populations of host insects exhibit decreased parasitism rates at the developmental stage that parasitoids undergo rapid growing due to excess superparasitism. Another possible explanation is that it is difficult for parasitized hosts to survive in stressful conditions, such as starvation due to a high density of host larvae (Morse and Chapman, 2015). These hypotheses should be tested using populations with a variety of parasitism rates and individuals with various levels of superparasitism in future studies.

The present parasitism rates of *A. philippinensis* in the wild are also very likely to be affected by other parasitoids that share *A. transecta* as a host insect. Leaf miners in the linear and flat mines are generally attacked more heavily by idiobionts (parasitoids that kill or inhibit the growth of hosts immediately after oviposition (Askew and Shaw, 1986)) than by koinobionts (Godfray, 1994; Kato, 1994; Sato, 1995; Ayabe and Ueno, 2012; Ayabe and Hiji, 2016). Indeed, we observed many early instars of *A. transecta*, which make linear mines, attacked by idiobionts in the wild, and it appears that it is difficult for the koinobiont parasitoid *A. philippinensis* to complete its growth in early host instars if they are superparasitized by idiobiont parasitoids. Therefore, there could be a trade-off between flat and three-dimensional mines in the fitness of *A. philippinensis*; flat mines are easy targets for oviposition, but competition with idiobionts is likely to occur, and it is difficult to lay eggs in three-dimensional mines, but superparasitism by idiobionts can be avoided.

The above discussion raises the question of why gracillariid larvae do not make three-dimensional mines in early instars. This is probably due to the phylogenetic and developmental constraints of Gracillariidae. Gracillariid larvae change their morphology drastically over the course of their development, and this is called larval hypermetamorphosis. Hypermetamorphosis is one autapomorphy of the superfamily Gracillarioidea (Scoble, 1992), which is composed mostly of gracillariid species. First to third instars lack spinnerets; thus, they cannot make tentiform mines, while they can feed within the epidermis of plants. The superfamily Gracillarioidea is the only insect taxon that can mine into the epidermis of plants; thus, the sap-feeding form enables gracillariid larvae to use an empty niche, although they suffer from high parasitization rates.

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COMPETING INTERESTS

The authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS

HA and IO conceived and designed the study. Both authors conducted sampling in the field and HA conducted the laboratory rearing and experiments. Both authors analyzed the data and wrote the paper.

SUPPLEMENTARY MATERIALS

Supplementary materials for this article are available online. (URL: <http://www.bioone.org/doi/suppl/10.2108/zs180062>).

Supplementary Table S1. *P*-values of pairwise comparisons of searching time among different mine-form and transplantation treatments. No *p*-values were lower than corresponding adjusted significant levels (in parentheses).

Supplementary Table S2. Results of pairwise comparisons of the parasitism rates among instars in Kiso (lower triangle) and Niimi (upper triangle) populations. *P*-values in bold letters are lower than adjusted *p*-values (in parentheses).

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