Intraspecific interaction of host plant influences local distribution of specialist herbivores through metabolic alterations in leaves

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20 COMPETING INTERESTS

21 The authors declare that they have no competing interests.

22

Abstract

1. Recent studies suggest that changes in leaf traits due to interactions between plants affect the resource utilisation and distribution of herbivores. However, this has not yet been confirmed experimentally. Here, we investigated the effects of phenotypic plasticity in leaf traits of *Rumex obtusifolius* (host plant) in response to the intra- and interspecific interaction on distribution of two leaf beetles, *Gastrophysa atrocyanea* (specialist herbivore) and *Galerucella grisescens* (generalist herbivore).
2. We investigated the local population density of *R. obtusifolius* plants and the presence of leaf beetles on the plants at five study sites. Leaf chemicals (condensed tannins and total phenolics) were compared between aggregated and solitary *R. obtusifolius* plants. To clarify the effects of the interaction environment of *R. obtusifolius* plants on their leaf traits and resource utilisation by leaf beetles, we conducted cultivation and preference experiments. Leaf chemicals (chlorophylls, organic acids, primary metabolites, condensed tannins and total phenolics) and preferences of adult leaf beetles were compared between intraspecific, interspecific plant interaction, or no-interaction treatments. Finally, we evaluated the effects of interaction between *R. obtusifolius* on leaf beetle distribution in mesocosm experiments.

3. In the field, the presence of the specialist leaf beetle, *G. atrocyanea*, was positively correlated with the local population density (rosette overlap ratio) of *R. obtusifolius* plants; however, no correlation was observed in the case of the generalist leaf beetle, *G. grisescens*. In the cultivation experiment, plants in the intraspecific interaction treatment increased their leaf contents of condensed tannins and total phenolics, and *G. atrocyanea* consumed more of these leaves than leaves in other treatments. Similar results were observed in the field. In the mesocosm experiment, larger numbers of *G. atrocyanea* were distributed on *R. obtusifolius* plants exposed to below-ground intraspecific interaction than on plants not exposed to intraspecific interaction.
4. Our results provide experimental evidence that leaf-trait changes in response to intraspecific interaction between host plants influence specialist herbivore distribution. This highlights the need to integrate plant–plant interactions into our understanding of plant–animal interactions.

Keywords: herbivory, host selection, phenotypic plasticity, plant–plant interactions, plant–herbivore interactions, resource concentration hypothesis, *Rumex*, secondary metabolites

INTRODUCTION

To improve our understanding of plant–animal interactions, numerous ecologists have tried to predict herbivorous insect distribution by using the local population density of host plants. Root (1973) predicted that herbivores would be concentrated on host plants growing in high-density populations or monocultures (resource concentration hypothesis). This prediction has been supported by several studies (e.g., Stephens & Myers, 2012; Nerlekar, 2018). On the other hand, the possibility of an inverse distribution pattern, in which herbivores are concentrated on low-density or solitary host plants (Yamamura, 1999; Otway et al., 2005), has been proposed as the resource dilution hypothesis, and these predictions have also been supported by several studies (e.g., Fagundes et al., 2019; Coutinho et al., 2019). These conflicting patterns have been reported for several herbivore species, and some species have even been found to be unresponsive to resource distribution (Rhainds and English-Loeb, 2003; Tuller et al., 2013). Regardless, the mechanism that produces the uneven distribution of each herbivore species has not been elucidated.

Differences in the local population density of host plants are likely to reflect differences in the quality of the host plants, because the local population density of plants is linked to their interaction environments; host plants present at high density are

exposed to direct intraspecific interaction. In contrast, host plants present at low density are exposed to direct interspecific interaction or no interaction. Many studies have reported that plant–plant direct interactions influence herbivory (Hambäck & Beckerman, 2003; Muiruri et al., 2019; Yamawo, 2021). For example, plant competition for resources induces plastic changes in the plants' resource allocation; these changes can affect root or shoot growth. The changes in resource allocation can also influence the expression of leaf thickness, leaf mass per area, and primary (essential nutrients) and secondary (potentially plant-protective compounds) metabolites in the leaves (Bartron & Bowers, 2006; Broz et al., 2010; Mraja et al., 2011; Takigahira & Yamawo, 2019; Yamawo, 2021). Therefore, variations in the interaction environment induce changes in expression of the chemical traits of host plant leaves (Barton & Bowers, 2006; Broz et al., 2010; Mraja et al., 2011; Muiruri et al., 2019), so they are likely to influence leaf herbivory and the distribution of herbivores. In fact, several studies have strongly suggested that changes in leaf traits in response to differences in the interaction environment of host plants influence herbivory or herbivore distribution (e.g., Broz et al. 2010; Muiruri et al., 2019; Yamawo, 2021). However, to our knowledge, no experimental evidence has yet been provided for this effect.

Specialist herbivores are often attracted by secondary metabolites in their host

plants; they adapt to these chemicals because they use them as cues to recognise the host plants (e.g., Wheat et al., 2007; Goodey et al., 2015). Brassicaceae plants produce glucosinolates to prevent herbivory by generalist herbivores; however, *Brevicoryne brassicae* (cabbage aphid), which specialises in Brassicaceae plants, prefers these glucosinolates (Titayavan & Altieri, 1990). Therefore, a high content of secondary metabolites induced in host plants by intraspecific interaction may attract the plants' specialist herbivores. In contrast, generalist herbivores avoid secondary metabolites (e.g., alkaloids, phenolics and condensed tannins) in the leaves of host plants (e.g., Schoonhoven et al., 2005; Macel, 2011; Jeschke et al., 2017). Here, we hypothesised that intraspecific interaction increases the content of secondary metabolites in plant leaves, and that this increase would lead to the aggregation of specialist herbivores. In contrast, generalist herbivores may gravitate towards low-density host plants to avoid high contents of secondary metabolites. Therefore, differences in resource quality due to variations in local population density within a plant population could induce either a concentrated or a low-density distribution of herbivores, depending on the resource concentration (Root, 1973) or resource dilution (Otway et al., 2005) hypothesis, when compared with the distribution predicted on the basis of resource quantity alone (Fretwell & Lucas, 1969).

Here, we focused on *Rumex obtusifolius* L. (broad-leaved dock; Polygonaceae) as a host plant, and two leaf beetles, *Gastrophysa atrocyanea* Motschulsky (Chrysomelidae), which is a specialist herbivore of *Rumex* plants, and *Galerucella grisescens* (Joannis) (Chrysomelidae), which is a generalist herbivore of Polygonaceae plants (see details in Supplemental methods). To test our hypothesis, we investigated the relationships between the local population density of *R. obtusifolius* plants and the herbivores' distributions in the field. Next, to clarify the effect of the interaction environment on leaf traits of *R. obtusifolius* plants and resource utilisation by the two leaf beetles, we conducted cultivation and preference experiments in adult leaf beetles. Finally, we evaluated the effects of intraspecific interaction of *R. obtusifolius* plants on the distribution of the leaf beetles by using a mesocosm experiment. On the basis of the results, we discuss the effects of plant–plant interaction on herbivore distributions.

MATERIALS AND METHODS

Field survey

To reveal the relationships between the local population density of *Rumex obtusifolius* and the distribution of leaf beetles, we conducted field surveys in April and May 2018,

130 at a time when the populations of both leaf beetles are large. Five grasslands were
131 selected as field-survey sites (5 April, Tomino-cho, Hirosaki City, Aomori Prefecture,
132 40°35′N 140°28′E; 13 April, Ozawa, Hirosaki City, Aomori Prefecture, 40°34′N
133 140°27′E; 28 April, Ohara, Hirosaki City, Aomori Prefecture, 40°34′N 140°26′E; 22
134 April, Nagoya City, Aichi Prefecture, 35°09′N 136°58′E; 5 May, Morioka City, Iwate
135 Prefecture, 39°42′N 141°08′E, Figure S1). These sites are all at least 2 km apart. At each
136 site we set up one square quadrat (Tomino-cho and Ohara, 10 × 10 m; Ozawa, 8 × 8 m;
137 Iwate, 4 × 6 m; Nagoya; 4 × 4 m) including varying local population densities and sizes
138 of *R. obtusifolius* plants. The maximum size of each quadrat was determined as 100 m^2;
139 in the case of small *R. obtusifolius* populations we adjusted the size of the quadrat
140 downward to include all *R. obtusifolius* individuals.
141
142 ***Survey of local population densities of R. obtusifolius and herbivore distributions on***
143 ***R. obtusifolius***
144 In each quadrat, a corner was used as the origin of two axes, x and y, which we used to
145 plot coordinates. From the origin, we described the positions of all *R. obtusifolius*
146 individuals, except for first-year seedlings that had cotyledons, to a precision of 1 cm by
147 using a ruler. The longest rosette diameters of the described *R. obtusifolius* plants were

recorded as the plant size. We also recorded the presence or absence of each herbivore species on each *R. obtusifolius* individual, regardless of the beetles' developmental stages (egg, larva or adult).

By using these data, a bubble chart was created by converting the positions of plants into distributions on a map and the rosette sizes into bubble sizes (Figure S2). As an indicator of the local population density of *R. obtusifolius*, the area of one rosette overlapping with the rosettes of neighbouring individuals was calculated by using image analysis software (Adobe Photoshop Elements 2.0, Adobe Systems, San Jose, CA, USA). The overlap ratio (overlapping area/total rosette area) was used to represent the population density for analytical purposes. Because leaf beetles often retire into the soil around the host plants, making it difficult to evaluate their numbers accurately, we used binomial data (presence or absence) to analyse their distribution. The correlations between the overlap ratio of the rosettes and the presence of *Gastrophysa atrocyanea* or *Galerucella grisescens* were examined.

Measurement of leaf traits in field plants

To reveal the effects of local population density on the secondary chemicals of *R. obtusifolius* in the field, we measured the leaf secondary metabolites of *R. obtusifolius* plants that grew alone or were aggregated. In April 2018, leaves of *R. obtusifolius* plants

were collected from three study sites in Aomori Prefecture, northern Japan (Hirosaki-city: 40° 35′N 140° 28′E, Fujisaki-city: 40° 39′ N 140° 29′ E, Itayanagi-city: 40° 40′ N 140°28′ E). Each site was at least 10 km apart from the next site. To exclude the effects of reproduction and leaf damage, we selected non-flowering individuals that had no herbivores and no leaf damage. An *R. obtusifolius* plant was defined as "Solitary" when there were no conspecific individuals within 30 cm from the edge of the widest rosette ($N = 15$), and *R. obtusifolius* plants with five or more conspecific individuals within a radius of 1 m from the centre of the plant were defined as "Aggregated" ($N = 25$). The widest rosettes of these plants were about 30 cm in diameter. We selected the youngest, fully expanded leaves. These leaves were analysed for secondary metabolites, namely the contents of total phenolics and condensed tannin, which are well known as major secondary metabolites in the *Rumex* genus (Feduraev et al., 2019). We measured the leaf contents of total phenolics and condensed tannins in accordance with the methods of Feeny (1970) and Dudt and Shure (1994).

Leaf beetle choice experiment using leaf sections from naturally growing plants

In April 2018, Solitary and Aggregated *R. obtusifolius* plants (85 individuals each) with rosette diameters of about 30 cm were selected at random in Hirosaki City. We collected the youngest fully expanded leaves from the plants. We cut one 2-cm piece from the

base of each collected leaf. A wet filter paper (8 cm in diameter) was placed in a covered Petri dish (8.5 cm in diameter), and a piece of leaf from a Solitary plant and a piece of leaf from an Aggregated plant were placed on it with one adult of *G. atrocyanea* or *G. grisescens*. The Petri dishes were kept in a growth chamber (25 °C, 12L, 12D). After 24 h, the damage to each leaf piece was estimated by image analysis. More details of the methods are given in the Supplementary methods.

Cultivation experiments

Cultivation design

To examine the effects of the interaction environment on leaf traits and leaf beetle preferences, we conducted cultivation experiments. To prepare enough samples to measure leaf traits and leaf beetle preferences, two experiments were conducted. Experiment 1 was conducted in 2017 to estimate the effects of interaction environment on leaf secondary metabolite contents and plant biomass. Experiment 2 was conducted in 2019 to estimate the effects of interaction environment on leaf primary metabolite and chlorophyll contents and leaf beetle preferences.

In September 2016, a total of more than 700 seeds of *R. obtusifolius* were

collected from four individual plants in the field in Hirosaki City. Each individual was separated by at least 2 km. As interspecific competitors, we focused on *Plantago asiatica* L., *Trifolium repens* L. and *Festuca ovina* L. These species are the dominant competitors of *R. obtusifolius* in Japan (Ohsaki, 2020). A total of 100 seeds of *P. asiatica* were collected from two individuals in the field in Aomori Prefecture. A total of 100 seeds of *T. repens* were collected from individuals in the field in Saga Prefecture. For *F. ovina*, commercially available seeds (Kaneko Seeds Co., Gunma, Japan) were used. The seeds were stored in a refrigerator at 4 °C until the experiments began. Seeds from each mother plant were mixed and sown on the surface of wet sand (2 cm deep) during March 2017 for Experiment 1 and during March 2019 for Experiment 2. The containers were kept in a growth chamber (25 °C, 12L, 12D). All plants had developed their first true leaves by the beginning of the experiment.

In April 2017 and 2019, to obtain the focal plants, we planted one *R. obtusifolius* seedling in each pot (10.5 cm diameter × 9 cm high) containing seed-free garden soil (Mori Sangyo Co., Hokkaido, Japan). These pots were assigned to three interaction treatments: no-interaction treatment as a control (2017, $N = 49$; 2019, $N = 35$), intraspecific interaction treatment (2017, $N = 66$; 2019, $N = 66$) and interspecific interaction treatment (2017, $N = 153$; 2019, $N = 98$). In the no-interaction treatment, to

220 provide a volume of soil similar to that used in the interaction treatment for each plant,
221 the pots were divided into halves with a plastic plate to block any below-ground
222 interaction, and one seedling of *R. obtusifolius* was planted in each half of the pot. In the
223 intraspecific interaction treatment, we planted another *R. obtusifolius* seedling beside
224 the focal plant as a competitor with no plastic plate. In the interspecific interaction
225 treatment, a seedling of another species (*P. asiatica*, 2017, $N = 58$; 2019, $N = 35$; *T.*
226 *repens,* 2017, $N = 41$; 2019, $N = 30$; *F. ovina*, 2017, $N = 54$; 2019, $N = 33$) was planted
227 next to the target *R. obtusifolius* seedling. In the interaction treatment, the distance
228 between seedlings was about 2 cm. All pots were placed randomly and maintained in
229 the growth chambers (25 °C, 12L, 12D) and watered once a day for 30 days.
230
231 ***Measurement of leaf traits in cultivated plants***
232 Experiment 1
233 After 30 days, we analysed total phenolics and condensed tannins. Plants were
234 harvested and dried at 50 °C for 3 days. The plants were then weighed on an electronic
235 balance to the nearest 0.1 mg. The leaves were used to analyse total phenolics and
236 condensed tannins by using the methods in field survey.
237

238 Experiment 2

239 After 30 days, we measured chlorophyll content and five organic acids as plant nutrients
240 (see details in Supplementary methods). The chlorophyll content reflects the plant's
241 nitrogen concentration and has been found to indirectly affect herbivore survival and
242 distribution (Scheirs & De Bruyn, 2004, Sousa-Souto et al., 2018). Also, organic acids
243 in the plant are necessary for the optimal development of phytophagous insects (Offor,
244 2010). Therefore, by measuring these, we examined changes in nutrient condition in
245 response to interactions between plants.

246

247 ***Leaf beetle choice experiment***

248 To reveal whether changes in leaf chemical contents induced in *R. obtusifolius* by
249 interaction influenced the preferences of leaf beetles, we conducted choice experiments
250 with the *R. obtusifolius* leaves used in cultivation experiment 2. The combinations of
251 leaf pairs were as follows: intraspecific interaction versus interspecific interaction;
252 interspecific interaction versus no-interaction treatment; and no-interaction treatment
253 versus intraspecific interaction. The experimental design and conditions were similar to
254 that described for the choice experiment using field leaves (see Supplementary
255 methods).

256

257 **Mesocosm experiments**

258 To determine the effects of the interaction environment of *R. obtusifolius* on the
259 distribution of *G. atrocyanea*, we conducted a mesocosm experiment in November 2019
260 and July 2020. In November 2019, we estimated the effects of intraspecific interaction
261 of *R. obtusifolius* plants on the distribution of leaf beetles by using plants of the same
262 patch size in a "one-to-one-pot experiment" (Figure 1). In July 2020, we conducted a
263 "one-to-three-pot experiment" to clarify the effects of intraspecific interaction of *R.*
264 *obtusifolius* on the distribution of leaf beetles by adding the effects of patch size (i.e.,
265 resource amount) of the host plants.

266 We used two types of pot, namely interaction-treatment pots and no-
267 interaction-treatment pots. In both types of treatment pot, two seedlings of *R.*
268 *obtusifolius* were planted. The no-interaction-treatment pot was divided in half by a
269 plastic plate to block below-ground interaction between *R. obtusifolius* plants. The
270 interaction-treatment pot allowed below-ground interaction between *R. obtusifolius*
271 plants, because this type of pot had no plastic plate (see Supplementary methods).

272 ***One-to-one-pot experiment***

273 We prepared 20 containers (911 mm × 602 mm × 207 mm). In each container, an

274 interaction-treatment pot and a no-interaction-treatment pot were placed 30 cm away
275 from the edge of the pot. The containers were surrounded by soil to a depth of 15 cm to
276 allow the beetles free access to the pods, as they would have in the field (Figure 1 a, b).
277 For data analysis, each container was allocated an ID. Five *G. atrocyanea* females were
278 released on the soil in the centre of each container, the top of which was then covered
279 with 1-mm-mesh white cloth. The containers were placed in a greenhouse (15 °C), and
280 the numbers of beetles on the plants were counted after 24 h.

281 ***One-to-three-pot experiment***

282 In this experiment, we set up two types of conditions, namely "quantity conditions" (25
283 containers) and "quantity + quality conditions" (24 containers). For the quantity
284 conditions, we set up patches of two sizes by using four no-interaction-treatment pots.
285 We placed three pots together to represent large patches and one pot by itself to
286 represent small patches (Figure 1c). For the quantity + quality conditions, we set up
287 patches of two sizes by using one no-interaction-treatment pot and three interaction-
288 treatment pots; the three interaction-treatment pots represented large patches and the
289 single no-interaction-treatment pot represented small patches. In all containers, pots
290 were set up as in the one-to-one-pot experiment and under the same controlled
291 conditions. For the quantity conditions 125 beetles were used, and 120 beetles were

used for the quantity + quality conditions.

Statistical analysis

All statistical analyses were performed by using R v.3.6.1 software (R Development Core Team, 2019). All data met the statistical assumptions of normality and homoscedasticity according to the Kolmogorov–Smirnov test and F-test, and statistical analyses were performed appropriately depending on the data set structure. All tests were two tailed. The significance level was set at 0.05.

Field survey data analysis

Survey of herbivore distribution on R. obtusifolius

We analysed the effects of the local population density of *R. obtusifolius* on the distribution of leaf beetles by using generalised linear mixed models (GLMMs) with a binomial distribution and logit function, followed by the Chi-square test. The models included presence or absence of leaf beetles as fixed terms and overlap ratio of *R. obtusifolius* rosettes for each plant, species of leaf beetles, and their interaction as explanatory variables. When the relationship between overlap ratio of rosette area and presence or absence of leaf beetles differed between leaf beetle species, the relationship

between these was analysed for each beetle species. Site ID was included as a random effect in these models. False discovery rate (FDR) correction for multiple comparisons was then applied.

Measurement of leaf traits in field plants

Leaf chemical traits (content of condensed tannin or total phenolics) were compared between Solitary and Aggregate plants by using GLMMs with Gaussian distribution and an identity link, followed by an F-test; the models included leaf chemical traits as fixed terms and plant density (Solitary or Aggregated) as an explanatory variable. Site ID was included as a random effect in the models. FDR correction for multiple comparisons was then applied.

Leaf beetle choice experiment using leaf sections from naturally growing plants

Consumed areas of leaves were compared between local *R. obtusifolius* population densities for each leaf beetle species. We used GLMMs with Gaussian distribution and an identity link, followed by an F-test; the models included area consumed by leaf beetles as a fixed term and plant density (Solitary or Aggregated) as an explanatory variable. Petri dish ID was included as a random effect in the models. FDR correction for multiple comparisons was then applied.

328

329 **Cultivation experiments data analysis**

330 ***Measurement of leaf traits in cultivated plants***

331 We used Gamma distributions for the dry weights of plants, Gaussian distributions for
332 the chlorophyll content of leaves, and Poisson distributions for the leaf contents of
333 condensed tannin and total phenolics. We compared plant dry weights and leaf traits
334 (condensed tannin, total phenolics and chlorophyll content) between the cultivation
335 treatments by using GLMMs. Gamma or Poisson distributions with a log link followed
336 by a Chi-square test were applied, and Gaussian distributions with an identifying link
337 followed by an F-test were applied. These models included each plant trait as fixed
338 terms and interaction treatment (no-, intraspecific or interspecific interaction treatment)
339 as an explanatory variable. Parent plant ID was included as a random effect in the
340 models. When there was an interaction effect between each plant trait and interaction
341 treatment, we conducted multiple comparisons by FDR correction.

342 Organic acids were analysed by using a principal component analysis (PCA)
343 based on the correlation matrix of variables. Scores on the first (PC1) and second (PC2)
344 axes of the PCA were compared between interaction treatments by using GLMMs with
345 a Gaussian distribution and an identity link, followed by an F-test. The models included

346 PC1 or PC2 as fixed terms, interaction treatment (no-, intraspecific or interspecific
347 interaction treatment) as an explanatory variable and parent plant ID as a random effect.
348 When there was an interaction between PC1 or PC2 and interaction treatments, we
349 conducted multiple comparison by using FDR correction.

350 ***Leaf beetle choice experiment***

351 The leaf area consumed by the leaf beetles was compared between interaction
352 treatments (no-, intraspecific or interspecific interaction). Data sets for female beetles
353 were analysed by using GLMMs with a Gamma distribution and a log link, followed by
354 a Chi-square test, and data sets of male beetles were analysed by using the Wilcoxon
355 signed-rank test because the data sets contained some 0 values. The analysis was
356 conducted for each species of leaf beetle and for each sex of each species. FDR
357 correction for multiple comparisons was then applied to each data set.

358

359 **Mesocosm experiments data analysis**

360 ***One-to-one-pot experiment***

361 The numbers of leaf beetles per patch were compared between cultivation treatments by
362 using GLMMs with Gaussian distributions and an identity link, followed by an F-test;
363 the models included number of leaf beetles on the patch as a fixed term and interaction

364 treatment (interaction or no- interaction) as an explanatory variable. Container ID was
365 included as a random effect in the models.

366 ***One-to-three-pot experiment***

367 Number of leaf beetles per patch or number of leaf beetles per pot (representing leaf
368 beetle density) was compared between patch sizes (quantity) and cultivation conditions
369 (quality) by using GLMMs with Poisson distributions and a log-link, followed by a Chi-
370 square test; the models included number of leaf beetles per patch or per pot as fixed
371 terms and patch size (small or large), cultivation conditions (quantity or quantity +
372 quality condition) and their interaction as an explanatory variable. Container ID was
373 included as a random effect in these models. When there was an interaction between
374 patch size and cultivation conditions, we conducted multiple comparisons by FDR
375 correction.

376

377

378 **RESULTS**

379 **Field survey**

380 More than 60 *R. obtusifolius* individuals were growing within each quadrat; the major
381 herbivores were *G. atrocyanea* and *G. grisescens* (Table 1). The relationship between

382 the overlap ratio of *R. obtusifolius* rosettes and the presence of leaf beetles differed
383 among leaf beetle species (x^2 = 81.032, *df* = 2, $P < 0.001$). There was a significant
384 positive correlation between the overlap ratio of *R. obtusifolius* rosettes and the
385 presence of *G. atrocyanea* (estimate coefficient = 0.786, x^2 = 12.764, *df* = 1, $P < 0.001$).
386 In contrast, the presence of *G. grisescens* was not significantly correlated with the
387 overlap ratio of *R. obtusifolius* rosettes (estimate coefficient = –0.456, x^2 = 2.451, *df* = 1,
388 $P = 0.117$).

389 Contents of total phenolics and condensed tannin tended to be higher in
390 Aggregated plants than in Solitary plants, but this relationship did not reach statistical
391 significance (total phenolics, $F = 3.910$, $P = 0.096$, Figure 2a; condensed tannin, F
392 =4.882 , $P = 0.067$, Figure 2b). Females of *G. atrocyanea* consumed significantly more
393 leaf tissue from Aggregated plants than from Solitary plants ($F = 7.837$, $P = 0.037$,
394 Figure 3a). In contrast, for males of *G. atrocyanea* ($F = 1.779$, $P = 0.323$, Figure 3b)
395 and for both sexes of *G. grisescens* (female, $F = 1.421$, $P = 0.323$, Figure 3c; male, $F =$
396 0.494, $P = 0.490$, Figure 3d), there were no differences in the area of feeding damage
397 between Aggregated and Solitary leaves.

398

399

Cultivation experiments

The biomass and chlorophyll content of *R. obtusifolius* did not differ among interaction treatments (biomass: $x^2 = 7.081$, $df = 4$, $P = 0.132$, Figure 4a; chlorophyll: $F = 1.444$, $P = 0.239$, Figure 4b). The contents of total phenolics of *R. obtusifolius* differed significantly among treatments; they were higher in the order of intraspecific, no-, and interspecific interaction treatment (Figure 4c). Plants subjected to the intraspecific interaction treatment had a significantly higher content of condensed tannins than those undergoing the no- or interspecific interaction treatments (Figure 4d).

We found that PC1 and PC2 explained 61.0% and 20.7%, respectively, of the total variance of the organic acid composition data. The PC1 value did not differ among interaction treatments ($F = 2.068$, $P = 0.154$, Figure S3a). Plants subjected to the no-interaction treatment had significantly lower PC2 values than those undergoing the intraspecific or interspecific interaction treatments (Figure S3b).

Females of *G. atrocyanea* consumed significantly more leaf tissue from the intraspecific interaction treatment plants than from the no-interaction plants ($x^2 = 5.470$, $df = 1$, $P = 0.029$, Figure 5a) and from the interspecific interaction plants ($x^2 = 6.064$, $df = 1$, $P = 0.029$, Figure 5b). There was no significant difference between the no interaction and interspecific interaction treatments in terms of the area of leaf eaten by

418 females of *G. atrocyanea* (x^2 = 1.832, *df* = 1, *P* = 0.176, Figure 5c). For males of *G.*
419 *atrocyanea*, there were no differences between treatments in the area of leaf consumed
420 (no-interaction versus intraspecific interaction, *z* = 0.329, *P* = 1, Figure 5d; interspecific
421 interaction versus intraspecific interaction, *z* = 2.139, *P* = 1, Figure 5e; no-interaction
422 versus interspecific interaction, *z* = 0, *P* = 0.097, Figure 5f).
423
424 **Mesocosm experiments**
425 In the one-to-one-pot experiment, a significantly greater number of *G. atrocyanea* were
426 distributed on the *R. obtusifolius* plants in the interaction treatment than in the no-
427 interaction treatment (*F* = 5.556, *P* = 0.030, Figure 6a). In the one-to-three-pot
428 experiment, the effect of patch size on the distribution of *G. atrocyanea* differed
429 significantly with the cultivation conditions (x^2 = 6.540, *df* = 2, *P* = 0.038, Figure 6b).
430 Under both types of cultivation condition, large patches had significantly more beetles
431 than small patches (quantity conditions, x^2 =18.301, *df* = 1, *P* < 0.001; quantity + quality
432 conditions, x^2 =55.474, *df* = 1, *P* < 0.001, Figure 6b). This trend was more pronounced
433 under quantity + quality conditions. Moreover, the effect of patch size on the number of
434 *G. atrocyanea* per pot (i.e., the leaf beetle density) differed significantly between
435 cultivation conditions (interaction treatment × patch size; *z* = –2.067, *P* = 0.039, Figure

436 6c). Although the densities of leaf beetles in small and large patches were similar under
437 quantity conditions (z = –0.308, P = 0.758), under quantity + quality conditions the
438 large patches had a greater density of leaf beetles than small patches (z = 2.118, P =
439 0.034; Figure 6c).
440

441 **DISCUSSION**

442 We found here that intraspecific interaction induced changes in the leaf metabolite
443 contents of *Rumex obtusifolius* and affected resource utilisation by the specialist leaf
444 beetle, *G. atrocyanea*, but not by the generalist leaf beetle, *G. grisescens*. In addition,
445 we showed experimentally that this type of resource utilisation affected the distribution
446 of *G. atrocyanea.* These results support our hypothesis, providing experimental
447 evidence that differences in the local population density of the host plant led to plastic
448 changes in leaf metabolite contents, affecting the resource utilisation and distribution
449 patterns of specialist herbivores.

450 ***Variations in leaf traits***

451 In the field, Aggregated *R. obtusifolius* plants tended to have higher contents of total
452 phenolics and condensed tannin than Solitary plants (Figure 2). This result suggests that
453 aggregation of *R. obtusifolius* plants induced changes in leaf chemical traits. In fact, in

454 the cultivation experiments, the contents of total phenolics and condensed tannin in the
455 leaves of *R. obtusifolius* were significantly higher under intraspecific interaction
456 conditions than under interspecific interaction or no-interaction conditions (Figure 4c,
457 d). Increased contents of secondary metabolites in the presence of a conspecific
458 neighbour have been reported in several plant species, and it has been suggested that
459 metabolic alterations in leaves in response to intraspecific interaction are common in
460 plants (Barton & Bowers, 2006; Ormeño et al., 2007; Broz et al., 2010, but see Kigathi
461 et al., 2013). In many plant species intraspecific competition is more intense than
462 interspecific competition (Adler et al., 2018), and such intraspecific competition causes
463 limitation of soil nutrients and water (Craine & Dybzinski, 2013; Takigahira &
464 Yamawo, 2019). It is well known that limitation of soil nutrients and water for plants
465 induces the accumulation of secondary metabolites in the leaves (reviewed in Akula &
466 Ravishankar, 2011). Thus, aggregation of *R. obtusifolius* plants may increase the leaf
467 contents of secondary metabolites, such as total phenolics and condensed tannin,
468 through soil resource competition.

469 Another possible hypothesis is that Aggregated plants invest more in defence
470 than do Solitary plants through recognition of con-specific neighbours, because
471 aggregated plants often consumed by specialist leaf beetles. Leaf-trait alteration based

472 on neighbour recognition has also been reported in several plant species (Yamawo &
473 Mukai 2020; Yamawo, 2015, 2021). Neighbour recognition can therefore be a cause of
474 leaf-trait alteration in *R. obtusifolius* plants. However, the history of interaction between
475 specialist leaf beetles and *R. obtusifolius* plants is weak, because *R. obtusifolius* is an
476 exotic species in Japan. To understand the adaptive importance of leaf-trait alteration in
477 *R. obtusifolius* plants, we would need to perform an additional study in a region to
478 which *R. obtusifolius* is native.

479 The contents of primary metabolites are strongly affected by light conditions
480 (Kitazaki et al., 2018). For example, experiments with lettuce, *Lactuca sativa*, have
481 shown that the pattern of accumulation of primary metabolites, such as sugars and
482 amino acids, is affected by light quality, intensity and exposure time (Kitazaki et al.,
483 2018). In fact, our cultivation experiment found differences in the content of primary
484 metabolites between no-interaction and interaction treatments (Figure S3b). Changes in
485 the contents of primary metabolites in leaves may depends on presence of neighbour
486 plants, regardless of the identity of neighbour.

487 ***Preferences and distribution of leaf beetles***

488 The local *R. obtusifolius* population density affected the amounts of leaf consumed by
489 the specialist leaf beetle, *G. atrocyanea*. In the experiment using leaves from the field,

females of *G. atrocyanea* preferred to consume the leaves of aggregated *R. obtusifolius* plants than of Solitary plants, despite similar quantities of leaves being provided for the beetles (Figure 3a). In the experiment using the leaves of cultivated plants, females of *G. atrocyanea* also preferred the leaves of *R. obtusifolius* plants exposed to intraspecific interaction over those of plants exposed to no interaction (Figure 5a). These preference pattern are consistent with the increases in the leaf contents of secondary metabolites (total phenolics and condensed tannins) (Figures 2 and 4c, d) but not with the variations in primary metabolites (Figures S3). Therefore, we concluded that females of *G. atrocyanea* selected leaves on the basis of increases induced in the leaf secondary metabolite content by the host plant's interactive environment. *G. atrocyanea* beetles are specialist herbivores of *Rumex* plants (Suzuki, 1985). Many herbivore specialists use host-specific secondary metabolites for host searching or detecting (Schoonhoven et al., 2005; Ômura, 2018). This type of host searching may reflect the feeding preferences of *G. atrocyanea*. Females of *G. atrocyanea* lay eggs on the plants on which they feed, and the hatched larvae feed on the same plants. The larvae of *G. atrocyanea* require large amounts of food, and plants are often completely consumed (Suzuki, 1985). For this reason, the selection of aggregated plant leaves by *G. atrocyanea* females during the reproductive season is linked to the securing of food resources for the next generation.

508 This may be associated with niche specialisation in coevolution among host plants and
509 specialist herbivores (Schoonhoven et al. 2005; Abrahamson, 2008). In contrast, no
510 preference was observed among males of *G. atrocyanea*, possibly because males use
511 fewer resources than females with egg masses.

512 Do the preferences of leaf beetles affect the beetles' distribution? Our
513 mesocosm experiment provided robust evidence that changes in leaf traits based on
514 intraspecific interaction can affect the distribution of the specialist leaf beetles, *G.*
515 *atrocyanea* (Figure 6). When the plant patch sizes were similar, approximately 1.7 times
516 more leaf beetles were distributed in the interaction treatment patch than in the no-
517 interaction treatment patch (Figure 6a). Effects of interaction between host plants were
518 also found in the one-to-three-pot experiment. Greater numbers of leaf beetles were
519 distributed on the large patches than on the small patches, and this trend was more
520 pronounced under quantity + quality conditions than under quantity conditions (Figure
521 6b). This finding is consistent with the distribution of *G. atrocyanea* in the field
522 (Suzuki, 1985).

523 Moreover, in the conditions under which both the patch size and the
524 competitive environment (and thereby leaf traits) differed, the leaf beetle density was
525 significantly higher on large patches than on small ones, but it did not differ in

526 conditions under which only the patch size differed (Figure 6c). Root (1973) predicted
527 that "Herbivores are more densely distributed as the patch size of the feeding site
528 increases (resource concentration hypothesis)." According to this prediction, the
529 response of *G. atrocyanea* to differences in leaf traits leads to the concentration of
530 beetles relative to the food resource. In other words, in this system, our results strongly
531 suggested that differences in metabolic alterations in leaves through intraspecific
532 interactions in plants induced the concentrated distribution of herbivores on resources.
533 In contrast, numbers of the generalist leaf beetle, *G. grisescens*, were not
534 correlated with the local population density of *R. obtusifolius* in the field, and these
535 beetles did not select the leaves of *R. obtusifolius* on the basis of the interaction
536 environment. These results did not support our hypothesis that generalist herbivores
537 accumulate on low-density host plants to avoid high levels of secondary metabolites.
538 Generalist herbivores respond to a variety of chemicals besides those measured as leaf
539 traits in this study (Schoonhoven et al., 2005; War et al., 2012). Perhaps other leaf traits,
540 which could not be measured here, may have been involved in the preferences of *G.*
541 *grisescens* and varied according to the interaction environment, thus masking the
542 avoidance effect of secondary metabolites on the generalist leaf beetles. Another
543 possible reason why the findings did not support our hypothesis is the effects of

resource competition among herbivores. In some cases, resource competition among herbivores influences herbivore distribution (e.g., Suzuki 1986; Schoonhoven et al., 2005; Godinho et al., 2020). A previous study pointed out that *G. grisescens* is vulnerable to resource competition from *G. atrocyanea* (Suzuki, 1986). It may therefore prioritise the avoidance of competitors over plant availability when deciding where to feed (Suzuki, 1985). Several studies, as well as the resource dilution hypothesis proposed by Otway et al. (2005), have pointed out that herbivore density per plant may be higher when the host density is low (e.g. Yamamura, 1999). Our results suggest that these phenomena may be caused not only by differences in the local population density of the host plants but also indirectly by interactions with other herbivorous insects. To determine whether these results are general or specific to certain herbivores, several species, including generalists, may need to be tested.

CONCLUSIONS

Our findings provide experimental evidence that intraspecific interaction between host plants affects specialist herbivore distribution. Many researchers have worked to unravel the relationship between the distribution of herbivores and the local population density of host plants. Some herbivores have shown a positive response to resource

563 abundance, as in the resource concentration hypothesis proposed by Root (1973),
564 whereas others, as in the resource dilution hypothesis proposed by Otway et al. (2005),
565 have shown a negative response. These studies have focused on the amount of food
566 available and have assumed that leaf traits are always constant. Our results indicate that
567 herbivore responses to resource quantity and quality may interact with each other as
568 factors governing herbivore distribution. Therefore, herbivore responses to the local
569 population density of host plants can be understood from a plant–plant interaction
570 perspective, highlighting the need to integrate plant–plant interactions into our
571 understanding of plant–animal interactions in nature.
572

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577

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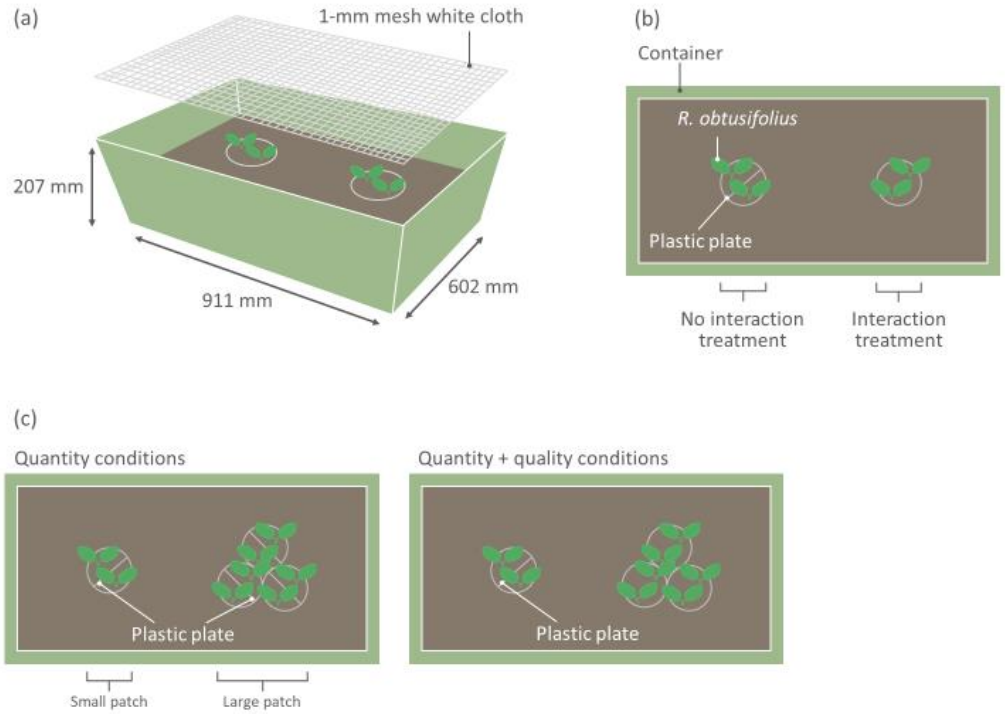
739 Table 1 Herbivores of *Rumex obtusifolius* and numbers and proportions of infested plants.
740

Taxon	Tomino-cho (N = 213)		Ozawa (N = 441)		Ohara (N = 112)		Iwate (N = 195)		Nagoya (N = 62)	
	No.	(%)	No.	(%)	No.	(%)	No.	(%)	No.	(%)
Gastrophysa atrocyanea Motschulsky (Chrysomelidae: Coleoptera)	14	6.57	195	44.22	84	75.00	49	25.13	44	22.56
Galerucella grisescens Joannis (Chrysomelidae: Coleoptera)	29	13.62	54	12.24	109	97.32	50	25.64		
Bothrogonia ferruginea Fabricius (Tettigellidae: Hemiptera)	1	0.47			1	0.89				
Mantura clavareaui Heikertinger (Chrysomelidae: Coleoptera)	1	0.47					1	0.51		
Aphis rumicis Linnaeus (Aphididae: Hemiptera)							15	7.69		
Dermaptera (Insecta)					1	0.89				
Helicoidea (Pulmonata)							60	30.77		
Lepidoptera (Insecta)							4	2.05		
Unknown	1	0.47								

741
742

743 **FIGURES**



744

745 Figure 1

746 (a) Experimental setup in mesocosm experiment. In all containers, the area around the
747 pots was filled with soil to a depth of 15 cm to allow the beetles free access to the
748 plants, as in the field. (b) In the one-to-one-pot experiment, the interaction and no-
749 interaction treatment pots were placed 30 cm away from each other in the container. (c)
750 In the one-to-three-pot experiment, two sets of conditions were set up, namely "quantity
751 conditions" and "quantity + quality conditions." Under quantity conditions, two patch
752 sizes were created by using four no-interaction pots. Under quantity + quality
753 conditions, two patch sizes were created by using one no-interaction pot and three
754 interaction pots. The distance between the large and small patches was 30 cm in each
755 container.

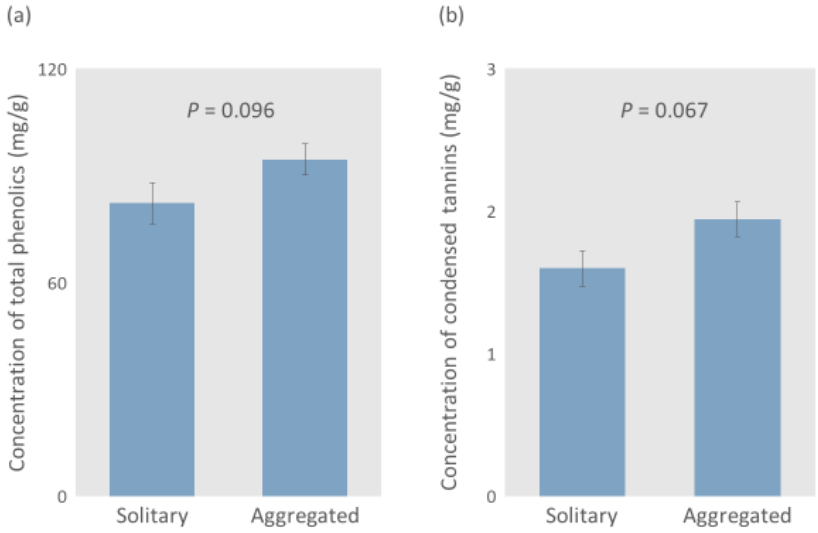
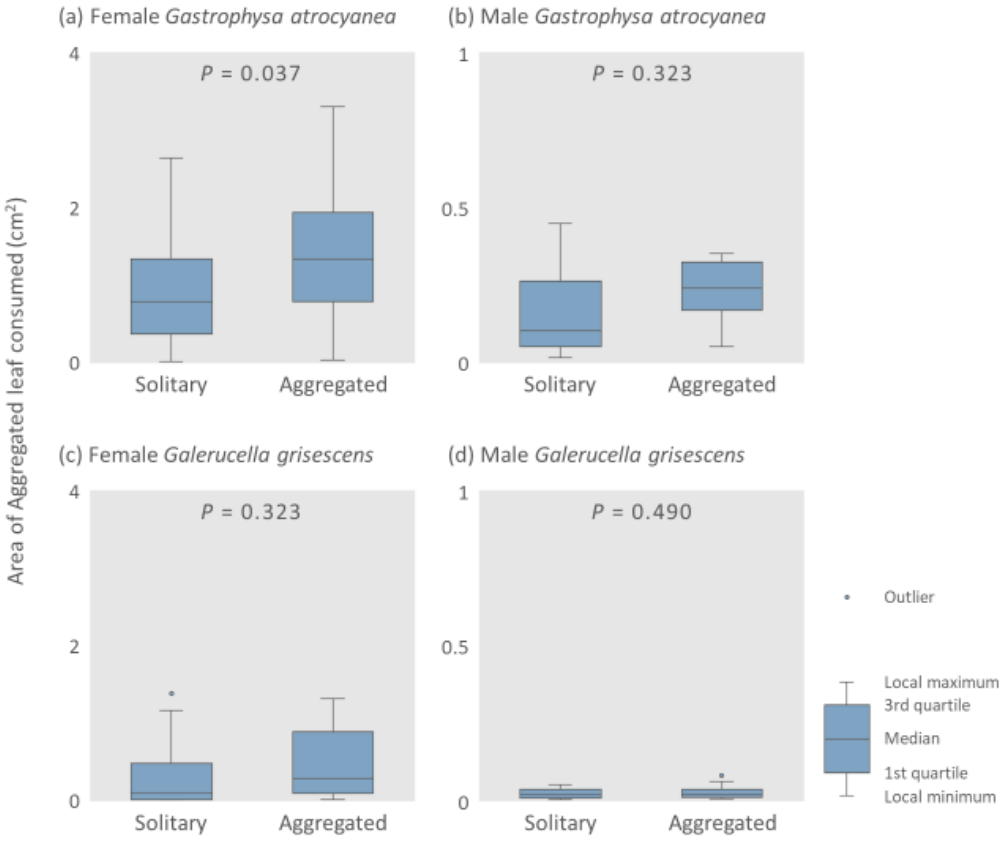


Figure 2

Contents of (a) total phenolics and (b) condensed tannin in Solitary ($N = 15$) and Aggregated ($N = 25$) plants. Bars represent SE. P-values are for the results of GLMM analysis.



761

762 Figure 3

763 Areas of leaf consumed by (a) female and (b) male *Gastrophysa atrocyanea* and (c)

764 female and (d) male *Galerucella grisescens* in the choice experiment using leaves from

765 the field. *P*-values are for the results of GLMM analysis.

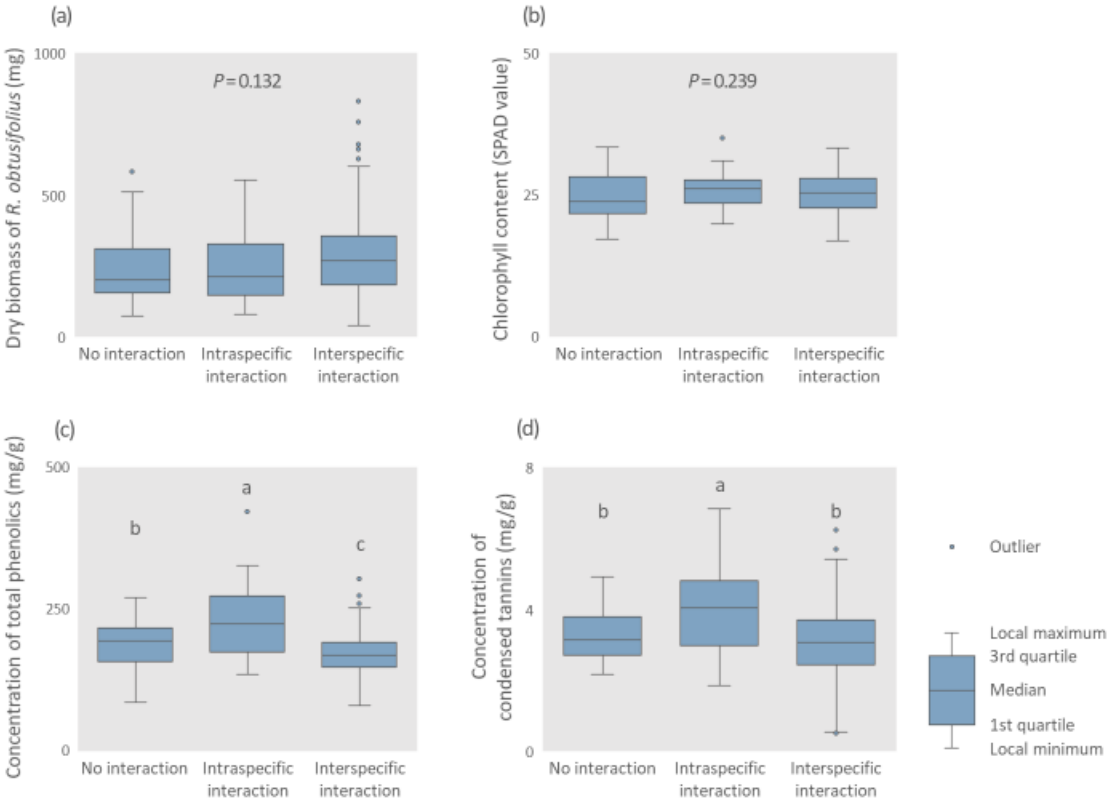
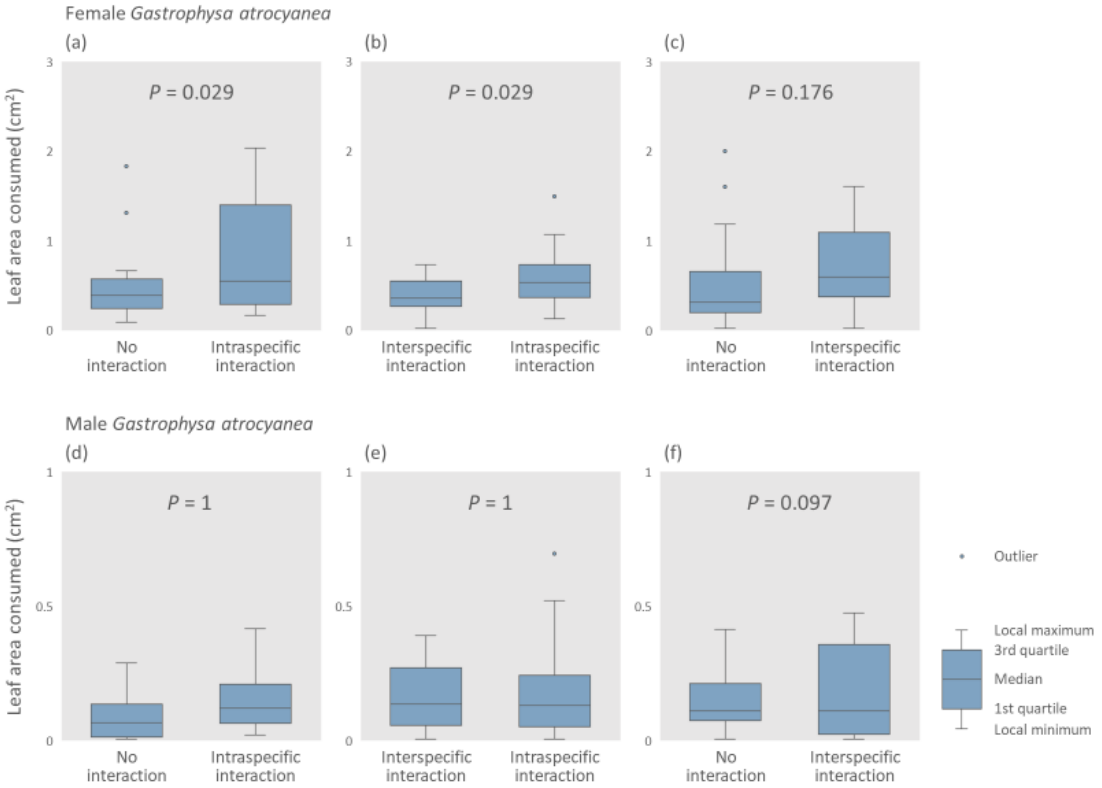


Figure 4

Dry biomass and leaf traits of *Rumex obtusifolius* in the cultivation experiment. (a) Dry biomass of whole plant, (b) chlorophyll content, (c) content of total phenolics and (d) content of condensed tannins in leaves. Different letters denote significant differences (GLMM, $P < 0.05$).



772

773 Figure 5

774 Leaf areas consumed by (a to c) female and (d to f) male *Gastrophysa atrocyanea* in the

775 choice experiment using cultivated plant leaves. The combinations of leaf pairs of

776 treatments were as follows: (a, d) no interaction versus intraspecific interaction; (b, e)

777 interspecific interaction versus intraspecific interaction; (c, f) no interaction versus

778 interspecific interaction.

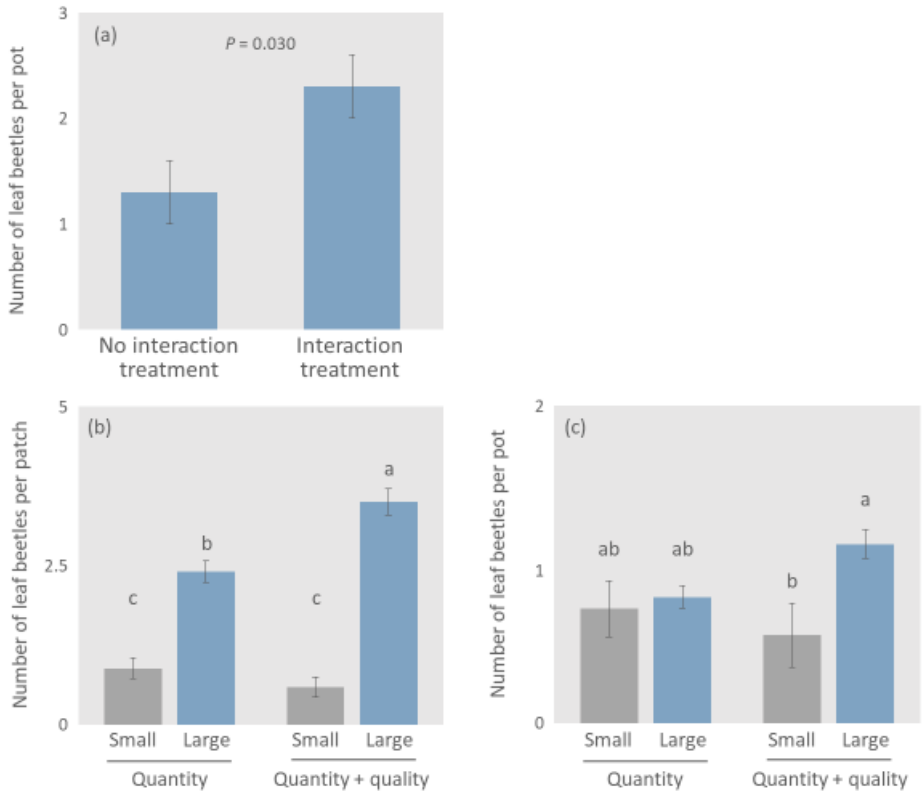


Figure 6

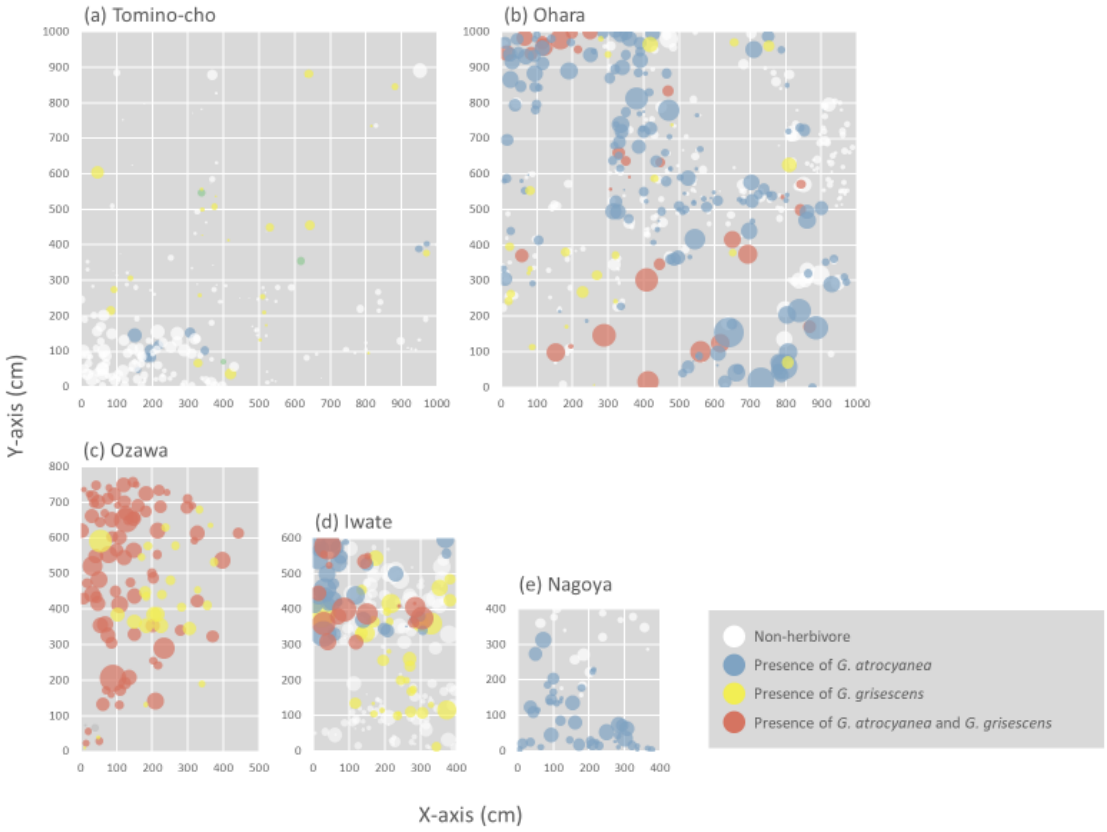
Numbers of leaf beetles in the mesocosm experiment. (a) Number of leaf beetles per pot in each treatment (no interaction or interaction treatment) in the one-to-one-pot experiment. (b) Number of leaf beetles per patch and (c) number of leaf beetles per pot in each patch (small and large patches) under each set of conditions (quantity and quantity + quality conditions) in the one-to-three-pot experiment. Bars represent SE. Different letters indicate significant differences (GLMM, $P < 0.05$).

787

788 Figure S1

789 Geographic locations of the five study sites used in the field survey in Japan. These sites

790 were at least 2 km apart.



791

792 Figure S2

793 Distributions of *Rumex obtusifolius*, the specialist leaf beetle *Gastrophysa atrocyanea*

794 and the generalist leaf beetle *Galerucella grisescens* at the five study sites. Bubble size

795 in the graphs represents the rosette size of each *R. obtusifolius* plant.

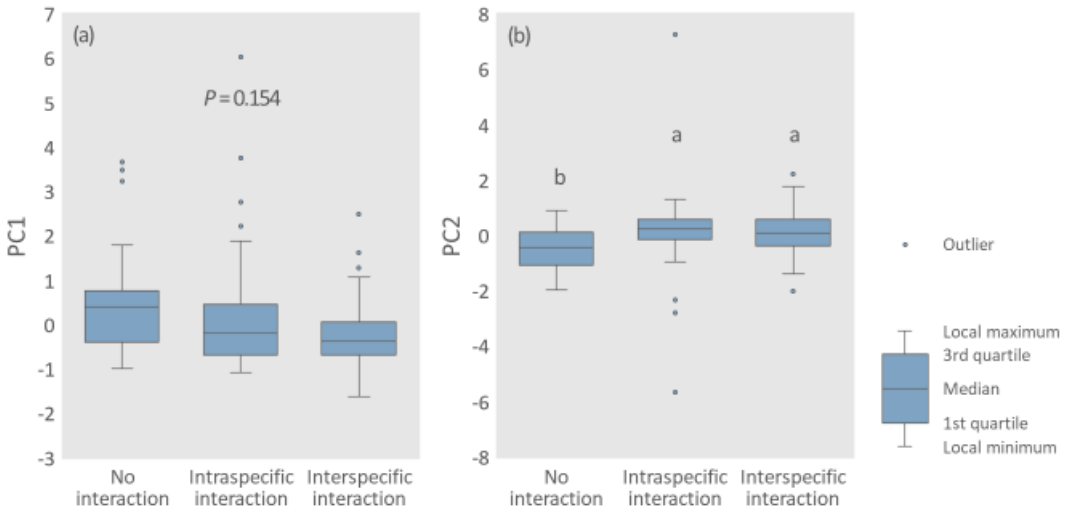


Figure S3

Boxplots of principal component values. (a) PC1 and (b) PC2 for organic acids in the cultivation experiment. Different letters indicate significant differences (GLMM, $P < 0.05$).

SUPPLEMENTARY METHODS

Study species

Rumex obtusifolius is a perennial herb native to Europe. In the early 1900s, *R. obtusifolius* plants were brought to Japan as contaminants of grasses imported from Europe (Makuchi & Sakai, 1984), and they invaded all regions of Japan except Okinawa. This species grows in wet grassland in Japan. The plants often aggregate with conspecific individuals, and they form patches of various sizes within the population (Van Evert et al., 2011) because their seed germination is promoted by the presence of the leaves of conspecifics via water-soluble-chemical exposure (Ohsaki et al., 2020). The species contains condensed tannins and phenolics as secondary metabolites in the leaves (Feduraev et al., 2019).

Rumex obtusifolius leaves are consumed mainly by two leaf beetles (Table 1), *Gastrophysa atrocyane* and *Galerucella grisescen*. *Gastrophysa atrocyanea* is a univoltine species and specialist herbivore of Rumex plants. It occurs on Rumex plant species from spring (March) to mid-summer (July) in Japan (Suzuki, 1985). *Galerucella grisescens* is a multivoltine species and a generalist herbivore of plants of the Polygonaceae family. It occurs on *Rumex* species from spring (March) to early winter (December) in Japan (Suzuki, 1985). Previous studies have shown that larger patches of Rumex plants are associated with *G. atrocyanea* and smaller patches with *G. grisescens* (Suzuki, 1985). This is related to differences in resource utilisation by the two species and to competition advantage: *G. atrocyanea* requires greater amounts of food resources than *G. grisescens*, and G. grisescens, which is less competitive, avoids competition with *G. atrocyanea* (Suzuki, 1985).

825

826 **Field survey**

827 ***Total phenolics and condensed tannins analysis***

828 We dried focal plants at 50 °C in an oven over 3 days. Dried leaf tissues were powdered
829 in a mill. Total phenolics were extracted from 20 mg of leaf powder in 10 mL of 50%
830 methanol for 1 h in an ultrasound bath at 40 °C. The content of total phenolics (mg/g)
831 was measured by using the Folin–Ciocalteu method (Julkunen-Tiitto, 1985). Condensed
832 tannins were extracted from 50 mg of dry leaves and were quantified by radial diffusion
833 assay with tannic acid as a standard (Hagerman, 1987).

834

835 ***Leaf beetle choice experiment using leaf sections from naturally growing plants***

836 In April 2018, Solitary and Aggregated *Rumex obtusifolius* plants (85 individuals each)
837 with rosette diameters of about 30 cm were selected at random in Hirosaki City. We
838 collected the youngest fully expanded leaves from the plants. These leaves had no
839 damage. We cut one 2-cm piece from the base of each collected leaf. A wet filter paper
840 (8 cm in diameter) was placed in a covered Petri dish (8.5 cm in diameter), and a piece
841 of leaf from a Solitary plant and a piece of leaf from an Aggregated plant were placed
842 on it. Adults of *G. atrocyanea* (36 females and 14 males) and *G. grisescens* (22 females
843 and 13 males) were also collected from the field in Hirosaki City. One beetle was placed
844 in the centre of the Petri dish, which was then kept in a growth chamber for 24 h at
845 25 °C, with a 12L 12D cycle. Each leaf piece was scanned by using an image scanner,
846 and the consumed area of the leaf was measured by using ImageJ bundled with 64-bit
847 Java 1.8.0_172 image analysis software (Abràmoff et al., 2004). This experimental
848 design is generally adopted for choice experiments with herbivores (e.g., Blüthgen,

849 2007; Sato et al., 2014; Shirahama et al., 2017; Lackner et al., 2019).

850

851

852 **Cultivation experiments**

853 ***Leaf beetle choice experiment***

854 To reveal whether changes in leaf chemical contents induced by interaction in *R.*
855 *obtusifolius* influenced the preferences of leaf beetles, we conducted choice experiments
856 with the *R. obtusifolius* leaves used in the cultivation experiment. The experimental
857 design was similar to that described for the choice experiment using field leaves. Leaf
858 pairs were cut into 1.5-cm pieces taken from the leaf base and placed on a Petri dish.
859 The combinations of leaf pairs were as follows: no-interaction treatment versus
860 intraspecific interaction; interspecific interaction versus intraspecific interaction; and
861 no-interaction versus interspecific treatment. In the interspecific interaction treatment,
862 treatments using three different species of plants *(P. asiatica, T. repens* and *F. ovina*)
863 were used equally. In this experiment, we used only *G. atrocyanea*, because in the
864 choice experiment using field leaves only *G. atrocyanea* had expressed a significant
865 preference for group leaves (see Results). Male ($N = 65$) and female ($N = 69$) beetles
866 were collected from Hirosaki City and assigned to the three different interaction
867 treatments (intraspecific interaction versus interspecific interaction, 25 males and 25
868 females; interspecific interaction versus no interaction, 15 males and 19 females; no
869 interaction versus intraspecific interaction, 25 males and 25 females). Experimental
870 conditions were the same as those in the choice experiment using field leaves.

871

872 ***Measurement of leaf traits in cultivated plants***

Chlorophyll content. First, a non-destructive chlorophyll meter (SPAD -502 Plus; Konica Minolta, Tokyo, Japan) was used to measure the chlorophyll content in the most recently fully expanded leaves of each cultivated *R. obtusifolius* on the final day of cultivation. A chlorophyll meter is one of the most commonly used diagnostic tools for rapid and non-destructive estimation of chlorophyll content in leaves; the resulting SPAD values are positively correlated with chlorophyll content (Shibaeva et al., 2020). Each leaf was measured twice—in the central part on both sides of the main vein—and the average value was determined. Next, one longitudinal half of each leaf was used to measure primary metabolites. The other halves of the leaves were used in the leaf beetle choice experiment.

Organic acids analysis

Organic acids were extracted according to the method of Miyagi et al. (2010). The leaf halves were frozen with liquid nitrogen and stored in a freezer at –80 °C until measurement. Organic acids were extracted according to the method of Miyagi et al. (2010). Frozen leaves (about 50 mg) were milled, and 50% methanol containing 50 mM PIPES (1,4-piperazineethanesulfonate) as an internal standard were added. After initial centrifugation (22,000*g*, 5 min, 4 °C), the supernatant was transferred to a 3-kDa cut-off filter (Millipore, Billerica, MA, USA) and recentrifuged (14,000*g*, 30 min, 4 °C). Five primary metabolites—oxalate, isocitrate, citrate, glycolate and threonate—were selected for analysis. These metabolites had been found in *R. obtusifolius* in our preliminary survey. The resulting filtrate was quantified by capillary electrophoresis triple quadrupole mass spectrometry (CE-QQQ-MS, CE; 7100, MS; 6420 Triple Quad LC/MS, Agilent Technologies, Santa Clara, CA, USA) with multi-reaction monitoring mode according to the method of Miyagi et al. (2019). Because of the small leaf size,

plants for which we did not have enough samples for analysis were excluded from the analysis, and the final number of plants analysed for organic acids was slightly smaller than the original collection (no interaction, $N = 35$; intraspecific interaction, $N = 63$; interspecific interaction, $N = 97$).

Mesocosm experiment

Preparation of plants and insects

In August 2018, seeds of *R. obtusifolius* were collected from three individuals in Hirosaki City. Individuals were separated by at least 2 km. In October 2019 and June 2020, seeds were germinated in the same way as described in the cultivation experiment and used for the mesocosm experiments. Two weeks later, we planted two *R. obtusifolius* seedlings in each pot (10.5 cm diameter × 9 cm high) containing seed-free garden soil (Mori Sangyo Co., Ltd, Japan). As a no-interaction treatment, pots were divided in half by a plastic plate to block below-ground interaction of the plants in each experiment (2019, $N = 35$; 2020, $N = 84$), because changes in leaf chemical content in response to conspecific neighbours depend on direct interaction below the ground (Ohsaki, 2020). The other pots were assigned to the interaction treatment, which consisted of two *R. obtusifolius* seedlings with no plastic plate (2019, $N = 35$; 2020, $N = 74$). All pots were watered once a day and maintained at 25 °C, 12L 12D for 30 days in growth chambers. *Gastrophysa atrocyanea* were collected from the field and kept in the laboratory until required for the experiment.

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