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Kyoto University
Frequency-dependent herbivory by a leaf beetle, *Phaedon brassicae*, on hairy and glabrous plants of *Arabidopsis halleri* subsp. *gemmifera*

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[65 characters]

Key-words: *Arabidopsis halleri* subsp. *gemmifera* • Frequency dependence • Herbivory • *Phaedon brassicae* • Resistance polymorphism • Trichome

The main text consists of 5972 words (excluding references, figures, and tables) including Abstract (271 words), Introduction (925 words), Materials and Methods (2779 words), Results (755 words), Discussion (1119 words), and Acknowledgements (122 words). The entire manuscript consists of the main text with 37 References, 4 figures (without colors), 2 tables, and 3 appendices.

Contribution of authors – Y. Sato collected the field data and performed laboratory experiments using insects. Y. Sawada and M. Y. Hirai performed the glucosinolate analysis. Y. Sato, T. Kawagoe, and H. Kudoh conceived the study and wrote the paper.
Abstract

Frequency-dependent prey choice by natural enemies may influence the coexistence of multiple prey types, but little is known about whether frequency-dependent foraging choice occurs in herbivory on plants showing resistance polymorphism within a single population. Here we examined frequency-dependent foraging by a crucifer-feeding leaf beetle, *Phaedon brassicae*, on trichome-producing (hairy) and trichomeless (glabrous) plants coexisting within a natural population of the perennial herb *Arabidopsis halleri* subsp. *gemmifera*. Larvae of *P. brassicae* fed on hairy leaves showed slower growth than those fed on glabrous leaves. Although adult beetles consumed similar amounts of leaves when they were fed either hairy or glabrous leaves in no-choice conditions, our choice experiment showed that adult beetles fed at less than the proportionally expected level on hairy leaves compared to glabrous leaves when the hairy leaves were less or equally abundant. Both types of leaves were consumed at the proportionally expected levels when the hairy leaves were more abundant than the glabrous leaves. In a natural population, the leaf damage on the hairy plants was negatively correlated with the local proportion of the glabrous plants in a 1-m diameter patch across two years, while correlations between the leaf damage on the glabrous plants and their proportion differed between the two years. Additionally, we found five glucosinolates in leaves of *A. halleri*, but their accumulation did not differ between hairy and glabrous plants. Our experimental results indicate that hairy plants incur less herbivory by *P. brassicae* when glabrous plants are abundant. The field pattern provides evidence suggestive of frequency-dependent herbivory acting on hairy plants. The present study highlights one of the putative mechanisms of maintaining plant resistance polymorphism.
Introduction

Natural enemies often alter their foraging tactics depending on the relative frequency of multiple prey or host types (Greenwood 1984; Endler 1991; Sherratt and Harvey 1993). Frequency-dependent foraging on various prey types has been reported for predators (Endler 1991; Sherratt and Harvey 1993), parasitoids (Sherratt and Harvey 1993) and herbivores (Cottam 1985; Behmer et al. 2001). The frequency dependence of foraging behaviour may be profitable when predators encounter multiple prey types that are distributed unevenly in their foraging environments. For example, if the cost of searching for a rare prey is large, a predator should increase foraging success by concentrating on major prey types (Greenwood 1984; Endler 1991). In a broad sense, frequency-dependent foraging can be defined as the behaviour by which predators feed on a given prey type at a disproportionately higher or lower rate. Although definitions of frequency-dependent foraging have been discussed in different publications (Greenwood 1984; Behmer et al. 2001; Bergvall and Leimar 2005), here we follow the above broad-sense definition.

Frequency-dependent foraging has long been investigated because of its potential impacts on the coexistence or extinction of multiple prey types (Greenwood 1984; Sherratt and Hervey 1993). If predators feed more on a major prey type than proportionally expected, rare prey types experience less predation risk as the frequency of the major type becomes larger. This may lead to negative frequency-dependent selection on multiple prey types, thereby allowing them to coexist (Greenwood 1984). Conversely, if predators feed less on a major prey type, positive frequency-dependent selection may occur and accordingly promote the extinction of the rare prey types (Greenwood 1984). Empirically, frequency-dependent foraging has
been studied with respect to anti-predator behaviour of prey such as warning
coloration or aggregation (reviewed by Endler 1991).

Frequency dependence can also occur regarding herbivory on multiple plant
types that share a common herbivore. Some insect and mammalian herbivores are
known to forage on multiple plant species (Chandra and Williams 1983; Cottam
1985) or diets containing different nutritional quality (Behmer et al. 2001; Bergvall
and Leimar 2005) in a frequency-dependent manner. Within a plant species, natural
populations often exhibit genetic polymorphism of chemical and physical resistance
traits against herbivores (e.g. Hughes 1991; Elle et al. 1999; Kivimaki et al. 2007). In
addition to frequency-dependent host choice, selectivity or host preference of
herbivores is also known with respect to anti-herbivore resistance polymorphism
(Burgess and Ennos 1987; Sletvold et al. 2010). Few attempts, however, have been
made to test a frequency-dependent host choice by a herbivore with respect to the
polymorphism within a single plant species (Wise et al. 2009).

The purpose of this study was to examine the existence of frequency-
dependent foraging of herbivores with respect to anti-herbivore resistance
polymorphism. To test this, we used the leaf beetle *Phaedon brassicae* Baley
[Coleoptera: Chrysomelidae] and natural variation in trichome production of
*Arabidopsis halleri* (L.) O’Kane & Al-Shehbaz subsp. *gemmifera* (Matsum.) O’Kane
& Al-Shehbaz [Brassicaceae/ Cruciferae] (referred to as *A. halleri* hereafter). Both
adults and larvae of *P. brassicae* forage on trichome-producing and trichomeless
plants (hereafter referred to as hairy and glabrous plants, respectively) in a natural
population of *A. halleri* (Kawagoe and Kudoh 2010; Kawagoe et al. 2011). This
system is suitable for testing frequency-dependent foraging of a herbivore on plants
showing resistance variation because, in our study site, interspecific interactions are specific between *P. brassicae* and *A. halleri*. As to the herbivore fauna, *P. brassicae* is the most influential insect herbivore of *A. halleri*, and other herbivorous insects are much less abundant (Kawagoe and Kudoh 2010). As to the vegetation, other cruciferous plants are absent and hence *P. brassicae* feeds exclusively on *A. halleri*. This simple interspecific interaction helps to exclude confounding effects of other crucifer-feeding herbivores or cruciferous plants.

In addition to the simplicity of species interactions, the plant and beetle characteristics allowed us to interpret and design our study straightforwardly. For *A. halleri* in our study site, trichome polymorphism is strongly associated with allelic variation in a single candidate gene, *GLABROUS1 (GL1)* (Kawagoe et al. 2011) and therefore we can assume that the visible phenotypes represent genetically determined strategies. For *P. brassicae*, the flightlessness of the beetle made it reasonable to ask whether the local frequency of hairy and glabrous plants affected foraging behaviour of the beetle. Furthermore, it has been reported that host choice by adults is a major determinant of the larvae distribution in *P. brassicae* (Ôtake and Funaki 1958). We have also observed migrations between plants by adults, but fewer by larvae in the field. Although larvae cause the majority of damage to plants during the flowering period in the study site, it can be plausibly assumed that adult behaviours play an important role in determining the distributions of damages among plants.

In this study, we performed three laboratory experiments and a field survey. First, to ascertain whether trichome production acts as a resistance trait against *P. brassicae*, we compared the growth of larvae fed on hairy or glabrous leaves. Second, to test whether the feeding preference of adult *P. brassicae* depended on the relative frequency of hairy and glabrous leaves, we conducted choice experiments.
manipulating the relative frequency of hairy and glabrous leaves. Third, the relationship between leaf damage and the proportion of hairy and glabrous plants within small patches was investigated in the field to examine whether frequency-dependent herbivory occurs in the natural habitat. Additionally, to examine whether the trichome phenotype was correlated with chemical resistance traits, we quantified glucosinolates, which are major secondary metabolites of Brassicaceae (Kliebenstein et al. 2001; Clauss et al. 2006), in hairy and glabrous leaves.

**Materials and Methods**

**Study system**

We conducted field surveys and collected materials in a natural population of *A. halleri* located in Hyogo prefecture in western Honshu, Japan (35°06′N, 134°56′E, ca. 200 m in altitude). The study species is a self-incompatible perennial distributed in Eastern Asia and the Russian Far East (Hoffmann 2005). The plant is a metallophyte and often inhabits soils contaminated by heavy metals (Kubota and Takenaka 2003). In the study site, *A. halleri* occurs near an abandoned mine, along a creek running through open secondary forest. Vegetation is sparse along the creek, probably due to heavy metal contamination of the soil, and no cruciferous species are observed except for *A. halleri*. Approximately half of the plants were hairy and the others were glabrous in this site (Kawagoe et al. 2011). The presence/absence of trichomes has been reported to be associated with the allelic status of a trichome-related gene, *GL1*, but not with its flanking regions and other genes (Kawagoe et al. 2011). Hairy plants produced fewer fruits than glabrous plants in an insect removal experiment (Kawagoe et al. 2011).
et al. 2011), indicating that there is a cost of the trichome production. In this study, the glabrous phenotype was defined as the absence of trichomes on leaves and stems. Because this species can reproduce clonally, we designated a plant with no vegetative connection with others as an individual in this study.

*Phaedon brassicae* is known to be a pest insect of cruciferous vegetables (Wang et al. 2007a). This species usually reaches the adult stage within 3 weeks after hatching, and adults survive for approximately 2 months under laboratory conditions with various ranges of temperature and photoperiod (Wang et al. 2007b). Adults and last-instar larvae are ca. 4-8 mm in body length. In our study site, larvae and adults mainly occur during the flowering period in spring, and severely damage leaves and inflorescences of *A. halleri*, while they also occur from summer to autumn with much lower abundance than in spring (Kawagoe and Kudoh 2010). We collected 31 adults of *P. brassicae* during May-July 2011 and established a laboratory-reared population (> 90 individuals of F1 to F2 generations). The beetles were reared on leaves of Chinese cabbage (*Brassica rapa* var. *glabra*) under 20°C, 12L:12D conditions with relative humidity of 40-70% in a growth chamber (Biotron NC-220, Nippon Medical & Chemical Instruments, Osaka, Japan). We pre-reared *P. brassicae* on *A. halleri*, Chinese cabbage, cabbage (*Brassica oleracea*) and radish leaves (*Raphanus sativus*). Because *P. brassicae* grew well on the Chinese cabbage and this cultivar had a moderate density of trichomes among the four host plants, Chinese cabbage was chosen to avoid pre-conditioning for hairy or glabrous *A. halleri*. The light intensity of the growth condition was 25.3 ± 2.08 μmol/m²s (LI-190 Quantum Sensor, LI-COR, Lincoln, NE, USA). The leaf diets were replaced every three or four days.

Other herbivorous insects also feed on *A. halleri* in the study site, including green-veined white butterflies, *Pieris napi* L., and diamondback moths, *Plutella*
xylostella L. However their abundance is much lower than that of *P. brassicae* throughout the year (Kawagoe and Kudoh 2010) and we found only a few *P. napi* and *P. xylostella* during the present study.

*Larval growth on hairy and glabrous leaves*

First-instar larvae were used within three days after hatching in the laboratory-reared population. Several hundred young radical leaves were harvested from approximately 100 intact hairy and glabrous plants growing in our study site. The hairy and glabrous leaves were kept separately in a plastic case filled with water. A petiole of a single leaf was wrapped with moistened paper and placed in the center of a Petri dish. Nineteen individual larvae were separately released onto the upper surface of either a hairy or a glabrous leaf. The larvae were allowed to infest the leaves for eight days under 20°C, 12L:12D conditions. The weight of larvae was measured before, four days, and eight days after release. Because adult beetles do not grow in size after emerging from pupae, the weight of larvae in the early developmental stage was used as an indicator of the herbivore performance.

Measurements for each larva were performed three times to the nearest 10^-2 mg (AEL-40SM, Shimadzu, Tokyo, Japan) and the average values were used for analyses. Four days after the first release, the leaves were replaced with fresh leaves that had been kept in a refrigerator.

*Choice experiments under different leaf frequencies*

We conducted choice or no-choice experiments under five leaf frequency conditions (Hairy: glabrous = 4:0, 3:1, 2:2, 1:3, 0:4). Adult beetles were used in the experiment within 1-2 months after emerging from pupae. To stimulate the feeding
motivation of beetles, they were starved for one day. Each beetle was randomly
chosen and returned to the colony after experiments. Each trial was performed in a
Petri dish (diameter 6 cm, depth 1.5 cm: Kord-Valmark Co., Ontario, Canada)
containing a moistened filter paper (diameter 5.5 cm: Toyo Roshi Kaisha, Ltd., Tokyo,
Japan). Leaves used for this experiment were harvested as described above and used
within 12 h after the harvest. Leaf discs (1 cm²) were made from the center of each
leaf, including a main vain. One disc from hairy plants had 101 ± 32 trichomes (sum
of adaxial and abaxial side, Mean ± SD, n = 24: counted using an 8× magnifying
glass). Four leaf discs were placed in each Petri dish in a four-way choice manner
(Raffa et al. 2002). We examined the five frequency conditions of hairy and glabrous
discs (hairy: glabrous = 4:0, 3:1, 2:2, 1:3, 0:4) and the location of hairy and glabrous
leaf discs was randomized. Three adult beetles were released into the center of each
dish because we often observed an individual plant being infested by multiple adult
beetles in the field. They were allowed to infest the leaf discs for 72 h under 20°C,
12L:12D conditions. The number of arenas analyzed (replicates of trials) was 15, 23,
18, 22 and 15 for hairy: glabrous = 4:0, 3:1, 2:2, 1:3 and 0:4 conditions, respectively.
We started 27 replicates per condition and removed arenas in which even one of the
four leaf disks showed signs of drying during the 72-h experimental period (22, 26, 23,
27, and 19 cases remained for hairy: glabrous = 4:0, 3:1, 2:2, 1:3 and 0:4 conditions,
respectively). We further excluded cases that involved a beetle death (one case) or no
leaf-infestation (see also Table S1).

The leaf discs that remained at 72 h were placed on 1-mm-grid paper and
converted into a digital image (scanned using MP-460, Cannon, Tokyo, Japan). We
used Image J (Abramoff et al. 2004) to estimate the remaining leaf area with the
accuracy of $10^{-3}$ cm$^2$. The leaf loss (cm$^2$) was calculated as [1.1 – the remaining leaf area (cm$^2$)].

Field survey

Field surveys were conducted for selected A. halleri patches along a creek (ca. 200 m in distance) that ran through the center of the study site. We arbitrarily set a circular patch (1 m in diameter) to record the trichome phenotype (hairy or glabrous) and the proportion of leaf area lost to herbivores for all individual plants in each patch. The proportion of leaf area lost by herbivory (referred to as the leaf damage hereafter) was evaluated by eye and recorded as one of 11 successive values, i.e. 0 (no damage), 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9, and 1.0 (complete leaf loss). A preliminary survey confirmed that the number of plants within circular patches approached a plateau with increasing patch size: 2.97 ± 0.32, 7.08 ± 0.99, and 8.83 ± 1.25 plants occurred within patches 0.5, 1, and 3 m in diameter, respectively (Mean ± SE, $n = 36$ patches examined). Therefore, we focused on the local interaction in 1-m-diameter patches. The surveys were conducted twice (on 12 July 2011 and 29 May 2012) after the peak abundance of P. brassicae had been observed. The number of hairy and glabrous plants examined was 318 and 232 in 2011; and 260 and 195 in 2012, respectively. At the peak abundance of P. brassicae, the number of beetles per plant was 0.18 ± 0.08 on 16 May 2011 and 0.20 ± 0.05 on 8 May 2012 (Mean ± SE, including both adults and larvae: $n = 100$ plants). We examined 60 patches for each survey while keeping the distance between neighboring patches greater than 3 m.

In addition to the patch-level survey, we collected subset data at the individual level with the following aims. First, to evaluate to what extent our method of
quantifying the leaf damage reflected the intensity of herbivory, we also recorded the
number of intact and damaged leaves for 40 plants as an independent estimate of
herbivory. This additional measurement confirmed that the leaf damage estimated by
our method was highly correlated with the proportion of leaves damaged (Pearson’s
product moment correlation, both variables were arcsine-transformed, $r = 0.93$, $t_{38} =
15.3$, $P < 0.0001$). Second, to examine whether a correlation between plant size and
leaf damage would bias our interpretation of frequency dependence based on trichome
phenotype, we measured the length of the longest leaf for the same 40 plants
mentioned above. Neither the total number of leaves nor the length of the longest
rosette leaf was significantly correlated with the leaf damage ($r = 0.19$, $t_{38} = 1.2$, $P =
0.25$; $r = -0.16$, $t_{38} = -1.0$, $P = 0.32$, respectively, where the leaf damage was arcsine-
transformed), indicating that effects of plant size on the leaf damage were negligible.

Glucosinolate analysis of hairy and glabrous leaves

Fully expanded leaves were harvested from flowering stems of intact hairy or
glabrous plants on 15 May 2013. Two or three leaves in proximate positions were
selected to minimize the within-individual variation of glucosinolate concentration.
Furthermore, pairs of a hairy and a glabrous plant (< 1m apart) were sampled to
control for micro-environmental variation. Leaves from each individual were
separately packed into a plastic bag. The bags were then immediately frozen using
70% ethanol cooled with dry ice at the field site. The leaf samples were stored at −80
°C until use. Glucosinolates were analyzed by liquid chromatography-tandem mass
spectrometry (LC-MS/MS) according to Sawada et al. (2009a, b, 2012) using 4 ± 0.4
mg crushed leaves per individual plant for nine pairs of hairy and glabrous plants.
Statistical analysis

For the data set from the larval growth experiment, the weights of larvae fed on the hairy and the glabrous leaves were compared with a Mann-Whitney $U$-test. The analysis was done separately for the weight before the release, four days, and eight days after the release. For the data set from the choice experiments, we calculated the average leaf loss (cm$^2$) for each trichome type per dish to analyze herbivory on each leaf type in the choice experiment. A Wilcoxon signed rank test was used to compare the average leaf loss between the hairy and glabrous leaf discs for choice conditions (Hairy: glabrous = 3:1, 2:2, 1:3). For no-choice conditions (Hairy: glabrous = 4:0, 0:4), the average leaf loss was compared between the hairy and glabrous leaf discs by a Mann-Whitney $U$-test. In all the analyses for the choice conditions, $P$-values were adjusted using sequential Bonferroni correction to control the risk of increased type I error due to multiple testing. To test whether the relative frequency of hairy and glabrous leaves affected the total amount of leaf loss (cm$^2$) in each arena, we analyzed the effect of the frequency conditions on the total amount of leaf loss in each arena with a Kruskal-Wallis test. Further, to analyze the preference of adult beetles in the choice conditions, Chesson’s selectivity index (Chesson 1978) was calculated for each preference arena for the three choice conditions. Chesson’s $\alpha$ for diet type $i$ is denoted as $\alpha_i = (r_i / P_i) / \Sigma (r_i / P_i)$, where $r$ indicates the relative frequency of diet $i$ in total consumption by predators and $P$ indicates the relative frequency of diet $i$ in the environment. When there are two types of diets, $\alpha > 1/2$ and $\alpha < 1/2$ mean positive and negative preference for the focal diet, respectively. The parameter $r$ for the hairy and glabrous leaf discs was estimated as the proportion of the hairy or glabrous leaf area consumed relative to the total leaf area consumed in each preference arena. The parameter $P$ was the relative frequency of the hairy or glabrous leaf discs in each Petri dish.
A Wilcoxon signed rank test was used to compare Chesson’s $\alpha$ between the hairy and glabrous leaf discs.

For the field data, we analyzed the trichome phenotype (hairy or glabrous), the proportion of glabrous plants in a patch (which represents the relative frequency of the two phenotypes), and the total number of *A. halleri* in a patch (which represents the density of *A. halleri*), and the study year as fixed effects explaining the leaf damage. We also analyzed up to three-way interaction terms among the fixed effects to test the dependency of the trichome phenotype on the other factors. However, interaction terms involving the proportion of glabrous plants and the total number of *A. halleri* were not analyzed, because this interaction term corresponded to the number of glabrous plants in a patch and was therefore strongly correlated with the main effect of the proportion of glabrous plants in a patch ($r = 0.67$, $t_{1003} = 28.5$, $P < 0.0001$). The patch ID was incorporated as a random effect in order not to treat multiple plants in a patch as independent samplings. These factors were analyzed using generalized linear mixed models (GLMMs: Bolker et al. 2009) with a normal error structure. The leaf damage (response variable) was arcsine-square-root transformed to improve the normality of residuals. The analysis of field data consisted of three steps. First, we performed a stepwise model selection procedure to search the best-fitted model from a number of possible combinations involving three-way interaction terms among the trichome phenotype, the proportion of glabrous plants in a patch, and the study year; and among the trichome phenotype, the total number of *A. halleri* in a patch, and the study year. We used Akaike’s information criteria (AIC) for the model selection criteria. Both forward and backward searches on the fixed effects were allowed in the stepwise model selection. Second, based on interactions between the study year and the other factors in the first analysis, we separately performed model selections for...
data collected in 2011 and 2012 to investigate whether the trichome phenotype and
the relative frequency of trichome dimorphism had interactive effects on the leaf
damage. In the second analysis, the full model included five fixed effects: (1)
trichome phenotype × proportion of glabrous plants in a patch, (2) trichome
phenotype × total number of A. halleri in a patch, (3) trichome phenotype, (4)
proportion of glabrous plants in a patch, and (5) total number of A. halleri in a patch.
Third, based on interactions between the trichome phenotype and the other fixed
effects in the second analysis, we estimated coefficients of the independent variables,
i.e., “proportion of glabrous plants in a patch” and “total number of A. halleri in a
patch”, to examine the sign and magnitude of the effects of the frequency of hairy and
glabrous plants and their density on the leaf damage. Additionally, to add trend lines
for figure presentation, we estimated coefficients of the variable “proportion of
glabrous plants in a patch” for models including this fixed effect alone.
For the data set from glucosinolate analysis, we analyzed glucosinolates
detected in more than eight out of nine sample pairs, in which individual
glucosinolates with peak area values of > 1.0 were regarded as detected for each
sample. The score of LC-MS/MS analysis was calculated as the peak area value of a
certain glucosinolate divided by that of the internal standard (10-camphorsulfonic
acid) for each sample. A Wilcoxon signed rank test was used to compare the peak
area values of the glucosinolates between hairy and glabrous leaves. In this analysis,
proximate hairy and glabrous plants were treated as a pair to control for spatial
heterogeneity of environmental conditions among plant patches. To control for the
risk of increased type I error due to multiple testing, P-values were adjusted with the
number of glucosinolates tested using sequential Bonferonni correction.
All statistical analyses were performed using R version 2.15.0 (R Development Core Team 2012). We used the lme function (in the nlme package) and the stepAIC function (in the MASS package) for the stepwise model selection; and the lmer function (in the lme4 package) for GLMM analyses. In all of the GLMM analyses, we used the maximum likelihood method to estimate AICs and coefficients.

**Results**

**Larval growth**

The initial weight did not differ significantly between the larvae released on the hairy and glabrous leaves (Fig. 1; $U = 157, n_1 = n_2 = 19, P = 0.49$). The weight of larvae four days after release also showed no significant difference between the hairy and glabrous leaves (Fig. 1; $U = 126, n_1 = n_2 = 18, P = 0.25$). The weight of larvae eight days after release on the hairy leaves was significantly lower than that on the glabrous leaves (Fig. 1; $U = 43, n_1 = 11, n_2 = 14, P < 0.05$). The reduction in sample size at later time points was due to mortality of larvae during the experiments.

**Choice experiments**

The average leaf loss of hairy leaves was significantly smaller than that of glabrous leaves under the hairy: glabrous = 1:3 condition (Fig. 2a; Wilcoxon signed rank test, $V = 224, n = 23, P < 0.05$ with sequential Bonferroni correction) and the hairy: glabrous = 2:2 condition (Fig. 2a; $V = 163, n = 18, P < 0.05$). The average leaf loss did not differ significantly between the hairy and glabrous leaves under the hairy: glabrous = 3:1 condition (Fig. 2a; $V = 161, n = 22, P = 0.26$). Under no-choice
conditions, no significant difference in leaf loss was found between the hairy and glabrous leaves (Fig. 2a; Mann-Whitney U-test, $U = 109$, $n_1 = n_2 = 15$, $P = 0.88$). The total leaf loss per dish did not differ significantly among the five frequency conditions (Kruskal-Wallis test, $X^2_4 = 5.30$, $P = 0.26$).

The selectivity index of hairy leaves was significantly smaller than that of glabrous leaves under the hairy: glabrous = 1:3 condition (Fig. 2b; $V = 239$, $n = 23$, $P < 0.01$) and the hairy: glabrous = 2:2 condition (Fig. 2b; $V = 153$, $n = 18$, $P < 0.01$). The selectivity index did not differ significantly between the hairy and glabrous leaves under the hairy: glabrous = 3:1 condition (Fig. 2b; $V = 162$, $n = 22$, $P = 0.26$).

We also performed the same statistical analyses including cases that involved no leaf-infestation or beetle death, but inclusion of these cases did not affect the conclusions (Table S1).

Field survey

A three-way interaction term among the trichome phenotype, the proportion of glabrous plants, and the study year was included as a result of the stepwise model selection (Table S2). Then, based on this year dependence, we separately analyzed data collected in 2011 and 2012. The interaction term between trichome phenotype of the focal plant and the proportion of glabrous plants was included in the best-fitted model explaining the leaf damage in 2011 and 2012 (Table 1), indicating that the trichome phenotype and the proportion of glabrous plants had interdependent effects on the leaf damage. Therefore, we separately analyzed the data set for each of hairy and glabrous plants for each of these study years, and estimated the coefficients of the terms of the proportion of glabrous plants and total number of plants for each data set.
Leaf damage of hairy plants tended to decrease concomitantly as the proportion of glabrous plants increased in a patch in both of these two years (Table 2, Fig. 3a, c), though the correlation was not significant in 2012 (Table 2). Leaf damage of glabrous plants decreased in 2011, while it increased in 2012, as the proportion of glabrous plants increased in a patch (Table 2, Fig. 3b, d). The leaf damage of glabrous plants increased significantly as the total number of *A. halleri* in a patch increased in 2012 (Table 2). The leaf damage of the hairy plants was $0.154 \pm 0.009$ in 2011 (Mean $\pm$ SE, $n = 318$) and $0.136 \pm 0.012$ in 2012 ($n = 260$), while the leaf damage of the glabrous plants was $0.134 \pm 0.009$ in 2011 ($n = 232$) and $0.163 \pm 0.011$ in 2012 ($n = 195$).

*Glucosinolate analysis of hairy and glabrous leaves*

The score of LC-MS/MS values of the five glucosinolates showed no significant difference between hairy and glabrous leaves (Fig. 4; 6-Methylsulfinyl-n-hexyl-glucosinolate, $n = 9$ pairs, $V = 38$, $P = 0.37$; 7-Methylsulfinyl-n-heptyl-glucosinolate, $n = 9$ pairs, $V = 23$, $P = 1$; 8-Methylsulfinyl-n-octyl-glucosinolate, $n = 9$ pairs, $V = 21$, $P = 1$; 7-Methylthio-n-heptyl-glucosinolate, $n = 8$ pairs, $V = 21$, $P = 1$; 8-Methylthio-n-octyl-glucosinolate, $n = 8$ pairs, $V = 18$, $P = 1$). The results for the other fifteen glucosinolates measured are given in supporting information (Table S3).

**Discussion**

The choice experiment demonstrated frequency-dependent herbivory by *P. brassicae* with respect to trichome polymorphism of *A. halleri*. We observed less herbivory on hairy leaves when they became a minority. Greenwood (1984) defined...
frequency-dependent predation to describe cases in which feeding preference changes inversely with the frequency of a given prey type (i.e. anti-apostatic or pro-apostatic predation: reviewed by Sherratt and Harvey 1993). When hairy leaves became abundant, we observed a disproportional increase of herbivory on them to levels equal to those found in glabrous leaves. Because we did not observe the inverse change in feeding preference, our results correspond to “potentially frequency-dependent predation” (Greenwood 1984). To our knowledge, the present results are one of a few reported examples of frequency-dependent herbivory with respect to plant resistance polymorphism within a single population. Behmer et al. (2001) documented that a locust, *Locusta migratoria*, consumed more of abundant but sub-optimal artificial foods. Wise et al. (2009) found frequency dependence in associational resistance between the erect-stemmed and candy-cane phenotype of *Solidago altissima* against a gall-fly, but they reported that increased frequency of the resistant phenotype lowered attacks by the herbivore for both phenotypes. Our growth experiment using larvae confirmed that trichome production of *A. halleri* reduced the larval performance, indicating that trichome production functioned as a resistance trait against *P. brassicae*. In our discussion, therefore, we could consider glabrous and hairy leaves as optimal and sub-optimal diets for *P. brassicae*, respectively.

The spatial structure of foraging patches relative to the searching area of predators can alter the consequences for foraging behaviour (Greenwood 1984; Endler 1991; Sherratt and Harvey 1993) and thus determine whether one detects frequency-dependent predation. In host plant choice by herbivores, for example, Janz et al. (2005) showed that frequency-dependent oviposition preference of the polyphagous butterfly *Polygonia c-album* for two host species was detected among plant patches, but not within a patch. In contrast, *Phaedon brassicae* is less mobile with regard to pg. 18
choosing host plants (Ôtake and Funaki 1958). Therefore the results of our choice
experiments presumably represent the feeding preference of \emph{P. brassicae} adults and
its frequency dependence within a single plant patch.

We found that leaf damage on hairy plants decreased as the proportion of
glabrous plants increased within local patches (1 m in diameter) in 2011. A similar
pattern was found in 2012, although it was not statistically significant. This tendency
is consistent with the frequency-dependent herbivory detected in the choice
experiments. We observed a positive correlation between leaf damage of glabrous
plants and the frequency of glabrous plants within patches in 2012. This pattern would
be expected according to the frequency-dependent preference changes observed in our
experiments. However, the negative correlation we observed between leaf damage
and frequency of glabrous plants in 2011 was inconsistent with the laboratory
evidence of frequency-dependent herbivory. We also observed significant density-
dependent herbivory on glabrous plants in 2012 (Table 2b). The effect of plant density
could not be tested in our choice experiments under the condition of equal leaf density.

Overall, our field observations support the existence of frequency-dependent
herbivory at least on hairy plants, but it remains unclear whether our experimental
evidence can account for the frequency-dependent herbivory on glabrous plants in the
field. We need further studies before we can reach a rigorous conclusion about how
important the frequency-dependent herbivory by adult beetles is under natural
conditions.

Our previous studies revealed that intensive leaf damage is predominantly
caused by larvae feeding in our field site (Kawagoe and Kudoh 2010, Kawagoe et al.
2011). In the flowering period, adult beetles were found on less than 2% of plants
censused, while ca. 0.5 larva was observed on a single plant (Kawagoe et al. 2011).
Active host choice by larvae, however, is unlikely to occur, since they feed on the
host plant upon which an adult female oviposits, and rarely move between plants.
Therefore, we assume that the frequency-dependent leaf damage in the field is
attributable to the frequency-dependent foraging and oviposition by adults. Given the
slow growth of larvae on hairy leaves (Fig. 1), the leaf damage in the field probably
reflected not only the adult choice but also the effects of trichomes on larval feeding
activity. Although it was difficult to distinguish whether plant injury was due to
feeding choice or oviposition choice in the field, the oviposition preference should
next be examined to determine the relative importance of adult host choice and larval
feeding in causing the frequency-dependent leaf damage.

One caveat is that other ecological functions or traits correlated with the
trichome phenotype may also influence the observed frequency of hairy and glabrous
plants. For instance, trichomes have been reported to reduce evapo-transpiration, and
to increase UV reflection and tolerance to drought (Wagner et al. 2004, Steets et al.
2010, Sletvold and Ågren 2012). At least within our study site, both hairy and
glabrous plants were observed without distinctive segregation throughout a range of
microhabitats that may have differed in droughtness and sun exposure. It has been
reported that the density of trichomes increases in response to damage in Arabidopsis
thaliana (Yoshida et al. 2009). Although the polymorphism examined in this study
(presence/absence of trichomes) is expected to be determined by a single locus, GL1
(Kawagoe et al. 2011), further study will be required to evaluate how variation in
trichome density among hairy plants is affected by herbivory. In leaves of A. halleri
we found glucosinolates that have also been found in leaves of related Arabidopsis
species (e.g. methylthio- and methylsulfinyl-glucosinolates: Kliebenstein et al. 2001
for A. thaliana; Clauss et al. 2006 for A. lyrata), but little association between
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trichome production and glucosinolate contents was observed during the flowering season, when *P. brassicae* infestation was most intensive. It is also known that *A. halleri* accumulates heavy metals in trichomes (Zhao et al. 2000). We do not have any evidence so far that *P. brassicae* discriminates hairy and glabrous plants by any correlated traits.

In summary, this study is one of the first examples to show frequency-dependent herbivory with respect to anti-herbivore resistance polymorphism coexisting within a natural population. Although frequency-dependent food choice by herbivores has been suggested to promote coexistence of multiple plant species at community levels (Chandra and Williams 1983; Cottam 1985), the same process can explain the maintenance of resistance polymorphism within a single species by incorporating a tradeoff between defense and growth (Pacala and Crawley 1992). Previous studies revealed that herbivory by *P. brassicae* greatly reduced fruit production (Kawagoe and Kudoh 2010). Therefore, the frequency-dependent herbivory found in this study could be a candidate mechanism that would result in frequency dependence of plant fitness. Future studies should especially focus on this point, because it may explain why hairy and glabrous plants coexist within a population.

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research assistant fellowship provided by the Center for Ecological Research and by the Grants for Excellent Graduate Schools Program, Kyoto University (Ministry of Education, Culture, Sports, Science and Technology, Japan). The authors declare that there is no conflict of interest regarding this work.

References


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Baly (Coleoptera: Chrysomelidae: Chrysomelinae). The Coleopterists Bulletin 61: 457–462


Table 1 AICs of generalized linear mixed models explaining the leaf damage (arcsine-transformed proportion of leaf area lost by herbivory) on Arabidopsis halleri subsp. gemmifera in the field. The AICs of models with and without trichome phenotype, frequency, and density terms were compared for each study year. Interaction terms were subtracted sequentially from the full model, and then models with or without each main term were compared. The smallest values of AIC (shown by bold letters) indicate the best-fitted model. The patch ID was incorporated as a random effect in these analyses (see text).

Abbreviations: T, Trichome phenotype; P, Proportion of glabrous plants in a patch; N, Total number of A. halleri in a patch.

<table>
<thead>
<tr>
<th>Fixed effects</th>
<th>Terms subtracted</th>
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<th>AIC 2012</th>
</tr>
</thead>
<tbody>
<tr>
<td>(T × P) + (T × N) + T + P + N</td>
<td>Full model</td>
<td>-214.5</td>
<td>-151.6</td>
</tr>
<tr>
<td>(T × P) + T + P + N</td>
<td>(T × N)</td>
<td>-216.5</td>
<td>-151.4</td>
</tr>
<tr>
<td>(T × N) + T + P + N</td>
<td>(T × P)</td>
<td>-213.2</td>
<td>-135.2</td>
</tr>
<tr>
<td>(T × P) + T + P</td>
<td>(T × N) + N</td>
<td>-217.8</td>
<td>-152.2</td>
</tr>
<tr>
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<td>(T × P) + P</td>
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<td>-137.0</td>
</tr>
<tr>
<td>T + P + N</td>
<td>(T × P) + (T × N)</td>
<td>-215.2</td>
<td>-136.0</td>
</tr>
<tr>
<td>T + P</td>
<td>(T × P) + (T × N) + N</td>
<td>-216.8</td>
<td>-137.4</td>
</tr>
<tr>
<td>T + N</td>
<td>(T × P) + (T × N) + P</td>
<td>-209.9</td>
<td>-137.6</td>
</tr>
<tr>
<td>P + N</td>
<td>(T × P) + (T × N) + T</td>
<td>-212.0</td>
<td>-129.9</td>
</tr>
</tbody>
</table>
Table 2 Coefficients and their standard error (SE) for terms of proportion of glabrous plants in a patch and total number of Arabidopsis halleri subsp. gemmifera in a patch in GLMMs explaining the leaf damage (arcsine-transformed proportion of leaf area lost by herbivory) in 2011 and 2012 in the field. Upper rows (a) present results of models including the proportion of glabrous plants, and lower rows (b) present results of models including both the proportion of glabrous plants and the total number of plants. Bold values indicate significant deviation of coefficients from zero (Wald tests). The patch ID was incorporated as a random effect in these analyses (see text).

<table>
<thead>
<tr>
<th>Fixed effect</th>
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<th>2012</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Hairy ( (n = 318) )</td>
<td>Glabrous ( (n = 232) )</td>
</tr>
<tr>
<td>(a) Single regression</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Proportion of glabrous plants</td>
<td>(-0.20 \pm 0.10)</td>
<td>(-0.26 \pm 0.10)</td>
</tr>
<tr>
<td>(b) Multiple regression</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Proportion of glabrous plants</td>
<td>(-0.20 \pm 0.10)</td>
<td>(-0.31 \pm 0.11)</td>
</tr>
<tr>
<td>Total number of plants in a patch</td>
<td>(-0.06 \pm 0.12)</td>
<td>(-0.12 \pm 0.12)</td>
</tr>
</tbody>
</table>
**Legends for figures**

**Fig. 1** Weight of larvae (Median ± 95% CI) fed hairy (H; filled bars) and glabrous (G; open bars) leaves before release, and four days and eight days after release. Asterisks indicate significant differences with Mann-Whitney $U$-test (n.s. not significant, * $P < 0.05$).

**Fig. 2** Frequency-dependent herbivory by adult beetles on hairy (H) and glabrous (G) leaves in choice experiments. The left panel (a) shows the average leaf loss (Median ± 95% CI) for each trichome type in the choice and no-choice conditions (Hairy: glabrous = 4:0, 3:1, 2:2, 1:3, 0:4), where filled and open bars indicate the hairy and glabrous leaf type, respectively. The right panel (b) shows Chesson’s selectivity index (Median ± 95% CI) for hairy leaf type under the three choice conditions (hairy: glabrous = 1:3, 2:2, 3:1). Asterisks indicate significant differences with Wilcoxon signed rank test or Mann-Whitney $U$-test (n.s. not significant, * $P < 0.05$, ** $P < 0.01$; see the Results section for details).

**Fig. 3** Average leaf damage (proportion of leaf area lost by herbivory) plotted against the proportion of glabrous plants growing in a 1-m-diameter patch. The leaf damage of hairy (closed circles) and glabrous (open circles) plants is shown separately for each survey (a-d). A circle represents a single patch and vertical bars indicate SE of average leaf damage within a patch. Darker tones of the circles indicate larger numbers of plants in a patch. Trend lines (dashed lines) were added based on the results of single regressions (also see Table 2 for the results of multiple regressions). Data are not transformed in the figures.

**Fig. 4** Score of LC-MS/MS analysis of five glucosinolates in hairy (H) and glabrous (G) leaves harvested in the field. Median and quartiles are shown for each leaf type (95% CI could not be calculated due to the sample size). n.s. indicates no significant difference between hairy and glabrous leaves with Wilcoxon signed rank test (see the Results section for details).
Fig. 1

Weight of larvae (mg)

<table>
<thead>
<tr>
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<th>8-days</th>
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<tr>
<td></td>
<td>H G</td>
<td>H G</td>
<td>H G</td>
</tr>
<tr>
<td>Level</td>
<td>n.s.</td>
<td>n.s.</td>
<td>*</td>
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</tbody>
</table>

H: High
G: Low
Fig. 2

(a) Average leaf loss (cm²)

(b) Selectivity index on hairy leaves

H  G
3 : 1  0.5
H  G
2 : 2  0.4
H  G
1 : 3  0.3
H  G
4 : 0  0 : 4
n.s.

H  G
3 : 1  0.5
H  G
2 : 2  0.4
H  G
1 : 3  0.3
n.s.

** **
Fig. 3

(a) 2011 Hairy plants  
(b) 2011 Glabrous plants

(c) 2012 Hairy plants  
(d) 2012 Glabrous plants

Leaf damage vs. Proportion of glabrous plants in a patch
Fig. 4

- 6-Methylsulfinyl-n-hexyl-glucosinolate
- 7-Methylsulfinyl-n-heptyl-glucosinolate
- 8-Methylsulfinyl-n-octyl-glucosinolate
- 7-Methylthio-n-heptyl-glucosinolate
- 8-Methylthio-n-octyl-glucosinolate
Table S1 Summary table showing the results of choice experiments when replicates with no leaf infestation were included in the analyses (these cases were excluded from the analyses presented in Figure 2 in the main text). Median and 95% CI values are listed for average leaf loss and the selectivity index for each leaf type. Bars (---) represent the values that are impossible to define. The sample number (n) indicates the total number of replicates analyzed.

<table>
<thead>
<tr>
<th>Condition</th>
<th>Trichome</th>
<th>n</th>
<th>Average leaf loss for each leaf type</th>
<th>Chesson's selectivity index</th>
</tr>
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<tr>
<td></td>
<td></td>
<td></td>
<td>Median</td>
<td>95% CI</td>
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<td>20</td>
<td>0.161</td>
<td>0.138-0.245</td>
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<td>26</td>
<td>0.206</td>
<td>0.185-0.328</td>
</tr>
<tr>
<td></td>
<td>Glabrous</td>
<td></td>
<td>0.239</td>
<td>0.195-0.374</td>
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<tr>
<td>H:G = 2:2</td>
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<td>0.179</td>
<td>0.151-0.229</td>
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<tr>
<td></td>
<td>Glabrous</td>
<td></td>
<td>0.240</td>
<td>0.192-0.323</td>
</tr>
<tr>
<td>H:G = 1:3</td>
<td>Hairy</td>
<td>27</td>
<td>0.168</td>
<td>0.143-0.223</td>
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<tr>
<td></td>
<td>Glabrous</td>
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<td>0.221</td>
<td>0.193-0.281</td>
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<tr>
<td>H:G = 0:4</td>
<td>Glabrous</td>
<td>19</td>
<td>0.185</td>
<td>0.141-0.229</td>
</tr>
</tbody>
</table>
Table S2 Results of the stepwise model selection for the full model that included three-way interaction terms, i.e., the trichome production, the proportion of glabrous plants in a patch, the total number of *A. halleri* subsp. *gemmifera* in a patch, and the study year. Backward and forward stepwise searches were allowed to minimize AICs. The model selection was performed using the stepAIC function implemented in R. The patch ID was incorporated as a random effect in these analyses (see text).

Abbreviations: T, Trichome phenotype; P, Proportion of glabrous plants in a patch; N, Total number of *A. halleri* in a patch; Y, Study year.

<table>
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<th>AIC</th>
</tr>
</thead>
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<td><em>Full model</em></td>
<td>-368.1</td>
</tr>
<tr>
<td>1</td>
<td><em>(T × P × Y) + (T × P) + (T × Y) + (P × Y) + (T × N) + (N × Y) + T + P + N + Y</em></td>
<td><em>(T × N × Y)</em></td>
<td>-368.6</td>
</tr>
<tr>
<td>2</td>
<td><em>(T × P × Y) + (T × P) + (T × Y) + (P × Y) + (N × Y) + T + P + N + Y</em></td>
<td><em>(T × N × Y) + (T × N)</em></td>
<td>-370.0</td>
</tr>
<tr>
<td>3</td>
<td><em>(T × P × Y) + (T × P) + (T × Y) + (P × Y) + T + P + N + Y</em></td>
<td><em>(T × N × Y) + (T × N) + (N × Y)</em></td>
<td>-370.4</td>
</tr>
</tbody>
</table>
Table S3. Peak area values of glucosinolates found in leaves of hairy and glabrous plants of *Arabidopsis halleri* subsp. *gemmifera* growing in the field. Search results of Kyoto Encyclopedia of Genes and Genomes (KEGG) are also presented.

| Name | KEGG | KEGG Name | Hairy_pair1 | Hairy_pair2 | Hairy_pair3 | Hairy_pair4 | Hairy_pair5 | Hairy_pair6 | Hairy_pair7 | Hairy_pair8 | Glabrous_pair1 | Glabrous_pair2 | Glabrous_pair3 | Glabrous_pair4 | Glabrous_pair5 | Glabrous_pair6 | Glabrous_pair7 | Glabrous_pair8 | Glabrous_pair9 |
|------|------|-----------|-------------|-------------|-------------|-------------|-------------|-------------|-------------|-------------|---------------|---------------|---------------|---------------|---------------|---------------|---------------|---------------|---------------|---------------|
| 10-Camphorsulfonic acid* | | | 31784.947 | 45899.02 | 47556.38 | 47888.285 | 30858.113 | 38237.468 | 36268.664 | 38007.168 | 33699.859 | 45198.031 | 42251.031 | 34571.953 |
| Sinigrin | C08427 | Sinigrin; 2-Propenyl glucosinolate | 1.011 | NA | NA | NA | NA | NA | NA | 16.353 | NA | NA | NA | NA | NA | NA | NA | NA | NA |
| 3-Methylallyl-n-propyl-glucosinolate | C08411 | Glucobrassicin; 3-Methylallyl-n-propyl glucosinolate | NA | NA | NA | NA | NA | 4.646 | NA | 0.208 | NA | NA | NA | NA | NA | NA | NA | NA | NA | NA |
| 4-Methylallyl-n-butyl-glucosinolate | C08419 | Gluconapin; 4-Methylallyl-n-butyl glucosinolate | NA | NA | 2.621 | 6.548 | 2.303 | NA | 23.08 | 65.522 | 4.818 | NA | 58.931 | NA | NA | NA | 59.223 | 0.646 | NA | NA |