Effects of plant-plant interactions on resource utilization by phytophagous animals

植物間相互作用が植食性動物の資源利用に与える影響の解明

岩手大学大学院　連合農学研究科 地域環境創生学専攻

大崎　晴菜

CONTENTS

1. General Introduction

Plant–herbivore interactions form the basis of the food web of terrestrial ecosystems and underpin

the foundations of ecosystem networks. Many ecologists have researched resource selection patterns

of herbivores to understand the plant–herbivore interaction. Thus, it has been shown that the

chemical traits of plants are used as critical cues for resource selection by herbivores. Particularly,

secondary metabolites, such as phenolics and alkaloids, have a role in defense against herbivores

(Bennett and Wallsgrove, 1994). Many generalist herbivore species have been reported to reject

plants with high concentrations of secondary metabolites (e.g., Jeschke et al., 2017; Macel, 2011;

Schoonhoven et al., 2005). Moreover, many specialist herbivores utilize secondary metabolites as

either host recognition cues or nutrients (Bennett and Wallsgrove, 1994). For example, Brassicaceae

plants produce glucosinolates to prevent consumption by dietary generalist herbivores, which

consume plants from multiple families. However, *Brevicoryne brassicae* (cabbage aphid), which

specializes in Brassicaceae plants, prefers these glucosinolates (Titayavan and Altieri, 1990).

Since 2000, a few studies have reported that secondary metabolites in leaves are influenced by

plant–plant direct interactions (Barton and Bowers, 2006; Mraja et al., 2011; Muiruri et al., 2019).

For example, *Plantago lanceolata* growing with conspecific neighbors had significantly higher

levels of aucubin, one of the most abundant iridoid glycosides, than plants growing with *P. major*

neighbors (Barton and Bowers, 2006). Such changes in the concentration of secondary metabolites

in leaves are thought to be caused by metabolic changes associated with competition for light or soil nutrients and kin recognition.

It is indicated in the above findings that changes in leaf chemical traits associated with plant–plant interactions may influence resource selection by herbivores. Here, I hypothesized that: (i) differences in plant–plant interactions (conspecific or heterospecific or no interaction) are dependent on vegetation lead to intraspecific variation in the concentrations of secondary metabolites in the leaves and (ii) plants with high concentrations of secondary metabolites are eaten by specialist herbivores and avoided by generalist herbivores. This integrated understanding of plant–plant and plant–herbivore interactions has considerable ecological implications. It has been considered that the distribution of plants in the field is uneven and that the herbivore distribution is affected by it. Typically, as represented by the resource concentration hypothesis (Root, 1973), it has been thought that the local population density of host plants regulates resource abundance, exploration efficiency, and residence time and determines the distribution of herbivores. Conversely, it is revealed in our hypothesis that leaf chemical traits, i.e., resource quality for herbivores, are regulated by plant distribution. This may be a novel mechanism for determining the distribution of herbivores, for which no direct evidence is available.

This study aimed to reveal the effects of changes in the concentration of secondary metabolites of leaves on herbivory as a result of plant–plant interactions. In chapter 2, I investigated the impact of

the interaction between host plants, *Rumex obtusifolius*, and neighbor plants on the resource

selection and distribution of specialist leaf beetle, *Gastrophysa atrocyanea*, using field investigation,

cultivation experiment, and mesocosm experiment. Thus, in chapter 2, I revealed that belowground

plant–plant interactions could affect the concentrations of phenols in leaves. In chapter 3, I

investigated the effects of intra and interspecific root exudates on the growth and leaf chemical

content of *R. obtusifolius* to experimentally tested the mechanisms that cause changes in leaf

chemical traits. In chapter 4, I focused on *Cervus nippon* as a large vertebrate and plants with high

concentrations of secondary metabolites, which generalist herbivores avoid. In chapter 5, as a

general discussion, I summarized the effects of plant–plant interactions on changes in leaf traits and

on resource selection by herbivores and future work in plant–herbivore interaction studies.

2. Intraspecific interaction of host plants leads to concentrated distribution of a specialist herbivore through metabolic alterations in the leaves

INTRODUCTION

To improve our understanding of plant–animal interactions, numerous ecologists have tried to predict herbivorous insect distribution from the local population density of host plants. Root (1973) proposed the 'resource concentration hypothesis' and the 'enemies hypothesis'. The resource concentration hypothesis predicted that herbivores would be concentrated on host plants growing in high-density populations or monocultures, because plants in large or dense patches of conspecifics are more easily found by herbivores and can be inhabited for a long time by the herbivores. The enemies hypothesis predicted that when low-density host plants were surrounded by a diverse range of other species, the low-density host plants would indirectly suppress herbivore populations by increasing natural enemy populations. Polycultures, unlike monocultures, provide a variety of habitats or prey resources, thereby herbivores concentrated on high-density host plant patches. These hypotheses have been supported by several studies (e.g. Nerlekar, 2018; Stephens and Myers, 2012).

On the other hand, the 'resource dilution hypothesis' has been proposed as the possibility of an inverse distribution pattern, in which herbivores are concentrated on low-population-density or solitary host plants (Otway et al., 2005; Yamamura, 1999). This distribution pattern may occur if

6

herbivores cannot migrate or find a more distant host and thus become concentrated on solitary or low-density host plants (Otway et al., 2005; Rhainds and English-Loeb, 2003). This prediction has also been supported by several studies (e.g. Coutinho et al., 2019; Fagundes 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.

Local population density of host plants likely affects host plant quality, because it is linked to the interaction environment: host plants present at high density are exposed to direct intraspecific interaction, whereas 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 leaf traits (Barton and Bowers, 2006; Mraja et al., 2011; Muiruri et al., 2019) and herbivory (Hambäck and 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 that occur in resource allocation as a result of intraspecific competition 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 (Barton and Bowers, 2006; Takigahira and Yamawo, 2019; Yamawo, 2021). A few studies have reported or

suggested that interspecific competition also affects the abundance of secondary metabolites (Barton and Bowers, 2006) and herbivory (Cipollini and Bergelson, 2002; Mraja et al., 2011). In general, intraspecific interactions are more likely to increase the abundance of leaf secondary metabolites because competition between plants can be more intense than with interspecific competition (Adler et al., 2018). Therefore, we predicted that changes in leaf traits would be more pronounced when plants were exposed to intraspecific than to interspecific interaction; variation in the interaction environment of the host plants would thus be likely to influence leaf herbivory and the distribution of herbivores.

Dietary specialist herbivores that consume particular plant families are often attracted by secondary metabolites in their host plants that they use to recognise the host plants (e.g. Goodey et al., 2015; Wheat et al., 2007). Brassicaceae plants produce glucosinolates to prevent herbivory by dietary generalist herbivores, which consume plants from multiple families; however, *Brevicoryne brassicae* (cabbage aphid), which specialises in Brassicaceae plants, prefers these glucosinolates (Titayavan and Altieri, 1990). Therefore, a high concentration of secondary metabolites induced in host plants by intraspecific interaction may attract the plants' specialist herbivores. In contrast, dietary generalist herbivores avoid secondary metabolites (e.g. alkaloids, phenolics and condensed tannins) in the leaves of host plants (e.g. Jeschke et al., 2017; Macel, 2011; Schoonhoven et al., 2005). We hypothesised that intraspecific interaction between plants would lead to a greater increase in the

concentration of secondary metabolites in plant leaves than would interspecific interaction, and that this increase would lead to the aggregation of specialist herbivores. In contrast, we anticipated that generalist herbivores would gravitate towards host plants present at low density to avoid high concentrations of secondary metabolites. Therefore, differences in resource quality due to variation 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 and 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 that consumes Polygonaceae and Rosaceae plants (Matsuda, 1974; Shirahama et al., 2017; Suzuki, 1985; see details in Supplementary 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 on resource utilisation by the two leaf beetles, we compared the leaf chemical concentrations and preferences of adult leaf beetles among treatments in which *R. obtusifolius* experienced intraspecific interaction, interspecific interaction, or no interaction in

cultivation and preference experiments with adult leaf beetles. Finally, we evaluated the independent

and combined effects of patch size and intraspecific interaction of *R. obtusifolius* plants on the

distribution of the leaf beetles using a mesocosm experiment. On the basis of these results, we

discuss the effects of plant–plant interaction on herbivore distributions.

MATERIALS AND METHODS

I.   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, at a time when

the populations of both leaf beetles at the study sites were large. Five grasslands were selected

as field-survey sites (5 April, Tomino-cho, Hirosaki City, Aomori Prefecture, 40°35′N

140°28′E; 13 April, Ozawa, Hirosaki City, Aomori Prefecture, 40°34′N 140°27′E; 28 April,

Ohara, Hirosaki City, Aomori Prefecture, 40°34′N 140°26′E; 22 April, Nagoya City, Aichi

Prefecture, 35°09′N 136°58′E; 5 May, Morioka City, Iwate Prefecture, 39°42′N 141°08′E,

Figure 1). These sites were all at least 2 km apart. At each site, we set up one square quadrat

(Tomino-cho and Ohara, 10 m × 10 m; Ozawa, 8 m × 8 m; Iwate, 4 m × 6 m; Nagoya; 4 m × 4

m) including varying local population densities and sizes of *R. obtusifolius* plants. The

maximum size of each quadrat was determined as 100 m2; in the case of small *R. obtusifolius*

populations, we adjusted the size of the quadrat downward to include all *R. obtusifolius*

individuals. All field investigations have been conducted after getting prior permission from

each landowner of ordinary citizens.

Figure 1. Geographic locations of the five study sites used in the field survey in Japan. These

sites were at least 2 km apart

Survey of local population densities of *R. obtusifolius* and herbivore distributions on *R.*

obtusifolius

In each quadrat, a corner was used as the origin of two axes, x and y, which we used to plot

coordinates. From the origin, we described the positions of all *R. obtusifolius* individuals,

except for first-year seedlings that had cotyledons, to a precision of 1 cm 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, distinguishing between developmental stages (egg, larva or adult) and sexes (adult male or female).

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 2). 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 using image analysis software (Adobe Photoshop Elements 2.0; Adobe Systems). 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 distributions. The correlations between the overlap ratio of the rosettes and the presence of the specialist leaf beetle *Gastrophysa atrocyanea*, or generalist leaf beetle, *Galerucella grisescens* were examined, distinguishing between developmental stages (larva or adult) and sexes (adult male or female). Eggs of *G. atrocyanea* and eggs and larvae of *G. grisescens* were excluded from the analysis because their frequencies of occurrence were very low.
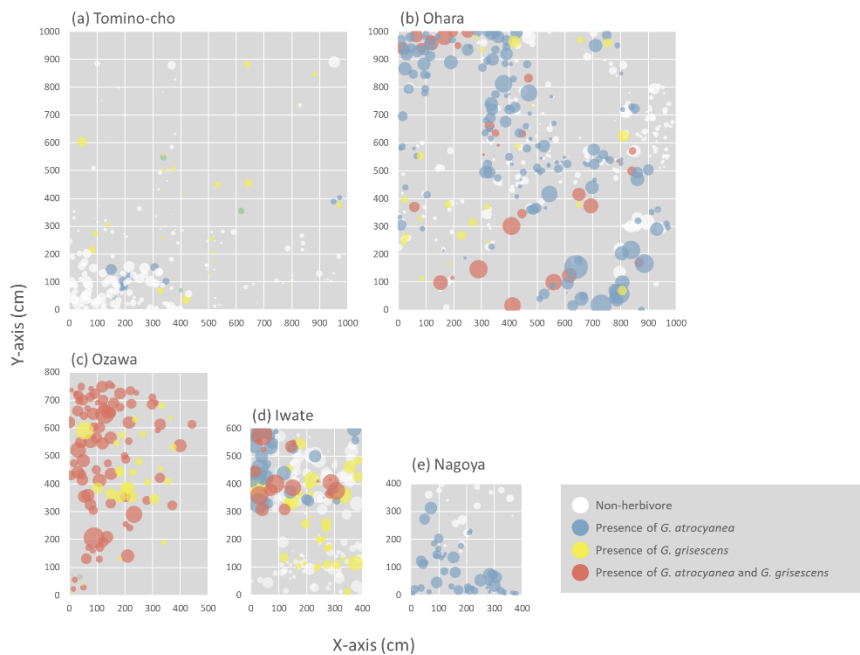
Figure 2. Distributions of *Rumex obtusifolius*, the specialist leaf beetle *Gastrophysa atrocyanea* and the generalist leaf beetle *Galerucella grisescens* at the five study sites. Bubble size in the graphs represents the rosette size of each *R. obtusifolius* plant.

Measurement of leaf traits in field plants

To reveal the effects of local population density on the concentrations of 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 four 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 from the next site. To exclude the effects of reproduction and leaf damage, we

13

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 ($N = 15$); *R. obtusifolius* plants with five or more conspecific individuals within 30 cm from the edge of the widest rosette were defined as 'Aggregated' ($N = 25$). The widest rosettes of these plants were about 30 cm in diameter. Therefore, there were no *R. obtusifolius* plants within a range of about one rosette diameter from the edge of a Solitary plant rosette. Although the distance covered in the horizontal plane by the roots of plants is sometimes larger than the rosette diameter, this distance still extends less than one rosette diameter from the edge of the plant rosette (H. Ohsaki, unpublished data). Therefore, Solitary plants do not interact directly with other *R. obtusifolius* plants, and instead they often grow directly alongside plants of other species, such as *Plantago asiatica* L., *Trifolium repens* L. and *Festuca ovina* L. We selected the youngest fully expanded leaves. These leaves were analysed for secondary metabolites, namely the concentrations of total phenolics and condensed tannins, which are well known as major secondary metabolites in the *Rumex* genus (Feduraev et al., 2019) and have been suggested to stimulate feeding by some leaf beetle species (Ikonen et al., 2002; Torp et al., 2013). We measured the leaf concentrations of these chemicals 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 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 hr, the damage to each leaf piece was estimated by image analysis. More details are given in the Supplementary Methods.

II. 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 the interaction environment on leaf secondary metabolite concentrations and plant biomass. Experiment 2 was conducted in 2019 to estimate the effects of the interaction environment on leaf primary metabolite and chlorophyll contents and leaf

15

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 *P. asiatica* L., *T. repens* L. and *F. ovina* L. These

species are the dominant competitors of *R. obtusifolius* in Japan (Ohsaki et al., 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.) 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.).

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 provide a volume of soil similar to that used in the interaction treatment for each

plant, the pots were divided into halves with a plastic plate to block any below-ground interaction, and one seedling of *R. obtusifolius* was planted in each half of the pot. In the intraspecific interaction treatment, we planted another *R. obtusifolius* seedling beside the focal plant as a competitor with no plastic plate. In the interspecific interaction treatment, a seedling of another species (*P. asiatica*, 2017, $N = 58$; 2019, $N = 35$; *T. repens*, 2017, $N = 41$; 2019, $N = 30$; *F. ovina*, 2017, $N = 54$; 2019, $N = 33$) was planted next to the target *R. obtusifolius* seedling. In the interaction treatment, the distance between seedlings was about 2 cm. All pots were placed randomly and maintained in the growth chambers (25°C, 12L/12D) and watered once a day for 30 days.

Measurement of leaf traits in cultivated plants

Experiment 1

After 30 days, we analysed total phenolics and condensed tannins. Plants were harvested and dried at 50°C for 3 days. The plants were then weighed on an electronic balance to the nearest 0.1 mg. The leaves were used to analyse total phenolics and condensed tannins using the methods in field survey.

Experiment 2

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

Leaf beetle choice experiment

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

III. Mesocosm experiments

To determine the effects of the interaction environment of *R. obtusifolius* on the distribution of *G. atrocyanea*, we conducted mesocosm experiments in November 2019 and July 2020. The experiments focused on the interaction environment below the ground, because our preliminary experiment had revealed that changes in the leaf concentrations of total phenolics and condensed tannins depended on below-ground interaction among *R. obtusifolius* plants (H. Ohsaki unpublished data). In November 2019, we estimated the effects of intraspecific below-ground interaction of *R. obtusifolius* plants on the distribution of leaf beetles using plants of the same patch size in a 'one-to-one-pot experiment' (Figure 3). In July 2020, we conducted a 'one-to-three-pot experiment' to clarify the effects of intraspecific below-ground interaction of *R. obtusifolius* on the distribution of leaf beetles by adding the effects of patch size (i.e. resource amount) of the host plants.
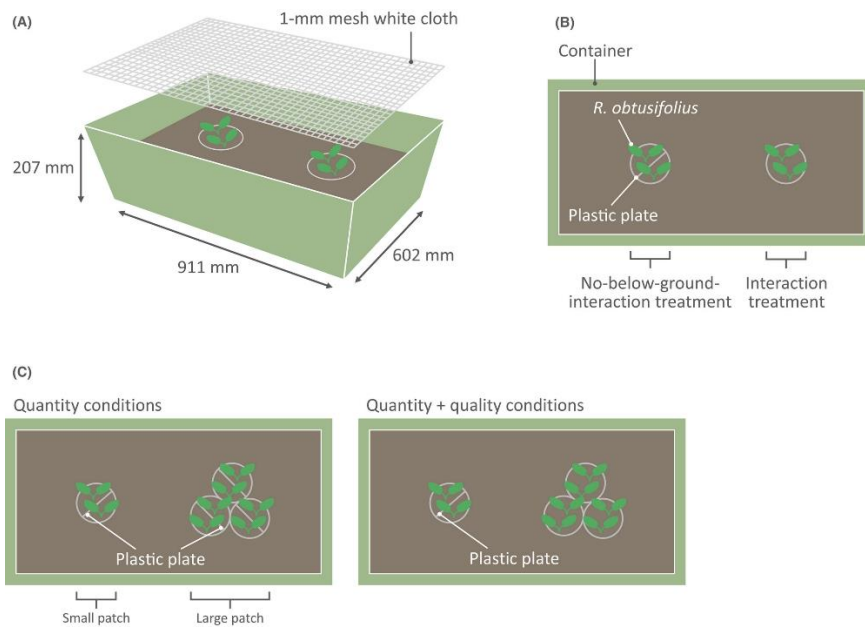
Figure 3. (A) Experimental setup in the mesocosm experiment. In all containers, the area around the pots was filled with soil to a depth of 15 cm to allow the beetles free access to the plants, as in the field. (B) In the one-to-one-pot experiment, the interaction and no-interaction treatment pots were placed 30 cm away from each other in the container. (C) In the one-to-three-pot experiment, two sets of conditions were set up, namely 'quantity conditions' and 'quantity + quality conditions'. Under quantity conditions, two patch sizes were created by using four no-interaction pots. Under quantity + quality conditions, two patch sizes were created using one no-interaction pot and three interaction pots. The distance between the large and small patches was 30 cm in each container

We used two types of pot, namely interaction-treatment pots and no-interaction-treatment pots. In both types of treatment pot, two seedlings of *R. obtusifolius* were planted. The

20

no-interaction-treatment pot was divided in half by a plastic plate to block below-ground interaction between *R. obtusifolius* plants. The interaction-treatment pot allowed below-ground interaction between *R. obtusifolius* plants because this type of pot had no plastic plate (see Supplementary Methods).

One-to-one-pot experiment

We prepared 10 containers (911 mm × 602 mm × 207 mm). In each container, an interaction-treatment pot and a no-interaction-treatment pot were placed 30 cm from the edge. The containers were surrounded by soil to a depth of 15 cm to allow the beetles free access to the pots, as they would have in the field (Figure 3A,B). For data analysis, each container was allocated an ID. Five *G. atrocyanea* females were released on the soil in the centre of each container, the top of which was then covered with 1-mm-mesh white cloth. The containers were placed in a greenhouse (15°C), and the beetles on the plants were counted after 24 hr.

One-to-three-pot experiment

In this experiment, we set up two types of conditions, namely 'quantity conditions' (25 containers) and 'quantity + quality conditions' (24 containers). For the quantity conditions, we set up patches of two sizes using four no-interaction-treatment pots. We placed three pots

together to represent large patches and one pot by itself to represent small patches (Figure 3C).

For the quantity + quality conditions, we set up patches of two sizes using one

no-interaction-treatment pot and three interaction-treatment pots; the three interaction-treatment

pots represented large patches and the single no-interaction-treatment pot represented small

patches. In all containers, pots were set up as in the one-to-one-pot experiment and under the

same controlled conditions.

IV. Statistical analysis

All statistical analyses were performed in 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 performed depended on the

dataset structure. All tests were two tailed, with $p < 0.05$ considered significant.

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 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 response variables and overlap ratio of *R. obtusifolius* rosettes for each plant, species

of leaf beetle 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 (concentration of condensed tannins or total phenolics) were compared

between Solitary and Aggregate plants using GLMMs with Gaussian distribution and an

identity link, followed by an F-test; the models included leaf chemical traits as response

variables and population density of host plants (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 response variable

and population density of host plants (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.

Cultivation experiments data analysis

Measurement of leaf traits in cultivated plants

We used Gamma distributions for the dry weights of plants, Gaussian distributions for the chlorophyll content of leaves and Poisson distributions for the leaf concentrations of condensed tannins and total phenolics. We compared plant dry weights and leaf traits (condensed tannins, total phenolics and chlorophyll content) between the cultivation treatments using GLMMs. Gamma or Poisson distributions with a log link followed by a Chi-square test were applied, and Gaussian distributions with an identifying link followed by an F-test were applied. These models included each plant trait as a response variable and interaction treatment (no, intraspecific or interspecific) as an explanatory variable. Parent plant ID was included as a random effect in the models. When there was a difference in plant traits among interaction treatments, we conducted multiple comparisons by FDR correction.

Organic acids were analysed using a principal component analysis (PCA) based on the correlation matrix of variables. Scores on the first (PC1) and second (PC2) axes of the PCA

were compared between interaction treatments using GLMMs with a Gaussian distribution and

an identity link, followed by an F-test. The models included PC1 or PC2 as a response variable,

interaction treatment (no-, intraspecific or interspecific) as an explanatory variable, and parent

plant ID as a random effect. When there was an interaction between PC1 or PC2 and interaction

treatments, we conducted multiple comparison using FDR correction.

Leaf beetle choice experiment

The leaf area consumed by the leaf beetles was compared between interaction treatments (no,

intraspecific or interspecific interaction). Datasets for female beetles were analysed using

GLMMs with a Gamma distribution and a log link, followed by a Chi-square test. The models

included the leaf area consumed as a response variable; interaction treatment (no-, intraspecific

or interspecific) and types of other plant species in the interspecific interaction treatment, and

their interactions, as explanatory variables, and Petri dish ID as a random effect. Datasets of

male beetles were analysed by Wilcoxon's signed-rank test because the datasets contained some

0 values. The analysis was conducted for each species of leaf beetle and for each sex of each

species. FDR correction for multiple comparisons was then applied to each dataset.

Mesocosm experiments data analysis

One-to-one-pot experiment

The numbers of leaf beetles per patch were compared between cultivation treatments using GLMMs with Gaussian distributions and an identity link, followed by an F-test; the models included number of leaf beetles on the patch as a response variable and interaction treatment (interaction or no below-ground interaction) as an explanatory variable. Container ID was included as a random effect in the models.

One-to-three-pot experiment

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

RESULTS

I.   Field survey

More than 60 *R. obtusifolius* individuals were growing within each quadrat; the major

herbivores were the specialist leaf beetle *Gastrophysa atrocyanea* and the generalist leaf beetle

Galerucella grisescens (Table 1). The relationship between the overlap ratio of *R. obtusifolius*

rosettes and the presence of leaf beetles differed among leaf beetle species (χ^2 = 81.032, dF = 2,

p < 0.001). There was a significant positive correlation between the overlap ratio and the

presence of larvae of *G. atrocyanea*. There was also a positive trend towards a correlation

between the overlap ratio and the presence of female adults of *G. atrocyanea*, but not of males

(Table 2). In contrast, the presence of *G. grisescens* (total, males or females) was not

significantly correlated with the overlap ratio (Table 2).

1 Table 1. Herbivore species of *Rumex obtusifolius* and numbers and proportions of infested plants. The lowest taxonomic level of identification for

2 some herbivores was higher than genus

| 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								

3

4

5   Table 2. Statistical results of generalised linear mixed model analyses of the effects of the local population density of *Rumex obtusifolius* on the

6   distribution of leaf beetles

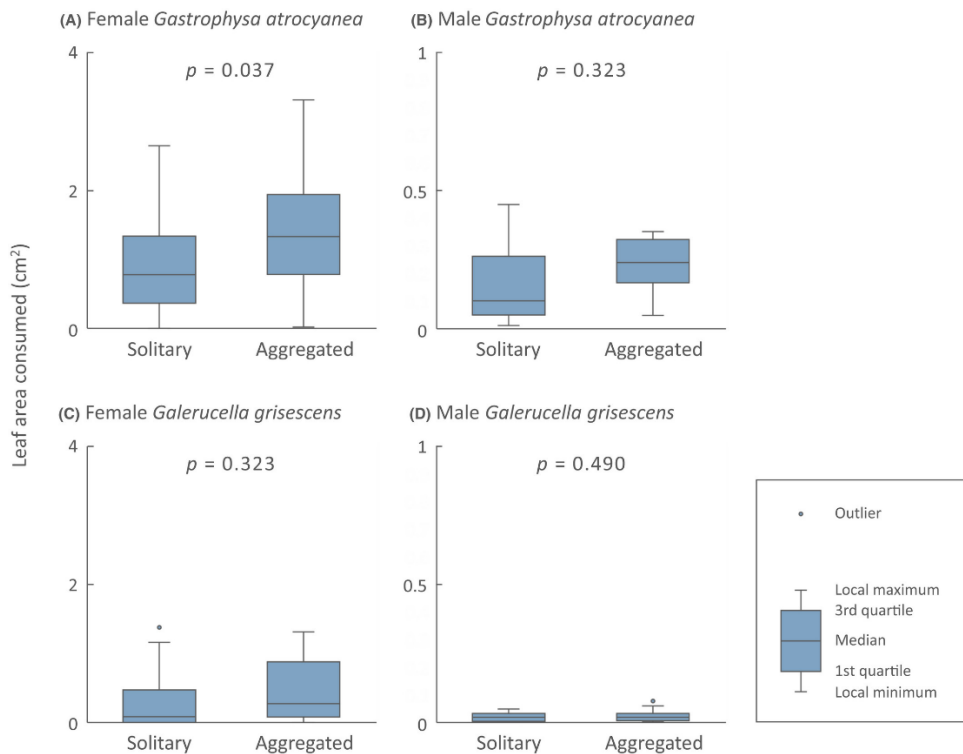|	*Gastrophysa atrocyanea*				*Galerucella grisescens*			
	estimate coefficient	x^2	df	P-value	estimate coefficient	x^2	df	P-value
all	0.786	12.764	1	< 0.001	-0.456	2.451	1	0.176
male	0.164	0.053	1	0.818	0.716	1.185	1	0.276
female	1.123	3.356	1	0.067	0.969	2.652	1	0.176
lavae	1.104	17.785	1	< 0.001				

7

8

9   Concentrations of total phenolics and condensed tannins in leaves collected in the field tended

10   to be higher in Aggregated plants than in Solitary plants, but not significantly (total phenolics, F

11     $= 3.910$, $P = 0.096$, Figure 4A; condensed tannins, $F = 4.882$, $P = 0.067$, Figure 4B). Females

12   of *G. atrocyanea* consumed significantly more leaf tissue from Aggregated plants than from

13   Solitary plants ($F = 7.837$, $P = 0.037$, Figure 5A). In contrast, for males of *G. atrocyanea* ($F =$

14   1.779, $P = 0.323$, Figure 5B) and for both sexes of *G. grisescens* (female, $F = 1.421$, $P = 0.323$,

15   Figure 5C; male, $F = 0.494$, $P = 0.490$, Figure 5D), there were no differences in the area of

16   feeding damage between Aggregated and Solitary leaves.

17
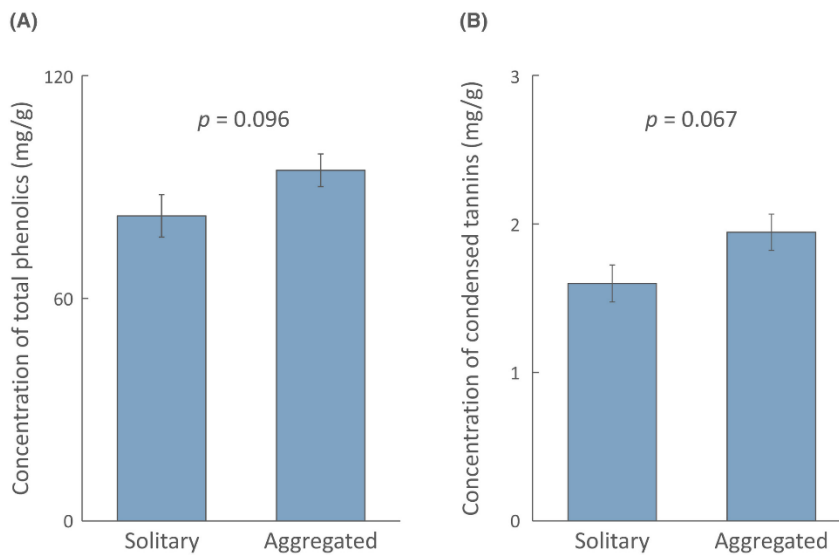
19   Figure 4. Concentrations of (A) total phenolics and (B) condensed tannins in Solitary ($N = 15$)

20   and Aggregated ($N = 25$) plants. Bars represent SE. p-values are for the results of GLMM

21   analysis

22

Figure 5. Areas of leaf consumed by (A) female and (B) male *Gastrophysa atrocyanea* and (C)

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

field. p-values are for the results of the GLMM analysis

II. Cultivation experiments

The biomass and chlorophyll content of *R. obtusifolius* did not differ among interaction

treatments (biomass: $\chi^2 = 7.081$, d$F = 4$, $P = 0.132$, Figure 6A; chlorophyll: $F = 1.444$, $P = 0.239$, Figure 6B). The concentrations of total phenolics of *R. obtusifolius* differed significantly

among treatments; they were higher in the order of intraspecific, no- and interspecific

31

interaction treatment (Figure 6C). Plants subjected to the intraspecific interaction treatment had

a significantly higher concentration of condensed tannins than those undergoing the no- or

interspecific interaction treatments (Figure 6D).
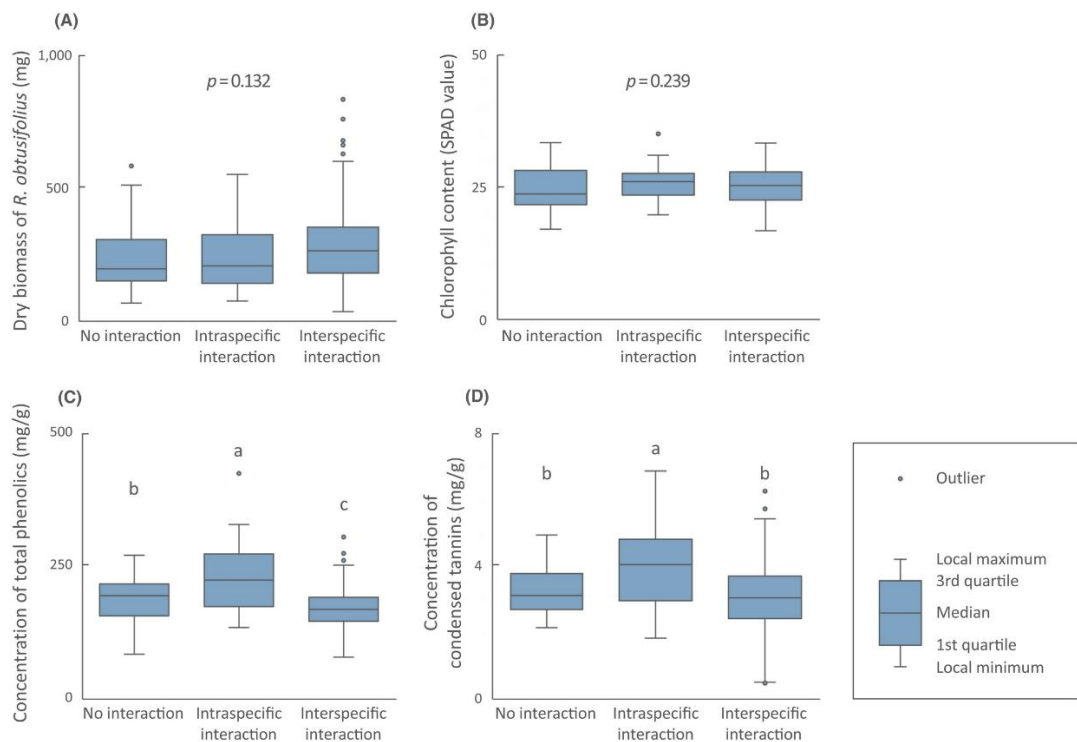
Figure 6. Dry biomass and leaf traits of *Rumex obtusifolius* in the cultivation experiment. (A)

Dry biomass of whole plant, (B) chlorophyll content, (C) concentration of total phenolics and

(D) concentration of condensed tannins in leaves. Different letters denote significant differences
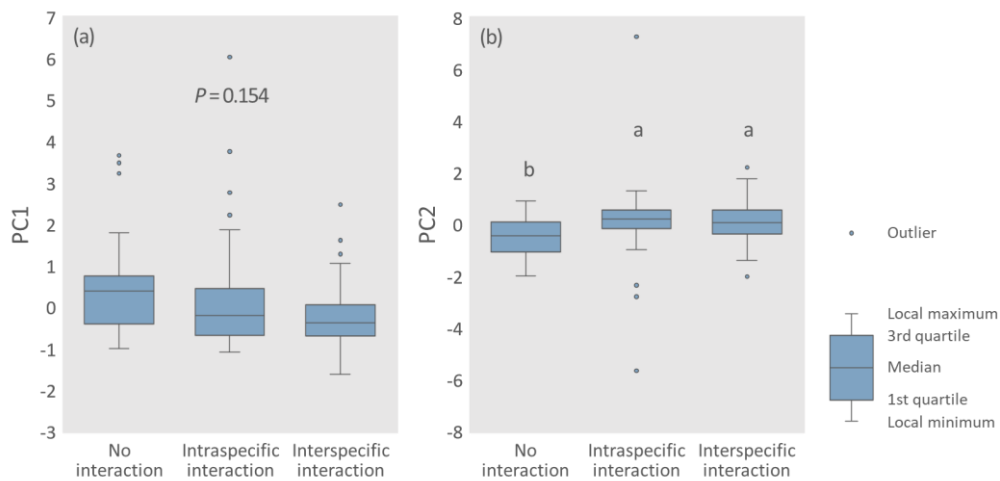
(GLMM, $p < 0.05$)

We found that PC1 and PC2 explained 61.0% and 20.7%, respectively, of the total variance of

44   the organic acid composition data. The PC1 value did not differ among interaction treatments

45   ($F = 2.068$, $P = 0.154$, Figure 7a). Plants subjected to the no-interaction treatment had

46   significantly lower PC2 values than those undergoing the intraspecific or interspecific
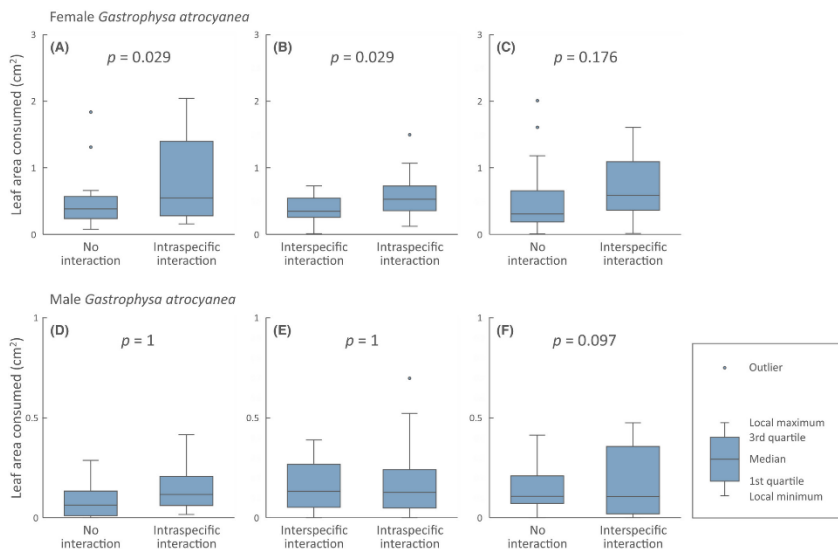
47   interaction treatments (Figure 7b).

48

50   Figure 7. Boxplots of principal component values. (a) PC1 and (b) PC2 for organic acids in the

51   cultivation experiment. Different letters indicate significant differences (GLMM, $P < 0.05$).

52

53   Females of *G. atrocyanea* consumed significantly more leaf tissue from the intraspecific

54   interaction treatment plants than from the no-interaction plants ($\chi^2 = 5.470$, d$F = 1$, $P = 0.029$,

55   Figure 8A) or from the interspecific interaction plants ($\chi^2 = 6.064$, d$F = 1$, $P = 0.029$, Figure

56   8B). There was no significant difference between the no-interaction and interspecific interaction

57   treatments in terms of the area of leaf eaten by females of *G. atrocyanea* ($\chi^2 = 1.832$, d$F = 1$, P

58   = 0.176, Figure 8C). These results did not depend on the identity of the other species in the

59   interspecific interaction treatments (intraspecific interaction vs. interspecific interaction

60   treatment, χ^2 = 0.913, dF = 2, P = 0.634; no-interaction treatment vs. interspecific treatment, χ^2

61   = 2.227, dF = 2, P = 0.328). For males of *G. atrocyanea*, there were no differences between

62   treatments in the area of leaf consumed (no interaction vs. intraspecific interaction, z = 0.329, P

63   = 1, Figure 8D; interspecific interaction vs. intraspecific interaction, z = 2.139, P = 1, Figure

64   8E; no interaction vs. interspecific interaction, z = 0, P = 0.097, Figure 8F).

65

67   Figure 8. Leaf areas consumed by (A–C) female and (D–F) male *Gastrophysa atrocyanea* in the

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

69   were as follows: (A, D) no interaction versus intraspecific interaction; (B, E) interspecific

70   interaction versus intraspecific interaction; (C, F) no interaction versus interspecific interaction

71

72   III. Mesocosm experiments

73   In the one-to-one-pot experiment, a significantly greater number of *G. atrocyanea* were

74   distributed on the *R. obtusifolius* plants in the interaction treatment than in the

75   no-below-ground-interaction treatment (F = 5.556, P = 0.030, Figure 9A). In the

76   one-to-three-pot experiment, the effect of patch size on the distribution of *G. atrocyanea*

77   differed significantly with the cultivation conditions (χ^2 = 6.540, dF = 2, P = 0.038, Figure 9B).

78   Under both types of cultivation condition, large patches had significantly more beetles than

79   small patches (quantity conditions, χ^2 = 18.301, dF = 1, p < 0.001; quantity + quality

80   conditions, χ^2 = 55.474, dF = 1, p < 0.001, Figure 9B). This trend was more pronounced under

81   quantity + quality conditions. Moreover, the effect of patch size on the number of *G.*

82   *atrocyanea* per pot (i.e. the leaf beetle density) differed significantly between cultivation

83   conditions (interaction treatment × patch size; z = –2.067, P = 0.039, Figure 9C). Although the

84   densities of leaf beetles in small and large patches were similar under quantity conditions (z = –

85   0.308, P = 0.758), under quantity + quality conditions, the large patches had a greater density

86   of leaf beetles than small patches (z = 2.118, P = 0.034; Figure 9C).

87
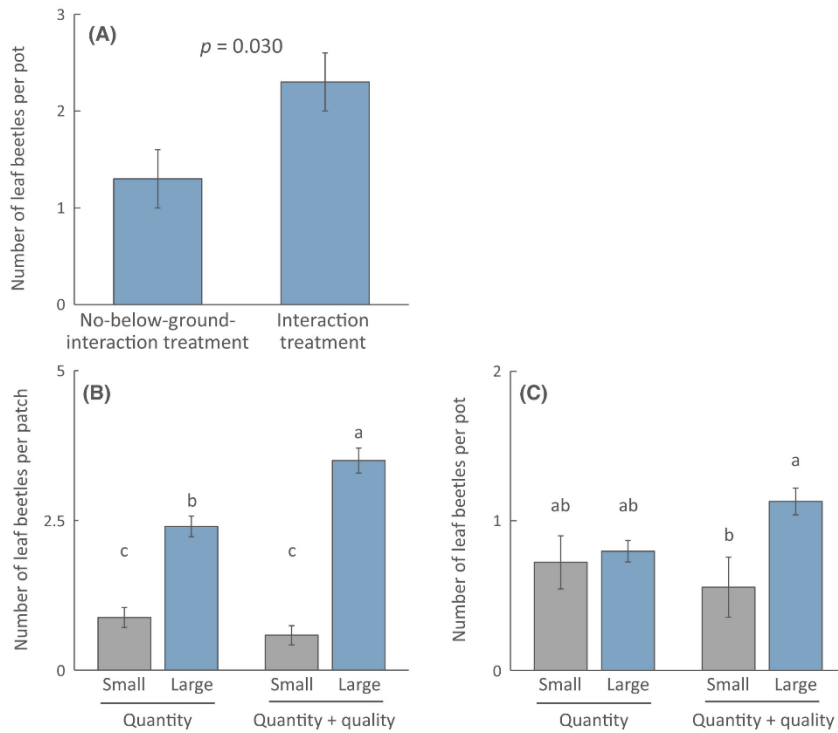
Figure 9. Numbers of leaf beetles in the mesocosm experiment. (A) Number of leaf beetles per pot in each treatment (no-below-ground-interaction treatment or interaction treatment) in the one-to-one-pot experiment. (B) Number of leaf beetles per patch and (C) number of leaf beetles per pot in each patch (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, $p < 0.05$)

DISCUSSION

We found that intraspecific interaction induced changes in the leaf metabolite concentrations of *Rumex obtusifolius* and affected resource utilisation by the specialist leaf beetle, *Gastrophysa*

36

99   *atrocyanea*, but not by the generalist leaf beetle, *Galerucella grisescens*. In addition, we showed

100   experimentally that this type of resource utilisation affected the distribution of *G. atrocyanea*. These

101   results support our hypothesis, providing experimental evidence that differences in the local

102   population density of the host plant led to plastic changes in leaf metabolite concentrations, affecting

103   the resource utilisation and distribution patterns of specialist herbivores.

104   Variation in leaf traits

105   In the field, Aggregated *R. obtusifolius* plants tended to have higher concentrations of total phenolics

106   and condensed tannins than Solitary plants (Figure 4). This result suggests that aggregation of *R.*

107   *obtusifolius* plants induced changes in leaf chemical traits. In fact, in the cultivation experiments, the

108   concentrations of total phenolics and condensed tannins in the leaves of *R. obtusifolius* were

109   significantly higher under intraspecific interaction conditions than under interspecific interaction or

110   no-interaction conditions (Figure 6C,D). Increased concentrations of secondary metabolites in the

111   presence of a conspecific neighbour have been reported in several plant species, and it has been

112   suggested that metabolic alterations in leaves in response to intraspecific interaction are common in

113   plants (Barton and Bowers, 2006; Ormeño et al., 2007; but see Kigathi et al., 2013). In many plant

114   species, intraspecific competition is more intense than interspecific competition (Adler et al., 2018),

115   and such intraspecific competition causes limitation of soil nutrients and water (Craine and

116   Dybzinski, 2013; Takigahira and Yamawo, 2019). It is well known that limitation of soil nutrients

117   and water for plants induces the accumulation of secondary metabolites in the leaves (reviewed by

118   Akula and Ravishankar, 2011). Thus, aggregation of *R. obtusifolius* plants may lead to a greater

119   increase in the leaf concentrations of secondary metabolites, such as total phenolics and condensed

120   tannins, than occurs in Solitary *R. obtusifolius* plants (no competition or competing with other

121   species) because of soil resource competition.

122   Another possible hypothesis is that Aggregated plants invest more in defence than do Solitary plants

123   through recognition of conspecific neighbours, because aggregated plants are more at risk of

124   consumption by specialist leaf beetles. Leaf-trait alteration based on neighbour recognition has been

125   reported in several plant species (Lepik et al., 2012; Yamawo, 2015, 2021; Yamawo and Mukai,

126   2020). Volatile organic compounds (VOCs) released from above-ground plant tissues (Karban and

127   Shiojiri, 2009) and root exudates below the ground (Biedrzycki et al., 2010) are used for recognition

128   by neighbouring plants. The results of our mesocosm experiments suggest that below-ground

129   interactions among *R. obtusifolius* plants have an important effect on the concentrations of secondary

130   metabolites. Although our experimental design did not reveal the importance of above-ground

131   interaction via VOCs, conspecific neighbour recognition via root exudates could have been the cause

132   of the changes in the leaf metabolite concentration of *R. obtusifolius* plants. However, the history of

133   interaction between specialist leaf beetles and *R. obtusifolius* plants is weak because *R. obtusifolius*

134   is an exotic species in Japan. To understand the adaptive importance of leaf-trait alteration in *R.*

135   *obtusifolius* plants, we would need to perform an additional study in a region to which *R. obtusifolius*

136   is native.

137   Our cultivation experiment revealed differences in the concentrations of primary metabolites

138   between the no-interaction treatment and the intraspecific or interspecific interaction treatments (see

139   Figure 7). This result means that changes in the leaf concentrations of primary metabolites depend

140   on the presence of neighbouring plants, regardless of the species of the neighbour. The

141   concentrations of primary metabolites are strongly affected by light conditions (Kitazaki et al., 2018).

142   For example, experiments with lettuce, Lactuca sativa, have shown that the pattern of accumulation

143   of primary metabolites, such as sugars and amino acids, is affected by light quality, intensity and

144   exposure time (Kitazaki et al., 2018). Therefore, the difference in leaf primary metabolite

145   concentration could be attributed to the likelihood that the light resources that could be acquired

146   under conditions without neighbouring plants were greater than under the other conditions.

147

148   Preferences and distribution of leaf beetles

149   The local *R. obtusifolius* population density affected the amounts of leaf consumed by the specialist

150   leaf beetle, *G. atrocyanea*. In the experiment using leaves from the field, females of *G. atrocyanea*

151   preferred to consume the leaves of aggregated *R. obtusifolius* plants than of Solitary plants, despite

152   similar quantities of leaves being provided for the beetles (Figure 5A). In the experiment using the

153   leaves of cultivated plants, females of *G. atrocyanea* also preferred the leaves of *R. obtusifolius*

154   plants exposed to intraspecific interaction over those of plants exposed to no interaction (Figure 8A).

155   These preference patterns are consistent with the increases in the leaf concentrations of secondary

156   metabolites (total phenolics and condensed tannins; Figures 4 and 6C,D) but not with the variations

157   in primary metabolite concentrations (Figures 7). Therefore, we concluded that females of *G.*

158   *atrocyanea* selected leaves on the basis of increases induced in the leaf secondary metabolite

159   concentration by the host plant's interactive environment. *Gastrophysa atrocyanea* beetles are

160   specialist herbivores of Rumex plants (Suzuki, 1985). Many herbivore specialists use host-specific

161   secondary metabolites for host searching or detecting (Ōmura, 2018; Schoonhoven et al., 2005). This

162   type of host searching may reflect the feeding preferences of *G. atrocyanea*. Females of *G.*

163   *atrocyanea* lay eggs on the plants on which they feed, and the hatched larvae feed on the same plants.

164   The larvae of *G. atrocyanea* require large amounts of food, and plants are often completely

165   consumed (Suzuki, 1985). For this reason, the selection of aggregated plant leaves by *G. atrocyanea*

166   females during the reproductive season is linked to the securing of food resources for the next

167   generation. This may be associated with niche specialisation in coevolution among host plants and

168   specialist herbivores (Abrahamson, 2008; Schoonhoven et al., 2005). In contrast, no preference was

169   observed among males of *G. atrocyanea*, possibly because males use fewer resources than females

170   with egg masses.

171   Do the preferences of leaf beetles affect the beetles' distribution? Our mesocosm experiment

172   provided robust evidence that changes in leaf traits based on below-ground intraspecific interaction

173   can affect the distribution of the specialist leaf beetle, *G. atrocyanea* (Figure 9). When the plant

174   patch sizes were similar, approximately 1.7 times more leaf beetles were distributed in the

175   interaction treatment patch than in the no-below-ground-interaction treatment patch (Figure 9A).

176   Effects of interaction between host plants were also found in the one-to-three-pot experiment.

177   Greater numbers of leaf beetles were distributed on the large patches than on the small patches, and

178   this trend was more pronounced under quantity + quality conditions than under quantity conditions

179   (Figure 9B). This finding is consistent with the distribution of *G. atrocyanea* in the field (Table 2;

180   Suzuki, 1985).

181   The resource concentration hypothesis predicts that herbivores would be concentrated on large

182   patches of host plants, because plants in such patches are easy to find and can be inhabited for a long

183   time by the herbivores (Root, 1973). This hypothesis assumes that the density of plants regulates the

184   amount of food available and affects the exploration behaviour and residence time of herbivores.

185   However, in our mesocosm experiments, leaf beetle density did not differ when only the patch size

186   (resource quantity) differed, whereas it was significantly higher on large patches than on small ones

187   when both the patch size and the competitive environment (and thereby the resource quality) differed

188   (Figure 6C). Our results demonstrate that differences in quantity alone do not explain the distribution

41

189　of *G. atrocyanea*, and that differences in quality are a key determinant.

190　The enemies hypothesis also explains the positive correlation between herbivore density and host

191　plant density, where the density of host plants is high, that of natural enemies is low (Root, 1973).

192　However, no parasitic wasp, pathogen or predator has yet been reported as a natural enemy of *G.*

193　*atrocyanea*. We have collected and kept more than 300 individuals from the field, but we have been

194　unable to find any parasitic natural enemy (H. Ohsaki, unpublished data). In addition, our mesocosm

195　experiment demonstrated that leaf beetles showed concentrated distribution even without natural

196　enemies. Interactions with competitors may also affect the distribution of *G. atrocyanea*. On the

197　populations of *R. obtusifolius* in the field, *G. grisescens* was the second most frequently observed

198　species after *G. atrocyanea*. However, the possibility of this interaction effect is also very unlikely,

199　because although a past study has suggested that *G. grisescens* is vulnerable to resource competition

200　from *G. atrocyanea* (Suzuki, 1986), our experiments produced a concentrated distribution of *G.*

201　*atrocyanea* in the absence of *G. grisescens*. Thus, the presence of natural enemies or competitors

202　may not, in fact, affect the concentration distribution of *G. atrocyanea*. We strongly suggest that,

203　instead, changes induced in the leaf metabolite concentrations through intraspecific interactions in

204　plants induced changes in the concentration distribution of the specialist herbivore *G. atrocyanea* on

205　resources in the field.

206　In contrast, numbers of the generalist leaf beetle, *G. grisescens*, were not correlated with the local

207   population density of *R. obtusifolius* in the field (Table 2), and these beetles did not select the leaves

208   of *R. obtusifolius* on the basis of the interaction environment (Figure 5). These results did not

209   support our hypothesis that generalist herbivores accumulate on low-density host plants to avoid

210   high levels of secondary metabolites. Generalist herbivores respond to a variety of chemicals besides

211   those measured as leaf traits in this study (Schoonhoven et al., 2005; War et al., 2012). It is possible

212   that leaf traits not analysed here are involved in the preference of *G. grisescens*, and were not altered

213   by the interaction treatments. Another possible reason why the findings did not support our

214   hypothesis is the effects of resource competition among herbivores. In some cases, resource

215   competition among herbivores influences herbivore distribution (e.g., Godinho et al., 2020;

216   Schoonhoven et al., 2005; Suzuki, 1986). A previous study pointed out that *G. grisescens* is

217   vulnerable to resource competition from *G. atrocyanea* (Suzuki, 1986). It may therefore prioritise

218   the avoidance of competitors over plant availability when deciding where to feed (Suzuki, 1985).

219   Several studies, as well as the resource dilution hypothesis proposed by Otway et al. (2005), have

220   pointed out that herbivore density per plant may be higher when the population density of hosts is

221   low (e.g. Yamamura, 1999). Our results suggest that these phenomena may be caused not only by

222   differences in the local population density of the host plants, but also indirectly by interactions with

223   other herbivorous insects. To determine whether these results are general or specific to certain

224   herbivores, several species, including generalists, may need to be tested.

225

CONCLUSIONS

227   Our findings provide experimental evidence that intraspecific interaction between host plants affects

228   the distribution of a specialist herbivore. Many researchers have worked to unravel the relationship

229   between the distribution of herbivores and the local population density of host plants. Some

230   herbivores have shown a positive response to resource abundance, as in the resource concentration

231   and enemies hypothesis proposed by Root (1973), whereas others, as in the resource dilution

232   hypothesis proposed by Otway et al. (2005), have shown a negative response. In these studies, it was

233   thought that herbivorous insect characteristics such as foraging behaviour, migration ability and

234   interaction with enemies determine the insects' distribution. In addition, these studies focused on the

235   amount of food available and assumed that leaf traits are always constant. In contrast, we focused on

236   interaction between plants. Our results indicate that herbivore responses to resource quantity and

237   quality may interact with each other as factors governing herbivore distribution. Therefore, herbivore

238   responses to the local population density of host plants can be understood from a plant–plant

239   interaction perspective, highlighting the need to integrate plant–plant interactions into our

240   understanding of plant–animal interactions in nature.

241

3. Effects of indirect plant–plant interaction via root exudate on growth and leaf chemical contents in *Rumex obtusifolius*

INTRODUCTION

Plant–plant interactions are an important part of terrestrial ecosystems because they affect not only the outcome of competition between plants (Yamawo and Mukai, 2020; Xu et al., 2021), but also functional leaf traits (Barton and Bowers, 2006; Mraja et al., 2011; Yamawo 2015; Takigahira and Yamawo, 2019; Muirui et al., 2019), herbivory (Yamawo, 2021; Ohsaki et al., 2022) and herbivore distributions (Ohsaki et al., 2022). In a previous study, we experimented with *Rumex obtusifolius* (Polygonaceae) to examine the effects of intraspecific, interspecific, and no belowground direct interactions on leaf chemical content and herbivore distribution (Ohsaki et al., 2022). Plants exposed to intraspecific direct interaction had increased total phenolic and condensed tannin concentrations in their leaves, and induced a concentrated specialist herbivore distribution on the leaves. A wide variety of plant parts (e.g., leaves, roots, and seeds) and media (e.g., volatile chemicals, nonvolatile chemicals, light, and soil microorganisms) are involved in plant–plant interactions (Karban, 2021). Detailed elucidation of the mechanisms of plant–plant interactions would greatly improve our understanding of not only how these interactions affect leaf traits, but also how they affect terrestrial ecosystems.

260   Kin and self-discrimination in plants occurs via root exudates (Biedrzycki et al., 2010; Semchenko et

261   al., 2014; Yamawo et al., 2017). Previous study reported that some plant species develop more roots

262   when growing in the vicinity of a non-self plant than when growing in the vicinity of a self plant

263   (Yamawo et al., 2017). Moreover, similar root behavior was observed in an experiment in which root

264   exudate reduced both root growth and clonal reproduction in non-self, competitor plants. On the

265   basis of these findings, we hypothesized that, if these results depend on indirect interactions

266   mediated by root exudates, *R. obtusifolius* leaf chemical contents, which are linked to herbivore

267   distribution in an ecosystem (Ohsaki et al., 2022), depend on the recognition of conspecific

268   neighbors via root exudates. More specifically, we hypothesized that the concentrations of secondary

269   chemicals in leaves of *R. obtusifolius* are increased in response to exposure to root exudates from

270   plants of the same species, but not to those from plants of other species. Conversely, if our previous

271   results9 depended not only on indirect interactions mediated by root exudates but also on other

272   interactions, such as direct contact and resource competition, the effects of these indirect

273   root-exudate-mediated interactions on leaf traits may differ from those of direct plant–plant

274   interactions.

275

276   MATERIALS AND METHODS

277   Cultivation

278   In September 2016, around 300 seeds of *R. obtusifolius* were collected from two plants about 2 km

279   apart in fields in Hirosaki City, Aomori Prefecture, Japan. As interspecific neighbors, we used

280   *Plantago asiatica* L. (Plantaginaceae), *Trifolium repens* L. (Fabaceae), and *Festuca ovina* L.

281   (Poaceae), which are the dominant competitors of *R. obtusifolius* in Japan (Ohsaki, 2018). Native to

282   Europe, the perennials *T. repens* and *F. ovina* now grow worldwide. *Plantago asiatica*, *T. repens*, and

283   *F. ovina* are sympatric with *R. obtusifolius* in Japan. A hundred seeds of *P. asiatica* were collected

284   from two plants in a field in Aomori Prefecture. A hundred seeds of *T. repens* were collected from

285   plants in a field in Saga Prefecture. Commercially available *F. ovina* seeds (Kaneko Seeds Co.,

286   Gunma, Japan) were bought. All seeds were stored in a refrigerator at 4°C until use. On 3 September

287   2017, all seeds from each species of mother plant were mixed and sown on the surface of wet sand

288   (2 cm deep) held in a container. The containers were then kept in a growth chamber at 25°C under a

289   12-h light/dark cycle until the plants had developed their first true leaves, then healthy of them were

290   randomly used in the subsequent experiment.

291   To obtain donor plants, we filled 350 plastic pots (10.5 cm diameter × 9 cm high) with sand (Sunday

292   Co., Ltd., Aomori, Japan), and on 13 September 2017 we planted seedlings of each species

293   individually in 280 of these pots (1 seedling/pot; 70 pots/species). The remaining 70 pots were left

294   unplanted as controls. All pots were watered once a day for 30 days. To obtain recipient plants, on 13

295   October 2017 we planted *R. obtusifolius* seedlings individually in 350 pots containing sand. After

296 sowing, the pots were arranged randomly in growth chambers and maintained at 25°C under a 12-h

297 light/dark cycle. Two days after planting, 0.5 g of solid fertilizer (ammonia nitrogen, 8.0%; soluble

298 phosphorus, 8.0%; water soluble potassium, 8.0%; Nichiryunagase Co., Ltd., Japan) was applied to

299 each pot. The experiment duration, water and soil conditions, and growth conditions were similar to

300 those in the direct interaction experiment in our previous work (Ohsaki et al., 2022).

301 Each of the 350 recipient plants was paired with one of the 350 donor plants and labeled accordingly.

302 Each day at 12:00, 40 mL of distilled water was added to the top of the sand containing the donor

303 plant and 20–25 mL of root exudate was collected from the bottom of the pot, and then added to the

304 top of the sand containing the recipient plant. As smaller plants could not produce enough root

305 exudates for the experiment, we planted the donor plants a month earlier than the recipient plants.

306 All donor plants were perennial species, and *R. obtusifolius* produces seeds every year. Consequently,

307 it is normal for seedlings of *R. obtusifolius* to be surrounded by mature conspecific plants and other

308 plant species in the field, and therefore their roots are exposed to those plants' exudates.

309 Thirty days after planting the recipient plants, we obtained a total of 233 recipient plants (control, N

310 = 66; intraspecific treatment, $N = 45$; interspecific treatments: *T. repens*, $N = 42$; *F. ovina*, $N = 58$; *P.*

311 *asiatica*, $N = 22$).

312

313 Measurement of leaf chemical contents

314   At 30 days, the leaves of the recipient plants were harvested. First, chlorophyll content in the most

315   recently fully expanded leaves was determined. Chlorophyll content reflects the plant's nitrogen

316   concentration and has been found to indirectly affect vertebrate and invertebrate herbivore survival

317   and distribution (Schai-Braun et al., 2015; Sousa-Souto et al., 2018). Therefore, we measured this to

318   examine changes in nutrient condition in response to exposure to root exudate. Measurements were

319   conducted with a chlorophyll meter (SPAD-502 Plus; Konica Minolta, Tokyo, Japan), which is a

320   commonly used tool for rapid and nondestructive estimation of leaf chlorophyll content; the

321   resulting SPAD values are positively correlated with chlorophyll content (Shibaeva et al., 2020).

322   Each leaf was measured twice – in the central part of the leaf on each side of the main vein – and the

323   average value per a leaf was determined.

324   Phenolics and condensed tannins are major secondary metabolites in genus Rumex18 and have been

325   suggested to stimulate feeding by some leaf beetle species (Ikonen et al. 2002; Torp et al., 2013).

326   Therefore, we also determined their contents in leaves. After determined, all recipient plants were

327   harvested and dried at 50°C for 3 days. The dried plants were weighed on an electronic balance to

328   the nearest 0.1 mg, and the total phenolic and condensed tannin contents of the leaves were

329   determined (Feeny 1970; Dudt and Shure 1994).

330

331   Statistical analysis

332   All statistical analyses were performed in R v. 4.0.2 software.[23] All data met the statistical

333   assumptions of normality and homoscedasticity according to the Kolmogorov–Smirnov test. The

334   chlorophyll, total phenolic, and condensed tannin contents in leaves were compared among root

335   exudate treatments by using a general linear model with a Gaussian distribution and an identity link

336   followed by an F-test; the models included each leaf chemical trait as a response variable and root

337   exudate treatment as the explanatory variable. The false discovery rate correction for multiple

338   comparisons was then applied. All tests were two-tailed, with $P < 0.05$ considered significant.

339

340   RESULTS AND DISCUSSION

341   The effect of root exudate on the leaf chemical content of *R. obtusifolius* differed according to the

342   species from which the root exudate was obtained (Figure 10). In *R. obtusifolius* exposed to

343   intraspecies root exudate, the total phenolic and condensed tannin concentrations in the leaves did

344   not differ from those in control leaves (total phenolics: $F = 1.581$, $P = 0.211$, Figure10b; condensed

345   tannins: $F = 0.217$, $P = 0.642$, Figure 10d). This result differs from those of previous study, in which

346   the leaves of *R. obtusifolius* exposed to intraspecific direct interaction had significantly higher total

347   phenolic and condensed tannin concentrations than those in control leaves (Figure 10a,c; Ohsaki et

348   al., 2022). Together, these findings indicate that total phenolic and condensed tannin concentrations

349   in the leaves of *R. obtusifolius* are altered in response to direct, but not indirect, intraspecies

350   interaction. In our previous study, *R. obtusifolius* exposed to intraspecific direct interaction had

351   increased total phenolic and condensed tannin concentrations in the leaves, and this induced a

352   concentrated specialist herbivore distribution on the leaves (Ohsaki et al., 2022). If these chemicals

353   directly induce a concentrated distribution of leaf beetles, then indirect interaction with *P. asiatica*

354   may affect this distribution in the field.

355
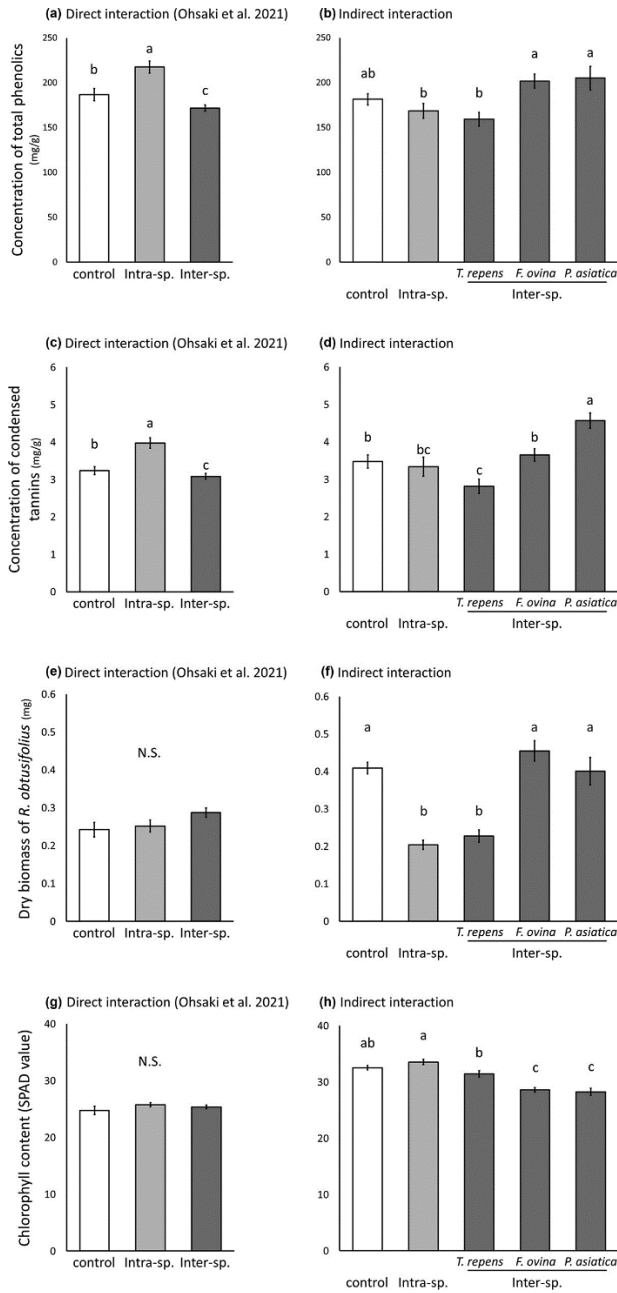
Figure 10. Total phenolic, condensed tannin, dry biomass, and chlorophyll contents in leaves of *Rumex obtusifolius* exposed to (a, c, e, g) direct interaction (grown in same pot)9 or to (b, d, f, h) indirect interaction via root exudate (present study). Intra-sp., intraspecific interaction; Inter-sp., interspecific interaction. N.S., non-significant. Bars indicate standard errors. Different letters denote significant differences (general linear model, $P < 0.05$).

362

363   The dry leaf biomass of *R. obtusifolius* exposed to intraspecies root exudate was significantly less

364   than control leaves ($F = 91.413$, $P = 0.001$, Figure 10f), indicating that the root exudate of *R.*

365   *obtusifolius* contains substances that inhibit the growth of conspecific plants (Young, 1984; Asao et

366   al. 2003). Phenolics and condensed tannins have allelopathic effects and are some of the most

367   abundant allelochemicals in higher plants (Cheema et al., 2012; Reigosa 1999). *Festuca ovina* and *P.*

368   *asiatica* are strong competitors of *R. obtusifolius*: grassland plant communities often shift from being

369   *R. obtusifolius* dominant to being *F. ovina* dominant (Kardol et al. 2006); and, like *R. obtusifolius*, *P.*

370   *asiatica* is a perennial herb that develops a leaf rosette. Leaf chemicals in *R. obtusifolius* inhibit the

371   germination of *F. ovina* (Ohsaki et al., 2020), and *R. obtusifolius* may increase them as a competitive

372   response.

373   Root exudates often include primary metabolites such as sugars and organic acids (Walker et al.,

374   2003). These metabolites may function as fertilizers, but we did not find more increase of biomass in

375   any condition than control condition (Figure 10f). We consider that any fertilization effect was

376   absent, or was canceled by the allelopathic effects of secondary metabolites.

377   The effect of root exudate on chlorophyll content also depended on the species from which the

378   exudate was collected (Figure 10h). Plants exposed to root exudate from *F. ovina* or *P. asiatica* had a

379   significantly higher chlorophyll content than plants exposed to that from *T. repens* or conspecific

380   plants (*F. ovina* vs. control, $F = 51.949$, $p < 0.001$; *F. ovina* vs. *R. obtusifolius*, $F = 65.628$, $p <$

381   0.001; *F. ovina* vs. *T. repens*, $F = 17.266$, $p < 0.001$; *P. asiatica* vs. control, $F = 34.138$, $p < 0.001$; *P.*

382   *asiatica* vs. *R. obtusifolius*, $F = 45.396$, $p < 0.001$; *P. asiatica* vs. *T. repens*, $F = 12.241$, $p < 0.001$).

383   The composition of root exudate is likely to be species specific (Bardri and Vivanco, 2009; Herz et

384   al. 2018). Here, the root exudate of conspecific plants reduced dry leaf biomass and that of *T. repens*

385   decreased condensed tannin concentration and dry leaf biomass in *R. obtusifolius*. The significant

386   reduction of chlorophyll content in plants exposed to root exudate from *F. ovina* or *P. asiatica*

387   suggests that these root exudates inhibit the uptake of constituents of chlorophyll (e.g., nitrogen and

388   magnesium) or increase the specific leaf area, which decreases aboveground competition (Knops et

389   al., 2000).

390   Recent studies have pointed out that root exudates or root chemicals alter the soil microbial

391   community and feedback for plant growth and resource allocation (Sugiyama, 2019; Takahashi et al.,

392   2021; Kong et al., 2021). The effects of root exudates on *R. obtusifolius* leaf traits may include those

393   caused by changes in soil microbial composition. To understand more about the specific effects of

394   root exudates, detailed analysis of the compositions of root exudates from different species and their

395   effects on soil microbes are needed.

396   Taking together these and our previous results (Ohsaki et al., 2022), we conclude that the effects of

397   indirect interaction via root exudates are different from those of direct interaction. In short, *R.*

398   *obtusifolius* may compete more strongly with *F. ovina* and *P. asiatica* by increasing the content of

399   total phenolics and reducing that of chlorophyll in leaves. These results suggest that *R. obtusifolius*

400   seedlings recognize other species via root exudates and express a competitive response, as do other

401   species (Kong et al. 2018). If so, leaf traits in *R. obtusifolius* are modulated in space. For example,

402   when plants are close together, the leaf chemical contents are affected by direct interactions, whereas

403   when plants are farther apart, they are affected by indirect interactions. These results highlight the

404   importance of distinguishing between direct and indirect belowground interactions between plants

405   for understanding the effects of plant–plant interactions not only on the plants themselves, but also

406   on the herbivores.

407

4. Plant–plant interaction by *Aster leiophyllus* affects herbivory by Sika deer, *Cervus nippon*

INTRODUCTION

Herbivory by large vertebrates such as deer and domestic ungulates greatly affects ecosystem functions, nutrient cycling, ecosystem resilience, and vegetation structure (Côté et al., 2004; Turkington, 2009; Verón et al., 2011; Forbes et al., 2019; Wakatsuki et al., 2021). Ungulate feeding directly or indirectly affects plant communities (Nopp-Mayr et al., 2020) and communities of animals such as arthropods and lizards (Pringle et al., 2007). Grazing by deer can lead to the disappearance of native, vulnerable, and sometimes rare plant species (Rooney and Waller, 2003). Grazing can alter many ecological processes, such as carbon and nutrient cycling (Hobbs, 1996; Augustine and McNaughton, 1998; Kasahara et al., 2016; Wakatsuki et al., 2021), as well as animal communities and populations (Okuda et al., 2014; Stephan et al., 2017; Nakahama et al., 2020; Seki et al., 2021). Therefore, many ecologists have questioned how the patterns of grazing by vertebrate herbivores are determined (e.g., McArthur et al. 1993; Foley and Moore 2005; Villalba et al., 2014). Although most large herbivores feed on more than one plant species (Freeland 1991), they have preferences (Augustine and McNaughton, 1998; Schai-Braun et al., 2015). For example, sika deer (*Cervus nippon*, Cervidae, Artiodactyla) in Japan have been reported to have 646 foraging plants and 135 unpalatable plants (Hashimoto and Fujiki, 2014). Such preferences of large herbivores for plants

56

426 are thought to be affected by the presence of secondary metabolites (e.g., phenols, Villalba et al.,

427 2014, Champagne et al., 2020; terpenes, Vourc'h et al. 2002) that have feeding inhibitory effects, and

428 by a combination of secondary metabolites and other nutritional factors (e.g., energy, proteins, and

429 minerals; Felton et al., 2018). Clearly, the concentrations and compositions of chemicals in plants

430 affect food choices.

431 Plant secondary metabolite production can be influenced by the plant neighborhood via plant–plant

432 interactions (Barton and Bowers, 2006; Mraja et al., 2011; Takigahira and Yamawo, 2019). For

433 example, Broz et al. (2010) found that total phenolics accumulation was lower in Centaurea

434 maculosa plants collected from heterospecific field stands than from conspecific ones. Plants with

435 heterospecific neighbors are considered to allocate more resources towards the production of

436 primary metabolites, which are crucial for plant growth, thereby reducing their allocation to

437 secondary metabolites (Broz et al. 2010); thus, changes in resource availability in response to

438 competition with neighboring plants may indirectly affect the synthesis of plant secondary

439 metabolites (e.g., Broz et al. 2010; Takigahira and Yamawo, 2019; Ohsaki et al., 2022).

440 The link between plant–plant interactions, plant secondary metabolite production, and herbivory has

441 been demonstrated in invertebrates (Ohsaki et al., 2022). Ohsaki et al. (2022) reported that *Rumex*

442 *obtusifolius* plants grown with a conspecific neighbor increased their leaf concentrations of total

443 phenolics, and a specialist leaf beetle (*Gastrophysa atrocyanea*) consumed more of these leaves than

444   those of plants grown with a heterospecific neighbor. This discrimination is thought to occur because

445   the leaf beetle is a specialist herbivore of genus *Rumex* plants that recognizes and searches for hosts

446   on the basis of the presence of secondary metabolites (Ohsaki et al., 2022). However, it is not known

447   whether plant–plant interactions affect resource utilization by vertebrates via variations in the

448   content of leaf secondary metabolites.

449   Here, our three research questions were as follows: (i) Do plants that belong to local populations

450   with different densities in the field have different concentrations of total phenolics, one of the

451   secondary metabolites? (ii) Do differences in plant–plant interaction treatments (inter- or

452   intraspecific) affect the leaf concentrations of secondary metabolites? and (iii) Do changes in leaf

453   traits caused by differences in plant–plant interactions affect leaf consumption by vertebrates? We

454   studied these questions by using *Aster leiophyllus* Franch. et Sav. var. leiophyllus (Asteraceae,

455   Astereae) as the plant and sika deer as a large vertebrate herbivore. In Asteraceae, changes in the leaf

456   total phenolics concentration in response to intra- and interspecific interactions have been reported,

457   suggesting that these changes have a defensive function against arthropods (Broz et al. 2010). To test

458   our hypotheses, we investigated the relationships among the interaction environment with neighbors

459   of *A. leiophyllus* plants, the leaf concentration of total phenolics, and the preference of sika deer in

460   the field. To clarify the effect of the interaction environment on leaf traits of *A. leiophyllus* and

461   resource utilization by sika deer, we grew plants in pots and examined deer preferences (Figure 11).

462
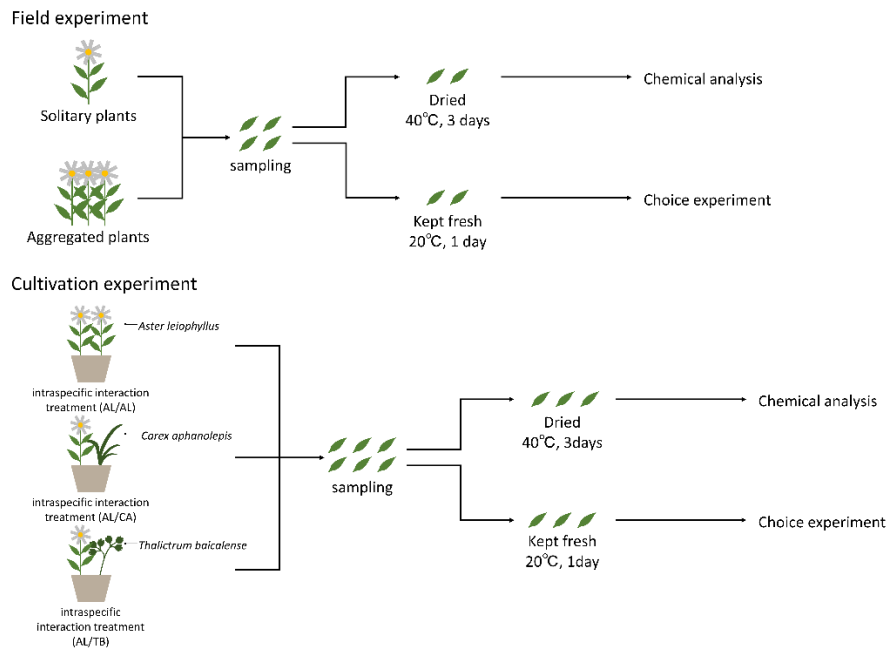
Figure 11. Flowchart outlining all the steps in the experiments.

464

MATERIALS AND METHODS

Study Species

Sika deer, also known as Japanese deer, are distributed extensively in the forests of East Asia (Goodman et al., 2001). Sika deer expanded their range in Japan by nearly 70% during the 1990s (Takatsuki, 2009). Grazing by sika deer affects the vegetation composition in both agricultural and forested habitats (Suzuki et al. 2008; Hashimoto and Fujiki, 2014; Ohashi et al. 2014; Wakatsuki et al., 2021). The deer feed on a variety of plant species (foraging plants) but reportedly avoid some species (unpalatable plants) (Takatsuki, 2009). From a literature analysis, Hashimoto and Fujiki (2014) listed the preferences of sika deer for plants in Japan; of 900 plant species, 646 were

59

474   categorized as foraging plants and 135 were categorized as unpalatable plants; another 119 species,

475   including *A. leiophyllus*, were reported as both foraging and unpalatable plants. *Aster leiophyllus* is a

476   perennial herb that grows in East Asia along forest edges and in the understory. It propagates via

477   wind-dispersed seeds and by stolons. Our unpublished preliminary experiment and a previous study

478   (Kawakami et al., 2020) showed that *A. leiophyllus* contains phenolic compounds. A comprehensive

479   analysis of chemicals from 11 species of the genus Aster, not including *A. leiophyllus*, revealed that

480   phenolic acids and flavonoids were widely present in these species (Li et al., 2022).

481

482   Study sites

483   Plant and seed samples were collected from an approximately 1-ha site in the city of Nikko (36°

484   48′ N, 139° 25′ E) in Tochigi Prefecture, Japan. The deer population in this area has increased

485   since the 1980s (Li et al. 1996). In some particularly severely damaged areas, dwarf bamboo species

486   have been nearly depleted (Koganezawa and Satake, 1996) and replaced with *A. leiophyllus*

487   understory (Seki and Koganezawa, 2010). At the study site, we found marks that seemed to have

488   been left on *A. leiophyllus* plants by foraging deer (K. O. and H. O. personal observations). A second

489   site, in Nara City, Nara Prefecture, Japan (34° 41′ N, 135° 50′ E), was used to present plants to

490   deer. At this site, the population density of deer was high (>200 deer/km^2), and many deer

491   individuals were familiar with humans. Plants were presented to the deer directly by hand, as

492   described below. The deer encountered *A. leiophyllus* for the first time during the experiment,

493   because it was not naturally distributed at this site.

494

495   Experiment with field-collected *A. leiophyllus*

496   On 16 September 2018, undamaged *A. leiophyllus* shoots were collected from the Nikko City study

497   site. The plants collected were approximately 30 cm tall and were at least 5 m apart from each other.

498   Plants with no conspecific individuals within a radius of 30 cm were defined as "solitary" ($N = 38$),

499   and those with five or more conspecific individuals within this radius were considered "aggregated"

500   ($N = 38$). One shoot was collected from each plant. The solitary plants that we collected were

501   surrounded by other species, such as *Carex aphanolepis* and *Thalictrum baicalense*, instead of

502   conspecific individuals. All focal plants were flowering. We cut the two youngest fully developed

503   leaves from the tip of the shoot of each focal plant and dried them at 40 °C in an oven over 3 days to

504   analyze the concentration of total phenolics (Julkunen-Tiitto and Tahvanainen, 1989). Preliminary

505   tests had revealed that the total phenolics content did not change over an approximately 3-day period

506   after the leaves had been picked (data not shown). Similarly, it has been reported that no differences

507   in the total phenolics concentration of cauliflower (*Brassica oleracea*) extracts were found between

508   three drying methods: air-dried (ambient conditions, 10 days), sun-dried (7 days), and oven-dried at

509   40 °C (3 days) (Anwar et al. 2013). After leaf harvest, the remaining shoots were individually kept in

510   moist plastic bags in a dark box at 20 °C for 24 h for use in the deer preference trials. Although we

511   recognize that there would have been some loss of phenolics during the 24 h (Julkunen-Tiitto and

512   Tahvanainen, 1989), we considered that the experimental treatments were comparable because the

513   experimental shoots from both solitary and aggregated plants were kept under the same conditions.

514   On 17 September 2018, the leaves of solitary and aggregated plants were presented to 38 randomly

515   selected deer at the Nara City study site. One of us carefully approached a deer, starting at 2 m to the

516   front of the animal. By using hands, a leaf from a solitary or aggregated plant was presented to the

517   deer for 30 s at 10 cm from the animal's snout. The same 30-s routine was then used with a leaf from

518   an aggregated or solitary plant, respectively, regardless of whether the deer had consumed the first

519   leaf. Each trial had two 30-second steps, thus allowing us to evaluate the deer's learning through the

520   consumption of *A. leiophyllus* leaves. In 20 of the trials, leaves of solitary plants were presented first,

521   followed by leaves of aggregated plants. Only one leaf per plant was used in the deer preference

522   trial; the other was used for analysis of the phenolics concentration (see below). All the trials were

523   performed on the same day. The order was reversed in the remaining 18 trials. The two types of trials

524   were alternated. Each deer selected was more than 20 m away from other deer. An individual deer

525   participated in one trial only. Each deer was identified by external characteristics such as body size,

526   color, and presence of horns or small wounds. Consumption was evaluated as (i) "completely

527   consumed" (deer consumed the leaf), (ii) "left" (deer took the leaf but spat it out), and (iii) "rejected"

528   (deer did not take the leaf). Deer were recorded as buck, doe, or fawn on the basis of body size and

529   the presence or absence of antlers.

530

531   Experiment with plants grown in pots

532   On 16 September 2018 in the city of Nikko, approximately 50 seeds were collected from each of

533   three *A. leiophyllus* plants that were at least 10 m apart. Seeds were kept in a refrigerator at 4 °C. On

534   6 May 2019, all seeds of each *A. leiophyllus* plant were germinated on the surface of wet sand (2 cm

535   deep) in the laboratory at 25 °C under a 12-h photoperiod. On 26 May 2019, six to 15 seedlings of

536   each *A. leiophyllus* plant that had true leaves (in total 28 seedlings) were transferred one by one to 28

537   pots (10.5 cm diameter × 9 cm height) filled with seed-free garden soil containing compost as a

538   major component (Mori Sangyo Co., Hokkaido Prefecture, Japan). These seedlings were defined as

539   focal plants and were allocated to intra- and interspecific interaction treatments. A neighboring plant

540   was planted in each pot: *A. leiophyllus* for the intraspecific interaction treatment ($N = 10$), or C.

541   aphanolepis or T. baicalense for the interspecific interaction treatments ($N = 9$ each). Both C.

542   aphanolepis and T. baicalense are predominant neighboring plants of *A. leiophyllus* in the field of the

543   city of Nikko (H. O. personal observation). These neighbor plants for the treatments were collected

544   from the city of Nikko and were individually trimmed to a weight of 5.0 ± 0.5 g on 25 May 2019.

545   The distance between plants in a pot was about 3 cm. All pots were placed randomly in a greenhouse

63

546   at Hirosaki University (40°59′N, 140°47′E) and watered once a day. To evaluate the change in

547   resource allocation in plants in each treatment, on 27 October 2019 we measured the diameter of the

548   major rosette and counted the leaves of each *A. leiophyllus*. The two youngest fully developed leaves

549   were collected and immediately dried at 40 °C in an oven over 3 days to analyze the concentration of

550   total phenolics. Each remaining leaf was kept as described above and presented to deer on 28

551   October 2019 at the Nara City study site. Each plant leaf was given to a different individual deer.

552   The approach to the deer was the same as above.

553

554   Concentration of total phenolics

555   We used the method of Dudt and Shure (1994) to quantify the total phenolics in the leaves of *A.*

556   *leiophyllus* collected in the field or grown in pots. Dried leaves were powdered in a mill. Total

557   phenolics were extracted from 20 mg of leaf powder in 10 mL 50% methanol for 1 h in an

558   ultrasound bath at 40 °C and quantified by using the Folin–Ciocalteu method (Julkunen-Tiitto 1985).

559

560   Statistical analysis

561   All statistical analyses were performed by using R v. 3.6.1 software (R Development Core Team,

562   2019). All data sets met the statistical assumptions of normality and homoscedasticity according to

563   the Kolmogorov–Smirnov test, and the statistical analyses performed were appropriate to the

564 structure of the data sets. All tests were two tailed. The significance level was set at 0.05.

565 The concentrations of total phenolics in the leaves of solitary and aggregated *A. leiophyllus* plants

566 collected in the field were compared by using generalized linear models with Gaussian distribution

567 and an identity link (McCullagh and Nelder, 2019), followed by an F-test; the models included the

568 concentration of total phenolics as a response variable and plant population density (solitary or

569 aggregated) as an explanatory variable.

570 The concentrations of total phenolics and the rosette diameters of *A. leiophyllus* grown in pots were

571 compared among neighboring plant species conditions (*A. leiophyllus*, C. aphanolepis, and T.

572 baicalense) by using generalized linear mixed models (GLMMs, R package "lmerTest", Kuznetsova

573 et al., 2015) with Gaussian distribution and an identity link, followed by an F-test; the models

574 included plant traits (total phenolics and rosette diameter) as response variables and the type of

575 neighboring plants as an explanatory variable. Numbers of leaves of *A. leiophyllus* were compared

576 among neighboring plant species conditions by using GLMMs (R package "glmmML", Broström,

577 2018) with Poisson distribution and a log link, followed by a likelihood ratio test; the models

578 included the number of leaves as a response variable and the type of neighboring plants as an

579 explanatory variable. Parent plant ID was included as a random effect in the models. When there was

580 a significant effect of the interaction environment on the deer response, we conducted multiple

581 comparisons by false discovery rate correction.

582 We used cumulative-link mixed models (CLMMs, R package "ordinal", Christensen 2015) with a

583 logit link to analyze the effects of the interaction with neighbors of *A. leiophyllus* collected in the

584 field or grown in pots on the consumption of leaves of focal plants by deer. The models included

585 deer response (completely consumed, left, or rejected) as a fixed term, and the density of plants

586 collected in the field (solitary or aggregated) or neighboring plant conditions as explanatory

587 variables. Deer sex (buck, doe, or fawn) was included as a random effect in these models. When

588 there was a significant effect of neighboring plant species conditions on deer response, we conducted

589 multiple comparisons by false discovery rate correction.

590 Relationships between consumption by deer and the concentration of total phenolics in the leaves of

591 *A. leiophyllus* collected in the field or grown in pots were analyzed by GLMMs (R package

592 "glmmML", Broström, 2018) with a binomial distribution and logit function, followed by the

593 Chi-square test; the models included consumption by deer as a response variable and the

594 concentration of total phenolics as an explanatory variable. In this analysis, we transformed the data

595 on deer response to binomial data (completely consumed or not). Deer sex ID or parent plant ID was

596 included as a random effect in the models.

597

598 RESULTS

599 Experiment with field-collected *A. leiophyllus*

600   The concentration of total phenolics was significantly higher in the leaves of aggregated plants than

601   in those of solitary plants (solitary or aggregated presented first: $F_{1,36} = 18.808$, $P < 0.001$, Figure

602   12a; solitary or aggregated presented second: $F_{1,36} = 25.116$, $P < 0.001$, Figure 12b). All of the deer

603   presented first with the leaves from solitary plants completely consumed them, whereas only about

604   50% of the deer presented first with the leaves of aggregated plants consumed them (Figure 13a).

605   There was a significant negative correlation between the concentration of total phenolics and

606   consumption by deer (estimated coefficient = -0.065, $\chi^2 = 5.797$, d$F = 1$, $P = 0.016$, Figure 14).

607   When deer were presented second with either solitary or aggregated leaves, about 40% of the deer

608   rejected or left the leaves (Figure 13b). The percentages of deer consuming the leaves of solitary or

609   aggregated plants did not differ significantly ($\chi^2 = 0.003$, d$F = 1$, $P = 0.960$, Figure 13b). There was

610   a significant negative correlation between the total concentration of phenolics and consumption by

611   deer (presented first, estimated coefficient = -0.065, $\chi^2 = 5.797$, d$F = 1$, $P = 0.016$; presented

612   second, estimated coefficient = -0.052, $\chi^2 = 4.439$, d$F = 1$, $P = 0.035$, Figure 14).

613
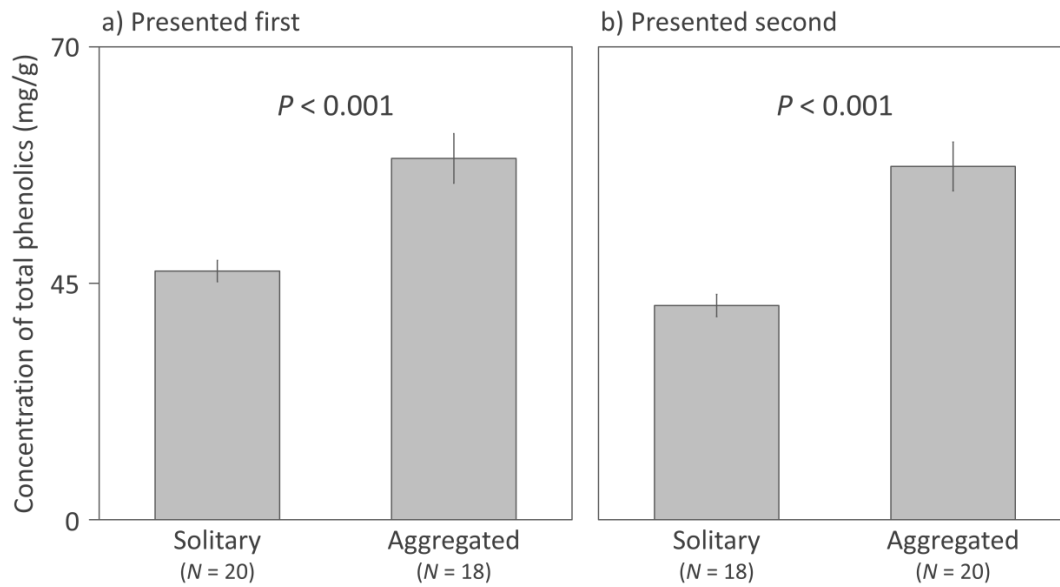
Figure 12. Concentrations of total phenolics in leaves of solitary and aggregated *Aster leiophyllus*

plants collected in the field and presented (a) first or (b) second to deer. All solitary *A. leiophyllus*

grew near *Carex aphanolepis* or *Thalictrum baicalense*. Bars represent SE. *P*-values are for the
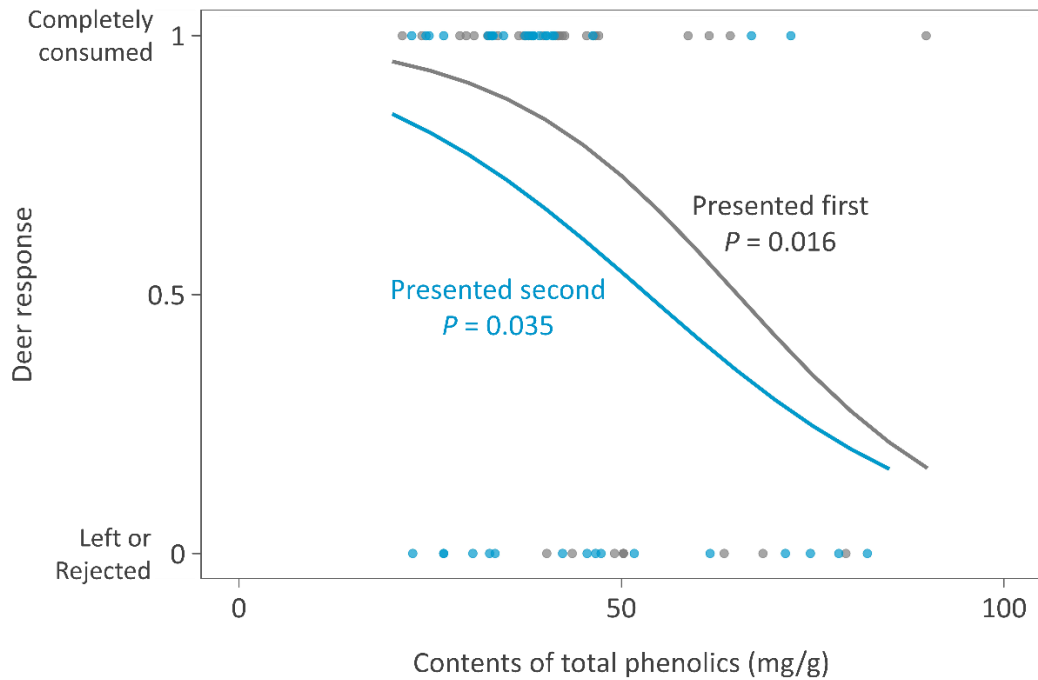
results of generalized linear model analysis.

618
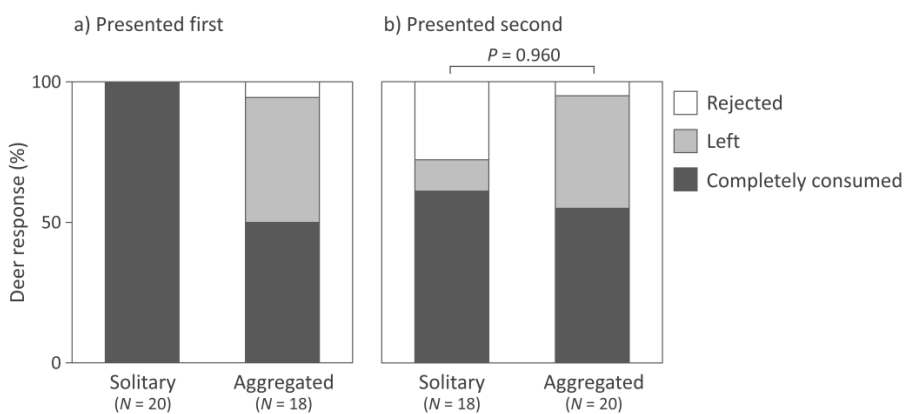
Figure 13. Deer responses to *Aster leiophyllus* leaves collected in the field and presented (a) first or

(b) second. Completely consumed: deer consumed the leaf; left: deer took the leaf but spat it out;

rejected: deer did not take the leaf. *P*-values are for the results of cumulative-link mixed-model

622   analysis.

623

624   Figure 14. Relationships between deer responses to field-collected *Aster leiophyllus* and

625   concentration of total phenolics in leaves of aggregated and solitary plants presented first (gray) and

626   second (blue).

627

628   Experiment with plants grown in pots

629   The concentration of total phenolics in the leaves of *A. leiophyllus* varied according to the type of

630   neighboring plant (χ^2 = 11.249, dF = 2, P = 0.004, Figure 15a). Plants subjected to interspecific

631   interaction treatment with *T. baicalense* had a significantly lower concentration of total phenolics

632   than those subjected to intraspecific interaction treatment (χ^2 = 11.114, dF = 1, P = 0.003, Figure

633   15a). The corresponding value in the *C. aphanolepis* interaction treatment did not differ significantly

634   from those in the two other treatments (compared with intraspecific interaction treatment, $\chi^2 = 3.056$,

635    $dF = 1$, $P = 0.123$; compared with interspecific interaction treatment using *T. baicalense*, $\chi^2 = 2.662$,

636    $dF = 1$, $P = 0.123$; Figure 15a). Rosette diameter and number of leaves of *A. leiophyllus* did not

637   differ among cultivation conditions (rosette diameter: $\chi^2 = 2.43$, $dF = 2$, $P = 0.297$, Figure 15b;

638   number of leaves: $\chi^2 = 0.041$, $dF = 2$, $P = 0.980$, Figure 15c).
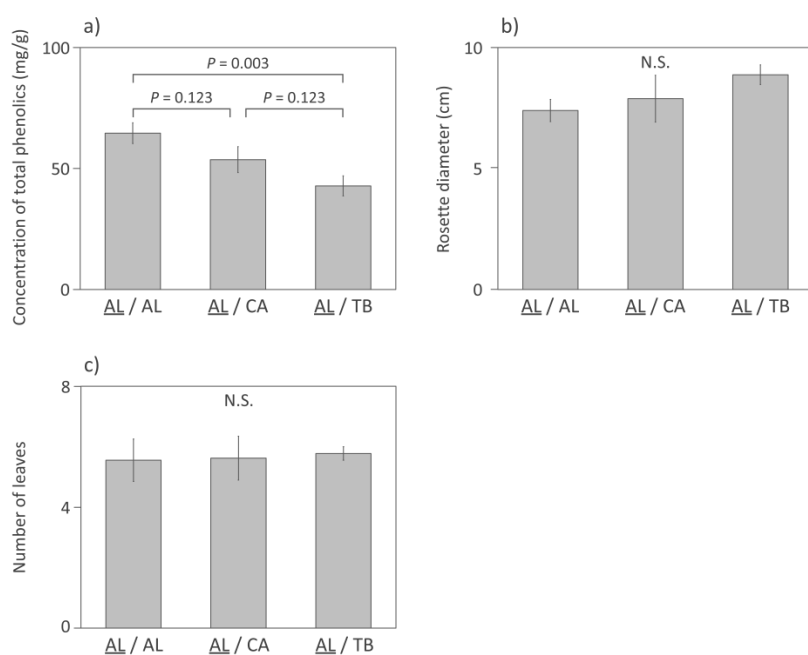
640   Figure 15. Leaf and growth traits of *Aster leiophyllus* grown in pots. (a) Concentration of total

641   phenolics; (b) rosette diameter; (c) number of leaves per plant. The focal plants, *A. leiophyllus*, were

642   grown in pots with the following neighbors: *A. leiophyllus* (AL/AL; $N = 10$) as an intraspecific

643   interaction treatment; and *Carex aphanolepis* (AL/CA; $N = 9$) or *Thalictrum baicalense* (AL/TB; N

644    $= 9$) as interspecific interaction treatments. Bars represent SE. *P*-values are for the results of

645   generalized linear mixed model analysis. N.S., not significant.

646

The differences in consumption of leaves of focal plants by deer showed a trend toward significance

among the different types of neighboring plants ($\chi^2 = 3.427$, d$F = 1$, $P = 0.064$, Figure 16). A

significantly greater percentage of deer completely consumed the leaves of *A. leiophyllus* plants

grown with *T. baicalense* than the percentage that consumed *A. leiophyllus* grown with conspecific

neighbors ($\chi^2 = 6.013$, d$F = 1$, $P = 0.043$, Figure 16). Rates of consumption of the leaves of *A.*

leiophyllus grown with *C. aphanolepis* did not differ from those of the other two groups (compared

with intraspecific, $\chi^2 = 1.146$, d$F = 1$, $P = 0.285$; with *T. baicalense*, $\chi^2 = 3.137$, d$F = 1$, $P = 0.115$;

Figure 16). There was a significant negative correlation between the total concentration of phenolics

and consumption by deer (estimated coefficient $= -0.061$, $\chi^2 = 4.486$, d$F = 1$, $P = 0.034$, Figure 17).

656

657
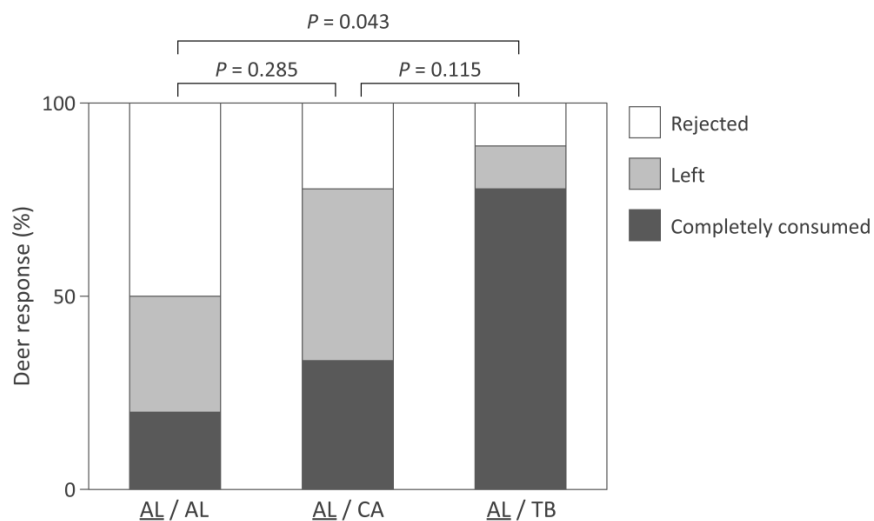
Figure 16. Deer responses to *Aster leiophyllus* grown in pots. The focal plants, *A. leiophyllus*, were

659   grown in pots with the following neighbors: *A. leiophyllus* (AL/AL; $N = 10$) as an intraspecific

660   interaction treatment; and Carex aphanolepis (AL/CA; $N = 9$) or Thalictrum baicalense (AL/TB; $N =$

661   9) as interspecific interaction treatments. Completely consumed: deer consumed the leaf; left: deer

662   took the leaf and spat it out; rejected: deer did not take the leaf. *P*-values are for the results of

663   cumulative-link mixed-model analysis.

664
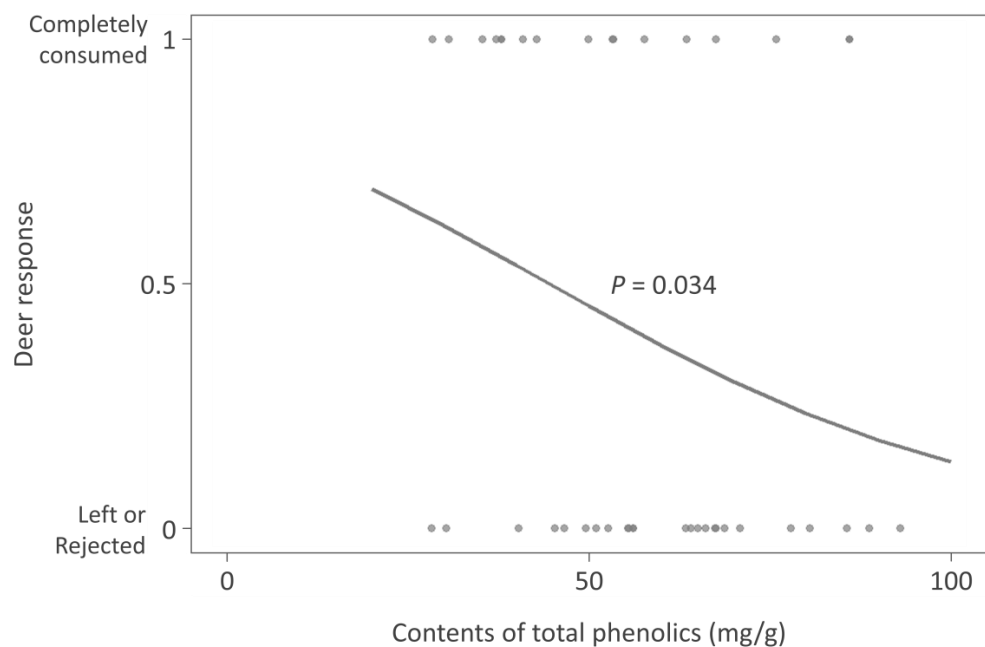
665

666   Figure 17. Relationships between deer responses to *Aster leiophyllus* grown in pots and

667   concentration of total phenolics in the leaves.

668

669   DISCUSSION

670   We found that *A. leiophyllus* plants from low-density populations had a lower concentration of total

671   phenolics than plants from high-density populations without changes in growth- traits, rosette size

672   and number of leaves. Moreover, interspecific interaction in *A. leiophyllus* reduced the leaf

673   concentration of total phenolics, but only in the case of interaction with *T. baicalense*. Significantly

674   more deer consumed the leaves of *A. leiophyllus* with interspecific interaction treatment with *T.*

675   *baicalense* than the leaves of *A. leiophyllus* given intraspecific interaction treatment. These results

676   indicate that variations in leaf traits associated with plant–plant interactions affect the deer foraging

677   response. Furthermore, the percentage of deer consuming the leaves was negatively correlated with

678   the leaf concentration of total phenolics. This result suggests that changes in the leaf concentrations

679   of total phenolics, or other chemicals, or both, in response to the plant interaction environment affect

680   deer feeding.

681   The concentration of total phenolics was significantly lower in solitary *A. leiophyllus* plants growing

682   in the field with other plant species (*C. aphanolepis* or *T. baicalense* or both) than in aggregated

683   plants (Figure 12a). This result suggests that the leaf chemical composition was changed by

684   interaction with neighboring plant species. The concentration of total phenolics in the leaves of *A.*

685   *leiophyllus* plants grown in pots with *T. baicalense* as the other species was significantly lower than

686   that in the leaves of those grown with the same species (Figure 15a). In contrast, the concentration of

687   total phenolics in the leaves of *A. leiophyllus* plants grown in pots with *C. aphanolepis* as the other

688   species did not differ significantly from that in the leaves of those grown with the same species,

689 although it was about 16% lower than that in the latter (Figure 15a). Furthermore, our preliminary

690 experiment revealed that the concentration of total phenolics in the leaves of *A. leiophyllus* grown

691 without neighbor plants was similar to that of *A. leiophyllus* grown with plants of the same species

692 (Figure 18). We consider that phenotypic plasticity in response to interspecific interaction may

693 contribute to the differences in the concentration of total phenolics or in related traits such as

694 primary and other secondary metabolites in leaves.

695
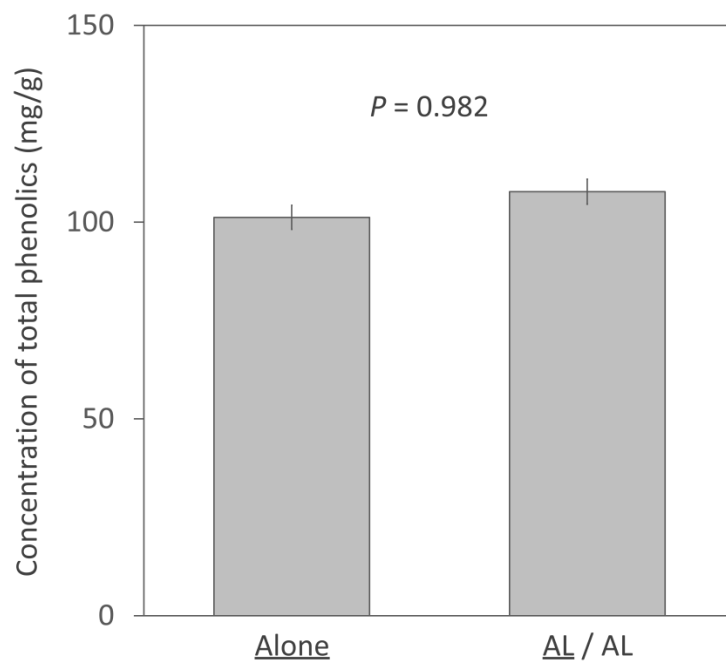
696

697 Figure 18.   Concentration of total phenolics in leaves of *Aster leiophyllus* grown in pots. The focal

698 plants, *A. leiophyllus*, were grown in pots alone ($N = 26$) or with conspecific neighbors (AL/AL; N

699 $= 40$). Bars represent SE. P-values are for the results of generalized linear mixed model analysis.

700

701   There are two main possible causes of the change in the concentration of total phenolics according to

702   the interaction environment. One is variation in competition intensity among treatments. Changes in

703   resource availability due to competition can affect leaf defense traits through the growth–defense

704   trade-off (Herms and Mattson, 1992; Karasov et al., 2017). Phenolics are also well known to be

705   defensive chemical compounds against herbivores (Forkner et al., 2004; Lattanzio et al., 2006;

706   Rehman et al. 2012), and negative correlations (growth–defense trade-off) between the concentration

707   of phenolics and growth are often found (e.g., Strauss et al., 2002; Rehman et al. 2012; Yamawo et

708   al., 2015). A decrease in the leaf concentration of phenolics suggests that a plant may be investing

709   more in growth for competition than in defense against herbivory. The cultivation period of our

710   experiment was short (about 5 months), and changes in the concentration of total phenolics did not

711   affect leaf number per plant or rosette diameter in plants grown in pots (Figure 15b, c). There may be

712   a time lag for the impact of interactions on growth to become apparent. We predict that growth traits

713   will be affected, but to show this would require a longer experimental period and measurements of

714   the belowground biomass.

715   The second possible cause of the change in leaf traits including total phenolics concentration is

716   leaf-trait alteration based on neighbor recognition. Generally, resource competition is intense among

717   conspecific individuals, because they require similar resources (Adler et al., 2018), but some plants

718   avoid such competition through the recognition of competitor identity, self, and kin (e.g., Dudley and

719   File, 2007; Yamawo et al., 2017). For example, the plant Cakile edentula avoids competition

720   belowground from kin individuals through kin recognition (Dudley and File, 2007). Although we

721   have no information on whether *A. leiophyllus* plants express self- or kin recognition, this

722   mechanism may have led to the differences in leaf traits (e.g. the concentration of phenolics) in our

723   experiments.

724   Some plant species, such as *Plantago lanceolata* (Plantaginaceae), *Quercus robur* (Fagaceae), and

725   *Rumex obtusifolius* (Polygonaceae), have higher leaf concentrations of secondary chemicals such as

726   lignins and phenolics when exposed to intraspecific interaction than when exposed to interspecific

727   interaction (Barton and Bowers, 2006; Moreira et al., 2017; Ohsaki et al., 2022). Thus, changes in

728   leaf chemical traits as a result of plant interaction with neighbors likely occur in many taxonomic

729   groups. In our pot experiments, we, too, found differences in the concentration of phenolics

730   depending on the species of the neighboring plant, so not only differences between intra- and

731   interspecific interactions but also the species identity of neighboring plants has to be considered in

732   future studies. It is important to clarify why the direction of change differs among plant species and

733   to verify these differences experimentally from the viewpoint of differences in taxonomic groups,

734   reproductive traits, and habitats.

735   Local *A. leiophyllus* population density (solitary vs. aggregated) affected the consumption response

736   by deer. Among deer presented first with the leaves of aggregated plants, about half completely

737   consumed the leaves, and when the deer were presented first with the leaves of solitary plants all of

738   the deer completely ate the leaves (Figure 3a). The consumption rate was negatively correlated with

739   the concentration of total phenolics (Figure 4). A similar negative correlation was observed in the

740   experiment with plants grown in pots (Figure 7). Phenolics may reduce herbivory not only by insects

741   (e.g., Howe and Jander, 2008; War et al., 2018), but also by deer. A reduction in deer feeding damage

742   in the presence of leaf phenolics has been reported (Felton et al., 2018; Champagne et al., 2020). Our

743   results suggest that food preference by deer for *A. leiophyllus* depends on phenolics concentration or

744   other related traits, or both. Extensive work by Salminen and colleagues over the past decade has

745   shown that the chemistry of specific phenolics in plants affects herbivory by both invertebrate and

746   vertebrate herbivores (e.g. Salminen and Karonen, 2011; Marsh et al. 2020). Nevertheless, there was

747   a correlation between deer foraging response and the total phenolics concentration in leaves (Figures

748   14, 17). Some phenolic substances (e.g. phenolic acids and flavonoids) that are dominant in leaves

749   may act in chemical defense against deer. In addition, preference-related substances in the leaves

750   may also affect the foraging response of deer. Energy and protein are the most limiting resources for

751   large herbivores, and resource selection is affected by these resources (Robbins, 1993, Spalinger,

752   2000). More detailed analyses of phenols, related chemicals, and nutrients are needed in future

753   studies.

754   In addition, consumption by deer may be influenced by conditioned food aversion. Conditioned food

755   aversion is a rejection response in which an animal associates the taste and scent of a food with the

756   negative effects of that food (mainly nausea or vomiting); the response is subsequently provoked

757   when the animal encounters the food again (Gustavson et al., 1974). For example, goats fed on

758   condensed tannins (phenolic compounds) associate the flavor of the plants with post-ingestive

759   aversion and learn to avoid them; the aversion is apparently not related to the inhibition of digestion

760   (Provenza et al., 1990). This feeding response is widespread among animals, including humans and

761   rats (Garcia et al. 1974; 1985): In our experiments, deer that were presented initially with aggregated

762   plants and were then presented with solitary plants rejected or left about 40% of the solitary plants

763   (Figure 13b). When presented with their first leaf of *A. leiophyllus*, the deer may have learned to

764   associate the flavor with aversion to phenols or other chemicals influencing subsequent foraging

765   choice and may therefore have not eaten the second leaf.

766   Our results also have important implications for environmental management. Grazing of vegetation

767   by deer has become a worldwide problem (Côté et al., 2004; Enright et al., 2014; Bernardo et al.,

768   2016; Shinoda et al., 2021). Our results imply that we can control deer damage to a certain extent by

769   manipulating interactions between plants. For example, aggregated transplants of focal plant species

770   may reduce deer herbivory by facilitating the accumulation of high levels of secondary chemicals in

771   leaves. However, on the other hand, aggregated planting may increase consumption by deer through

772   resource concentration effects (Holík and Janík, 2022). In our deer foraging experiment we focused

773   on the short-term response of the deer, and this limits our discussion of these issues. Future work

774   could confirm these possibilities by testing whether plastic chemical changes in the leaves have

775   long-term effects on feeding by deer. In conclusion, in order to accurately estimate the effect of

776   herbivory by large vertebrates on ecosystem, we will need to understand the effects of phenotypic

777   changes in leaf traits in response to plant–plant interactions.

778

5. General discussion

780   I demonstrated that population density of plants shape the types of plant-plant interaction, intra- or

781   inter-specific interaction, and the differences in types of plant-plant interaction induce changes in the

782   concentrations of secondary metabolites in leaves. These phenomena were observed in different

783   plant species belong to Polygonaceae and Asteraceae. Furthermore, I showed that the resource

784   utilization and distribution by several taxonomic groups of herbivores are affected by these chemical

785   changes in leaves. These results supported my hypothesis.

786   In chapter 2, I revealed that the accumulation of condensed tannins and total phenolics in leaves

787   occurred in response to intraspecific interactions in *R. obtusifolius*. On the other hands, in chapter 4,

788   a reduction in total leaf phenolics occurred as a response to interspecific interaction in *Aster*

789   *ageratoides*. Thus, responses to plant-plant interactions may differ among plant species. Moreover,

790   previous studies have shown several different responses in several plant species. Studies in which

791   plant-plant interaction treatments were experimentally manipulated and the concentrations of

792   secondary metabolites in leaves analyzed are summarized in Table 3. These research include

793   experimental designs where many plant individuals are grown together, such as monocultures and

794   polyculture. I excluded experiments that manipulated species diversity around the focal plants and

795   experiments without control treatment, i.e., conditions that do not expose the plants to interaction.

796   The studies reviewed in Table 3 were divided into the following six patterns. The first is the pattern

797   that plant-plant interaction did not affect changes in concentrations of secondary metabolites in

798   leaves (Figure 19a). The second is the pattern that plant-plant interaction increased concentrations of

799   them, whether intra- or interspecific interaction (Figure 19b). The third and fourth are the patterns

800   that either intraspecific (Figure 19c) or interspecific interaction (Figure 19d) increased

801   concentrations of them. The results of *R. obtusifolius* in Chapter 2 falls under the Figure 19c. The

802   fifth and sixth are patterns that plant-plant interactions increased and the extent of the increase varied

803   between intra- (Figure 19e) and interspecific interaction (Figure 19f). Furthermore, as far as I had

804   been able to find, there is no trend that the interaction treatment reduces the concentration of

805   secondary metabolites in leaves more than the control, i.e., no interaction treatment. Therefore,

806   decreased concentrations of total phenolics in leaves associated with interspecies interaction that

807   occurred in the *A, ageratoides* in Chapter 4 may be an extremely rare case. In any case, there was no

808   consistency in the pattern of changes in concentration of secondary metabolites according to type of

809   taxonomic group or secondary metabolite.

810   Additionally, clarifying the mechanisms of change of concentration of secondary metabolites in

811   leaves with plant-plant interactions may help in understanding the differences in their patterning. I

812   considered two possible factors to the increase of concentration of secondary metabolites in leaves

813   with plan-plant interaction: interference with neighboring plants via resources and recognition by the

814   focal plant. The former hypothesis is that the presence of neighboring plants has changed the

815   composition of resources in the environment and caused some metabolic change in the focal plant. In

816   other words, the focal plant may have been interfered with by the behavior of neighboring plants,

817   which resulted in changes in secondary metabolites. For example, Barton and Bowers (2006) cite the

818   carbon–nutrient balance hypothesis (Bryant et al., 1983) and discuss the possibility that competition

819   for nutrient in soil may allocate more photosynthate to carbon-based defenses.

820   The latter hypothesis is that the focal plant recognized the presence of neighboring plants. In

821   other words, the focal plant may have been responded actively to neighboring plants, resulting in

822   changes in secondary metabolites. Alternatively, the focal plant may have been responded actively to

823   neighboring plants, resulting in changes in secondary metabolites. Variation in leaf traits such as leaf

824   area (Lepik et al., 2012) and extrafloral nectar (Yamawo, 2021) based on recognition of neighboring

825   plant has been reported in several plant species.

826

827   Table 3. List of variation of concentration of leaf secondary metabolites in response to plant-plant interaction treatments

| Types of changes in secondary metabolites based on Figure 19	Family	Focal species	Secondary metabolites	Mecanisms	Reference	Comparison with controls
a	Cucurbitaceae	*Cucurbita pepo* L.	catalase	unknown	Di N. et al. (2018)	Yes
a	Fabaceae	*Phaseolus vulgaris* L.	superoxide dismutases	unknown	Di N. et al. (2018)	Yes
a	Brassicaceae	*Brassica rapa* L.	superoxide dismutases	unknown	Di N. et al. (2018)	Yes
b	Plantaginaceae	*Plantago major* L.	iridoid glycosides	The carbon–nutrient balance hypothesis, the adaptive plasticity hypothesis or dilution effects	Barton & Bowers (2006)	Yes
b	Brassicaceae	*Brassica rapa* L.	peroxidase	unknown	Di N. et al. (2018)	Yes
b	Cucurbitaceae	*Cucurbita pepo* L.	superoxide dismutases	unknown	Di N. et al. (2018)	Yes
c	Solanaceae	*Solanum lycopersicum* L.	catalase	unknown	Di N. et al. (2018)	Yes
c	Fabaceae	*Phaseolus vulgaris* L.	peroxidase	unknown	Di N. et al. (2018)	Yes
c	Cucurbitaceae	*Cucurbita pepo* L.	peroxidase	unknown	Di N. et al. (2018)	Yes
c	Solanaceae	*Solanum lycopersicum* L.	peroxidase	unknown	Di N. et al. (2018)	Yes
c	Fabaceae	*Phaseolus vulgaris* L.	polyphenol oxidase	unknown	Di N. et al. (2018)	Yes
c	Brassicaceae	*Brassica rapa* L.	polyphenol oxidase	unknown	Di N. et al. (2018)	Yes
c	Solanaceae	*Solanum lycopersicum* L.	polyphenol oxidase	unknown	Di N. et al. (2018)	Yes
c	Solanaceae	*Solanum lycopersicum* L.	superoxide dismutases	unknown	Di N. et al. (2018)	Yes
e	Plantaginaceae	*Plantago lanceolata* L.	iridoid glycosides	The carbon–nutrient balance hypothesis, the adaptive plasticity hypothesis or dilution effects	Barton & Bowers (2006)	Yes
e	Fabaceae	*Phaseolus vulgaris* L.	catalase	unknown	Di N. et al. (2018)	Yes
e	Brassicaceae	*Brassica rapa* L.	catalase	unknown	Di N. et al. (2018)	Yes
e	Cucurbitaceae	*Cucurbita pepo* L.	polyphenol oxidase	unknown	Di N. et al. (2018)	Yes

828
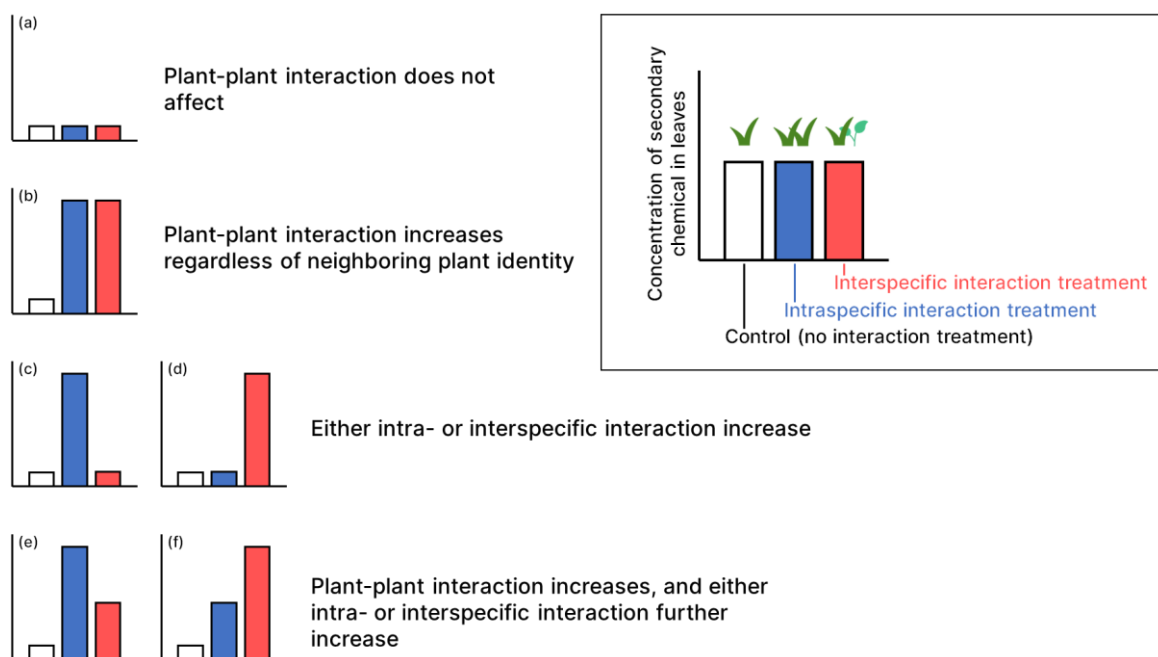
Figure 19. Six patterns of variation of concentration of leaf secondary metabolites in response to plant-plant interaction.

Thus, even though plant-plant interactions involve at least two or more phenomena, such as resource competition and neighbor recognition, most previous studies have regarded plant-plant interaction as competition (e.g., Barton and Bowers, 2006; Bustos-Segura et al., 2017). Distinguishing and clarifying competition and neighbor recognition may improve our understanding of the complex patterning of changes in the concentration of secondary metabolites. We need to objectively examine plant-plant interactions using detailed chemical analysis sach as gene expression analysis in plant, metabolome analysis in plant and resource composition analysis in environment. For example, it is useful to analyse components of soil nutrients and lights in the

cultivation experiments and to experimentally manipulate soil nutrient and light conditions in order

to determine whether resource availability affects changes in leaf chemicals. Competition for water

is unlikely to influence change in leaf chemicals, as all studies referenced in Table 3 showed that

water was provided adequately. To determine whether recognition of neigbouring plants occurs

through plant-plant interactions, it is necessary to examine the response of the focal plants to the

presence of neighbouring plants when light and soil nutrient conditions are held constant. The

Chapter 3 research was a useful to certain the effects of recognition. In this experiment, only the

presence of neighbouring plants could be presented to the for plants using root exudate with keeping

the same conditions of resource and light. Then, we detected effects of recognition through root

exudate chemicals in Chapter 3, but which differed from the results of experiments with direct

plant-plant interaction in Chapter 2. *Rumex obtusifolius* recognize neighboring plants and occur

chemical responses of leaves, but this effect of recognition is thought to be masked by the effects of

resource competition in field. Thus, we must not forget that both recognition and competition may

arise, which collectively influence changes in leaf chemicals. To distinguish the effects of resource

competition and neighbour recognition, it may be useful to focus on the spatial scale at which each

phenomenon can occur. Competition occurs to the area that plants can share resources with

neighbours, whereas neighbour recognition occurs to the area that plants can give and receive the

mediums with neighbours. Root exudates and volatile organic compounds are diffused extensively,

so recognition may arise more extensively than competition. Future experiments should therefore focus on distance-dependent responses.

Resource utilization by herbivores was affected by changes in leaf secondary metabolites following plant–plant interaction. Plants with high concentrations of secondary metabolites were preferred by the specialist leaf beetle in Chapter 2 and rejected by the generalist deer in Chapter 4. The first experimental evidence is that plant–plant interactions and associated variations in leaf chemical traits are involved in resource selection by herbivores were provided by these results. As mentioned in the General Introduction, secondary metabolites in plants act as a defense against herbivory by generalists (e.g., Jeschke et al., 2017; Macel, 2011; Schoonhoven et al., 2005), while specialists can overcome and use secondary metabolites for foraging (e.g. Goodey et al., 2015; Wheat et al., 2007). These ecological characteristics of herbivores may have made the difference between the results of Chapters 2 and 3. However, *Galerucella grisescens* did not respond to secondary metabolites despite being generalist herbivores. Accumulation of further empirical studies will help understand the impact of host range on foraging and hence distribution patterns of herbivores.

In previous study, ecological researchers interpreted the local population density of plants as patch size and resource abundance (e.g., Root, 1973; Otway et al., 2005). The density has then been thought to influence the resource selection of herbivores by controlling foraging efficiency and associational effects with neighboring plants. Conversely, our results provide evidence that the local

population density of host plants can influence the resource selection of herbivores by causing changes in the concentration of leaf chemicals, i.e., resource quality. A unified understanding of the multiple mechanisms for determining herbivore distribution, such as the resource concentration hypothesis (Root, 1973) and resource dilution (Otway et al., 2005), may be provided by our hypothesis. Further studies with herbivores from a wide variety of taxa are needed to evaluate the generality of this mechanism.

Our findings may also help to understand the formation mechanisms of the herbivore community. It has been tried in many studies to predict herbivore distribution and community structure from plant communities (e.g., Scherber et al., 2010; Moreira et al., 2012; Bustos et al., 2017). Sherber et al. (2010) reported that as the number of species in plant communities increases the amount and number of species of herbivores also increases. In these studies, researchers focused on parameters such as species diversity or composition and the density or biomass of each plant species in plant communities. However, variations in the local population density of host plants within their habitat or spatial structure have rarely been accounted for and have been regarded as uniform. Our results reveal that the spatial structure of plant communities may result in spatial heterogeneity in the chemicals used by herbivores, which may influence arthropod community formation. More comprehensive research, including multiple plants and herbivore species, should be included in future studies to determine how the herbivore community is affected by the spatial structure of the

plant community.

References

Abrahamson WG (2008) Specialization, speciation, and radiation: The evolutionary biology of

   herbivorous insects. University of California Press.

Adler PB, Smull D, Beard KH, Choi RT, Furniss T, KulmatiskiA, Meiners JM, Tredennick AT and

   Veblen KE (2018) Competition and coexistence in plant communities: Intraspecific

   competition is stronger than interspecific competition. Ecology Letters, 21(9):1319–

   1329. https://doi.org/10.1111/ele.13098

Akula R and Ravishankar GA (2011) Influence of abiotic stress signals on secondary metabolites in

   plants. Plant Signaling & Behavior, 6(11):1720–1731.

   https://doi.org/10.4161/psb.6.11.17613

Asao T, Hasegawa K, Sueda Y, Tomita K, Taniguchi K, Hosoki T, Pramanik MHR and Matsui Y

   (2003) Autotoxicity of root exudates from taro. Scientia Horticulturae (Amsterdam),

   97(3–4):389–396. https://doi.org/10.1016/S0304- 4238(02)00197-8.

Augustine DJ and McNaughton SJ (1998) Ungulate Effects on the Functional Species Composition

   of Plant Communities: Herbivore Selectivity and Plant Tolerance. The Journal of

   Wildlife Management, 62:1165–1183. https://doi.org/10.2307/3801981

Anwar F, Kalsoom U, Sultana B, Mushtaq M, Mehmood T and Arshad HA (2013) Effect of drying

   method and extraction solvent on the total phenolics and antioxidant activity of

cauliflower *(Brassica oleracea* L.) extracts. International Food Research Journal, 20(2):653.

Badri DV and Vivanco (2009) JM Regulation and function of root exudates. Plant Cell Environ, 32(6):666–681. https://doi.org/10.1111/j.1365- 3040.2009.01926.x.

Barton KE and Bowers MD (2006) Neighbor species differentially alter resistance phenotypes in Plantago. Oecologia, 150(3):442–452. https://doi.org/10.1007/s0044 2-006-0531-z

Bennett RN and Wallsgrove RM (1994) Secondary metabolites in plant defence mechanisms. New phytologist, 127(4):617-633. https://doi.org/10.1111/j.1469-8137.1994.tb02968.x

Bernardo HL, Albrecht MA and Knight TM (2016) Increased drought frequency alters the optimal management strategy of an endangered plant. Biological Conservation, 203:243–251. https://doi.org/10.1016/j.biocon.2016.09.030

Biedrzycki ML, Jilany TA, Dudley SA and Bais HP (2010) Root exudates mediate kin recognition in plants. Communicative & Integrative Biology, 3(1):28–35. https://doi.org/10.4161/cib.3.1.10118

Broström G (2018) glmmML: generalized linear models with clustering. R package version 1.0.3. https://CRAN.R-project.org/package=glmmML

Broz AK, Broeckling CD, De-la-Peña C, Lewis MR, Greene E, Callaway RM, et al. (2010) Plant

neighbor identity influences plant biochemistry and physiology related to defense. BMC

Plant Biology, 10(1):1–14. https://doi.org/10.1186/1471-2229-10-115

Bryant JP, Chapin III FS and Klein DR (1983) Carbon/nutrient balance of boreal plants in relation to

vertebrate herbivory. Oikos, 40(3): 357-368. https://doi.org/10.2307/3544308

Bustos‐Segura C, Poelman EH, Reichelt M, Gershenzon J and Gols R (2017) Intraspecific

chemical diversity among neighbouring plants correlates positively with plant size and

herbivore load but negatively with herbivore damage. Ecology Letters, 20(1):87-97.

https://doi.org/10.1111/ele.12713

Champagne E, Royo AA, Tremblay JP and Raymond P (2020) Phytochemicals involved in plant

resistance to leporids and cervids: a systematic review. Journal of Chemical Ecology,

46(1):84–98. https://doi.org/10.1007/s10886-019-01130-z

Cheema ZA, Farooq M and Wahid A, editors. Allelopathy: current trends and future applications.

Berlin/Heidelberg: Springer- Verlag; 2012.

Christensen RHB (2015) Package ordinal. Available at:

cran.r-project.org/web/packages/ordinal/ordinal.pdf

Cipollini DF and Bergelson J (2002) Interspecific competition affects growth and herbivore damage of Brassica napus in the field. Plant Ecology, 162(2):227–231. https://doi.org/10.1023/A:10203 77627529

Coutinho RD, Cuevas-Reyes P, Fernandes GW and Fagundes M (2019) Community structure of gall-inducing insects associated with a tropical shrub: Regional, local and individual patterns. Tropical Ecology, 60(1):74–82. https://doi.org/10.1007/s4296 5-019-00010-7

Craine JM and Dybzinski R (2013) Mechanisms of plant competition for nutrients, water and light. Functional Ecology, 27(4):833–840. https://doi.org/10.1111/1365-2435.12081

Côté SD, Rooney TP, Tremblay JP, Dussault C and Waller DM (2004) Ecological Impacts of Deer Overabundance. Annual Review of Ecology, Evolution, and Systematics, 35:113–147. https://doi.org/10.1146/annurev.ecolsys.35.021103.105725

Dudley SA and File AL (2007) Kin recognition in an annual plant. Biology Letters, 3(4):435–438. https://doi.org/10.1098/rsbl.2007.0232

Dudt JF and Shure DJ (1994) The influence of light and nutrients on foliar phenolics and insect herbivory. Ecology, 75(1):86–98. https://doi.org/10.2307/1939385

Enright NJ, Fontaine JB Lamont BB., Miller BP and Westcott VC (2014) Resistance and resilience to changing climate and fire regime depend on plant functional traits. Journal of Ecology, 102(6):1572–1581. https://doi.org/10.1111/1365-2745.12306

Fagundes M, Barbosa EM, Oliveira JB, Brito BG, Freitas KT, Freitas KF and Reis-Junior R (2019)

Galling inducing Insects associated with a tropical shrub: The role of resource

concentration and species interactions. Ecología Austral, 29(1):12–19.

https://doi.org/10.25260/ EA.19.29.1.0.751

Feduraev P, Chupakhina G, Maslennikov P, Tacenko N and Skrypnik L (2019) Variation in phenolic

compounds content and antioxidant activity of different plant organs from *Rumex*

crispus L. and *Rumex obtusifolius* L. at different growth stages. Antioxidants, 8(7):237.

https://doi.org/10.3390/antio x8070237

Feeny P (1970) Seasonal changes in oak leaf tannins and nutrients as a cause of spring feeding by

winter moth caterpillars. Ecology, 51(4):565–581. https://doi.org/10.2307/1934037

Felton AM, Wam HK, Stolter C, Mathisen KM and Wallgren M (2018) The complexity of

interacting nutritional drivers behind food selection, a review of northern cervids.

Ecosphere, 9(5):e02230. https://doi.org/10.1002/ecs2.2230

Foley WJ, Moore BD (2005) Plant secondary metabolites and vertebrate herbivores–from

physiological regulation to ecosystem function. Current Opinion in Plant Biology,

8(4):430–435. https://doi.org/10.1016/j.pbi.2005.05.009

Forbes ES, Cushman JH, Burkepile DE, Young TP, Klope M and Young HS (2019) Synthesizing the

effects of large, wild herbivore exclusion on ecosystem function. Functional Ecology,

33(9):1597–1610. https://doi.org/10.1111/1365-2435.13376

Forkner RE, Marquis RJ, Lill JT (2004) Feeny revisited: condensed tannins as anti-herbivore

defences in leaf-chewing herbivore communities of Quercus. Ecological Entomology,

29(2):174–187. https://doi.org/10.1111/j.1365-2311.2004.0590.x

Freeland WJ (1991) Plant secondary metabolites: Biochemical coevolution with herbivores. In RT

Palo, CT Robbins (Eds.), Plant defenses against mammalian herbivory (pp. 62–79).

Boca Raton, FL: CRC Press.

Fretwell DS and Lucas HLJ (1969) On territorial behavior and other factors influencing habitat

distribution in birds. Acta Biotheoretica, 19(1):16–36. https://doi.org/10.1007/BF016

01953

Garcia J, Hankins WG and Rusiniak KW (1974) Behavioral Regulation of the Milieu Interne in Man

and Rat: Food preferences set by delayed visceral effects facilitate memory research and

predator control. Science, 185(4154):824-831.

https://doi.org/10.1126/science.185.4154.824

Garcia J, Lasiter PS, Bermudez-Rattoni F and Deems DA (1985) A general theory of aversion

learning. Annals of the New York Academy of Sciences, 443(1):8-21.

https://doi.org/10.1111/j.1749-6632.1985.tb27060.x

Godinho DP, Janssen A, Li D, Cruz C and Magalhães S (2020). The distribution of herbivores

between leaves matches their performance only in the absence of competitors. Ecology

and Evolution, 10(15):8405–8415. https://doi.org/10.1002/ece3.6547

Goodey NA, Florance HV, Smirnoff N and Hodgson DJ (2015) Aphids pick their poison: Selective

sequestration of plant chemicals affects host plant use in a specialist herbivore. Journal

of Chemical Ecology, 41(10):956–964. https://doi.org/10.1007/s10886-015-0634-2

Goodman SJ, Tamate HB, Wilson R, Nagata J, Tatsuzawa S, Swanson GM, Pemberton JM and

McCullough DR (2001) Bottlenecks, drift and differentiation: the population structure

and demographic history of sika deer (*Cervus nippon*) in the Japanese archipelago.

Molecular Ecology, 10(6):1357–1370.

https://doi.org/10.1046/j.1365-294X.2001.01277.x

Gustavson CR, Garcia J, Hankins WG and Rusiniak KW (1974) Coyote predation control by

aversive conditioning. Science, 184(4136):581-583.

https://doi.org/10.1126/science.184.4136.581

Hambäck PA and Beckerman AP (2003) Herbivory and plant resource competition: A review of two

interacting interactions. Oikos, 101(1):26–37.

https://doi.org/10.1034/j.1600-0706.2003.12568.x

Hashimoto Y and Fujiki D (2014) List of food plants and unpalatable plants of sika deer (*Cervus

nippon*) in Japan. Humans and Nature, 25:133–160.

https://doi.org/10.24713/hitotoshizen.25.0_133

Herms DA and Mattson WJ (1992) The dilemma of plants: to grow or defend. The Quarterly Review

of Biology, 67(3):83–335.

Herz K, Dietz S, Gorzolka K, Haider S, Jandt U, Scheel D and Bruelheide H. (2018) Linking root

exudates to functional plant traits. Plos One, 13(10):e0204128.

doi:10.1371/journal.pone.0204128.

Hobbs NT (1996) Modification of Ecosystems by Ungulates. The Journal of Wildlife Management,

60:695. https://doi.org/10.2307/3802368

Holík J and Janík D (2022) Spatial patterns in neighbourhood effects on woody plant selection and

bark stripping by deer in a lowland alluvial forest. Journal of Vegetation Science,

33(1):e13114. https://doi.org/10.1111/jvs.13114f

Howe GA and Jander G (2008) Plant Immunity to Insect Herbivores. Annual Review of Plant

Biology, 59(1):41–66. https://doi.org/10.1146/annurev.arplant.59.032607.092825

Ikonen A, Tahvanainen J and Roininen H (2002) Phenolic secondary compounds as determinants of

the host plant preferences of the leaf beetle *Agelastica alni*. Chemoecology, 12(3):125–

131. https://doi.org/10.1007/s0001 2-002-8337-2

Jeschke V, Kearney EE, Schramm K, Kunert G, Shekhov A, Gershenzon J and Vassão DG (2017)

How glucosinolates affect generalist lepidopteran larvae: Growth, development and

glucosinolate metabolism. Frontiers in Plant Science, 8:1995.

https://doi.org/10.3389/fpls.2017.01995

Julkunen-Tiitto R (1985) Phenolic constituents in the leaves of northern willows: methods for the

analysis of certain phenolics. Journal of Agricultural and Food Chemistry 33(2):213–

217. https://doi.org/10.1021/jf00062a013

Julkunen-Tiitto R and Tahvanainen J (1989) The effect of the sample preparation method of

extractable phenolics of Salicaceae species. Planta Medica, 55(01):55-58.

Karasov TL, Chae E, Herman JJ and Bergelson J (2017) Mechanisms to mitigate the trade-off

between growth and defense. The Plant Cell, 29(4):666–680.

https://doi.org/10.1105/tpc.16.00931

Karban R (2021) Plant Communication. Annual Review of Ecology, Evolution, and Systematics,

52(1):1–24. https://doi.org/10.1146/annurev-ecolsys-010421-020045.

Karban R and Shiojiri K (2009) Self-recognition affects plant communication and defense. Ecology

Letters, 12(6):502–506. https://doi.org/10.1111/j.1461-0248.2009.01313.x

Kardol P, Martijn Bezemer T and van der Putten WH (2006) Temporal variation in plant–soil

feedback controls succession. Ecology Letters, 9(9):1080–1088.

https://doi.org/10.1111/j.1461-0248.2006.00953.x.

Kasahara M, Fujii S, Tanikawa T and Mori AS (2016) Ungulates decelerate litter decomposition by

altering litter quality above and below ground. European Journal of Forest Research,

135(5):849–856. https://doi.org/10.1007/s10342-016-0978-3

Kawakami H, Fuchino H and Kawahara N (2020) Endotoxin contamination and reaction interfering

substances in the plant extract library. Biological and Pharmaceutical Bulletin

43(11):1767–1775.

Kigathi RN, Weisser WW, Veit D, Gershenzon J and Unsicker SB (2013) Plants suppress their

emission of volatiles when growing with conspecifics. Journal of Chemical Ecology,

39(4):537–545. https://doi.org/10.1007/s1088 6-013-0275-2

Kitazaki K, Fukushima A, Nakabayashi R, Okazaki Y, Kobayashi M, Mori T, Nishizawa T,

Reyes-Chin-Wo S, Michelmore RW, Saito K, Shoji K and Kusano M (2018) Metabolic

reprogramming in leaf lettuce grown under different light quality and intensity

conditions using narrow-band LEDs. Scientific Reports, 8(1):1–12.

https://doi.org/10.1038/s4159 8-018-25686-0

Knops JM, Reinhart K. (2000) Specific leaf area along a nitrogen fertilization gradient. The

American Midland Naturalist, 144(2):265–272.

https://doi.org/10.1674/0003-0031(2000)144[0265:SLAAAN]2.0.CO;2.

Koganezawa M and Satake C (1996) Effects of grazing by sika-deer on the vegetation of Oku-Nikko

and their management. Trans Nat Found Proj, 5:57–66.

Kong C, Zhang S, Li Y, Xia Z, Yang X, Meiners S J and Wang P (2018) Plant neighbor detection and

allelochemical response are driven by root-secreted signaling chemicals. Nature

Communications, 9(1):10.1038/s41467-018-06429-1

Kong HG, Song GC, Sim HJ and Ryu CM (2021) Achieving similar root microbiota composition in

neighbouring plants through airborne signalling. The ISME Journal, 15(2):397–408.

https://doi.org/10.1038/s41396-020-00759-z.

Kuznetsova A, Brockhoff PB and Christensen RHB (2015) Package 'lmertest'. R package version

2(0):734.

Lattanzio V, Lattanzio VM and Cardinali A (2006) Role of phenolics in the resistance mechanisms of

plants against fungal pathogens and insects. Phytochemistry: Advances in research,

661(2):23-67.

Lepik A, Abakumova M, Zobel K and Semchenko M (2012) Kin recognition is density-dependent

and uncommon among temperate grassland plants. Functional Ecology, 26(5):1214–

1220. https://doi. org/10.1111/j.1365-2435.2012.02037.x

Li L, He L, Su X, Amu H, Li J and Zhang Z (2022) Chemotaxonomy of Aster species from the

Qinghai‐Tibetan Plateau based on metabolomics. Phytochemical Analysis, 33(1):23-39.

https://doi.org/10.1002/pca.3058

Li Y, Maruyama N, Koganezawa M, Kanzaki N (1996) Wintering range expansion and increase of

sika deer in Nikko in relation to global warming. Wildlife Conservation Japan, 2(1):23–

35. https://doi.org/10.20798/wildlifeconsjp.2.1_23

Macel M (2011) Attract and deter: A dual role for pyrrolizidine alkaloids in plant–insect interactions.

Phytochemistry Reviews, 10(1):75–82. https://doi.org/10.1007/s1110 1-010-9181-1

Marsh KJ, Wallis IR, Kulheim C, Clark R, Nicolle D, Foley WJ and Salminen JP (2020) New

approaches to tannin analysis of leaves can be used to explain in vitro biological

activities associated with herbivore defence. New Phytologist, 225(1):488-498.

https://doi.org/10.1111/nph.16117

Matsuda K (1974) Feeding stimulation of nutrient chemicals for the strawberry leaf beetle,

Galerucella vittaticollis Baly (Coleoptera: Chrysomelidae). Japanese Journal of Applied

Entomology and Zoology, 18:14–20. https://doi.org/10.1303/jjaez.18.14

McArthur C, Robbins CT, Hagerman AE, Hanley TA (1993) Diet selection by a ruminant generalist

browser in relation to plant chemistry. Canadian Journal of Zoology, 71(11):2236–2243.

https://doi.org/10.1139/z93-314

McCullagh and Nelder JA (2019) Generalized linear models. Routledge.

Moreira X, Glauser G, Abdala-Roberts L (2017) Interactive effects of plant neighbourhood and

ontogeny on insect herbivory and plant defensive traits. Scientific Reports, 7(1):1–9.

https://doi.org/10.1038/s41598-017-04314-3

Moreira X, Mooney KA, Zas R and Sampedro L (2012) Bottom-up effects of host-plant species

diversity and top-down effects of ants interactively increase plant performance.

Proceedings of the Royal Society B: Biological Sciences, 279(1746):4464-4472.

https://doi.org/10.1098/rspb.2012.0893

Mraja A, Unsicker SB, Reichelt M, Gershenzon J and Roscher C (2011) Plant community diversity

influences allocation to directchemical defence in *Plantago lanceolata*. PLoS ONE,

6(12):e28055. https://doi.org/10.1371/journ al.pone.0028055

Muiruri EW, Barantal S, Iason GR, Salminen JP, Perez-Fernandez E and Koricheva J (2019) Forest

diversity effects on insect herbivores: Do leaf traits matter? New Phytologist,

221(4):2250–2260. https://doi.org/10.1111/nph.15558

Nakahama N, Uchida K, Koyama A, Iwasaki T, Ozeki M and Suka T (2020) Construction of deer

fences restores the diversity of butterflies and bumblebees as well as flowering plants in

semi-natural grassland. Biodiversity and Conservation, 29(7):2201–2215.

https://doi.org/10.1007/s10531-020-01969-9

Nerlekar AN (2018) Seasonally dependent relationship between insect herbivores and host plant

density in Jatropha nana, a tropical perennial herb. Biology Open, 7(8):1–7.

https://doi.org/10.1242/bio.035071

Nopp-Mayr U, Reimoser S, Reimoser F, Sachser F, Obermair L and Gratzer G (2020) Analyzing

long-term impacts of ungulate herbivory on forest-recruitment dynamics at community

and species level contrasting tree densities versus maximum heights. Scientific Reports,

10(1):1–13. https://doi.org/10.1038/s41598-020-76843-3

Offor E (2010) The Nutritional Requirements of phytophagous insects: Why do insects feed on

plants? Available at SSRN, 1535274.

Ohashi H, Yoshikawa M, Oono K, Tanaka N, Hatase Y and Murakami Y (2014) The impact of sika

deer on vegetation in Japan: Setting management priorities on a national scale.

Environmental Management, 54(3):631–640.

https://doi.org/10.1007/s00267-014-0326-7

Ohsaki H, Miyagi A, Kawai-Yamada M and Yamawo A (2022) Intraspecific interaction of host

plants leads to concentrated distribution of a specialist herbivore through metabolic

alterations in the leaves. Functional Ecology, 36(3):779-793.

https://doi.org/10.1111/1365-2435.13988

Ohsaki H, Mukai H and Yamowo A (2020) Biochemical recognition in seeds: germination of *Rumex*

obtusifolius is promoted by leaves of facilitative adult conspecifics. Plant Species

Biology, 35(3):233–242. https://doi.org/10.1111/1442-1984.12275.

Ohsaki H (2018) Phenotypic plasticity in leaf traits in response to intraspecific competition in host

plants promotes resource partitioning in herbivores. Aomori, Japan: Graduation thesis of

Hirosaki University. in Japanese.

Okuda K, Tamura Y, Seki Y, Yamawo A, Koganezawa M (2014) Effects of a deer-proof fence in a

high-deer-density area on the recovery of bumblebee communities in Oku-Nikko, Japan.

Japanese Journal of Conservation Ecology, 19(2):109–118.

https://doi.org/10.18960/hozen.19.2_109

Ormeño E, Bousquet-Mélou A, Mévy JP, Greff S, Robles C, Bonin G and Fernandez C (2007) Effect

of intraspecific competition and substrate type on terpene emissions from some

Mediterranean plant species. Journal of Chemical Ecology, 33(2):277–286.

https://doi.org/10.1007/s1088 6-006-9219-4

Otway SJ, Hector A and Lawton JH (2005) Resource dilution effects on specialist insect herbivores in a grassland biodiversity experiment. Journal of Animal Ecology, 74(2):234–240. https://doi.org/10.1111/j.1365-2656.2005.00913.x

Pringle RM, Young TP, Rubenstein DI and McCauley DJ (2007) Herbivore-initiated interaction cascades and their modulation by productivity in an African savanna. Proceedings of the National Academy of Sciences USA, 104(1):193–197. https://doi.org/10.1073/pnas.0609840104

Provenza FD, Burritt EA, Clausen TP, Bryant JP, Reichardt PB and Distel RA (1990) Conditioned flavor aversion: a mechanism for goats to avoid condensed tannins in blackbrush. The American Naturalist, 136(6):810-828. https://doi.org/10.1086/285133

R Development Core Team (2019) R: A Language and Environment for Statistical Computing.

Rehman F, Khan FA, Badruddin SMA (2012) Role of phenolics in plant defense against Insect herbivory. In Chemistry of Phytopotentials: Health, Energy and Environmental Perspectives. Springer Berlin Heidelberg, Berlin, Heidelberg (pp 309–313). https://doi.org/10.1007/978-3-642-23394-4_65

Reigosa MJ, Souto XC and González L (1999) Effect of phenolic compounds on the germination of six weeds species. Plant Growth Regulation, 28(2):83–88. https://doi.org/10.1023/A:1006269716762.

Rhainds M and English-Loeb G (2003) Testing the resource concentration hypothesis with tarnished

plant bug on strawberry: Density of hosts and patch size influence the interaction

between abundance of nymphs and incidence of damage. Ecological Entomology,

28(3):348–358. https://doi.org/10.1046/j.1365-2311.2003.00508.x

Robbins CT (1993) Wildlife feeding and nutrition. Second edition. Academic Press, New York, New

York, USA.

Rooney TP, Waller DM (2003) Direct and indirect effects of white-tailed deer in forest ecosystems.

Forest Ecology and Management, 181(1–2):165–176.

https://doi.org/10.1016/S0378-1127(03)00130-0

Root RB (1973) Organization of a plant–arthropod association in simple and diverse habitats: The

fauna of collards (*Brassica oleracea*). Ecological Monographs, 43(1):95–124.

https://doi.org/10.2307/1942161

Salminen JP and Karonen M (2011) Chemical ecology of tannins and other phenolics: we need a

change in approach. Functional Ecology, 25(2):325-338.

https://doi.org/10.1111/j.1365-2435.2010.01826.x

Schai-Braun SC, Reichlin TS, Ruf T, Klansek E, Tataruch F, Arnold W and Hackländer K (2015)

The european hare (*Lepus europaeus*): a picky herbivore searching for plant parts rich in

fat. Plos One, 10(7):e0134278. https://doi.org/10.1371/journal.pone.0134278.

Scherber C, Eisenhauer N, Weisser WW, Schmid B, Voigt W, Fischer M, ... and Tscharntke T (2010)

Bottom-up effects of plant diversity on multitrophic interactions in a biodiversity

experiment. Nature, 468(7323):553-556. https://doi.org/10.1038/nature09492

Scheirs J and De Bruyn L (2004) Excess of nutrients results in plant stress and decreased grass miner

performance. Entomologia Experimentalis et Applicata, 113(2):109–116.

https://doi.org/10.1111/ j.0013-8703.2004.00215.x

Schoonhoven LM, van Loon JJA and Dicke M (2005) Insect–plant biology (2nd ed.). Oxford

University Press.

Seki Y, Ito M, Okuda K, Koganezawa M (2021) Effects of sika deer density on the diet and

population of red foxes. European Journal of Wildlife Research 67(2):1–13.

https://doi.org/10.1007/s10344-021-01475-5

Seki Y, Koganezawa M (2010) Factors influencing the increase in earthworms outside deer proof

fences in Oku-Nikko, central Japan: The influence of the modification of understory

vegetation by sika deer. Journal of the Japanese Forest Society, 92(5):241–246.

https://doi.org/10.4005/jjfs.92.241

Semchenko M, Saar S and Lepik A (2014) Plant root exudates mediate neighbour recognition and

trigger complex behavioural changes. New Phytolgist, 204(3):631–637.

https://doi.org/10.1111/nph.12930

Shibaeva TG, Mamaev AV and Sherudilo EG (2020) Evaluation of a SPAD-502 Plus chlorophyll

meter to estimate chlorophyll content in leaves with interveinal chlorosis. Russian

Journal of Plant Physiology, 67(4):690–696.

https://doi.org/10.1134/S1021443720040160.

Shinoda Y, Uchida K, Koyama A and Akasaka M (2021) Deer grazing changes seed traits and

functions of grazing-intolerant plants. Plant Species Biology, 36(1):126–132.

https://doi.org/10.1111/1442-1984.12300

Shirahama S, Yamawo A and Tokuda M (2017) Dimorphism in trichome production of *Persicaria

lapathifolia* var. lapathifolia and its multiple effects on a leaf beetle. Arthropod–plant

Interactions, 11(5):683–690. https://doi.org/10.1007/s1182 9-017-9520-x

Sousa-Souto L, Bocchiglieri A, Dias DDM, Ferreira AS and José Filho PDL (2018) Changes in leaf

chlorophyll content associated with flowering and its role in the diversity of

phytophagous insects in a tree species from a semiarid Caatinga. PeerJ, 6:e5059.

https://doi.org/10.7717/peerj.5059

Spalinger DE (2000) Nutritional ecology. Pages 108–139 inS. Demarais and P. R. Krausman, editors.

Ecology and management of large mammals in North America. Prentice-Hall, Upper

Saddle River, New Jersey, USA.

Stephan JG, Pourazari F, Tattersdill K, Kobayashi T, Nishizawa K and de Long JR (2017) Long-term

deer exclosure alters soil properties, plant traits, understory plant community and insect

herbivory, but not the functional relationships among them. Oecologia, 184(3):685–699.

https://doi.org/10.1007/s00442-017-3895-3

Stephens AE and Myers JH (2012) Resource concentration by insects and implications for plant

populations. Journal of Ecology, 100(4):923–931.

https://doi.org/10.1111/j.1365-2745.2012.01971.x

Strauss SY, Rudgers JA, Lau JA and Irwin RE (2002) Direct and ecological costs of resistance to

herbivory. Trends in Ecology & Evolution, 17(6):278–285.

https://doi.org/10.1016/S0169-5347(02)02483-7

Sugiyama A (2019) The soybean rhizosphere: metabolites, microbes, and beyond—A review. Journal

of Advanced Research, 19:67-73. https://doi.org/10.1016/j.jare.2019.03.005

Suzuki M, Miyashita T, Kabaya H, Ochiai K, Asada M and Tange T (2008) Deer density affects

ground-layer vegetation differently in conifer plantations and hardwood forests on the

Boso Peninsula, Japan. Ecological Research, 23(1):151–158.

https://doi.org/10.1007/s11284-007-0348-1

Suzuki N (1985) Resource utilization of three chrysomelid beetles feeding on Rumex plants with

diverse vegetational background. Japanese Journal of Ecology, 35(2):225–234.

https://doi.org/10.18960/seitai.35.2_225

Suzuki N (1986) Interspecific competition and coexistence of the two chrysomelids, *Gastrophysa*

atrocyanea Motschulsky and Galerucella vittaticollis Baly (Coleoptera: Chrysomelidae),

under limited food resource conditions. Ecological Research, 1(3):259–268.

https://doi.org/10.1007/BF023 48683

Takahashi Y, Shiojiri K and Yamawo A (2021) Aboveground plant-to-plant communication reduces

root nodule symbiosis and soil nutrient concentrations. Scientific Reports, 11(1):1–6.

https://doi.org/10.1038/s41598-021- 92123-0.

Takatsuki S (2009) Effects of sika deer on vegetation in Japan: A review. Biological Conservation,

142(9):1922–1929. https://doi.org/10.1016/j.biocon.2009.02.011

Takigahira H and Yamawo A (2019) Competitive responses based on kin-discrimination underlie

variations in leaf functional traits in Japanese beech (*Fagus crenata*) seedlings.

Evolutionary Ecology, 33(4):521–531. https://doi.org/10.1007/s10682-019-09990-3.

Titayavan M and Altieri MA (1990) Synomone-mediated interactions between the parasitoid

Diaeretiella rapae and *Brevicoryne brassicae* under field conditions. Entomophaga,

35(4):499–507. https://doi.org/10.1007/BF023 75084

Torp M, Lehrman A, Stenberg JA, Julkunen-Tiitto R and Björkman C (2013) Performance of an

herbivorous leaf beetle (*Phratora vulgatissima*) on Salix F2 hybrids: The importance of

phenolics. Journal of Chemical Ecology, 39(4):516–524.

https://doi.org/10.1007/s10886-013-0266-3

Tuller J, Queiroz ACM, Luz GR and Silva JO (2013) Gall-forming insect attack patterns: A test of

the Plant Vigor and the Resource Concentration Hypotheses. Biotemas, 26(1):45–51.

https://doi.org/10.5007/2175-7925.2013v26n1p45

Turkington R (2009) Top-down and bottom-up forces in mammalian herbivore – vegetation systems:

an essay review. Botany, 87(8):723-739. https://doi.org/10.1139/B09-035

Verón SR, Paruelo JM and Oesterheld M (2011) Grazing-induced losses of biodiversity affect the

transpiration of an arid ecosystem. Oecologia, 165(2):501–511.

https://doi.org/10.1007/s00442-010-1780-4

Villalba JJ, Burritt EA and Clair SBS (2014) Aspen (*Populus tremuloides* Michx.) Intake and

Preference by Mammalian Herbivores: The Role of Plant Secondary Compounds and

Nutritional Context. Journal of Chemical Ecology, 40(10):1135–1145.

https://doi.org/10.1007/s10886-014-0507-0

Vourc'h G, Vila B, Gillon D, Escarré J, Guibal F, Fritz H, Clausen TP and Martin JL (2002)

Disentangling the causes of damage variation by deer browsing on young Thuja plicata.

Oikos 98(2):271–283. https://doi.org/10.1034/j.1600-0706.2002.980209.x

Wakatsuki Y, Nishizawa K and Mori AS (2021) Leaf trait variability explains how plant community

composition changes under the intense pressure of deer herbivory. Ecological Research,

36(3):521–532. https://doi.org/10.1111/1440-1703.12224

Walker TS, Bais HP, Halligan KM, Stermitz FR and Vivanco JM (2003) Metabolic profiling of root

exudates of arabidopsisthaliana. J Agric Food Chem, 51(9):2548–2554.

https://doi.org/10.1021/jf021166h

War AR, Taggar GK, Hussain B, Taggar MS, Nair RM and Sharma HC (2018) Plant defence against

herbivory and insect adaptations. AoB PLANTS, 10(4):ply037.

https://doi.org/10.1093/aobpla/ply037

War AR, Paulraj MG, Ahmad T, Buhroo AA, Hussain B, Ignacimuthu S and Sharma HC (2012)

Mechanisms of plant defense against insect herbivores. Plant Signaling & Behavior,

7(10):1306–1320. https://doi.org/10.4161/psb.21663

Wheat CW, Vogel H, Wittstock U, Braby MF, Underwood D and Mitchell-Olds T (2007) The genetic

basis of a plant–insect coevolutionary key innovation. Proceedings of the National

Academy of Sciences of the United States of America, 104(51):20427–20431.

https://doi.org/10.1073/pnas.07062 29104

Xu Y, Cheng HF, Kong CH and Meiners SJ (2021) Intra-specific kin recognition contributes to

inter-specific allelopathy: a case study of allelopathic rice interference with paddy

weeds. Plant Cell Environ. 44(12):3709–3721. https://doi.org/10.1111/pce.14083.

Yamamura, K. (1999). Relation between plant density and arthropod density in cabbage fields.

Population Ecology, 41, 177–182. https://doi.org/10.1007/s1014 40050020

Yamawo A, Sato M and Mukai H (2017) Experimental evidence for benefit of self discrimination in

roots of a clonal plant. AoB Plants, 9(6):plx049. https://doi.org/10.1093/aobpla/plx049

Yamawo A, Tokuda M, Katayama N, Yahara T and Tagawa J (2015) Ant-Attendance in Extrafloral

Nectar-Bearing Plants Promotes Growth and Decreases the Expression of Traits Related

to Direct Defenses. Evolutionary Biology, 42(2):191–198.

https://doi.org/10.1007/s11692-015-9310-2

Yamawo A (2021) Intraspecific competition favors ant-plant protective mutualism. Plant Species

Biology, 36(3):372–378. https://doi.org/10.1111/1442-1984.12331.

Yamawo A (2015) Relatedness of neighboring plants alters the expression of indirect defense traits

in an extrafloral nectary-bearing plant. Evolutionary Biology, 42(1):12–19.

https://doi.org/10.1007/s11692-014-9295-2.

Yamawo A and Mukai H (2020) Outcome of interspecific competition depends on genotype of

conspecific neighbours. Oecologia, 193(2):415–423. https://doi.org/10.1007/s0044

2-020-04694-w

Young CC (1984) Autointoxication in root exudates of *Asparagus officinalis* L. Plant and Soil,

82(2):247–253. https://doi.org/10.1007/BF02220251.

Ōmura H (2018) Plant secondary metabolites in host selection of butterfly. In J. Tabata (Ed.),

Chemical ecology of insects (pp. 3–27). CRC Press.

List of publications

<u>Ohsaki H*</u>, Miyamoto R, Tajima M, Sakamoto Y, Okuda K and Yamawo A* (2022) Plant–plant interaction by *Aster leiophyllus* affects herbivory by Sika deer, *Cervus nippon*. The Science of Nature, 109(6), 54. https://doi.org/10.1007/s00114-022-01827-4

<u>Ohsaki H*</u> and Yamawo A (2022) Effects of indirect plant–plant interaction via root exudate on growth and leaf chemical contents in *Rumex obtusifolius*. Plant Signaling & Behavior, 17(1), 2050628. https://doi.org/10.1080/15592324.2022.2050628

<u>Ohsaki H*</u>, Miyagi A, Kawai-Yamada M and Yamawo A* (2022) Intraspecific interaction of host plants leads to concentrated distribution of a specialist herbivore through metabolic alterations in the leaves. Functional Ecology, 36(3), 779-793. https://doi.org/10.1111/1365-2435.13988

<u>Ohsaki H*</u>, Mukai H and Yamawo A* (2020) Biochemical Recognition in Seeds: Germination of *Rumex obtusifolius* is promoted by leaves of facilitative adult conspecifics. Plant Species Biology. Plant Species Biology, 35(3), 233-242. https://doi.org/10.1111/1442-1984.12275

Yamawo A*, **<u>Ohsaki H</u>**, Cahill Jr JF (2019) Damage to leaf veins suppresses root foraging precision. American Journal of Botany, 106(8), 1126-1130. https://doi.org/10.1002/ajb2.1338

Sato M, **Ohsaki H**, Fukano Y and Yamawo A* (2018) Self-discrimination in vine tendrils of

different plant families. Plant signaling & behavior, 13(4), e1451710.

https://doi.org/10.1080/15592324.2018.1451710

Acknowledgements