

1 **Avoidance of parasitoid attack is associated with the spatial use**
2 **within a leaf by a lepidopteran leafminer**

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1 **Abstract**

2 In prey-predator systems, top-down effects can be a powerful determinant for spatial
3 distributions of prey through their search for enemy-free space. Leafminers live and eat
4 within leaves, making feeding tracks called mines, and mine conspicuousness exposes them
5 to a high risk of parasitism. Those lepidopteran leafminers that use lower leaf surfaces as
6 mining sites show wide evolutionary radiation. We hypothesized that leafminers making
7 mines on the lower surface are less often detected by parasitoids and thus have a selective
8 advantage in avoiding parasitism compared to those on the upper surface. To investigate the
9 adaptiveness of lower-surface mining, we examined the relationship between parasitism and
10 within-leaf mine distribution for 3 years using a field population of the leafminer
11 *Phyllocnistis spec. Zeller* (Lepidoptera: Gracirallidae, Phyllocnistinae), which prefers the
12 lower surface of leaves of the Japanese privet, *Ligustrum japonicum* Thunb. (Oleaceae).
13 Parasitoid attack was more frequent in the upper-surface mines than in the lower-surface
14 mines and on leaves with upper-surface mines than on leaves with only lower-surface mines.
15 When both surfaces were mined, leafminers on the lower surface could avoid parasitism.
16 Upper-surface mines were attacked by more parasitoid species as compared to lower-surface
17 mines. Although the results demonstrated that mining on the lower surface was advantageous
18 in avoiding parasitism, the vulnerability of lower-surface mines to parasitism varied
19 depending on their abundance. When many lower-surface mines were present, lower-surface
20 mines suffered a higher parasitism rate than upper-surface mines, probably because
21 parasitoids formed search images for and concentrated on lower-surface mines. This study
22 suggests that the preferential use of the lower leaf surface by leafminers is in part attributed
23 to interactions with parasitoids.

1 **Introduction**

2 Interactions with natural enemies may be a selective force underlying the wide diversity of
3 insect herbivores, which have evolved a broad array of life-history and ecological traits.
4 Consequently, insect herbivores have also developed a wide variety of defensive strategies,
5 ranging from indirect ones, such as mimicry and cryptic coloration, to direct ones, such as
6 biting and escaping (Evans & Schmidt, 1990). The major direction of adaptive radiation with
7 regard to defense strategy varies among insect taxonomies because a species' defense
8 strategy depends on its ecological traits per se (Evans & Schmidt, 1990). Because defense is
9 associated with other adaptations in morphology, physiology, niche use, behavior, and so on
10 (Whitman et al., 1990; Singer & Stireman, 2005), the direction of the defense in one group of
11 insect herbivores may be based on some radiation patterns of other non-defensive traits.
12 Recently, such top-down effects have been proposed as a powerful determinant for host-plant
13 use in insect herbivores through their search for enemy-free space (Singer et al., 2004; Singer
14 & Stireman, 2005; Bailey et al., 2009).

15 The leafminer–parasitoid system is an example of an antagonistic interaction that
16 evolves over time in response to intense selection pressure for both the leafminer and the
17 parasitoid. Leafminers live within plant leaves during the larval stage (and at the pupal stage
18 in some species) and acquire nutrients by consuming inner-leaf tissues or by sucking tissue
19 fluid. The tissues that the various taxa mine are species-specific, and their feeding track,
20 called a mine, is conspicuous due to the white color against the green background of the leaf.
21 The leaf-mining habit allows leafminers to escape from predation (Connor & Taverner, 1997).
22 However, the conspicuousness of the mines attracts insect parasitoids that can pierce leaves
23 with the ovipositor (Casas, 1989; Salvo & Valladares, 2004; Low, 2008) and consequently
24 makes leafminers vulnerable to parasitoid attack (Hawkins & Lawton, 1987; Hochberg &
25 Hawkins, 1992; Connor & Taverner, 1997). In this situation, compensating for the
26 disadvantage caused by mine conspicuousness can alleviate parasitism and improve the
27 fitness of leafminers and, therefore, can direct the evolutionary path of their defensive
28 strategies.

29 A variety of defensive strategies to compensate for the conspicuousness of mines
30 has been reported. For example, complicated patterns of mines with crosses (Kato, 1985;
31 Ayabe et al., 2008; Ayabe & Ueno, 2012), shield-like structures in mines (Djemai et al.,
32 2000), and tent-like-structured mines (Brandl & Vidal, 1987) can have defensive functions to
33 reduce parasitism rates even after mines are detected by parasitoids, which still have to locate

1 a tiny host larva hiding inside the mine. These represent post-detection defense strategies.
2 Leafminers also have developed pre-detection defenses against parasitoids, i.e., defenses
3 before their mines are detected. Leafminer species that form mines of light green or brown,
4 which are optically less conspicuous on the green background of the leaves, are less attractive
5 to generalist parasitoids than leafminers that form white mines, which are conspicuous (Salvo
6 & Valladares, 2004).

7 The directions of the evolution of leafminer defenses against parasitoids may have
8 affected the patterns of their use of host plants. In general, this is because patterns of
9 host-plant use (a niche component) could have been shaped by interactions not only with
10 conspecific leafminers for resource and space but also with natural enemies for enemy-free
11 space (Jeffries & Lawton, 1984). Enemy-free space can occur even at the within-plant scale,
12 as exemplified by the butterfly *Pieris brassicae* L. which obtains the advantage of reduced
13 parasitism by moving from leaves to flowers of the host plant during the larval stage
14 (Lucas-Barbosa et al., 2014). Leafminers may also obtain some advantages by preferentially
15 using a local space within a leaf.

16 Leafminer species can be divided into three groups with respect to within-leaf
17 spatial use: those using only the upper (adaxial) surface tissue, those using only the lower
18 (abaxial) surface tissue, and those using both surfaces. Among lepidopteran leafminers, the
19 species that use the lower leaf surface have radiated more widely phylogenetically than
20 species that use the upper surface (Reavey & Gaston, 1991; Lopez-Vaamonde et al., 2003),
21 which would strongly suggest the adaptiveness of mining the lower leaf surface. It has been
22 suggested that lower-surface mines were less likely to be attacked by parasitoids than
23 upper-surface mines because mines on the lower surface are hidden and more difficult for
24 parasitoids to locate visually (Reavey & Gaston, 1991; Yamazaki, 2010). In fact, the dipteran
25 leafminer *Phytomyza cytisi* Brischke, which spends its early stages on the lower leaf surface
26 but later moves to the upper surface, was parasitized to a lesser extent than *Agromyza*
27 *demeijerei* Hendel, which uses the upper surface during the entire mining stage (Askew,
28 1968). However, the decrease of parasitism in *P. cytisi* was likely also caused by another
29 factor: *P. cytisi* may have been less preferred by parasitoids due to its smaller size than *A.*
30 *demeijerei*. Thus, it is still unclear whether spatial use at the within-leaf scale – upper- vs.
31 lower-surface mining – can cause differences in the pattern of parasitism. Leafminers are
32 sedentary and not able to move freely to seek a better position to attain higher survival and,
33 therefore, positions of mines within a leaf would be a critical component of the occupants’

1 fitness.

2 In the present study, we examined the relationships between within-leaf positions
3 of mines and the susceptibility of the mines to parasitism, to investigate the adaptiveness of
4 mining in lower-surface tissue. This would allow us to explore the possibility that evolution
5 of a defense strategy against parasitism affected the adaptive radiation of lower-surface
6 mining across the lepidopteran leafminer species. We used the leafminer moth *Phyllocnistis*
7 spec. Zeller (Lepidoptera: Gracirallidae, Phyllocnistinae). *Phyllocnistis* spec. is multivoltine,
8 active from May to October (Ayabe et al., 2015), and forms mines on both the upper and
9 lower surfaces of leaves of the Japanese privet, *Ligustrum japonicum* Thunb. (Oleaceae), but
10 shows a preference for the lower surface (relative to the upper surface) throughout the
11 occurrence period (Ayabe et al., 2015). Only about 30% of mines were formed on the upper
12 surface (Ayabe et al., 2015). This preferential use of the lower surface makes *Phyllocnistis*
13 spec. suitable for detecting the adaptive significance of lower-surface mining.

14 If the lower-surface mining functions as a pre-detection defense, we can
15 hypothesize (1) that *Phyllocnistis* spec. individuals on the lower surface are less readily
16 recognized by parasitoids and thus have an advantage over those on the upper surface with
17 regard to avoiding parasitism. This simple hypothesis can provide a straightforward
18 interpretation of the relationship between intra-leaf mining positions and the fitness of
19 leafminers. However, when two or more *Phyllocnistis* individuals simultaneously use both
20 surfaces of a single leaf, a parasitoid may locate a mine on the upper surface first, parasitize it,
21 and then move to the lower surface. If this is true, parasitoid attack can occur on miners on
22 both surfaces despite lower-surface mines are not visually located by parasitoids. Therefore,
23 we proposed another hypothesis (2): if upper-surface mines are more attractive for parasitoids,
24 then parasitoid attack will be more likely to occur on leaves with upper-surface mines than on
25 leaves without upper-surface mines. We tested the second hypothesis by comparing per-leaf
26 parasitism between leaves with and without upper-surface mines. Finally, we hypothesized
27 (3) that individuals on leaves with only lower-surface mines will be parasitized by fewer
28 parasitoid species than those on leaves with upper-surface mines. To test the three hypotheses,
29 we performed a nondestructive survey by following the fate of individuals in a field
30 population of *Phyllocnistis* spec. for 3 years and a destructive survey of mines by collecting
31 mines and the associated parasitoids. We then examined whether the use of lower leaf
32 surfaces could be advantageous in parasitism avoidance.

33

1 **Materials and methods**

2 **Study system**

3 The study was conducted in a secondary forest at Nagoya University, central Japan (35°9'N,
4 136°58'E), from 2010 to 2012. We selected 26 *L. japonicum* trees (3.5–11.8 cm trunk
5 diameter at the base, 1.2–6.7 m high). Of the 26 sample trees, one sample tree was excluded
6 in 2012 because it was damaged by fallen branches from the surrounding *Quercus* trees. At
7 the site, *L. japonicum* trees flushed leaves synchronously from April to early May (primary
8 shoots) and successively produced new foliage as midsummer shoots and compensatory
9 shoots in response to herbivory (lammas shoots). *Phyllocnistis* spec. initially uses leaves of
10 primary shoots, then shifts to young leaves of lammas shoots (Ayabe et al., 2015). It has 4-5
11 generations from May to October and mines on the lower surface in preference to the upper
12 surface (Ayabe et al., 2015). A female adult oviposits on either surface of a leaf, and the
13 hatched larva tunnels through the cuticle layer and then feeds within the epidermal layer,
14 making a serpentine mine. Larvae never change surfaces to mine. *Phyllocnistis* spec. pupates
15 inside the mine at the edge of the leaf, bending a small piece of leaf to make a structured
16 pupation site. These behaviors enable us to identify the developmental stages of the occupant
17 miners. The duration of the larval stage is usually 3–6 days, and the pupal stage is
18 approximately 10–14 days, depending on air temperature. The details of the study system
19 were described by Ayabe et al. (2015). The leafminer of the present study is an undescribed
20 species. We confirmed that leafminer individuals examined consisted of a single species, on
21 the basis of the larval and adult morphology and shape of mines.

22

23 **Nondestructive survey of parasitism in *Phyllocnistis* spec.**

24 Field censuses were conducted about every 10–14 days from May to November in the 3 years,
25 yielding 14 or 15 censuses per year. On each census date, we carefully observed the foliage
26 of the sample trees at 0–2 m above the ground level to identify mines that had newly formed.
27 For each identified mine, we recorded (1) leaf surface (i.e., upper or lower) on which the
28 focal mine formed, (2) development stage (i.e., larval or pupal), (3) whether other conspecific
29 miner individuals were in the leaf, and (4) if conspecific miners were observed, their position
30 within the leaf (i.e., upper or lower surface). We further monitored the identified mines until
31 we could see the final fate of the occupants (i.e., successfully emerged or pupated, dead from
32 unknown causes, or parasitized). Fate of leafminers was identified by inspecting occupants
33 through the leaf epidermis with a loupe. When a mined leaf abscised, the fate of the

1 occupants was identified as dead from unknown causes. When a larva had been attacked by a
2 koinobiont parasitoid that could allow further development of the host, we might have
3 incorrectly ranked the larval fate as ‘successfully pupated’. We could not distinguish attack
4 by koinobiont parasitoids from attack by idiobionts because we could not identify parasitoid
5 species responsible for the attack to the focal host. However, koinobiont parasitoids are
6 generally in the minority of leafminer parasitoid assemblages (e.g., Kato, 1994; Sato, 1995;
7 Ayabe & Ueno, 2012), and this was true in the present study (see the result section). We
8 determined the timing of parasitism based on the host stage at parasitoid emergence. We also
9 recorded the presence or absence of conspecific miners on the opposite leaf surface to that
10 mined by the focal miner. When conspecific leafminers simultaneously used the opposite
11 surface, the leaf utilization pattern was defined as ‘both-surfaces use’, and otherwise as
12 ‘one-surface use.’

13

14 **Destructive survey of parasitism in *Phyllocnistis spec.***

15 We collected a total of 85 mines from *L. japonicum* trees that were different from those used
16 in the nondestructive survey. These mines were collected on 13 July 2012, when many mines
17 were formed on both surfaces of leaves. (For details of the seasonal occurrence of
18 *Phyllocnistis spec.* mines, see Ayabe et al., 2015.) We recorded within-leaf positions of
19 mines, the presence or absence of conspecific mines in the opposite surface to that mined by
20 the focal miner, and then inspected the fate of each individual under a microscope.

21

22 **Collection of parasitoids**

23 In the field observation, some mined leaves were accidentally removed from trees, and some
24 other mined leaves dropped naturally, probably due to the damage caused by the leafminer.
25 We brought such leaves to the laboratory (the total number of collected mines was not
26 recorded), kept them, and obtained a total of 59 parasitoid individuals over the 3 years. We
27 identified the species of the parasitoids and which surface mine they had emerged from and
28 then compared the parasitoid species composition between the two within-leaf positions of
29 mines. We also determined the type of parasitoids (i.e., idiobiont or koinobiont) by
30 scrutinizing the development of the mines and the occupant individuals.

31

32 **Data analysis**

33 All statistical tests were performed using the R v.3.1.0 software (R Development Core Team,

1 2014). We used the package ‘stats’ for GLMM and ‘epiR’ for the Breslow-Day test.
2
3 *Nondestructive survey*. Prior to testing our hypotheses, we analyzed whether intensity of
4 parasitism varied depending on the mine abundance on each census date for each year using a
5 linear regression analysis, with the number of mines on each census date included as an
6 explanatory variable and the number of mines the fates of which were determined as
7 parasitized as a response variable. We then analyzed whether the susceptibility of
8 *Phyllocnistis spec.* to parasitism varied between upper- and lower-surface mines, using two
9 statistical tests: the Mantel–Haenszel χ^2 test (MH test) and the generalized linear mixed
10 model (GLMM). Using the MH test, we performed a direct and simple comparison of
11 parasitism rates between upper and lower mine positions to test our first hypothesis, and we
12 used a GLMM to examine the occurrence of parasitism at the leaf scale to test our second
13 hypothesis. For our third hypothesis, we compared parasitoid species compositions between
14 upper and lower mine positions. No statistical analyses could be applied to the data set,
15 instead we used the Jaccard index for larval and pupal parasitoids assemblages together,
16 regardless of whether one surface or both surfaces of leaves were mined, to evaluate
17 dissimilarity of the species composition of the parasitoid assemblages between the two
18 within-leaf positions of mines. Two assemblages tend to be structurally equivalent as the
19 index value increases to 1.

20 In the MH test, we simply divided miner individuals into two groups (i.e., miners
21 on the upper surface and those on the lower surface), regardless of whether other conspecific
22 miners mined in the opposite surface of the leaf, and then tested for the association of
23 occurrence of parasitism (i.e., parasitized or not) and intra-leaf positions of mines (i.e., upper
24 or lower surface) across the censuses in each year. We excluded some census dates from the
25 MH test, viz. those dates when no parasitism was observed, when only the lower surfaces of
26 the leaves were mined (this happened early in the occurrence season; for details, see Ayabe et
27 al., 2015), or when mine abundance across all leaves was < 40 . The census dates excluded
28 from the MH test were either at the beginning or the end of the occurrence season of
29 *Phyllocnistis spec.* We performed the MH test separately for larval and pupal stages and for
30 the entire mining stage (larval + pupal stages) in each year. Prior to the MH test, we
31 performed the Breslow–Day test to verify whether these data satisfied the MH test’s
32 assumption of homogeneity of odds ratios among census dates. We calculated
33 Mantel–Haenszel adjusted odds ratios.

1 In the GLMM, the per-leaf parasitism was compared between leaves with only
2 lower-surface mines (i.e., without upper-surface mines) and those with upper-surface mines.
3 We used the data set derived from a series of census dates when statistically sufficient
4 numbers of mined leaves were observed, and then performed GLMM tests with a binomial
5 distribution and a log-link function for each developmental stage and for the total mining
6 stage in each year. The two variables (i.e., the types of leaves and per-leaf mine density) were
7 included in the model as fixed effects, and census dates were included as a random effect. P
8 values were obtained with the likelihood ratio test. Rates of successful emergence of
9 *Phyllocnistis spec.* were not analyzed in the present study because of low emergence rates
10 (only 1% of the total leafminer individuals).

11

12 *Destructive survey.* We tested the associations between occurrence of parasitism and
13 within-leaf positions of mines using Fisher's exact test, with the data stratified into two
14 groups: mines on leaves of 'both-surfaces use' and mines on leaves of 'one-surface use'.

15

16 **Results**

17 **Nondestructive survey**

18 We identified 585 mines on 497 mined leaves in 2010, 1 119 mines on 908 mined leaves in
19 2011, and 1 119 mines on 773 mined leaves in 2012. *Phyllocnistis spec.* formed mines on the
20 upper surfaces in less than 30% of the cases (black and dark gray bars in Figure 1). Many of
21 the upper-surface mines were formed on leaves whose lower surfaces were also mined. In
22 2012 a large fraction of mines were formed simultaneously on both surfaces of a leaf, as
23 compared to those in the other 2 years (Figure 1). In 2011, many lower-surface mines were
24 observed.

25 Parasitism showed a significant relationship with the abundance of mines (linear
26 regression analyses, 2010: $t = 5.34$, $P < 0.001$; 2011: $t = 27.43$, $P < 0.0001$; 2012: $t = 16.91$,
27 $P < 0.0001$) (Figure 2). The results indicate that the occurrence of parasitism depended on the
28 abundance of hosts, and on each census date, about 30% of mines were parasitized. In the
29 MH tests, we used data derived from four census dates in 2010, five in 2011, and eight in
30 2012. These census dates included 117 parasitized leafminers in 2010 (rate of parasitism =
31 24.4%), 374 in 2011 (33.4%), and 300 in 2012 (26.8%). Breslow–Day tests confirmed that
32 the data satisfied the MH test's assumption of the homogeneity of odds ratios among census
33 dates ($P > 0.22$ for the 3 years). The MH tests showed different rates of parasitism among

1 developmental stages and among years (Table 1, Figure 3). In 2010, throughout the mining
2 stage (larva + pupal stages in Table 1), leafminers in upper-surface mines were about 2× as
3 often parasitized as those in lower-surface mines. Likewise, in 2012, larvae in upper-surface
4 mines were approximately 1.5× as often parasitized as those in lower-surface mines. In 2011,
5 however, parasitism occurred more frequently on leafminers in the lower-surface mines than
6 those in the upper-surface mines throughout the entire mining stage. Thus, our first
7 hypothesis was supported in 2010 and 2012, but in 2011 we obtained the opposite result.

8 Per-leaf parasitism also differed among developmental stages and among years
9 (Table 2). Contrary to the results of the direct comparison of parasitism between within-leaf
10 positions, we obtained no evidence opposing our second hypothesis. Especially in 2012,
11 parasitism during the larval stage and throughout the entire mining stage occurred more
12 frequently on leaves with upper-surface mines than on leaves without them. The odds ratio
13 was 1.67 for larval parasitism and 1.45 for the entire mining stage; these odds ratios were
14 calculated with the formula $\exp(\text{correlation coefficients of the GLMM})$. Density of mines per
15 leaf had statistically significant effects on the susceptibility of *Phyllocnistis* spec. larvae to
16 parasitism in all 3 years (Table 2). Thus, parasitoid attack of larvae occurred more frequently
17 on leaves with more mines. No significant effect was detected in pupal parasitism in all 3
18 years.

19

20 **Destructive survey**

21 We collected 38 upper-surface mines and 47 lower-surface mines. The destructive survey
22 showed that leafminers in upper-surface mines were more frequently parasitized (39%) than
23 those in lower-surface mines (13%; Fisher's exact test: $P < 0.01$; $n = 85$). The comparison
24 stratified with the presence or absence of other conspecifics on the opposite surface showed
25 that leafminers in lower-surface mines could avoid parasitoid attack especially when
26 conspecifics mined on the opposite upper surface (Figure 4; Fisher's exact test, family-wise α
27 = 0.05). The destructive survey demonstrated the disadvantage of mining on the upper
28 surface in avoiding parasitoid attack.

29

30 **Parasitoid assemblage**

31 We identified a total of eight parasitoid species in this system, all belonging to Eulophidae
32 (Table 3). Five parasitoid species were obtained from larval mines, and three were obtained
33 from pupal mines. *Neochrysocharis* spec. was obtained from both larval and pupal mines.

1 The larval parasitoid species were all idiobiont. The two pupal parasitoids, *Cirrospilus* spec.
2 and *Neochrysocharis* spec., were koinobiont and idiobiont, respectively. The dominant pupal
3 parasitoid, Entedontinae spec., was probably idiobiont because parasitoids did not emerge
4 from pupal mines that had been collected during the larval stage.

5 When comparing parasitoid assemblages between the upper- and lower-surface
6 mines regardless of whether one surface or both surfaces of leaves were mined, the value of
7 the Jaccard index between the two parasitoid assemblages was 0.375. Seven parasitoid
8 species emerged from upper-surface mines and six emerged from lower-surface mines. Five
9 species were common to both, whereas the remaining three were position-specific:
10 *Quadrastichus* spec. and *Cirrospilus* spec. emerged only from upper-surface mines, and
11 *Stenomesus japonicus* only from lower-surface mines. Upper-surface mines were more
12 attractive to parasitoids, which supported our third hypothesis.

13

14 **Discussion**

15 **Parasitism associated with the utilization pattern of a leaf by *Phyllocnistis* spec.**

16 Parasitoids imposed constant parasitic pressure on the dynamics of *Phyllocnistis* spec.
17 Patterns of the parasitoid attack, however, varied among years and among host stages, which
18 made the interpretation of the results complicated. Most importantly, the present results
19 indicated the disadvantage of forming a mine on the upper leaf surface, supporting our three
20 hypotheses. However, in the MH test in 2011, lower-surface mines were attacked more
21 frequently by parasitoids than were upper-surface mines over the entire mining stage, which
22 apparently opposed our first hypothesis. This opposite result could be explained by
23 search-image formation by parasitoids. Parasitoids can increase host-search efficiency by
24 forming search images for a specific type of high-density host, and search images are more
25 likely to be used for cryptic hosts (Ishii & Shimada, 2010). *Phyllocnistis* spec. formed mines
26 only on the lower surface of leaves early in the occurrence season (Ayabe et al., 2015), which
27 may dispose parasitoids toward search-image formation or make them oviposit exclusively in
28 the lower-surface mines. In 2011, more mines were formed on the lower surfaces of leaves
29 compared to the other 2 years. Therefore, search-image formation for lower-surface mines
30 could have been reinforced in 2011, and the effects of upper-surface mines as a parasitoid
31 attractant might not have been strong enough to surpass the search image. The extent to
32 which the attractant effect of upper-surface mines on parasitoids exceeds parasitoids'
33 search-image formation for lower-surface mines could determine the attack patterns of the

1 parasitoids, whereby the different attack patterns among 3 years could be explained. Thus,
2 mining on the upper surface is not always negative for *Phyllocnistis spec.*, but it can elicit
3 attractant effects on parasitoids at a relatively low percentage of upper-surface mines (i.e., at
4 least 30% in the present study).

5 The result of the destructive survey also demonstrated the disadvantage of mining
6 on the upper surfaces and helped us to interpret the results of the nondestructive survey.
7 When both surfaces were mined, parasitoid attack occurred on leafminers in the upper
8 surfaces, but not on those in the lower surfaces. Thus, individuals in the lower-surface mines
9 could avoid parasitism even when the leaf was located by parasitoids. This could explain the
10 lower rate of parasitism in lower-surface leafminers in 2012, when a large fraction of mines
11 was simultaneously formed on both surfaces and many lower-surface leafminers were
12 expected to avoid parasitism. In addition, the fact that parasitoids had attacked only
13 leafminers in the upper surface rejected the second hypothesis' assumption that a parasitoid
14 would search both surfaces. A field observation found that leafminer parasitoids examined
15 for mines from above or from underneath during flight, suggesting they may subsequently
16 land on the upper or the lower side of the leaf (Casas, 1989). Parasitoids can move quickly to
17 the other side from the side they land on (J Casas, pers. obs.). However, in the present study
18 system, when parasitoids encountered leaves with both surfaces mined, they might have
19 landed on the upper surface, parasitized the host miners and then left the leaf without further
20 searching hosts in the lower surface.

21 Our finding of higher rates of parasitoid attack on leaves with higher leafminer
22 densities agreed with results of density-dependent parasitism at the leaf scale reported in the
23 previous studies (Connor & Cargain, 1994; Patel et al., 2003; Eber, 2004; Low, 2008). In
24 *Phyllocnistis spec.*, leaves with higher mine densities involved the simultaneous use of both
25 surfaces of a leaf by several individuals (Ayabe et al., 2015). This implies that leaves mined
26 on both surfaces are more likely to be located by parasitoids. For larval parasitism in 2010
27 and 2011, however, the results of GLMM suggested density-dependent patterns of parasitism
28 whereas the presence of upper-surface mines had no significant effect on the parasitism
29 patterns. This was probably because the number of upper-surface mines was relatively low in
30 these 2 years, and thus parasitism might have been concentrated on lower-surface mines, as
31 noted as above, through the parasitoids' search-image formation.

32 Our survey of the parasitoid assemblage in this system also indicated the
33 disadvantage of mining on the upper surfaces, where leafminers had the risk of being

1 attacked by more parasitoid species, although the Jaccard index value indicated that the
2 species composition of parasitoid assemblages did not differ so much between the two
3 within-leaf positions of mines. Among them, *Neochrysocharis* spec. and Entedontinae spec.
4 are probably undescribed species (T Kawano, pers. comm.), and their host ranges are
5 unknown. The other parasitoid species are generalists and can attack agromyzid leafminer
6 species that form mines on the upper surface of leaves (e.g., Konishi, 1998). Generalist
7 parasitoids are more strongly attracted by visibly conspicuous mines than by inconspicuous
8 ones (Salvo & Valladares, 2004), which could explain the increased susceptibility of
9 upper-surface mines to parasitism in the present study. Although generalist parasitoids can
10 innately locate a variety of hosts with different mining patterns and positions, they may
11 specifically attack a particular host species at a local scale. *Phyllocnistis* spec. is multivoltine
12 and active from early spring until late autumn (Ayabe et al., 2015); thus, it provides
13 oviposition opportunities to generalist parasitoids for a long period. Therefore, it is likely that
14 parasitoids would be specialized and adapted to using *Phyllocnistis* spec. at the study site.
15 Because the sample size of the present study was small, however, we need further
16 investigation to reveal the relationships between within-leaf mine positions and the parasitoid
17 assemblages, through experimental, comparative, or theoretical approaches.

18 Relationships between leafminers and parasitoid assemblages are complex and vary
19 greatly (Hochberg & Hawkins, 1992, 1993). Phylogenetically unrelated leafminer species in
20 a habitat can be attacked by a common parasitoid assemblage (Memmott et al., 1994; Hirao
21 & Murakami, 2008), whereas congeneric leafminer species may be parasitized by different
22 dominant parasitoid species (Nakamura & Kimura, 2009). Also, for gall insects living inside
23 plant tissues, species with more similar gall morphology and spatiotemporal niche traits were
24 attacked by parasitoid assemblages with a more similar community structure (Bailey et al.,
25 2009). Further studies will be required to determine the extent to which a parasitoid
26 community is shared among various leafminer species that are similar to or different from
27 *Phyllocnistis* spec. in mining habit.

28

29 **Effects of other factors on the resource use of *Phyllocnistis* spec.**

30 Leaf traits and phylogenetic constraints of host plants also should be considered as factors in
31 the preferential use of the lower leaf surface by *Phyllocnistis* spec. The host plant *L.*
32 *japonicum* has a thicker cuticle layer on the upper surface than on the lower surface, and the
33 trait can make it difficult for hatched larvae to enter the leaf and suck the upper-surface

1 epidermal layer (Y Ayabe, T Minoura, and N Hijii, unpubl. data). However, whether physical
2 traits of leaves could affect the within-leaf positions of mines has yet to be clarified (Reavey
3 & Gaston, 1991). If mining on the lower surface is an ancestral character, it would be
4 inherited by many species, including *Phyllocnistis spec.*, in the course of adaptive radiation.
5 However, mining sites in ancestral lineages are still unknown because the genus *Phyllocnistis*
6 has been poorly studied (De Prins & Kawahara, 2009; Kawahara et al., 2009). Our findings
7 suggested that the interactions with parasitoids have encouraged *Phyllocnistis spec.* to prefer
8 the lower surface over the upper surface of a leaf, but also raised another question: why does
9 *Phyllocnistis spec.* still use the upper leaf surfaces as mining sites? There are three possible
10 reasons. First, as demonstrated in the present study, parasitism does not always impose a
11 disadvantage on miners in the upper surface; that is, interactions with parasitoids would be a
12 weak selective pressure. Second, upper-surface mining could provide *Phyllocnistis spec.* with
13 the benefit of avoiding direct competition for resources with a conspecific larva mining in the
14 lower surface (Ayabe et al., 2015). Third, laying eggs on the upper surface increases
15 parasitism on that surface, thereby increasing survival on the lower surface. If the net effect
16 (increase in survival on the lower surface minus decrease in survival on the upper surface) is
17 greater than 0, this would increase the fitness of the species by increasing its survival. For
18 most lepidopteran leafminers, including the Gracillariidae, an adaptive radiation of mining on
19 the lower surface appears to be associated with leaf traits and phylogenetic constraints of host
20 plants (Reavey & Gaston, 1991; Lopez-Vaamonde et al., 2003). Our results suggest the
21 possibility that the adaptive radiation of lower-surface mining could be related to the defense
22 against parasitoids.

23 It is known that leafminer larvae inside mines can often die due to being overheated
24 depending on their positions within the canopy and the levels of exposure to sunlight
25 (Pincebourde & Casas, 2006; Pincebourde et al., 2007). Thus, microclimates would be one of
26 the selective pressures on within-leaf site selections by leafminers. Reavey & Gaston (1991)
27 also suggested that the upper leaf surface often has unsuitable microclimates for egg
28 development, but using that surface allows leafminer females to save time during oviposition,
29 which may have made about 20% of the leafminer species to use only the upper surface. In
30 our study, upper-surface mining was not disadvantageous with respect to parasitism, as long
31 as it occurred at low frequency. This could explain, in part, why upper-surface mining is still
32 observed in lepidopteran leafminers, although in a minority of such species.

33 Under field conditions, many factors can contribute to the variability in fitness

1 among individuals in a population. By carefully monitoring every miner individual until its
2 fate was determined, we demonstrated that the patterns of parasitoid attack could affect the
3 fitness of leafminers at a local (within-leaf) scale and the attack patterns varied depending on
4 mine density and ratios of upper- to lower-surface mines. Studies have demonstrated that
5 mine distribution at a within-tree scale can cause specific patterns of parasitoid attack (Barrett,
6 1994; Jahnke et al., 2008). Our findings suggest that the within-leaf spatial distribution of
7 mines also can be used to evaluate the effects of oviposition behavior of parasitoids on
8 leafminer fitness and the susceptibility of leafminers to parasitism.

9

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15

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16
17

18 **Figure captions**

19 **Figure 1** Within-leaf distribution of *Phyllocnistis* spec. mines, stratified by the presence or
20 absence of mines on the opposite leaf surface. Values inside bars indicate the percentage of
21 each type of mine based on the total mine abundance in each year. Data were derived from
22 the census dates when mines were formed on both the upper and lower leaf surfaces.

23

24 **Figure 2** Relationships between parasitism and mine abundance on each census date from
25 2010 to 2012.

26

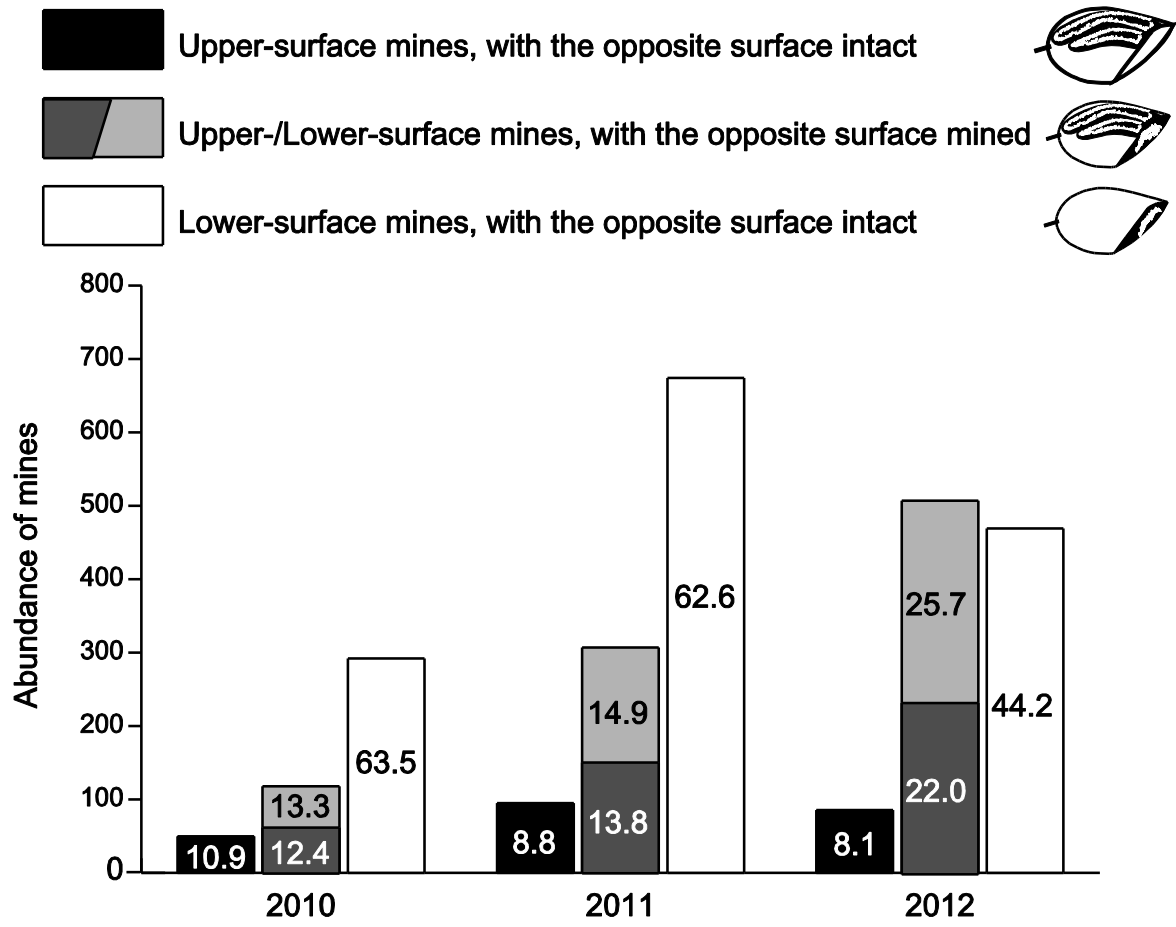
27 **Figure 3** Parasitism rates (the ratio of no. parasitism to no. mines) of the upper- and
28 lower-surface mines during the entire mining stage on each census date from 2010 to 2012.

29

30 **Figure 4** Susceptibility to parasitism of *Phyllocnistis* spec. mines at various within-leaf
31 positions. Multiple statistical comparisons were performed, so the family-wise α was
32 corrected to be 0.05. An asterisk indicates a significant between-group difference (Fisher's
33 exact test: $P < 0.05$); ns, not significant.

34

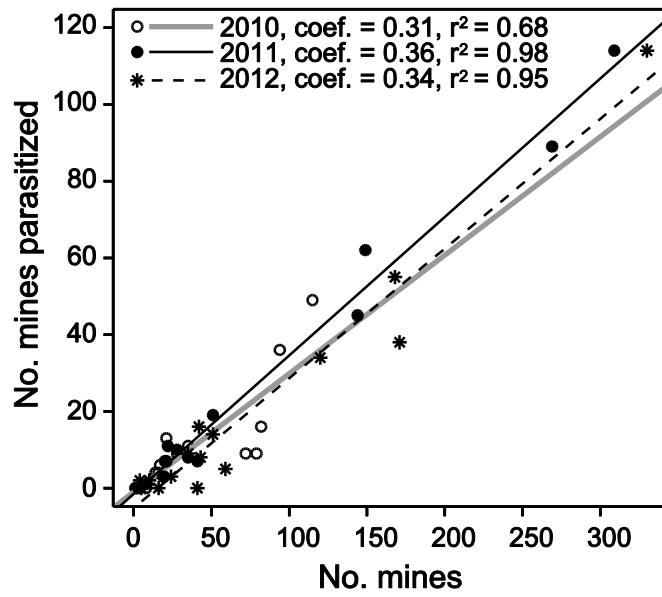
1 Fig 1



2

3

1 Fig 2



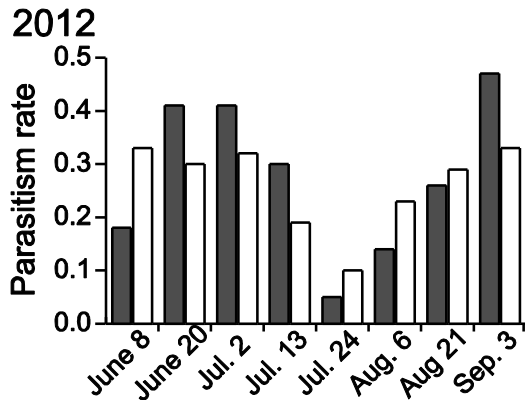
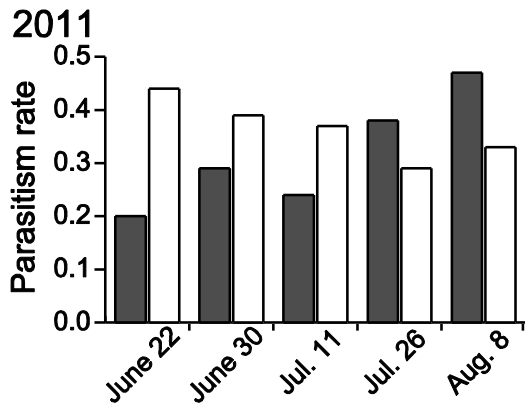
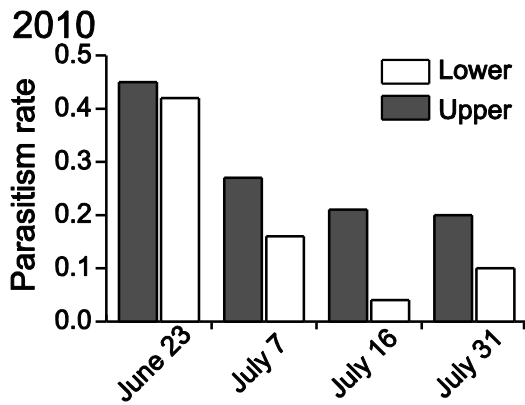
2

3 Remove the top and right hand axes please <avoid 'closed box' appearance>

4 Horizontal axis: center the numbers 50-300 relative to the respective tick marks, pls

5

1 Fig 3



2

3 Move '2010/2011/2012' to the middle please <center>

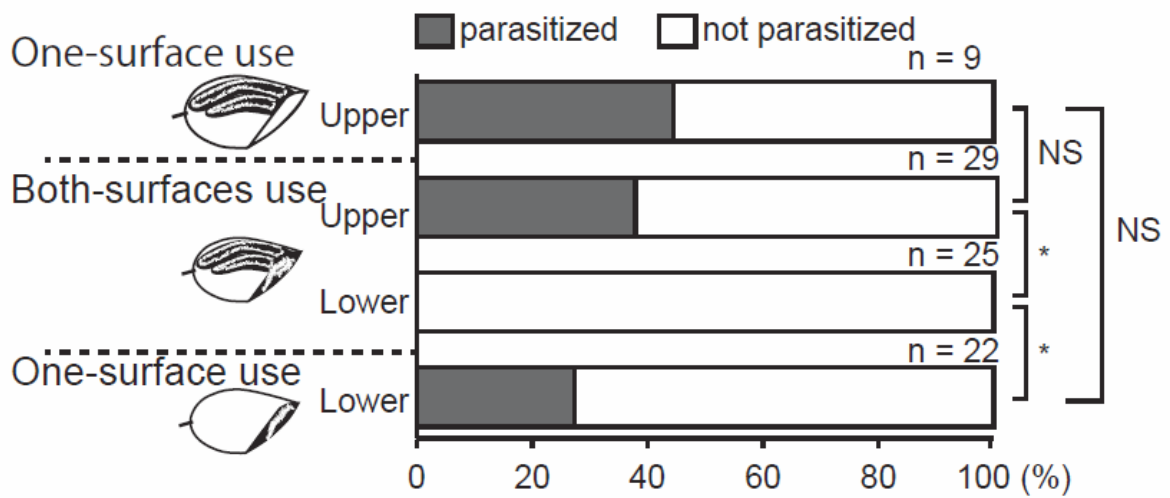
4 Remove 'Parasitism rate' from panels '2010' and '2012'

5 Panels 2011 & 2012: please write 'July' in full <as in '2010'>

6 Panel 2012: add '.' in 'Aug 21', pls

7 Please change date format to 23 June etc.

1 Fig 4



2

3 Legend: entries should be in 'Sentence style' <i.e., with capital initial; 'Parasitized', 'Not
4 parasitized'>

5 Replace 'NS' with 'ns' <lower case letters; 2x

6 Replace '(%)' with '%' <remove parentheses>

7

1 **Table 1** Susceptibility to parasitism of *Phyllocnistis* spec. mining on the upper surface of
 2 leaves compared to those mining on the lower surface, expressed as Mantel–Haenszel
 3 adjusted odds ratios

Year	Stage								
	Larva			Pupa			Larva + pupa		
	Odds ratio	P	n	Odds ratio	P	n	Odds ratio	P	n
2010	0.87	0.88	347	0.36	0.37	37	2.11	0.03	348
2011	0.78	0.20	921	0.41	0.08	207	0.66	0.017	922
2012	1.46	0.02	984	na			1.22	0.23	984

4 When an odds ratio is significantly larger than 1, it means that parasitism occurred more
 5 frequently on leafminers in the upper surface, and when <1, parasitism occurred more
 6 frequently on those in the lower surface. ‘n’ indicates the number of mines analyzed. Pupal
 7 parasitism in 2012 was not analyzed (na) because we did not observe a sufficient number of
 8 parasitized pupae to conduct the MH test.

9
 10

1 **Table 2** The effects of density of mines and the presence of upper-surface mines on the
 2 susceptibility of *Phyllocnistis spec.* to per-leaf parasitism in the 3 years, as analyzed by
 3 GLMM

Stages	Year	Effects	χ^2	Coefficient	P
Larva	2010	With upper mines ¹	2.20	-0.63	0.14
		Density of mines	7.45	0.79	0.006
	2011	With upper mines	0.02	-0.02	0.90
		Density of mines	6.35	0.39	0.012
	2012	With upper mines	6.95	0.51	0.0084
		Density of mines	53.68	0.54	<0.0001
Pupa	2010	With upper mines	1.06	0.79	0.30
		Density of mines	0.26	-0.25	0.61
	2011	With upper mines	1.72	-0.49	0.20
		Density of mines	1.10	0.30	0.30
	2012	With upper mines	2.00	-1.05	0.16
		Density of mines	1.93	-0.52	0.16
Larva + pupa	2010	With upper mines	1.31	0.43	0.25
		Density of mines	1.90	0.37	0.16
	2011	With upper mines	0.07	-0.05	0.80
		Density of mines	2.89	0.25	0.09
	2012	With upper mines	3.82	0.37	0.05
		Density of mines	15.03	0.44	<0.001

4 Parasitism was treated as a dummy variable; it was set to 1 when a mined leaf had at least one
 5 parasitized *Phyllocnistis spec.* individual and set to 0 otherwise.

6 ¹The effect of the presence of upper-surface mines was treated as a dummy variable; it was
 7 set to 1 if the upper surface of a leaf was mined by a *Phyllocnistis spec.* and set to 0 otherwise.
 8 A significantly positive coefficient means that parasitism occurred more frequently when the
 9 leaves had upper-surface mines.

10

11

1 **Table 3** Number of larval and pupal parasitoid (A) specimens and (B) species of the
 2 leafminer *Phyllocnistis* spec. and the positions of the mines they attacked

A		One-surface use		Both-surfaces use		Total
		Upper	Lower	Upper	Lower	
Parasitoids ¹⁾						
Larval	<i>Asecodes erxias</i> (Walker)	2	6	7	-	15
	<i>Neochrysocharis</i> spec. ²	1	4	7	1	13
	<i>Quadrastichus liriomyzae</i> Hansson & LaSalle	-	3	4	1	8
	<i>Pnigalio katonis</i> (Ishii)	1	1	2	1	5
	<i>Quadrastichus</i> spec.	-	-	1	-	1
	<i>Stenomesus japonicus</i> (Ashmead)	-	1	-	-	1
Pupal	Entedontinae spec.	1	11	-	2	14
	<i>Cirrospilus</i> spec.	1	-	-	-	1
	<i>Neochrysocharis</i> spec. ²	-	-	-	1	1
Total		6	26	21	6	59
B		Host stages				
Positions of mines		Larva	Pupa			
Upper surface		5	2			7
Lower surface		5	2			6 ³

3 ¹All species belong to Eulophidae. *Cirrospilus* spec. is koinobiont, the others are idiobiont.
 4 Voucher specimens are stored in the collections of the Entomological Laboratory, Faculty of
 5 Agriculture, Kyushu University, Fukuoka, Japan.

6 ²*Neochrysocharis* spec. emerged from both larval and pupal stages of the host leafminer

7 ³Double count of the parasitism by *Neochrysocharis* spec. on larval and pupal stages of the
 8 host leafminer was avoided.