# Associational effects against a leaf beetle mediate a minority advantage in defense and growth between hairy and glabrous plants

YASUHIRO SATO[1*] and HIROSHI KUDOH[1]

[1]Center for Ecological Research, Kyoto University, Hirano 2-509-3, Otsu, Shiga 520-2113, Japan

Y. Sato (*Author correspondence)
*Center for Ecological Research, Kyoto University*
*Hirano 2-509-3, Otsu, Shiga 520-2113, Japan*
*Phone: +81-77-549-8018; Fax: +81-77-549-8201*
*E-mail:* yassato@ecology.kyoto-u.ac.jp

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## Abstract

Based on the accumulation of evidence, the risk of herbivory depends not only on the traits of a plant but also on those of neighboring plants. Despite the potential importance of frequency-dependent interactions in the evolutionary stability of anti-herbivore defense, we know little about such associational effects between defended and undefended plants within a species. In this study, we determined whether the intraspecific associational effects against the oligophagous leaf beetle, *Phaedon brassicae*, caused a minority advantage in defense and growth between trichome-producing (hairy) and trichomeless (glabrous) plants of *Arabidopsis halleri* subsp. *gemmifera*. We experimentally demonstrated that the magnitude of herbivory and the number of adult beetles on hairy plants decreased when hairy plants were a minority, whereas the leaf damage and the beetle abundance did not differ between hairy and glabrous plants when glabrous plants were a minority. By contrast, the larvae of *P. brassicae* occurred less when hairy plants were a majority. We also found a reciprocal minority advantage in the biomass production for both hairy and glabrous plants. Additionally, the adults tended to attack glabrous leaves more rapidly than hairy ones, particularly when the beetles were starved or experienced glabrous diets. Furthermore, in the absence of herbivory, the growth of hairy plants tended to be slower than glabrous plants, which indicated a cost for the production of trichomes. Our study suggests that associational effects are a mechanism for the maintenance of trichome dimorphism by contributing to negative frequency-dependent growth. [241 words]

Keywords: Anti-herbivore defense, *Arabidopsis halleri* subsp. *gemmifera*, Associational effects, Frequency dependence, Minority advantage, Polymorphism

## Introduction

As sessile organisms, plants are exposed to threats of resource loss by herbivory. To mitigate the negative effects of losses from herbivory, many plant species develop defensive traits (e.g., toxins and spines) against herbivores. Based on resource allocation theory of plant defense, the assumption has long been that the magnitude of herbivory on an individual plant depends on the investment of that plant in defensive traits (Rhoades 1979; Simms and Fritz 1992; Stamp 2003). However, the accumulation of evidence now indicates that herbivory risk depends not only on the traits of an individual plant but also on those of neighboring plants (Agrawal et al. 2006; Barbosa et al. 2009). Some researchers have noted the crucial role of such ‘associational effects’ in determining the coexistence of multiple plant types because the associational effects may mediate the local frequency or the density dependence of herbivory (Underwood et al. 2014; Kim and Underwood 2015). Although several terminologies have been proposed for associational effects (reviewed by Barbosa et al. 2009; Rautio et al. 2012; Underwood et al. 2014), we followed Underwood et al.'s (2014) definition which is "Associational effects occur when consumer effects on individuals of one resource organism type, at a given density of that type, are a function of the neighborhood composition of other resource types at particular spatial scales."

Although associational effects are investigated as a type of interspecific interaction (e.g., Root 1973; White and Whitham 2000; Callaway et al. 2005), the effects can also be applied to anti-herbivore defense polymorphism within a single species (Tuomi et al. 1999; Rautio et al. 2012). Based on evolutionary game theory, several authors discuss how defended and undefended plants coexist under associational interactions (Sabelis and de Jong 1988; Till-Bottraud and Gouyon 1992; Tuomi et al. 1999). One possible explanation is negative frequency-dependent selection in which associational effects may allow multiple plant types to persist within a population when herbivory decreases plant fitness and rare types can escape from herbivory (Tuomi et al. 1999). As a type of associational effect, Bergvall et al. (2006) introduced the term ‘neighbor contrast defense and susceptibility’ to denote, respectively, the minority advantage or disadvantage in herbivory on a given plant type. Additionally, these preceding studies suggest that herbivore perception on contrasting diets is a pivotal mechanism for that type of associational effect (Bergvall et al. 2006; Rautio et al. 2012). However, empirically, little is known about whether and how associational effects mediate frequency dependence in defense and growth for defended and undefended plants within a species (Wise et al. 2009).

From previous research on anti-herbivore defense, defensive traits often incur a cost on the growth and/or reproduction of a plant (Elle et al. 1999; Koricheva 2002; Züst et al. 2011), and optimal defense theory predicts a single defense strategy balanced between defense and growth (Simms and Fritz 1992; Stamp 2003). In contrast to this prediction, natural plant populations exhibit genetic polymorphisms in both chemical (Hughes 1991; Windsor et al. 2005) and physical defenses (Kivimäki et al. 2007; Wise et al. 2009). Generally, in prey-predator interactions, (1) some fraction of prey abandon defensive traits to avoid the cost, and (2) predation risks for defended and undefended prey sometimes depends on the composition of the neighborhood prey (Brower et al. 1970; Speed and Ruxton 2005; Svennungsen and Holen 2007). At least theoretically, a stable dimorphism of defended and undefended prey can evolve under predator-mediated interactions (Svennungsen and Holen 2007). Thus, associational effects may provide insights into why anti-herbivore defense polymorphism is maintained under plant-herbivore interactions.

Trichomes (plant epidermal hairs) are a physical defense trait with distinct phenotypes that are governed by a few primary genes (Kivimäki et al. 2007; Grebe 2012).

Plant trichomes are a mechanical barrier against feeding by adult and larval insects and against ovipositing by adults (e.g., Zvereva et al. 1998; Chu et al. 2001; Sletvold et al. 2010; Yamawo et al. 2012). Trichomes also function in the tolerance to abiotic stresses (Wagner et al. 2004). Moreover, trichomes impose a cost on plant growth and/or reproduction (Sletvold et al. 2010; Kawagoe et al. 2011; Züst et al. 2011). To test intraspecific associational effects and their frequency dependence, we focused on trichome production as a representative trait of plant defense that exhibited genetic polymorphism.

*Arabidopsis halleri* (L.) O'Kane & Al-Shehbaz subsp. *gemmifera* (Matsum.) O'Kane & Al-Shehbaz [Brassicaceae/Cruciferae: referred to as *A. halleri*, hereafter] possesses a genetic dimorphism of trichome-producing (hairy) and trichomeless (glabrous) morphs (Kawagoe et al. 2011). For this subspecies, Kawagoe et al. (2011) determined the reproductive cost for the production of trichomes. In an evaluation of the anti-herbivore resistance conferred by trichomes, Sato et al. (2014) reports that an oligophagous beetle *Phaedon brassicae* Baly [Coleoptera: Chrysomelidae] preferred to feed on glabrous leaves and avoided hairy leaves only when the latter leaf type was rare. However, Sato et al. (2014) used leaf-discs in the feeding experiment, and whether associational effects can be detected at the whole-plant level is unknown.

The purpose of this study was to test whether the associational effects resulted in a minority advantage in the defense and the growth for the two plant morphs. We experimentally tested intraspecific associational effects in anti-herbivore defense against a leaf beetle on *A. halleri* in which leaf damage, herbivore abundance, and plant biomass were evaluated under contrasting neighborhood conditions of hairy and glabrous plants. We also performed two additional experiments. First, to examine a possible cause of the associational effects, we observed the biting behaviors of the leaf beetles on hairy and glabrous leaves because biting is a useful measure of host preference in *Phaedon* species (Kühnle and Müller 2012). In this additional experiment, we preconditioned adult beetles on the two leaf types with the aim to simulate beetles with experience feeding on hairy-abundant and glabrous-abundant plants. Second, hairy and glabrous plants were cultivated without herbivory to ascertain the growth and/or reproductive cost of trichome production. Because the cost of trichome production can differ depending on plant ontogeny in *Arabidopsis* (Züst et al. 2011), we measured the growth and reproduction at multiple points in time.

## Materials & Methods

### *Study system*

*Arabidopsis halleri* is a self-incompatible perennial herb that is distributed across Japan and the Russian Far East. In the lowlands of western Japan, plants begin flowering in late March or early April. After flowering, plants develop leaves and roots on the primary and axillary meristems of the flowering stems, and these new rosettes establish as clonal offspring by rooting into the ground. The presence/absence of trichomes is associated with the allelic status of a trichome-related gene, *GL1*, but not with its flanking regions or other genes (Kawagoe et al. 2011). Hairy plants develop nonglandular trichomes on the surfaces of leaves and stems, whereas glabrous plants have no trichomes except for on the leaf margins. From a natural population that was previously studied (located in central Japan, 35°06′ N, 134°56′ E, ca. 200 m in altitude; Kawagoe et al. 2011; Sato et al. 2014), the seeds of *A. halleri* were collected. The glucosinolate profiles were not associated with the trichome phenotype in the young leaves collected from our study population (Sato et al. 2014).

*Phaedon brassicae* is a pest of cruciferous vegetables (Wang et al. 2007a). Adults of *P. brassicae* are flightless and access host plants exclusively by walking. The larvae are far less mobile than the adults (Ôtake and Funaki 1958), and therefore, the host plant on which a larva feeds is determined by adult choice. This species of leaf beetle typically reaches the adult stage within 3 weeks after hatching, and adults survive for approximately 2 months under laboratory conditions across various ranges of temperature and photoperiod (Wang et al. 2007a, b). The adults and the last-instar larvae are ca. 4-8 mm in body length. We used a laboratory-reared population that was previously established and maintained the population at a temperature of 20˚C and a photoperiod of 12:12 h L:D (Sato et al. 2014). The sex of the beetles was determined based on the body size (males, 0.5 mm smaller in abdomen height and width than females). To equalize the likelihood of oviposition, we used the sex ratio of 50:50 within each treatment in all of the experiments described below.

### *Greenhouse test of minority advantage in defense and growth*

We conducted a greenhouse experiment that used two contrasting neighborhoods of hairy and glabrous plants to test for the intraspecific associational effects (hairy plants:glabrous plants = 3:1 and 1:3 individuals; referred to hereafter, as hairy-abundant and glabrous-abundant conditions, respectively). These two contrasting neighborhood conditions were adopted because the feeding responses of *P. brassica*e did not differ between the glabrous-abundant condition and the condition with even proportions of the two morphs (i.e., hairy leaves:glabrous leaves = 2:2; Sato et al. 2014).

The mature fruits were harvested from 14 maternal plants (seven hairy and seven glabrous plants) in early July 2013 at our study site; the maternal plants were separated by at least 5 m to minimize the likelihood of multiple sampling of a single clone. Hundreds of seeds from each maternal plant were separately sown on Petri dishes (diameter 9 cm, depth 1.5 cm; Kord-Valmark Co., Ontario, Canada) filled with quartz sand. The seeds were germinated under 12:12 h L:D conditions with the respective air temperatures of 25°C and 15°C; in these conditions, > 80% of the seeds germinated successfully. We transplanted three seedlings that each had 2-4 leaves into a plastic pot (diameter 9.0 cm, depth 8.0 cm, Y-pot; SAKATA SEED CO., Yokohama, Japan) filled with seedling-raising compost (Cat. No. 776-274-01; TAKII & CO., Ltd., Kyoto, Japan). The compost contained nutrients (N:P:K = 320:210:300 mg/L, respectively). The plants were grown under 12:12 h L:D with a constant air temperature of 20°C. Water was supplied every 3-4 days during the experiment. The two smallest plants were harvested from each pot after three months of cultivation to avoid competition within the pots. The photosynthetically active radiation ranged from 40 to 60 μmol/m$^2$/s on the surface of the pots (LI-190 Quantum Sensor; LI-COR, Lincoln, NE, USA) for which the red:far-red ratio was 2.1–2.4.

The experiment began 3.5 months after the seedling transplantations: 120 plants were transferred to 30 nylon nets (23 cm diameter by 33 cm height, supported by a 30-cm long wood pole, with 75% light-transmittance), with four individuals per net. Fifteen replicates were established for each neighborhood condition (4 individuals × 2 neighborhood conditions × 15 replicates = 120 plants in total). The pots were adjoined to allow leaf beetle movement between plants without the necessity of first climbing down to the ground. The four individual plants within each net were arbitrarily chosen from different maternal plants to minimize the frequency of bias of the maternal family within a replicate. The smallest plants (length of largest leaf < 80 mm) were not selected to control for the effects of initial size on the magnitude of herbivory. The adult beetles were allowed to feed on the *A. halleri* individuals under the two neighborhood conditions, with two males and two females randomly chosen from the laboratory-reared population released per net after being starved for a day.

We scored the damage level to each leaf as 0 (intact), 1 (ca. 0% < area loss ≤ 25%), 2 (25% < loss ≤ 50%), 3 (50% < loss ≤ 75%), 4 (75% < loss < 100%), or 5 (100% loss or only a petiole remained) at 1, 2, 3, and 4 weeks after the release of the beetles. The damage levels were evaluated separately for mature and young leaves because these leaf types differed in the density of trichomes (mean ± SE = 2.3 ± 0.5 and 16.0 ± 1.4 trichomes/0.25 $cm^2$ for mature and young leaves, respectively; Wilcoxon signed rank tests, $V = 0$, $n = 9$ plants, $P < 0.01$) and in the leaf thickness (specific leaf area, mean ± SE = 252 ± 18 and 380 ± 39 $cm^2/g$ for mature and young leaves, respectively; $V = 55$, $n = 16$ plants, $P < 0.01$). For the growth stages, the radial leaves developed first at the core of the rosette toward the vertical axis and then expanded outward toward the horizontal axis. Thus, we used the angle of the leaf petiole as an indicator of leaf age: the mature leaves were classified as leaves with a petiole angle < 45°, whereas the young leaves were those with angles ≥ 45°. The larval and adult leaf beetles on each plant were counted every 3-4 days. Because the eggs of *P. brassicae* are embedded in plant tissue by adults, it was difficult to distinguish oviposition in plants from injuries; thus, the newly hatched larvae were retrieved after each count to evaluate oviposition by adult females in this early period of the greenhouse experiment. For the larvae, we also recorded the leaf type on which the larvae were found (i.e., young or mature leaf) to evaluate which type of leaf was chosen for oviposition by the females. To analyze both the intensity and the duration of the cumulative effects of herbivory, all measurements were repeated until a month after the release of adult beetles.

To examine the effects of trichome phenotype and neighborhood condition on plant growth, we measured the plant biomass (including the aboveground vegetative organs and the primary root) of the experimental plants at the conclusion. All plants were harvested 2 months after the release of the adult beetles. The samples were oven-dried overnight (50˚C, DRK633DB; Toyo Roshi Kaisha, Ltd., Tokyo, Japan). In this later period of the greenhouse experiment, to include the effects of larval feeding on plant growth, we did not remove the larvae after the last measurement of leaf damage that was referred to above. The dried plants were weighed to a precision of $10^{-4}$ g using an electronic analytical scale (GR-60; A&D Company, Ltd., Tokyo, Japan). We recorded the weight of a dead plant as zero (1 of the 120 plants). The flower production was not evaluated because the plants did not produce flowers at this stage. No plants and only one beetle died during the first month after the release of the beetles.

Additionally, in the greenhouse experiment, we determined whether the trichome density of hairy plants was altered by wounding. We cut 50% of the leaf area from 16 hairy plants that were not used in the aforementioned experiment. A month after the damage, we counted the trichomes on newly expanded leaves under an 8× magnifying glass. However, the trichome density of the hairy plants did not increase (intact plant, mean ± SE = 87.8 ± 14.2 trichomes/$\mathrm{cm}^2$, $n = 8$; damaged plant, 81.5 ± 10.6 trichomes/$\mathrm{cm}^2$, $n = 8$; Mann-Whitney *U*-test, $U = 34$, $P = 1$).

*Feeding preference of adult beetles on hairy and glabrous leaves*

We observed the feeding preference of adult beetles on hairy and glabrous leaves as a complement to the greenhouse experiment. In this observation, we also tested whether short-term experience with abundant hairy and glabrous diets could enhance/inhibit the host preference by beetles. Young leaves (1.5-2.0 cm in length) were used that were randomly harvested from plants grown under the identical conditions described above. Forty-five male and female adults of *P. brassicae* were randomly selected from the laboratory-reared population. We placed thirty beetles in each of three transparent plastic cases (diameter 8.0 cm, depth 4.5 cm), and these beetles were preconditioned with hairy or glabrous leaves or were starved. Ten young leaves of either hairy or glabrous plants were supplied to the pre-hairy and pre-glabrous conditions, respectively. After the preconditioning, each beetle fed on either a hairy or glabrous leaf within a Petri dish (diameter 6 cm, depth 1.5 cm; Kord-Valmark Co., Ontario, Canada) with a moistened filter paper (diameter 5.5 cm; Toyo Roshi Kaisha, Ltd., Tokyo, Japan). We recorded the feeding marks on the leaves every thirty minutes for six hours after the start of the observations. The presence/absence of feeding marks was then checked a day after the beginning of the observations. Twenty-four male and female beetles randomly chosen from each preconditioning treatment were used per observation (8 beetles × 2 leaf types × 3 preconditions). This procedure was repeated three times (i.e., 144 beetles were examined in total). The observations were conducted under the identical conditions used to cultivate the experimental plants, as described above.

### *Performance of hairy and glabrous plants without herbivory*

We cultivated hairy and glabrous plants without herbivory to compare the growth and reproductive potentials. The mature fruits were collected from 14 maternal plants (seven hairy and seven glabrous plants) from our study population in early July 2011. The maternal plants were separated from each other by at least 5 m. We prepared 5 to11 plants from each maternal family in an indoor space without herbivory. Fifty seeds from each maternal family were sown on a Petri dish (diameter 9 cm, depth 1.5 cm) filled with moistened quartz sand on August 15, 2011, to germinate at room temperature (ca. 80% of the seeds germinated). Three seedlings from the identical family were transplanted to the identical pot on November 25, 2011. The plastic pots (diameter 10.5 cm, depth 9 cm) were filled with mixed soil (pumice:leaf mold:peat moss = 1:1:1). All seedlings had 2 to 4 leaves when transplanted. These plants were grown inside a transparent plastic case (75 cm × 45 cm with a depth of 18 cm; 28 pots per case) for four months with natural sunlight (10–12 h in day length) at room temperature (average 10–30°C per day). The locations of the cases were rotated monthly. Liquid fertilizer (N:P:K ratio = 6:10:5, Hyponex; Hyponex, Osaka, Japan) was diluted 1000-fold and supplied monthly. The photosynthetically active radiation was 800-1100 and 100-300 μmol/m$^2$/s on sunny and cloudy days, respectively. The red:far-red ratio ranged from 1.0 to 1.3 (LI-190 Quantum Sensor; LI-COR, Lincoln, NE, USA).

The largest plant of the three in each pot was identified with a wooden pin 1 month after the transplant, and the marked plants were measured. We recorded the length of the largest leaf and the numbers of leaves and flowers to assess plant growth and reproduction. The number of leaves and the leaf lengths were recorded ca. 1, 4, and 6 months after the transplant (corresponding to December 30, 2011, March 2, 2012, and May 3, 2012, respectively). Of the three plants in each pot, the smallest two (i.e., the unmarked plants) were removed 4 months after the transplant to avoid competition within the pots. The flowers were counted ca. 6 and 7 months after the transplant (corresponding to May 3, and May 26, 2012, respectively). The fruits were not evaluated because the plants did not produce any fruits because of self-incompatibility. All plants except for two individuals survived to the end of the study. The trichome phenotype was identified 6 months after the transplants ($n_{\text{hairy}} = 68$ and $n_{\text{glabrous}} = 42$) because hairs on the stem identified the phenotype more easily. To follow the individual plants without destructive sampling, we estimated plant biomass using plant allometry based on measurements at ca. 4 months after transplant for this growth condition. We used the length of the largest leaf (cm) × ln[no. of leaves] to estimate ln[biomass (mg)] because this metric explained a large proportion of the variation in biomass (linear regression, $Y = 0.13X + 2.48$, $R^2 = 0.80$; see Figure S1 in Supporting Information).

*Statistical analyses*

We used cumulative link mixed models (Agresti 2002) to analyze the effects of the trichome phenotype, the neighborhood condition, and the initial plant size on the leaf damage score. The cumulative model is used to fit ordinal response data (Agresti 2002), and therefore, the model was applied to the ordinal response of the leaf damage score. We treated the trichome phenotype, neighborhood condition, and initial plant size as fixed effects. Additionally, we included an interaction between the trichome phenotype and the neighborhood condition in the model as a fixed effect to test whether the trichome phenotype differentially influenced leaf damage depending on the neighborhood conditions of the two plant types. The length of the largest radial leaf represented the initial plant size, which was an approximation of the radius of the rosette. We included two clustering variables as random effects. First, the maternal plant ID was incorporated to consider potential heritable variation among the maternal families. Second, we considered the individual plant ID among repeated measures as another random effect. The significance of the fixed effects was analyzed using likelihood ratio tests in which we first compared the models with or without an interaction term and then tested each fixed effect using the models without interaction terms. The coefficients of fixed effects were also examined when performing the likelihood ratio tests. These approaches of cumulative model analyses were performed separately for the young and the mature leaves and for the pooled data on the two leaf types to examine whether the resulting herbivory patterns were specific to the leaf types. Although the damage scores of individual leaves were used for the statistical analyses, we calculated the proportion of leaf area loss of an entire plant by averaging the midpoints of the corresponding area loss of the damage score of the leaves (i.e., damage scores of 0, 1, 2, 3, 4, and 5 = 0.0, 0.125, 0.375, 0.625, 0.875, and 1.0, respectively).

We used generalized linear mixed models to analyze the numbers of adult and larval beetles and linear mixed models (LMMs) to analyze plant biomass. We selected a Poisson error structure for the generalized LMMs to analyze the count response (i.e., the numbers of beetles and larvae). According to these cumulative model analyses, likelihood ratio tests were used to analyze the trichome phenotype, neighborhood condition, interaction between the trichome phenotype and the neighborhood condition, and initial plant size as fixed effects. The cumulative number of beetles was analyzed as the response variable to reflect both the intensity and the duration of herbivory on the plants (Ruppel 1983). For the data on larvae, we separately analyzed the numbers of larvae on the young, mature and all leaves on an individual plant. The maternal plant ID was incorporated as a random effect in these analyses. The biomass data were ln($x$ + 0.1)-transformed to improve normality. Additionally, for the data of the greenhouse experiment, we compared each pair of the two trichome phenotypes and the neighborhood conditions using pairwise likelihood ratio tests. The sample size of the minority phenotypes was only fifteen in our greenhouse experiment; and therefore, the $p$-values are provided without any correction because of the low statistical power (see also Nakagawa 2004 for an explanation of multiple comparisons).

The time until the initial bite was also analyzed using the cumulative model. The response, i.e., time until the initial bite, was an ordinal variable, and therefore, it was converted to scores of 1-14. The scores of 1-12 corresponded to every < 30-minute time interval up to 6 h, and the scores of 13 and 14 indicated the presence and absence of feeding after a day, respectively. The trichome phenotype, precondition, and interaction between these two factors were analyzed as explanatory variables using likelihood ratio tests. We incorporated the experiment ID as a random effect, but the estimated variance was small ($< 10^{-8}$), and several models did not converge. Thus, the random effect was not included in the cumulative model used to explain the timing of bite initiation.

For the data from the indoor cultivation, we used LMMs for the estimated biomass (4 and 6 months after the transplant) and generalized LMMs with a Poisson error structure for the number of flowers (6 and 7 months after the transplant). The estimated biomass was ln-transformed to improve normality. The trichome phenotype and maternal plant ID were analyzed as fixed and random effects, respectively. To incorporate potential size biases in the initial state, we considered the initial biomass (estimated 1 month after the transplant) as a covariate for the analysis of the estimated biomass. For the number of flowers, to distinguish the direct effects of the trichome phenotype on flower production from those mediated by a growth difference, we also analyzed models with or without a fixed effect of the estimated biomass 4 months after the transplant. We tested the significance of each fixed effect using likelihood ratio tests.

All statistical analyses were performed using the R statistical software package version 3.0.0 (R Core Team 2013). We used the clmm function (in the ordinal package; Christensen 2013) for the cumulative link mixed models, the glmer function (in the lme4 package; Bates et al. 2014) for the generalized or ordinal LMMs, and the vglm function (in the VGAM package: Yee 2013) for the cumulative models with no random effects.

## Results

### *Minority advantage in defense and growth for hairy and glabrous plants*

The magnitude of leaf damage on hairy plants was lower than that on glabrous plants under the glabrous-abundant condition (LR-$\chi^2_1 = 14.5$, $P < 0.001$), whereas the leaf damage was not significantly different between hairy and glabrous plants under the hairy-abundant condition (LR-$\chi^2_1 = 0.6$, $P = 0.44$; Fig. 1a). At the end of the experiment, the less abundant type of plant accumulated a larger biomass than that of the abundant type in the identical neighborhood condition (hairy-abundant, LR-$\chi^2_1 = 5.97$, $P < 0.05$; glabrous-abundant, LR-$\chi^2_1 = 3.89$, $P < 0.05$) or in the identical type in the other neighborhood condition (hairy plants, LR-$\chi^2_1 = 4.77$, $P < 0.05$; glabrous plants, LR-$\chi^2_1 = 7.66$, $P < 0.01$; Fig. 1b). The result that less abundant hairy plants had less leaf damage compared with the other three treatments was prominent in young leaves (LR-$\chi^2_1 > 9.0$, $P < 0.01$; Table S1 and Fig. 1c). The differential patterns between the neighborhood conditions were confirmed by a significant trichome × neighborhood interaction for the pooled data of damage on young and mature leaves, damage on young leaves, and plant biomass (Table 1a). The leaf damage on mature leaves was far less compared with that on young leaves (Fig. 1d) in which no significant effects were detected for the trichome phenotype or the neighborhood conditions on the damage (Table 1a). These results for the leaf damage on pooled, young and mature leaf types were detected across all points in time (after 1-4 weeks after the release of the beetles; Figure S2). The initial plant size did not differ between the trichome phenotypes or the neighborhood conditions (generalized LMM, $P > 0.2$ for the trichome phenotype, neighborhood condition, and the interaction). The details for the coefficients of the intercepts of the cumulative models are in the supporting information (Table S2).

Adults occurred less frequently on hairy plants under the glabrous-abundant condition (LR-$\chi^2_1 = 19.7$, $P < 0.0001$), whereas the cumulative number of adults did not differ significantly (< 1 beetle, on average) between hairy and glabrous plants under the hairy-abundant condition (LR-$\chi^2_1 = 0.93$, $P = 0.33$; Fig. 1g). The difference in occurrence of adults on hairy and glabrous plants between the neighborhood conditions was confirmed by a significant trichome × neighborhood interaction (Table 1b). More larvae occurred on hairy and glabrous plants under the glabrous-abundant condition than those on the identical plant type under the hairy-abundant condition (hairy plants, LR-$\chi^2_1 = 4.54$, $P < 0.05$; glabrous plants, LR-$\chi^2_1 = 17.2$, $P < 0.0001$; Fig. 1h), which was a result that was supported by a significant effect of the neighborhood condition on the abundance of larvae (Table 1b). The larvae occurred more frequently on the young leaves of glabrous plants under the glabrous-abundant condition than those under the hairy-abundant condition (LR-$\chi^2_1 > 6.0$, $P < 0.05$; Table S1 and Fig. 1e), and the larvae occurred less often on hairy leaves than glabrous leaves under the glabrous-abundant condition (although the difference was not significant, LR-$\chi^2_1 = 0.6$, $P = 0.7$; Fig. 1e). We observed a larger number of larvae on the mature leaves of hairy plants under the glabrous-abundant condition than those on the other three treatments (LR-$\chi^2_1 > 9.0$, $P < 0.01$: Table S1; Fig. 1f). For the herbivore abundance, no significant effect of initial plant size was detected (Table 1b).

*Feeding preference of adult beetles on hairy and glabrous leaves*

The glabrous leaves were attacked more rapidly than the hairy ones by adult beetles, and a larger number of hairy leaves remained intact than that of glabrous ones (Fig. 2). We also detected a significant effect of the trichome phenotype on the timing of bite initiation of adult beetles (cumulative link models, LR-$\chi^2_1 = 4.13$, $P < 0.05$). Neither the main effect of the precondition nor the interaction between the trichome phenotype and the precondition had a significant effect on the timing of the bite initiation (LR-$\chi^2_2 = 0.48$, $P = 0.79$; LR-$\chi^2_2 = 3.73$, $P = 0.15$, respectively). However, the timing of the bite initiation suggested no difference between hairy and glabrous leaves for the hairy-preconditioned beetles (upper row, Fig. 2). To further examine whether the hairy or glabrous preconditioning affected the bite initiation, we performed the identical analysis but excluded the data set of the starved precondition. In this case, the biting initiation of adult beetles was affected by the interaction of the leaf type and the preconditioning at a marginally significant level (LR-$\chi^2_1 = 3.2$, $P = 0.08$). Detailed results for the coefficients of explanatory variables and intercepts are given in the supporting information (Table S3).

*Performance of hairy and glabrous plants without herbivory*

The estimated biomass of hairy plants was smaller than that of the glabrous ones 4 and 6 months after the transplants (Table 2; Figs. 3a, b). The estimated biomass was not significantly affected by the initial biomass ($P > 0.1$, both for 4 and 6 months; Table 2). The hairy plants produced fewer flowers than those of glabrous plants 6 months after the transplant at a marginally significant level ($P = 0.07$; Table 2 and Fig. 3c), although the hairy plants did not have significantly fewer flowers 7 months after the transplant ($P = 0.63$; Table 2 and Fig. 3d). Trichome production had no significant effect on flower production when the initial biomass (i.e., estimated biomass at 4 months after the transplant) was incorporated as a covariate ($P > 0.05$, both for 6 and 7 months; Table 2). The initial biomass was positively correlated to flower production 6 and 7 months after the transplant, at least at a marginally significant level (Table 2).

## Discussion

We demonstrated experimentally that hairy plants incurred less herbivory by *P. brassicae* when they were surrounded by glabrous plants. This pattern has been reported as a type of associational effect; specifically, our example corresponded with the ‘neighbor contrast defense’ (Bergvall et al. 2006; Rautio et al. 2012) because we observed less herbivory on the defended phenotype (hairy plants, in our case) when surrounded by undefended individuals. We also found a reciprocal minority advantage in biomass production for the hairy and glabrous plants, which might serve as a potential mechanism to maintain the polymorphism through negative frequency-dependent selection.

### *Mechanisms responsible for the pattern of associational effects*

As a possible mechanism for these associational effects, Bergvall et al. (2006) suggest that the contrast of food types perceived by herbivores increases their feeding preference for the more palatable food. In *Phaedon* species, the host preference of adult beetles was less for *Brassica* cultivars that produced dense trichomes (Kühnle and Müller 2012). Our observation that adult beetles were inclined to attack glabrous leaves more rapidly than hairy ones also provided support for the negative influence of trichomes on host preference in *P. brassicae*. Furthermore, when the weak difference in adult abundance between the two plant morphs under the hairy-abundant condition was compared, the feeding preference did not differ considerably between hairy and glabrous leaves when adult *P. brassicae* had been preconditioned with hairy diets. Provided with this circumstantial evidence for feeding preference determined by diet experience, the behavioral response of adult beetles indicated that plant damage was likely dependent on the neighborhood conditions for hairy and glabrous plants. The relevance of this mechanism is also apparent because *P. brassicae* are flightless and access their hosts exclusively by walking (Ôtake and Funaki 1958), which forces the beetles to choose plants within a small-scale area. It has been argued that the spillover of herbivores from undefended to defended plants can alter damage on defended neighbors (spillover effects; White and Whitham 2000); however, in our study, spillover was not likely because the adult beetles actively avoided hairy plants.

Trichome density is often high on young leaves (e.g., Chu et al. 2001; Yamawo et al. 2012), and consequently, leaf age can influence the effectiveness of trichomes as a defense against herbivory (Chu et al. 2001; Puentes and Ågren 2013). In our study, the pattern that less abundant hairy plants suffered less herbivory was the most prominent for the young leaves because the young leaves of hairy plants produced denser trichomes than those of mature leaves. Therefore, trichomes might play an important role in defending the younger leaves that contribute to the future growth of *A. halleri*. Although the pattern of larval abundance between hairy and glabrous plants was not clear, the larvae tended to occur more often on the young leaves of glabrous plants under the glabrous-abundant condition. Because the larvae of *P. brassicae* rarely move between plants, the abundance of newly hatched larvae likely reflected the oviposition patterns of adult females. Thus, the tendency for a larger number of larvae to occur on young glabrous leaves led to the presumption that the adult *P. brassicae* preferred to oviposit on glabrous leaves.

At least at the scale of our experiment, we did not detect significant effects of plant size on the number of *P. brassicae* for either the larvae or the adults; thus, we hypothesized that *P. brassicae* responded to the trichome phenotype rather than the resource abundance. However, it was still possible that density as well as frequency of hairy and glabrous plants might result in the pattern of leaf damage, because our experiment with two neighborhood conditions could not determine the relative importance of density- and frequency-dependent damage on the two plant types (but see Kim and Underwood 2015). We should also note that when herbivores respond nonadditively to plant density (i.e., group effects; Rautio et al. 2012) it may lead to similar patterns of damage for an individual plant as those caused by associational effects (see also Hambäck et al. 2014 for a theoretical consideration). In future studies, the density and the frequency of the two plant types should be manipulated simultaneously (i.e., response surface design; Damgaard 1998; Inouye 2001) to examine potential interactions between the associational and the resource concentration/dilution effects on plant damage (Underwood et al. 2014; Hambäck et al. 2014).

### *Maintenance of polymorphism under associational effects*

Classically, a reciprocal minority advantage (or negative frequency-dependent selection) leads to the coexistence of multiple species/genotypes because a reciprocal minority advantage can prevent rare types from going extinct (reviewed by Ayala and Campbell 1974). Our study uncovered a reciprocal minority advantage in plant growth between the hairy and glabrous morphs, which might be a mechanism that maintains the dimorphism within populations. The minority advantage could have a number of possible explanations. First, as assumed in earlier game theories related to associational effects (Augner et al. 1991; Tuomi et al. 1999), the defended plants might be at a disadvantage in growth because of the cost of defense. In the indoor cultivation study, we also found that the growth and reproduction of glabrous plants were slightly but consistently higher than those of hairy plants in the absence of herbivores. The potential costs of trichomes may even out defense superiority of hairy plants when the defense is weakened under the hairy-abundant condition. However, under the glabrous-abundant condition, the escape from herbivory benefited hairy plants. The associational effects and the costs of defense presumably led to the reciprocal minority advantage in biomass for the two morphs, although these two factors are mutually nonexclusive. Second, the larvae became less abundant when hairy plants were a majority, whereby the less abundant glabrous plants might be protected by the abundant hairy plants (i.e., associational resistance) and therefore grow faster than hairy ones under the hairy-abundant condition. To distinguish the associational effects and the plant growth potentials, studies must manipulate the presence/absence of herbivores as well as the neighborhood condition.

Hairy and glabrous plants coexist in a natural population in which *P. brassicae* is the primary consumer of *A. halleri* (Kawagoe et al. 2011; Sato et al. 2014). In this field site of our previous study, we found that leaf damage on hairy plants decreased as the frequency of neighboring glabrous plants within a 1-m patch scale increased (Sato et al. 2014). Although it remains unknown how important biomass production is in the lifetime fitness of *A. halleri* in the field, the growth before flowering could be a major component of fitness because the fecundity of hairy plants depends on their size before flowering (Kawagoe and Kudoh 2010). In the indoor cultivation described in the present study, the biomass in the growth stage was also positively correlated with flower production. In addition to sexual reproduction, *A. halleri* produces clonal rosettes after flowering, and this clonal propagation is expected to depend on biomass at the growth stage. There are no trichomes on the flowers of hairy and glabrous plants, and if associational effects influence pollinator attraction, it will occur indirectly through flower production. If plant neighbor affects host-searching abilities and colonization rates of herbivores between patches in the field, then we must test whether associational effects result in negative frequency-dependent selection in a natural population.

### *Conclusions and implications*

Our study suggests that intraspecific associational effects serve as a mechanism for the maintenance of defense dimorphism by contributing to negative frequency-dependent growth between defended and undefended plants. Notably, associational effects may cause frequency-dependent selection not only in plant-herbivore interactions but also in plant-pollinator and prey-predator interactions (Underwood et al. 2014). For example, the learning behaviors of pollinators can maintain flower color polymorphism via negative frequency-dependent selection (Gigord et al. 2001). As for plant-pathogen interactions, the evidence is suggestive for frequency-dependent infection between resistant and susceptible plants (e.g., Brunet and Mundt 2000; Siemens and Roy 2005). Thus, the findings of this study position associational anti-herbivore defense in the broad context of mutualistic and antagonistic interactions involving plants. Future studies should incorporate such intraspecific associational effects because these effects might explain how genetic polymorphism is maintained under plant-consumer interactions.

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**Table 1** Effects of trichome phenotype, neighborhood condition, and initial plant size on traits related to plant damage and growth (a) and herbivore abundance (b).

(a) Plant damage and growth

| Fixed effects | *df* | Damage on pooled leaf types* | | | End-point biomass | | |
|---|---|---|---|---|---|---|---|
| | | Coef. ± SE | LR-$\chi^2$ | *P* | Coef. ± SE | LR-$\chi^2$ | *P* |
| Trichome (Hairy) | 1 | **-0.39 ± 0.11** | **13.09** | **< 0.001** | -0.03 ± 0.08 | 0.11 | 0.74 |
| Neighborhood (H>G) | 1 | 0.12 ± 0.10 | 1.41 | 0.23 | 0.032 ± 0.09 | 0.03 | 0.86 |
| Initial leaf length | 1 | 0.002 ± 0.003 | 0.59 | 0.44 | **0.008 ± 0.002** | **12.9** | **< 0.001** |
| Tri. × Neigh. | 1 | **0.56 ± 0.20** | **7.67** | **< 0.01** | **-0.55 ± 0.17** | **9.9** | **< 0.01** |

| Fixed effects | *df* | Damage on young leaves* | | | Damage on matured leaves* | | |
|---|---|---|---|---|---|---|---|
| | | Coef. ± SE | LR-$\chi^2$ | *P* | Coef. ± SE | LR-$\chi^2$ | *P* |
| Trichome (Hairy) | 1 | **-0.52 ± 0.13** | **15.15** | **< $10^{-4}$** | 0.02 ± 0.32 | 0.004 | 0.95 |
| Neighborhood (H>G) | 1 | 0.20 ± 0.13 | 2.27 | 0.13 | -0.58 ± 0.33 | 3.15 | 0.08 |
| Initial leaf length | 1 | 0.003 ± 0.003 | 0.83 | 0.36 | -0.002 ± 0.008 | 0.05 | 0.82 |
| Tri. × Neigh. | 1 | **0.86 ± 0.24** | **11.93** | **< 0.001** | -0.14 ± 0.65 | 0.04 | 0.83 |

(b) Herbivore abundance

| Fixed effects | *df* | Larvae on young leaves | | | Larvae on matured leaves | | |
|---|---|---|---|---|---|---|---|
| | | Coef. ± SE | LR-$\chi^2$ | *P* | Coef. ± SE | LR-$\chi^2$ | *P* |
| Trichome (Hairy) | 1 | 0.004 ± 0.123 | 0.0009 | 0.98 | **0.53 ± 0.18** | **9.07** | **< 0.01** |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| Neighborhood (H>G) | 1 | **-0.24 ± 0.12** | **4.43** | **< 0.05** | **-0.95 ± 0.18** | **29.9** | **$< 10^{-7}$** |
| Initial leaf length | 1 | -0.001 ± 0.003 | 0.16 | 0.69 | -0.001 ± 0.005 | 0.37 | 0.85 |
| Tri. × Neigh. | 1 | 0.47 ± 0.24 | 3.84 | 0.050 | **-1.06 ± 0.34** | **9.31** | **< 0.01** |

| Fixed effects | *df* | Adults per plant | | | Larvae per plant | | |
|---|---|---|---|---|---|---|---|
| | | Coef. ± SE | LR-$\chi^2$ | *P* | Coef. ± SE | LR-$\chi^2$ | *P* |
| Trichome (Hairy) | 1 | **-0.23 ± 0.11** | **4.00** | **< 0.05** | 0.20 ± 0.10 | 3.72 | 0.054 |
| Neighborhood (H>G) | 1 | 0.09 ± 0.11 | 0.59 | 0.44 | **-0.46 ± 0.10** | **22.8** | **$< 10^{-5}$** |
| Initial leaf length | 1 | 0.003 ± 0.003 | 1.25 | 0.26 | -0.002 ± 0.002 | 0.41 | 0.52 |
| Tri. × Neigh. | 1 | **1.05 ± 0.26** | **18.5** | **$< 10^{-4}$** | -0.10 ± 0.19 | 0.25 | 0.62 |

**Notes:** Results of mixed models (coefficients and their standard errors) and likelihood ratio (LR-$\chi^2$) are shown for each fixed effect with its degree of freedom (*df*), where bold values indicate < 5% significance. Coefficients of the neighborhood condition were estimated for the hairy-abundant condition (H>G). We tested the interaction term firstly and then analyzed the main effects of each factor. The initial plant size was represented by the length of the largest leaf. An asterisk (*) indicates response variables analyzed by cumulative link models, whereas no mark indicates those analyzed by generalized or ordinal linear mixed models.

**Table 2** Effects of trichome phenotype on growth and reproduction of *Arabidopsis halleri* subsp. *gemmifera* cultivated under no herbivory.

| Fixed effects | *df* | 4 months | | | 6 months | | | | | | 7 months | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Estimatd biomass | | | Estimated biomass | | | No. of flowers | | | No. of flowers | | |
| | | Coef ± SE | LR-$\chi^2$ | *P* | Coef ± SE | LR-$\chi^2$ | *P* | Coef ± SE | LR-$\chi^2$ | *P* | Coef ± SE | LR-$\chi^2$ | *P* |
| Trichome (single) | 1 | **-0.36 ± 0.09** | **16.3** | **< 0.001** | **-0.25 ± 0.12** | **4.2** | **< 0.05** | -0.13 ± 0.07 | 3.3 | 0.07 | 0.02 ± 0.05 | 0.23 | 0.63 |
| Trichome (multiple) | 1 | **-0.36 ± 0.09** | **16.8** | **< 0.001** | **-0.26 ± 0.12** | **4.4** | **< 0.05** | 0.12 ± 0.07 | 2.7 | 0.10 | 0.06 ± 0.05 | 1.2 | 0.27 |
| Initial biomass* | 1 | -0.56 ± 0.56 | 1.01 | 0.31 | -0.87 ± 0.76 | 1.32 | 0.25 | **0.71 ± 0.07** | **108** | **$< 10^{-16}$** | 0.09 ± 0.05 | 3.2 | 0.07 |

**Notes:** Results of mixed models (coefficients and their standard errors) and likelihood ratio (LR-$\chi^2$) are shown for each fixed effect with its degree of freedom (*df*). Bold values indicate < 5% significance with likelihood ratio tests. The effect of trichome phenotypes was tested with or without the covariate of initial biomass (denoted as single and multiple, respectively). Biomass and flowers were evaluated 4, 6, and 7 months after transplant, where the biomass was estimated on the basis of plant allometry (see Materials and Method for details). Estimated biomass 1 or 4 months after transplant was used as the initial biomass for the analyses of biomass or flowers, respectively.

**Figure legends**

**Fig. 1** Associational effects in defense and growth of hairy (H; grey) and glabrous (G; white) plants under hairy-abundant (H > G) and glabrous-abundant (H < G) conditions. Shown are leaf damage (proportion of leaf area loss) for pooled data of young and mature leaves (a), plant biomass (b), damage for young (c) and mature leaves (d), the cumulative number of larvae on young leaves (e), larvae on mature leaves (f), adult beetles per plant (g), and larvae per plant (h). Leaf damage was presented as values at four weeks after the release of beetles. The number of larvae reflected oviposition by female adults because newly hatched larvae were retrieved after each count. The plant biomass was measured two months after the release of adult beetles. Different letters indicate < 5% significant differences with pairwise likelihood ratio tests by mixed models that incorporated maternal plant ID as a random factor. Error bars indicate SEM of the raw data. Sample sizes are given within parentheses.

**Fig. 2** Time to initial biting by adult *Phaedon brassicae* on hairy (a) and glabrous (b) leaves. Histograms are shown for overnight preconditioning on hairy (Precon. = Hairy, upper row) and glabrous leaves (Precon. = Glabrous, middle row) and for beetles starved overnight (Precon. = None, lower row). Grey and black bars indicate the presence and absence of feeding marks a day after the release, respectively.

**Fig. 3** Estimated biomass (mg) and the number of flowers of hairy (H; grey) and glabrous (G; white) plants under no herbivory. Figures are shown for plant growth 4 and 6 months after the transplant (a and b) and flowering 6 and 7 months after the transplant (c and d). The biomass was estimated based on plant allometry at ca. 4 months after transplant. Error bars indicate SEM of the raw data. Data are not transformed in these figures. Sample sizes are given within parentheses. Asterisks and dots indicate statistical significances ($^{***}$ $P <$ 0.001, $^{*}$ $P < 0.05$, $^{\bullet}$ $P < 0.1$).

**Figure 1**

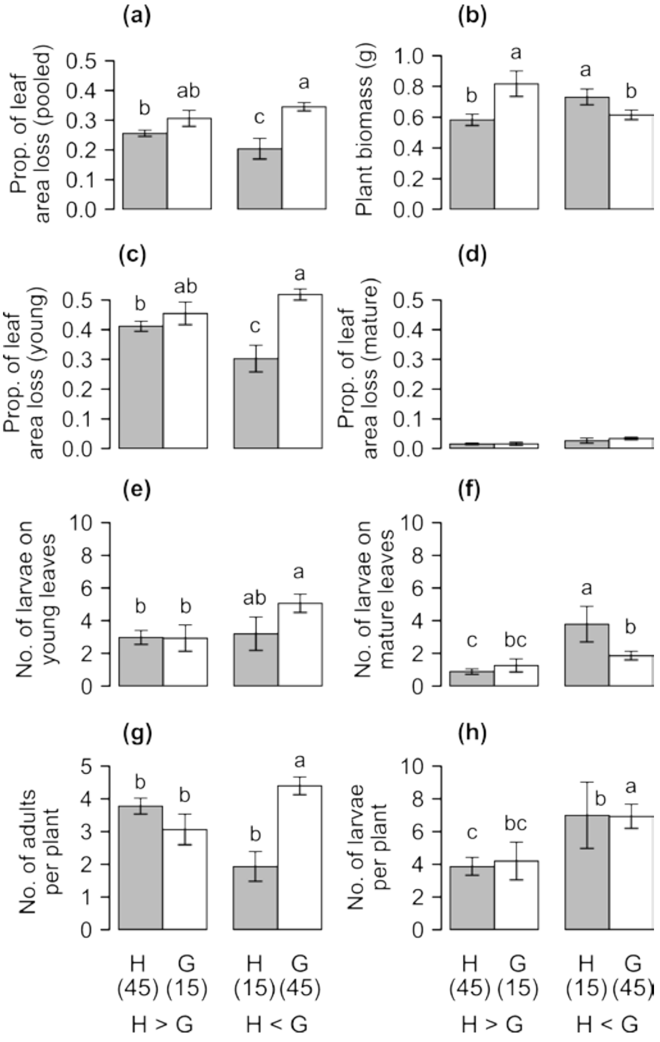

(a)
Prop. of leaf area loss (pooled)
0.0
0.1
0.2
0.3
0.4
0.5
b
ab
c
a
(b)
Plant biomass (g)
0.0
0.2
0.4
0.6
0.8
1.0
b
a
a
b
(c)
Prop. of leaf area loss (young)
0.0
0.1
0.2
0.3
0.4
0.5
b
ab
c
a
(d)
Prop. of leaf area loss (mature)
0.0
0.1
0.2
0.3
0.4
0.5
(e)
No. of larvae on young leaves
0
2
4
6
8
10
b
b
ab
a
(f)
No. of larvae on mature leaves
0
2
4
6
8
10
c
bc
a
b
(g)
No. of adults per plant
0
1
2
3
4
5
b
b
b
a
(h)
No. of larvae per plant
0
2
4
6
8
10
c
bc
b
a
H (45)
G (15)
H (15)
G (45)
H > G
H < G
H (45)
G (15)
H (15)
G (45)
H > G
H < G

**Figure 2**

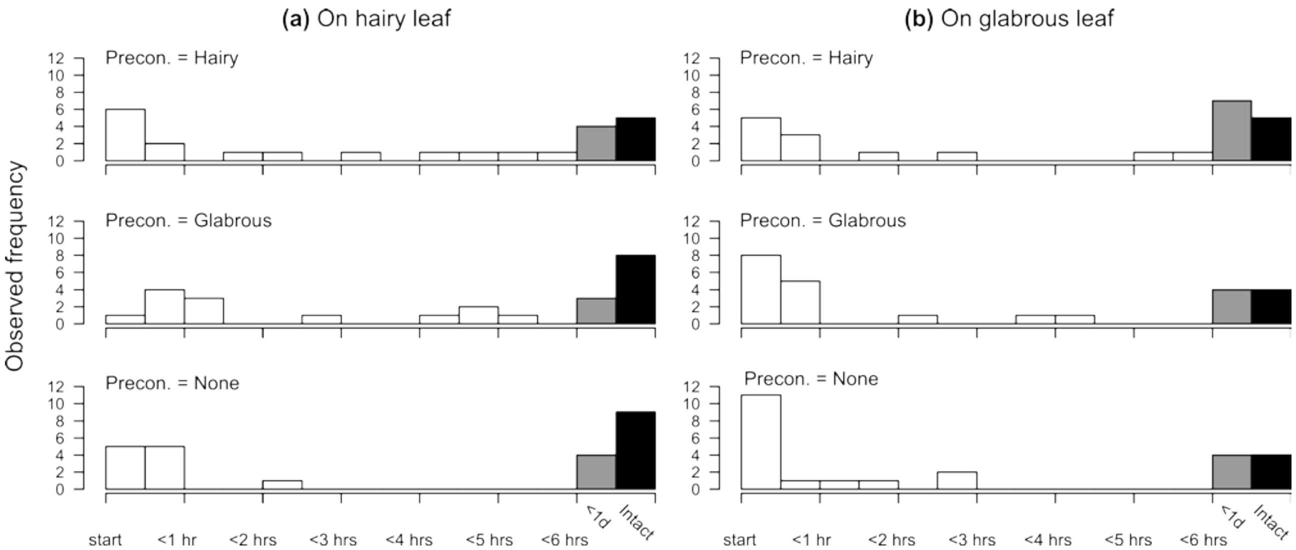
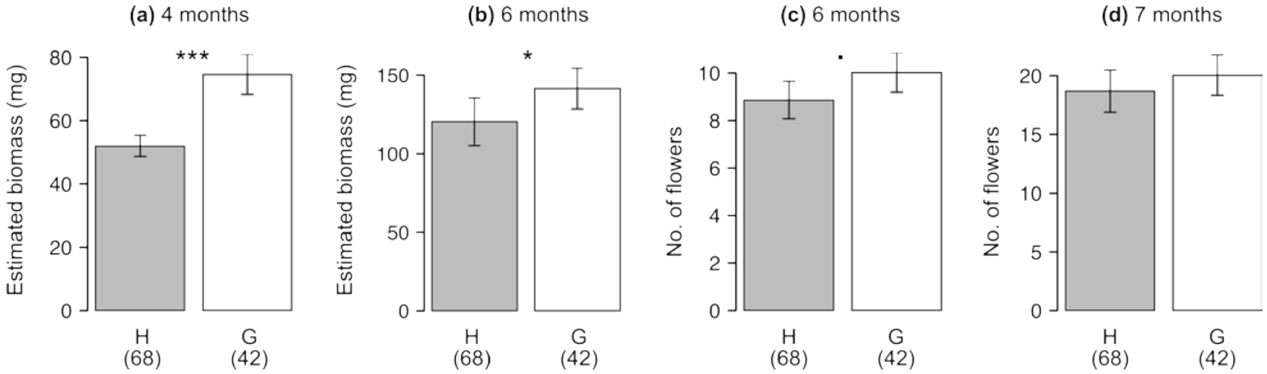

(a) On hairy leaf
(b) On glabrous leaf
Observed frequency
Precon. = Hairy
Precon. = Glabrous
Precon. = None
12
10
8
6
4
2
0
start
<1 hr
<2 hrs
<3 hrs
<4 hrs
<5 hrs
<6 hrs
<1d
Intact

**Figure 3**


(a) 4 months
Estimated biomass (mg)
0
20
40
60
80
***
H
(68)
G
(42)
(b) 6 months
Estimated biomass (mg)
0
50
100
150
*
H
(68)
G
(42)
(c) 6 months
No. of flowers
0
2
4
6
8
10
.
H
(68)
G
(42)
(d) 7 months
No. of flowers
0
5
10
15
20
H
(68)
G
(42)

## SUPPORTING INFORMATION

**Table S1** Results of pairwise likelihood ratio tests for the greenhouse experiment data.

**Table S2** Intercepts for the leaf damage scores in cumulative link mixed models.

**Table S3** Coefficients and intercepts of the time to initial biting by an adult beetle in the cumulative models.

**Figure S1** Biomass (mg) estimation of *Arabidopsis halleri* subsp. *gemmifera* by the number and length of leaves.

**Figure S2** Leaf damage data (proportion of leaf area loss) until four weeks after the release of leaf beetles.

**Table S1** Results of pairwise likelihood ratio tests for the greenhouse experiment data including four groups of the trichome phenotypes (H: hairy plants; G: glabrous plants) plants under two neighborhood conditions (H>G: hairy-abundant condition; H<G: glabrous-abundant condition).

| Response | Group 1 | | Group 2 | | LR-$\chi^2$ | *P* |
|---|---|---|---|---|---|---|
| | Tri. | Neigh. | Tri. | Neigh. | | |
| Damage (pooled) | **H** | **H>G** | **H** | **H<G** | **7.69** | **<0.01** |
| | G | H>G | G | H<G | 1.53 | 0.22 |
| | H | H>G | G | H>G | 0.60 | 0.44 |
| | **G** | **H<G** | **H** | **H<G** | **14.5** | **<0.001** |
| | **H** | **H<G** | **G** | **H>G** | **7.86** | **<0.01** |
| | **G** | **H<G** | **H** | **H>G** | **4.86** | **<0.05** |
| Damage (young) | **H** | **H>G** | **H** | **H<G** | **11.7** | **<0.001** |
| | G | H>G | G | H<G | 2.24 | 0.13 |
| | H | H>G | G | H>G | 0.03 | 0.86 |
| | **G** | **H<G** | **H** | **H<G** | **20.9** | $\mathbf{<10^{-5}}$ |
| | **H** | **H<G** | **G** | **H>G** | **9.15** | **<0.01** |
| | **G** | **H<G** | **H** | **H>G** | **4.52** | **<0.05** |
| Damage (mature) | H | H>G | H | H<G | 1.97 | 0.16 |
| | G | H>G | G | H<G | 1.19 | 0.28 |
| | H | H>G | G | H>G | 0.02 | 0.90 |
| | G | H<G | H | H<G | 0.19 | 0.66 |
| | H | H<G | G | H>G | 1.10 | 0.29 |
| | G | H<G | H | H>G | 2.49 | 0.11 |
| Plant Biomass | **H** | **H>G** | **H** | **H<G** | **4.77** | **<0.05** |
| | **G** | **H>G** | **G** | **H<G** | **7.66** | **<0.01** |
| | **H** | **H>G** | **G** | **H>G** | **5.98** | **<0.05** |
| | **G** | **H<G** | **H** | **H<G** | **3.89** | **<0.05** |
| | H | H<G | G | H>G | 0.37 | 0.55 |
| | G | H<G | H | H>G | 0.13 | 0.91 |
| Adult beetles | **H** | **H>G** | **H** | **H<G** | **14.1** | **<0.001** |

|  |  |  |  |  |  |  |
|---|---|---|---|---|---|---|
|  | **G** | **H>G** | **G** | **H<G** | **5.42** | **<0.05** |
|  | H | H>G | G | H>G | 0.94 | 0.33 |
|  | **G** | **H<G** | **H** | **H<G** | **19.7** | **$<10^{-5}$** |
|  | H | H<G | G | H>G | 3.24 | 0.07 |
|  | G | H<G | H | H>G | 1.60 | 0.21 |
| Larvae per plant | **H** | **H>G** | **H** | **H<G** | **4.54** | **<0.05** |
|  | **G** | **H>G** | **G** | **H<G** | **17.2** | **$<10^{-4}$** |
|  | H | H>G | G | H>G | <0.001 | 0.98 |
|  | **G** | **H<G** | **H** | **H<G** | **7.67** | **<0.01** |
|  | H | H<G | G | H>G | 3.66 | 0.56 |
|  | **G** | **H<G** | **H** | **H>G** | **12.4** | **<0.001** |
| Larvae on young leaves | H | H>G | H | H<G | 1.02 | 0.31 |
|  | **G** | **H>G** | **G** | **H<G** | **14.2** | **<0.001** |
|  | H | H>G | G | H>G | 0.62 | 0.43 |
|  | G | H<G | H | H<G | 0.10 | 0.76 |
|  | H | H<G | G | H>G | 0.04 | 0.84 |
|  | **G** | **H<G** | **H** | **H>G** | **6.21** | **<0.05** |
| Larvae on mature leaves | **H** | **H>G** | **H** | **H<G** | **26.0** | **$<10^{-6}$** |
|  | G | H>G | G | H<G | 2.25 | 0.13 |
|  | H | H>G | G | H>G | 1.49 | 0.22 |
|  | **G** | **H<G** | **H** | **H<G** | **19.1** | **$<10^{-4}$** |
|  | **H** | **H<G** | **G** | **H>G** | **9.52** | **<0.01** |
|  | **G** | **H<G** | **H** | **H>G** | **9.22** | **<0.01** |

**Notes:** Effects of initial plant size and maternal plants were considered as a covariate and random factor in mixed models, respectively (see text for details). Bold values indicate <5% significant differences between groups.

**Table S2** Intercepts for the leaf damage scores in cumulative link mixed models.

| Intercept | (a) Pooled leaf types | | | (b) Young leaves | | | (c) Matured leaves | | |
|---|---|---|---|---|---|---|---|---|---|
| | Coefficients | SE | *Z* | Coefficients | SE | *Z* | Coefficients | SE | *Z* |
| 0 \| 1 | 0.48 | 0.29 | 1.68 | -0.23 | 0.35 | -0.65 | **2.76** | **0.89** | **3.10** |
| 1 \| 2 | **1.32** | **0.29** | **4.58** | **0.75** | **0.35** | **2.16** | **6.12** | **0.93** | **6.59** |
| 2 \| 3 | **1.82** | **0.29** | **6.32** | **1.33** | **0.35** | **3.83** | NA | NA | NA |
| 3 \| 4 | **2.16** | **0.29** | **7.52** | **1.72** | **0.35** | **4.95** | NA | NA | NA |
| 4 \| 5 | **2.54** | **0.29** | **8.83** | **2.13** | **0.35** | **6.12** | NA | NA | NA |

**Notes:** Numbers of intercept column indicate the damage score 0 - 5 that correspond to intact leaves - complete area loss, respectively. For example, 0|1 indicates a ratio of probability of being the damage score 0 on that of the score 1. The estimates were calculated using models including all of the fixed effects. Bold values indicate a deviation of coefficients from zero significant at $P < 0.05$ (Wald test). NA indicates no data available.

**Table S3** Coefficients and intercepts of the time to initial biting by an adult beetle (order 1 - 14) in the cumulative models.

| Factors | Coefficient | SE | *Z* |
|---|---|---|---|
| Trichome (Hairy) | **-0.60** | **0.30** | **-2.02** |
| Precon. (Hairy) | -0.04 | 0.36 | -0.11 |
| Precon. (None) | 0.20 | 0.36 | 0.56 |
| Tri. × Precon. (Hairy) | 1.18 | 0.73 | 1.62 |
| Tri. × Precon. (None) | -0.05 | 0.73 | -0.07 |
| Intercept (1\|2) | -0.67 | 0.38 | -1.77 |
| Intercept (2\|3) | 0.01 | 0.37 | 0.03 |
| Intercept (3\|4) | 0.13 | 0.37 | 0.35 |
| Intercept (4\|5) | 0.22 | 0.37 | 0.58 |
| Intercept (5\|6) | 0.30 | 0.37 | 0.82 |
| Intercept (6\|7) | 0.42 | 0.37 | 1.12 |
| Intercept (7\|8) | 0.45 | 0.37 | 1.20 |
| Intercept (8\|9) | 0.48 | 0.37 | 1.28 |
| Intercept (9\|10) | 0.56 | 0.37 | 1.51 |
| Intercept (10\|11) | 0.65 | 0.38 | 1.73 |
| Intercept (11\|12) | **0.74** | **0.38** | **1.96** |
| Intercept (12\|13) | **0.80** | **0.38** | **2.11** |
| Intercept (13\|14) | **1.64** | **0.40** | **4.15** |

**Notes:** Ordered score 1 - 12 correspond to 30-minute intervals after the beginning of observation, respectively. Score 13 and 14 indicates the presence and absence of biting marks after one day, respectively. For example, 0|1 indicates a ratio of probability of being the damage score 0 on that of the score 1. These intercept estimates were calculated using models including all of the explanatory variables. Bold values indicate a deviation of coefficients from zero significant at $P < 0.05$ (Wald test).

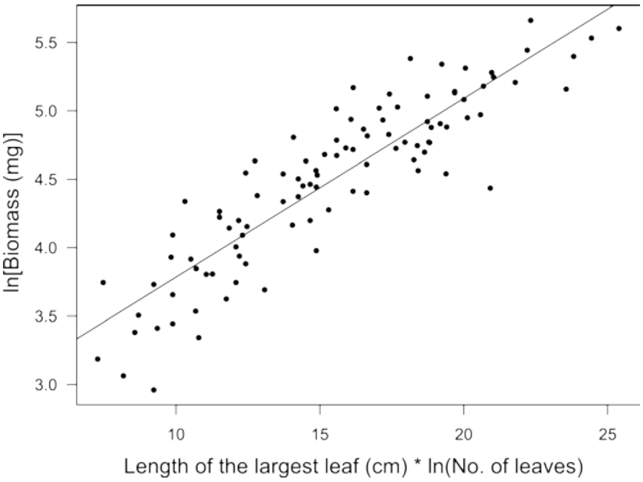


**Figure S1** Biomass (mg) estimation of *Arabidopsis halleri* subsp. *gemmifera* by the number and length of leaves. The biomass, leaf number, and leaf length were evaluated at ca. 4 months after transplant. Solid line represents the prediction by simple linear regression ($Y = 0.13X + 2.48$, $R^2 = 0.80$). Details of the growth conditions are described in the main text (see Materials & Methods).

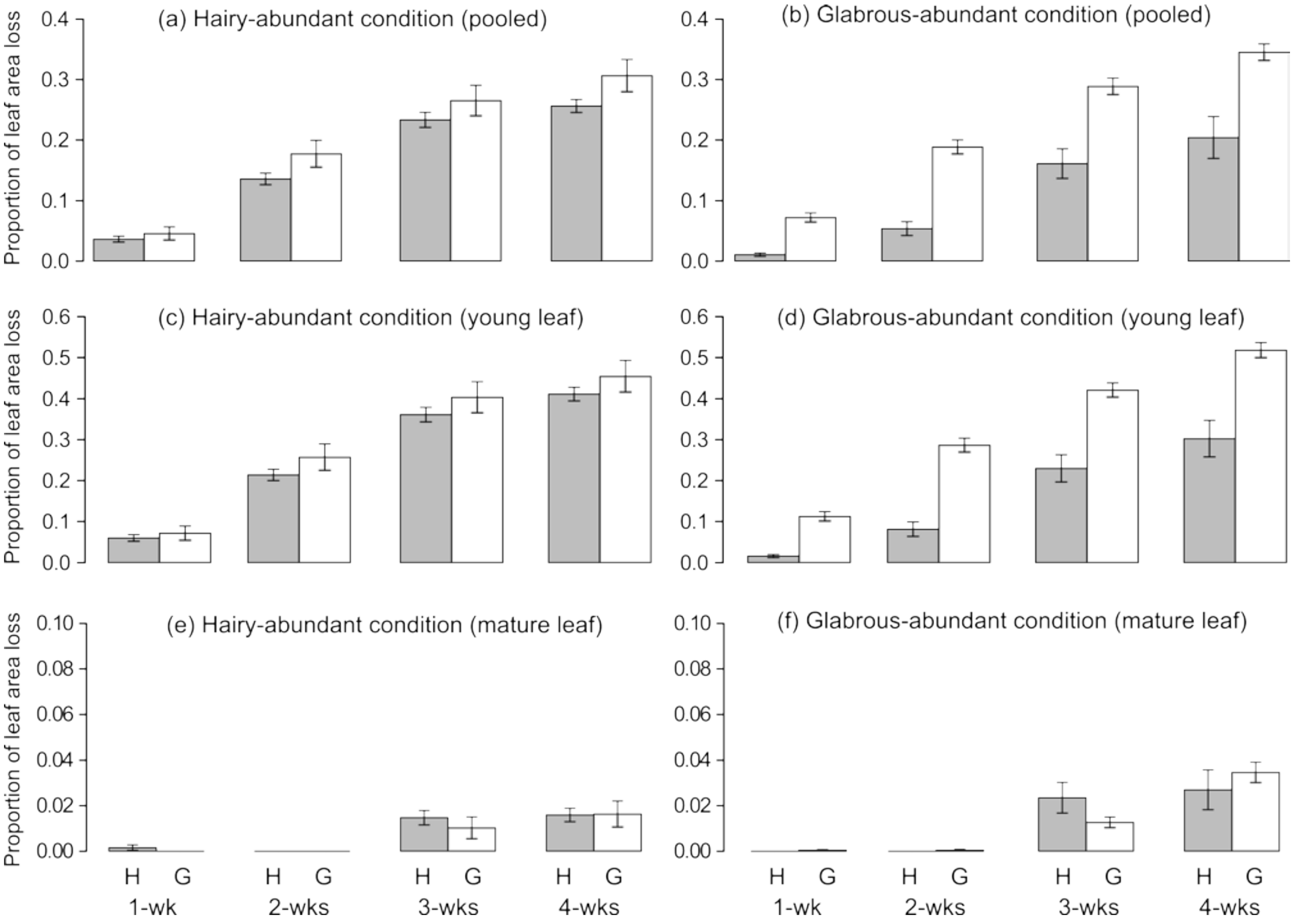


**Figure S2** Leaf damage data (proportion of leaf area loss) until four weeks after the release of adult beetles. Data are separately presented for pooled dataset of young and mature leaves (a, b), young (c, d), or mature (e, f) leaf type. Mean ± SE values of the raw data are shown for hairy (H; grey) and glabrous (G; white) plants under the hairy-abundant and glabrous-abundant conditions.